Development of new technological applications for post- and prenatal diagnosis of the hemoglobinopathies

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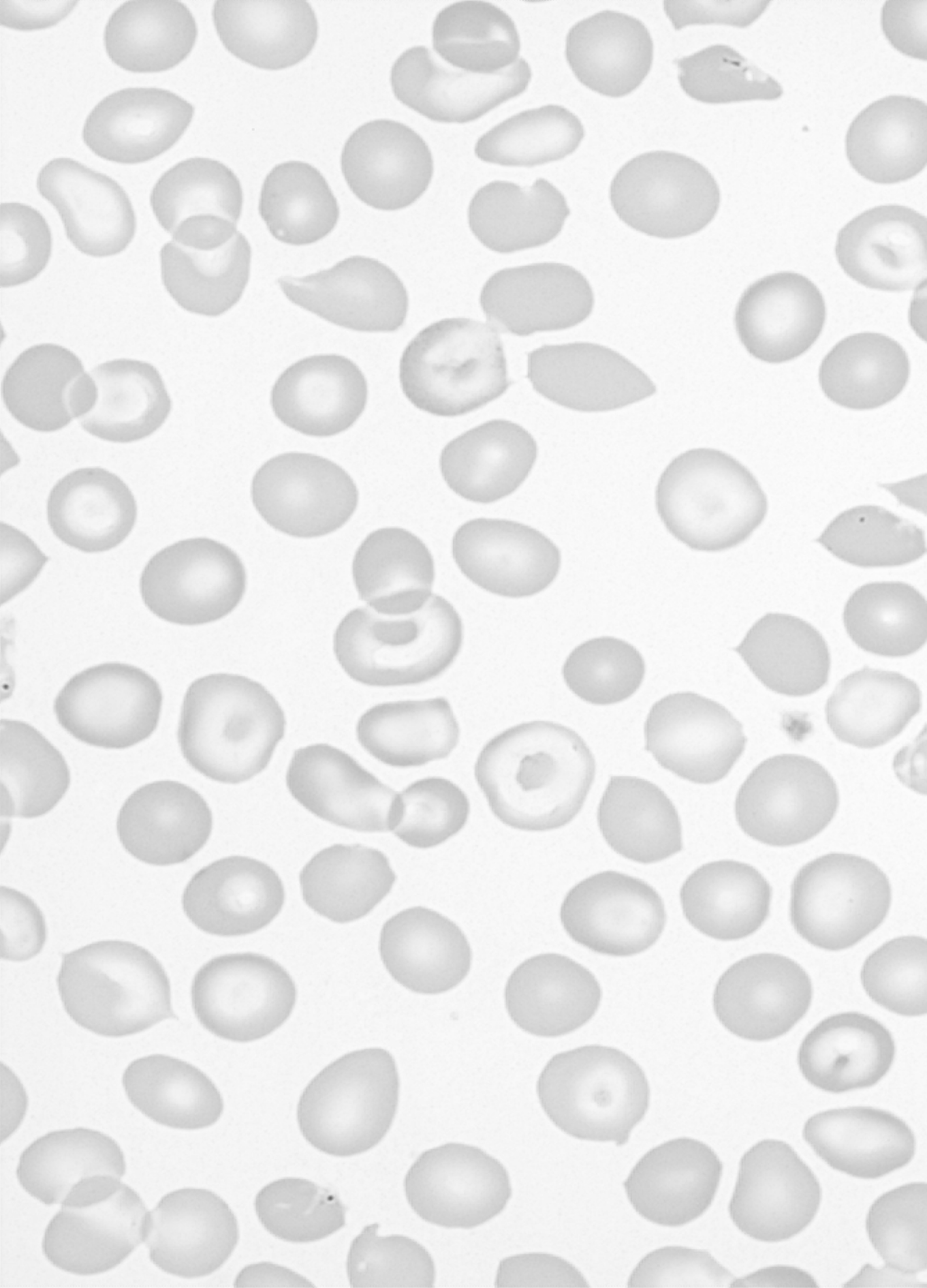


Chapter 1

Introduction

A. Hemoglobin and the hemoglobinopathies

Hemoglobin

Mature red blood cells (RBC), or erythrocytes, are nucleus-free cells with a life span of approximately 120 days. In postnatal life, RBCs are produced in the bone marrow from multipotent stem cells under the influence of different hematopoietic cytokines. They reside in the peripheral blood circulation as membrane-surrounded flexible bags of protein, of which approximately 70% is accounted for by hemoglobin. Hemoglobin (Hb) is a tetramer with a molecular weight of 64,450 Dalton. It is responsible for transporting oxygen from the lungs to the tissues and carbon dioxide from the tissues to the lungs.

The hemoglobin molecule is composed of two alpha and two non-alpha globin chains (Figure 1), which form a stable structure by strong hydrogen bonding. Each chain contains a heme group, consisting of a protoporphyrin IX ring and an iron atom (Fe^{2+}) occupying the center of the ring, which is able to bind oxygen. A shift of 15° between the dimers allows a gradual oxygenation or de-oxygenation of the hemoglobin molecule, depending on the oxygen tension of the surrounding area (1).

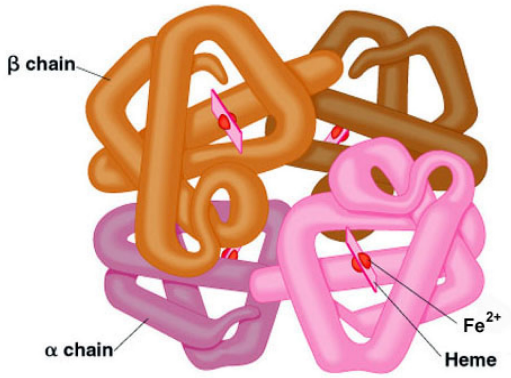


Figure 1 Schematic representation of the hemoglobin molecule. The protein consists of 4 subunits, each containing a heme group with an iron atom (Adapted from Michael W. King, PhD, themedicalbiochemistrypage.org, LLC).

Evolution of the globin gene clusters

Hemoglobin is one of the best studied proteins. Similar forms of this basic structure are found in bacteria, fungi, plants and animals, serving physiological roles ranging from oxygen transport in the blood of vertebrates to catalyzing the combination of oxygen and nitric oxide to form nitrate in bacteria, yeast and worms. The structural features of the globin genes are highly conserved across species. For these reasons, hemoglobins and the genes encoding them have been an important system for investigating many biochemical and evolutionary issues (2-4).

The very first globin-like molecule, a primordial oxygen carrier, existed approximately four billion years ago, at the time when life originated on Earth. A proto-myoglobin gene arose from this protein by different mutations and selection mechanisms of evolution. Approximately 500 million years ago, this proto-myoglobin differentiated into hemoglobin and myoglobin, which are responsible for the oxygen transport and storage in muscle tissue. About 100 million years later, the ancestral globin gene started to evolve through duplications, mutations, deletions, gene conversions and transpositions events into the current globin gene clusters (5;6).

Genetics of human hemoglobin

The genes that code for the different hemoglobin chains are located in clusters, with the β-globin gene cluster on the short arm of chromosome 11 and the α-globin gene cluster on the tip of the short arm of chromosome 16. In addition to the α- and β-globin genes, both clusters contain several other globin genes which are expressed at different stages of development in a tissue specific manner. Both clusters contain pseudogenes (ψ), which are remnants of once functioning genes that have undergone mutations rendering them no longer capable of producing proteins. The θ-gene in the β-globin gene cluster is being expressed, but its function is still unknown. All genes are transcribed in the same direction; towards the centromere, in order of expression during development (1) (Figure 2).

In the first weeks of embryonic development, hemoglobin synthesis starts in the yolk sac. At this time, only the embryonic ζ- and ε-globin genes are expressed producing Hb Gower I ($\zeta_2\varepsilon_2$). Shortly thereafter, the α- and γ-globin genes are expressed, giving rise to the embryonic hemoglobins Gower II ($\alpha_2\varepsilon_2$) and Portland ($\zeta_2\gamma_2$). After 8 to 10 weeks, the site of hemoglobin synthesis changes from the yolk sac to the fetal liver and spleen. The ε- and ζ-globin chains are gradually replaced by the α- and γ-globin chains, which give rise to the fetal hemoglobin HbF ($\alpha_2\gamma_2$). HbF has a somewhat higher oxygen affinity than adult hemoglobin and is thus capable of extracting oxygen more efficiently across the placenta from the maternal circulation. Shortly before birth, a gradual switch from γ-globin expression to β-globin expression occurs. The adult hemoglobin HbA ($\alpha_2\beta_2$) starts replacing the HbF a few months before birth, a process which is almost completed 6 months after birth. At this time, the bone marrow is the major site of hematopoiesis and hemoglobin synthesis. Another adult hemoglobin, HbA_2, consists of two α-chains and two δ-chains. The expression of the δ-globin gene occurs at approximately the same time as the β-globin gene. The δ-globin gene has acquired a number of alterations in its promoter, particularly in the CCAAT-box region, which makes transcription relatively inefficient. A healthy adult individual has approximately 97% HbA, 2.5% HbA_2 and 0.5% HbF (1) (Figure 3).

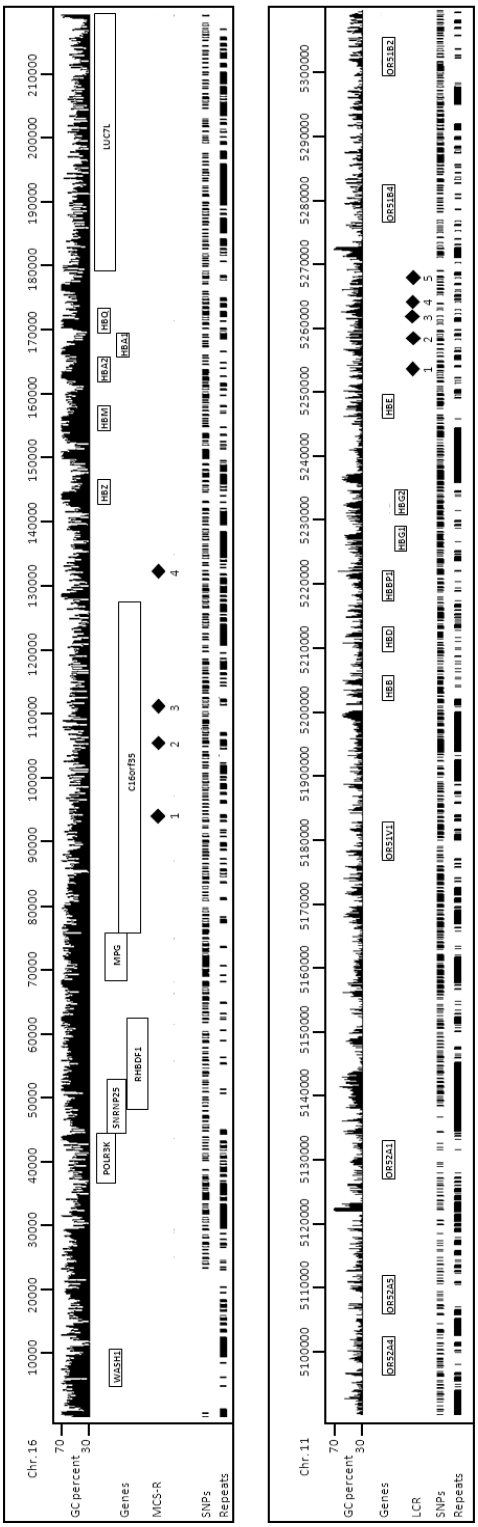
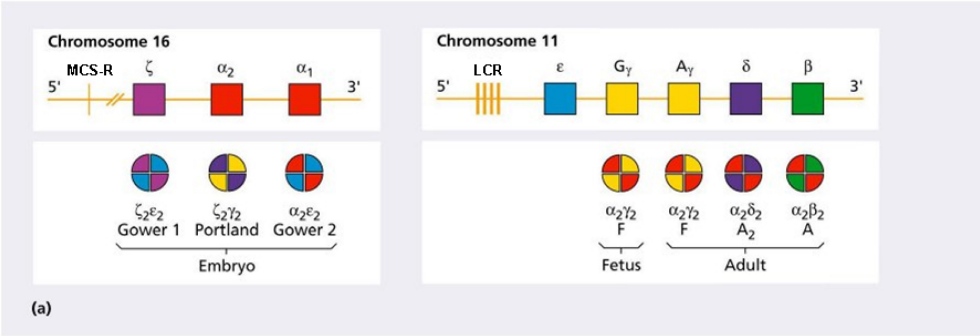


Figure 2 Overview of the α- (top panel) and β-globin gene cluster (bottom panel) and surrounding region. Locations along chromosomes 16 and 11, respectively, are indicated at the top of each panel and are according to the UCSC Genome Browser 2006 (hg18). GC-percentage differs between the globin gene clusters; the α-globin gene cluster is GC-rich, whereas the β-globin gene cluster is AT-rich. The area surrounding the α-globin gene cluster contains many other genes with various functions. A few genes surround the β-globin gene cluster, they all belong to the family of olfactory receptor genes. Both clusters contain regulatory elements which are responsible for correct expression of the globin genes. The α-globin gene cluster contains 4 multi-species conserved sequence regions (MCS-R 1-4). The locus control region (LCR) of the β-globin gene cluster consists of 5 hypersensitive sites. The majority of the repetitive sequences in the α-globin gene cluster belongs to the Alu-family, while the β-globin cluster mainly contains LINE-repetitive sequences (Adapted from UCSC Genome Browser, http://genome.ucsc.edu).



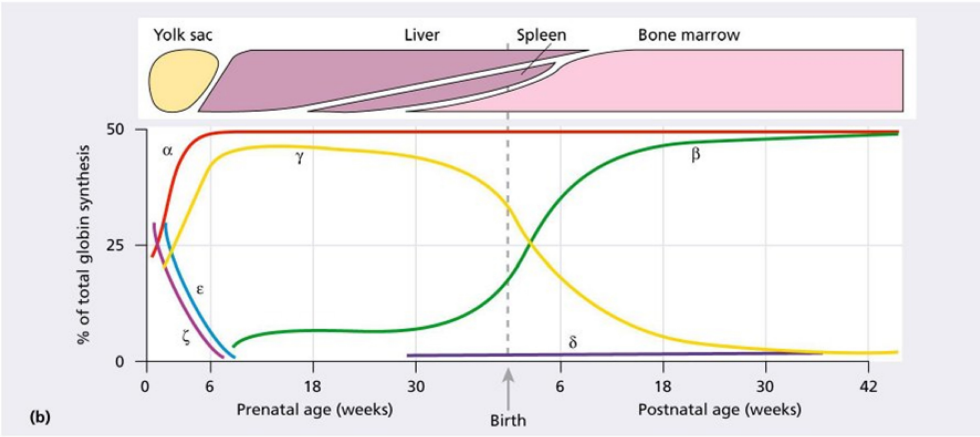


Figure 3 (a) The globin gene clusters on chromosomes 16 and 11. In embryonic, fetal and adult life different genes are activated or suppressed. The different globin chains are synthesized independently and then combined with each other to produce the different hemoglobins. LCR, locus control region, MCS-R, multi-species conserved sequence region. **(b)** Synthesis of individual globin chains in prenatal and postnatal life (From: *Essential Haematology*, 6th Edn. © A.V. Hoffbrand & P.A.H. Moss, Blackwell Publishing Ltd 2011).

Structure of the globin genes

The globin genes vary in size between 1 and 2 kb and they all consist of three coding regions (exons) and two non-coding regions (intervening sequences (IVS) or introns). Mutations in the coding regions of the globin gene result in the synthesis of abnormal hemoglobins. The introns are transcribed into precursor mRNA molecules, but they are subsequently excised (splicing) and the ends of the coding sequences joined to yield the mature mRNA. Presence of specific nucleotide sequences at the junctions between exons and introns is required for proper splicing of the introns. These consensus sequences are almost universally found at the 5' and 3' end of the introns and are called donor and acceptor sites, respectively. Mutations that either alter or create consensus sequences can lead to abnormal globin mRNA precursors and thus abnormal hemoglobins.

All globin genes are preceded by a promoter, a region of DNA that facilitates transcription by interaction with the upstream locus control region (LCR). The promoter sequence consists of a transcription start site (TSS), a binding site for RNA polymerase and binding sites for general transcription factors. A polyadenylation site is located at the 3' end of each gene (Figure 4). The function of this sequence is to add multiple adenosine monophosphates (poly-A tail) to an mRNA molecule, which is important for nuclear export, translation and stability of mRNA. Mutations in the LCR, promoter or polyadenylation site might inhibit proper transcription of the globin gene, leading to a thalassemia phenotype.

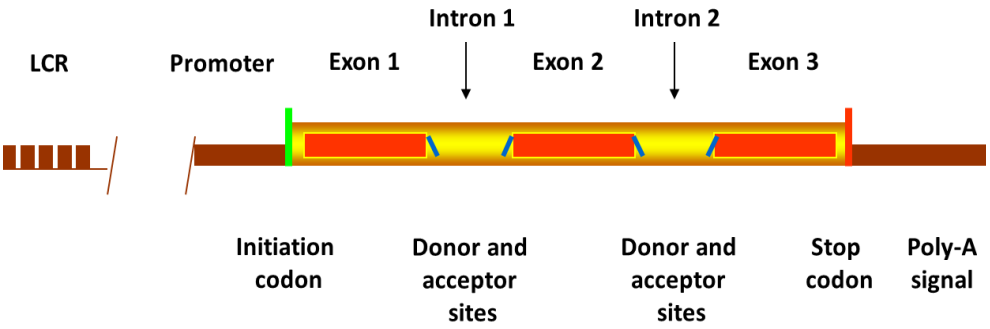


Figure 4 Schematic overview of the globin gene structure (courtesy of P.C. Giordano).

Hemoglobinopathies and the public health problem

Hemoglobinopathies are hereditary blood disorders caused by mutations affecting the globin genes. Mutations may cause changes in the globin chain structure leading to abnormal hemoglobins (Hb variants), while mutations affecting the expression of the genes result in thalassemias (α or β, according to the gene affected). The hemoglobinopathies represent a major public health problem particularly in the Mediterranean area, the Middle East, India, Southeast Asia and Africa. Due to migration, however, hemoglobinopathies are also becoming a public health problem in non-endemic immigration countries, including northern Europe (Figure 5).

Globally, the inherited disorders of hemoglobin are by far the most common recessive diseases, partially as a consequence of selective advantage for carriers of these disorders against malaria. Other factors that play a role in the spreading of these traits and in the high incidence of the diseases include the widespread practice of consanguineous marriage, founder effects, increased maternal age and genetic drift (7).

It is estimated that approximately 7% of the world population is a healthy carrier of a hemoglobinopathy, resulting in ~350,000 severely affected newborns each year, mainly in the emerging countries with poor health care and prevention programs. However, reviews of these data suggest a possible underestimation, as a lack of adequate population data is one of the major problems in assessing the global health burden posed by these diseases (8-10).

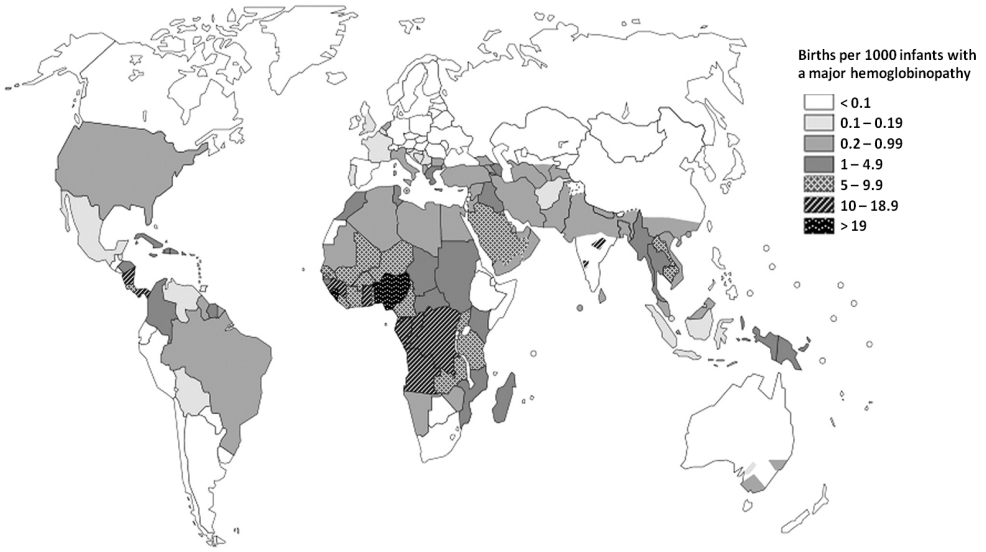


Figure 5 Global incidence of hemoglobin disorders (adapted from the World Health Organization, http://www.who.int)

To date, the improvements of hygiene, nutrition and the control of infectious diseases in developing countries have significantly reduced the childhood mortality rate. As a result, babies born with a severe hemoglobin disorder survive long enough to present for diagnosis and treatment, which will increase the effect of hemoglobinopathies on the burden of health care in these countries. Therefore, it is important that international healthcare agencies and authorities of countries where the hemoglobin disorders occur at a high frequency become aware of the future extent of this problem and develop programs for their control and management (8).

Nowadays, national programs to identify carriers of hemoglobinopathy have been implemented in many endemic countries to facilitate primary prevention, which is based on information, carrier screening and counseling (11-16). Carrier screening can take place at several stages, including the premarital or preconceptional phase, during early pregnancy and after newborn screening (17-19). Couples at risk can be offered an informed reproductive choice, including termination of an affected pregnancy after prenatal diagnosis. Since 2007, sickle cell disease has been included in the national newborn screening program in the Netherlands. However, a better preventative strategy is urgently needed to detect carriers before pregnancy or at the first pregnancy visit, as is commonly done in other countries (20). Therefore, a study has been performed to examine the feasibility of standardized hemoglobinopathy carrier testing for pregnant women in the Netherlands. This study showed that the prevalence of hemoglobinopathy carriers is high enough in the Dutch population to warrant testing for the entire multiethnic population during early pregnancy. Such an intervention could be easily performed at the national level when included in the ongoing early pregnancy screening (PSIE) for Rhesus antagonism and infectious diseases (21;22).

Abnormal hemoglobins and sickle cell disease

Abnormal hemoglobins arise due to mutations within the coding region of one of the globin genes. The majority of the abnormal hemoglobins is the result of point mutations causing one or more amino acid substitutions. Other abnormal hemoglobins arise due to small insertions or deletions or a mutation in the stop codon, leading to an extension of the protein (Hb Constant-Spring, Hb Koya Dora, Hb Icaria, Hb Seal Rock and Hb Paksé). In addition, unequal crossing over can give rise to fusion proteins like Hb Lepore, Hb anti-Lepore, Hb Kenya or Hb anti-Kenya (Figure 6).

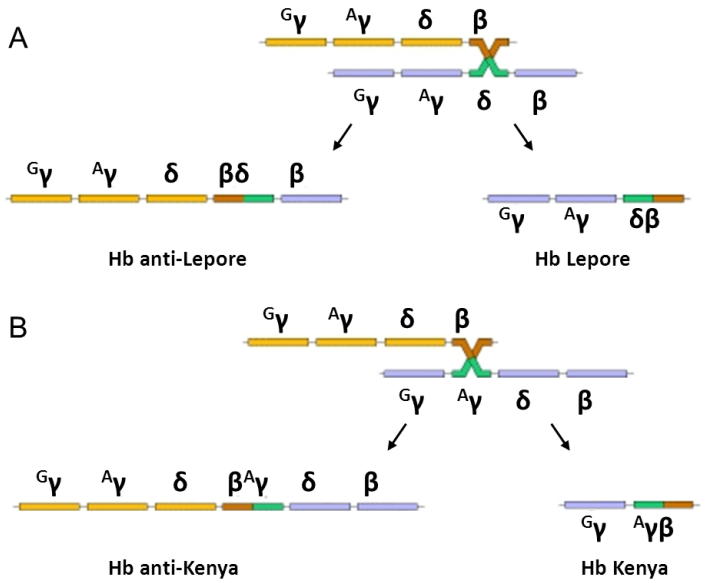


Figure 6 Crossover mechanisms for hemoglobins Lepore and Kenya. Misalignment of the two alleles results in hybrid genes. **(A)** A δβ-hybrid gene is present in Hb Lepore. The normal δ- and β- globin genes are deleted. The Hb anti-Lepore allele consists of a βδ-hybrid gene located between the normal δ- and β-genes. **(B)** In the case of Hb Kenya, recombination occurs between the β- and $^{A}\gamma$-globin genes, resulting in a $^{A}\gamma\beta$-hybrid gene. The normal $^{A}\gamma$-, δ- and β-globin genes are deleted on this allele. Hb anti-Kenya contains two δ-globin genes, with a $\beta^{A}\gamma$-hybrid gene in between (From: "Recent Developments in Molecular Genetics of Human Hemoglobin," Cell 16, 1979, D. J. Weatherall and J. B. Clegg).

Until now, more than 1100 hemoglobin variants have been described and reported in HbVar, which is a locus specific database developed in 2001 to provide information on the genomic sequence changes leading to Hb variants, thalassemias and hemoglobinopathies (23-25). Most Hb variants, unless associated with molecular instability, are recessive and only a relatively small number of these abnormal hemoglobins can cause severe diseases in homozygous or compound heterozygous combinations. The most frequently occurring mutation is associated with sickle cell disease (SCD). The main mutation responsible for SCD arises from an A>T transition at position 20 in the β-globin gene, which results in a Glu>Val amino acid substitution

in codon 6 (HBB:c.20A>T, p.Glu7Val), leading to the HbS variant. It is known that carriers of HbS are protected against malaria tropica, caused by the parasite *Plasmodium falciparum*. Every year about 225 million people, mostly children, get infected with malaria and about 800,000 die from the consequences of this disease (World Malaria Report 2010, WHO). Carriers of HbS have a higher survival rate than non-carriers. Thus, the presence of the trait will lead to higher survival rates and, over time, selection of the trait that is transmitted to their offspring (26).

However, homozygosity for HbS or combined heterozygosity with a β-thalassemia mutation or with one of the frequently occurring variants (HbC, HbE, HbD^{Punjab}, HbO-Arab) and with a few more less frequent variants causes the severe SCD. The valine for glutamic acid replacement results in hemoglobin tetramers that, when deoxygenated, can aggregate into polymers rather than remaining soluble. This will lead to deformation of the erythrocytes, which will become unable to flow through the postcapillary veins. After repeated deoxygenation and reoxygenation, some cells become irreversibly sickled, which will lead to splenic infarctions (Figure 7). Bones are particularly affected in SCD, leading to the frequent and severe pain episodes known as sickle cell 'crises'. Over the long term, internal organs, especially the heart, spleen, lungs, and kidneys, are progressively damaged and hemolysis caused by the destruction of the erythrocytes that contain aggregated hemoglobin contributes to cardiac stress. In addition, the immune system of a sickle cell patient does not function properly because of spleen damage, which makes patients - especially infants and young children - vulnerable to infections with bacteria and viruses. These life-threatening infections, including pneumonia, sepsis, meningitis, osteomyelitis and influenza, are the commonest cause of death in sickle cell disease patients (27;28). Another important fatal consequence is acute chest syndrome, caused by vaso-occlusion of the capillaries in the lungs.

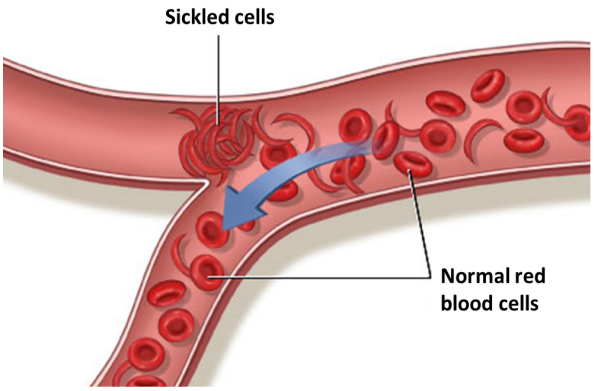


Figure 7 Vaso-occlusion of the microcirculation. When oxygen tension drops, erythrocytes containing hemoglobin S will deform due to aggregation of the HbS molecules. These sickled cells are relatively inflexible, resulting in vaso-occlusion of the postcapillary veins and oxygen deprivation of the downstream tissue (Adapted from Healthwise Inc.).

Beta-thalassemia

In β-thalassemia, the expression of the β-gene on one or both alleles is absent or suppressed, which leads to a deficiency of normal β-globin chains. Depending on the kind of mutation or deletion, β-thalassemia can be classified clinically into three types: minor, intermedia and major. In β-thalassemia minor, one of the alleles is mutated or deleted, resulting in reduced (β^+) or no expression (β^0) of the β-globin gene. Carriers of such a mutated allele present with a mild chronic microcytic hypochromic anemia.

Thalassemia intermedia (TI) arises when the expression of both β-genes is reduced, but one or both of the mutations is relatively mild so that a significant amount of β-globin is still produced. Moreover, the phenotype of thalassemia major patients can be influenced by ameliorating factors such as increased levels of HbF or a coexisting α-thalassemia resulting in TI. In addition, triplication or quadruplication of the α-globin genes in a β-thalassemia minor patient can result in an intermedia phenotype (described below). Beta-thalassemia intermedia patients are variably symptomatic, experiencing anemia, but may not require regular blood transfusion.
In β-thalassemia major, also called Cooley's anemia, no or very little β-globin is produced due to mutations or defects on both alleles. Fetal globin production progressively drops after birth and by 6 months of life symptoms of severe anemia become apparent. The bone marrow becomes hyperactive to compensate for severe hemolytic anemia. However, the majority of the erythrocytes is destroyed before being released into the blood circulation due to β/α globin chain imbalance. If patients do not receive regular blood transfusions, ineffective erythropoiesis expands to all bones leading to fractures and malformation of the long and the flat bones of the face and skull. This causes the typical facies thalassemica with expansion of the trabeculae on the skull (hair-on-end). Due to erythropoietic stress, the liver and spleen become markedly enlarged because of erythrocyte production (extra-medullary erythropoiesis). If not properly treated this condition becomes lethal due to severe anemia, debilitation and infection. In order to survive, patients are lifelong dependent on blood transfusions and iron chelation therapy (1;29;30). The only possibility of cure is a successful hematopoietic stem cell transplantation in young non-compromised patients with an HLA matching donor (31-33).

Genetic modifiers of beta-thalassemia

Although the phenotypes of thalassemia minor, intermedia and major are classic, the clinical manifestations of β-thalassemia can also be extremely diverse, ranging from the almost asymptomatic status to severe anemia and transfusion-dependency for a number of genetic modifiers. The degree of globin chain imbalance and the excess of α-globin chains play the key role in the pathophysiology of β-thalassemia (34;35). Free α-globin chains aggregate in the erythrocyte precursors, precipitate on the cell membrane interacting with band 3 protein which causes hemolysis. Consequently, a coexisting α-thalassemia which reduces the amount of free α-globin chains has an ameliorating effect on the phenotype of a β-thalassemia major patient (36;37). The degree of amelioration is dependent on the severity of the β-thalassemia mutation and the number of functional α-globin genes left (38). A single α-globin gene deletion has very little effect on β^0-thalassemia, whereas patients with a deletion of two α-globin genes and β^+-thalassemia may experience milder anemia. Patients with HbH disease and homozygous β-thalassemia have moderate to severe anemia (39).

Furthermore, an inherent capacity to produce γ-globin chains, which form HbF with the excess of α-globin chains, may also reduce the globin chain imbalance in β-thalassemia. The ameliorating effect of increased levels of HbF is not only due to less excess of α-globin chains, but also to an higher amount of total hemoglobin. Several cis- and trans-acting factors can influence the expression of the γ-globin genes. For instance, the common C>T transition at the polymorphic XmnI-site in the $^{G}\gamma$-gene promoter is associated with increased HbF production (40;41). The expression of the γ-globin genes can also be related to the β-globin gene mutation itself. Mutations or deletions in the promoter region, or larger deletions involving the δ- and

β-globin genes, inhibit interaction of the β-globin gene with the locus control region (LCR, described below). These deletions are associated with the removal of silencer elements in the region between γ- and δ-globin genes or the removal of enhancer elements in the proximity of the γ-globin genes (42;43). This eliminates the competition between the γ-globin genes and the β-globin gene to interact with the LCR, leading to a higher γ-globin gene expression (44). Because of the ameliorating effect of increased HbF level, patients carrying a deletion of the δ- and β-globin genes usually present with a milder phenotype than patients with a point mutation, who have normal (<1%) HbF.

Linkage studies and genome wide association studies have been performed to identify trans-acting factors influencing the γ-globin gene expression. Loci linked to increased HbF production include the HBS1L-MYB intergenic region on chromosome 6q23 (45), the BCL11A gene on chromosome 2p15 (46;47) and the KLF1 gene on chromosome 19p (48). HBS1L and MYB play a role in hematopoiesis, and it has been shown that this region contains multiple regulatory elements (49). However, it is still unknown how variants in the HBS1L-MYB region influence the activity of these regulatory elements, and how this relates to the activity of HBS1L, MYB and eventually the γ-globin genes (50). The BCL11A gene has been shown to serve as a direct transcriptional regulator of the fetal to adult hemoglobin switch in humans (51). BCL11A cooperates with other factors such as GATA-1 and SOX-6 to repress γ-globin gene expression (52). KLF1 encodes a key erythroid transcriptional regulator which plays a crucial role in the controlled events that lead to developmental regulation of the β-globin gene cluster (53). Functional studies suggested that KLF1 is also a key activator of the BCL11A gene (48).

Lastly, the pathophysiology of β-thalassemia can also be influenced by co-inheritance of triplicated or quadruplicated α-globin genes. In this way, the globin chain imbalance is even more tilted, causing β-thalassemia intermedia phenotypes in β-thalassemia heterozygous patients. These patients present with more severe symptoms and may be transfusion dependent at regular intervals. The severity of this condition is determined by the type of β-globin gene mutation (β^+ or β^0) and the number of functional α-globin genes. A triplicated α-globin gene on one allele (ααα/αα) in combination with a β^+-thalassemia leads to a slightly more severe phenotype (54). On the other extreme, a combination of a triplicated α-globin gene on one allele, a duplication of the whole α-globin gene cluster on the other allele (ααα/αααα) and a β^0-thalassemia results in a severe phenotype and transfusion dependency (55).

Hereditary persistence of fetal hemoglobin (HPFH)

Hereditary persistence of fetal hemoglobin (HPFH) is a phenotypic condition characterized by an abnormal high presence of fetal hemoglobin during adulthood. The precise molecular mechanism causing HPFH is still unclear. However, in many cases the condition is associated with large deletions involving the δ- and β-globin genes, sometimes including the ^{G}γ-globin gene as well (described below) and / or extending a considerable distance into the 3'-side. The fetal Hb expression remains high in adult life because of the suppression of a regulatory element 5' to the γ-globin genes which normally switches the expression of these genes off during the first six months after birth. Deletional HPFH alleles that do not have β-globin gene expression, are in fact δβ-thalassemias, phenotypically compensated by the high HbF expression and it is becoming increasingly doubtful whether some of the better defined forms of HPFH should be separated from the δβ-thalassemias (56). For example, the Sicilian-type δβ-thalassemia (57) also

causes higher levels of HbF, but is not classified as a HPFH syndrome.

Another cause of HPFH is an overproduction of γ-globin due to mutations in the promoter region of either the $^{G}\gamma$- or $^{A}\gamma$-globin gene (non-deletion type of HPFH). One hypothesis for the mechanism of these mutations would be that they alter a binding site for an erythroid-specific repressor protein that is normally involved in switching off fetal globin production but which cannot bind as efficient due to the presence of a mutation.

Non-deletional HPFH is a benign condition while deletional defects may result into thalassemia intermedia when combined with β^0-thalassemia defects. Carriers will often remain unaware of their condition because of their normal Hb levels. A conscious GP might decide to investigate their erythrocytosis, caused by the higher O_2 affinity of fetal hemoglobin, and consequent mild chronic hypoxia (58;59).

The εγδβ- and γδβ-thalassemias

A more severe situation may occur when deletion defects involve one or both fetal genes, resulting in a $(\varepsilon\gamma\delta\beta)^0$- or $(\gamma\delta\beta)^0$-thalassemia. The reduced expression of the ε- and γ-genes induces a severe pre- and perinatal anemia in the carrier for which intra-uterine care is required in order to overcome the critical phase. The defects may be associated with complications including hydropic conditions, a low hemoglobin concentration and hemolysis at birth. The severity of the disease in embryonic and fetal life is probably due to the excess of non-bound ζ- and α-chains causing globin chain imbalance and hemolysis in fetal cells. Furthermore, the cells that do not hemolyze contain not enough HbF to provide the necessary oxygen. After birth, when the normal allele has started to transcribe β-globin, the hematological condition of an $(\varepsilon\gamma\delta\beta)^0$-thalassemia carrier is that of a β-thalassemia trait with a normal HbA_2 level (60;61).

At the molecular level the $(\varepsilon\gamma\delta\beta)^0$-thalassemias can be roughly divided into two categories. The first group includes large deletions, which have removed all genes located in the β–globin gene cluster; the second group has been found in association with intact β-globin genes. These deletions silence β-globin gene expression and led to the discovery of the upstream regulatory element or locus control region (βLCR). Several deletions of this kind have been described and three of them were found in Dutch Caucasian families. The Dutch II deletion (62) (causing $(\varepsilon\gamma\delta\beta)^0$-thalassemia) belongs to the group of large deletions, involving the whole β-globin gene cluster, whereas the Dutch I (63;64) leaves the β-gene intact, leading to an $(\varepsilon\gamma\delta)^0$ β-thalassemia. The Dutch III deletion (65) gives rise to an $(\varepsilon\gamma)^0$ δβ-thalassemia, indicating that the δ- and β-genes are both intact. However, no globin is produced from the affected chromosome in all cases, irrespective of the presence of the intact δ- and β-globin genes. An overview of deletions in the β-globin gene cluster is shown in Figure 8. For a list of β-thalassemia causing point mutations is referred to the online database of human hemoglobin variants and thalassemias (http://globin.bx.psu.edu/cgi-bin/hbvar/).

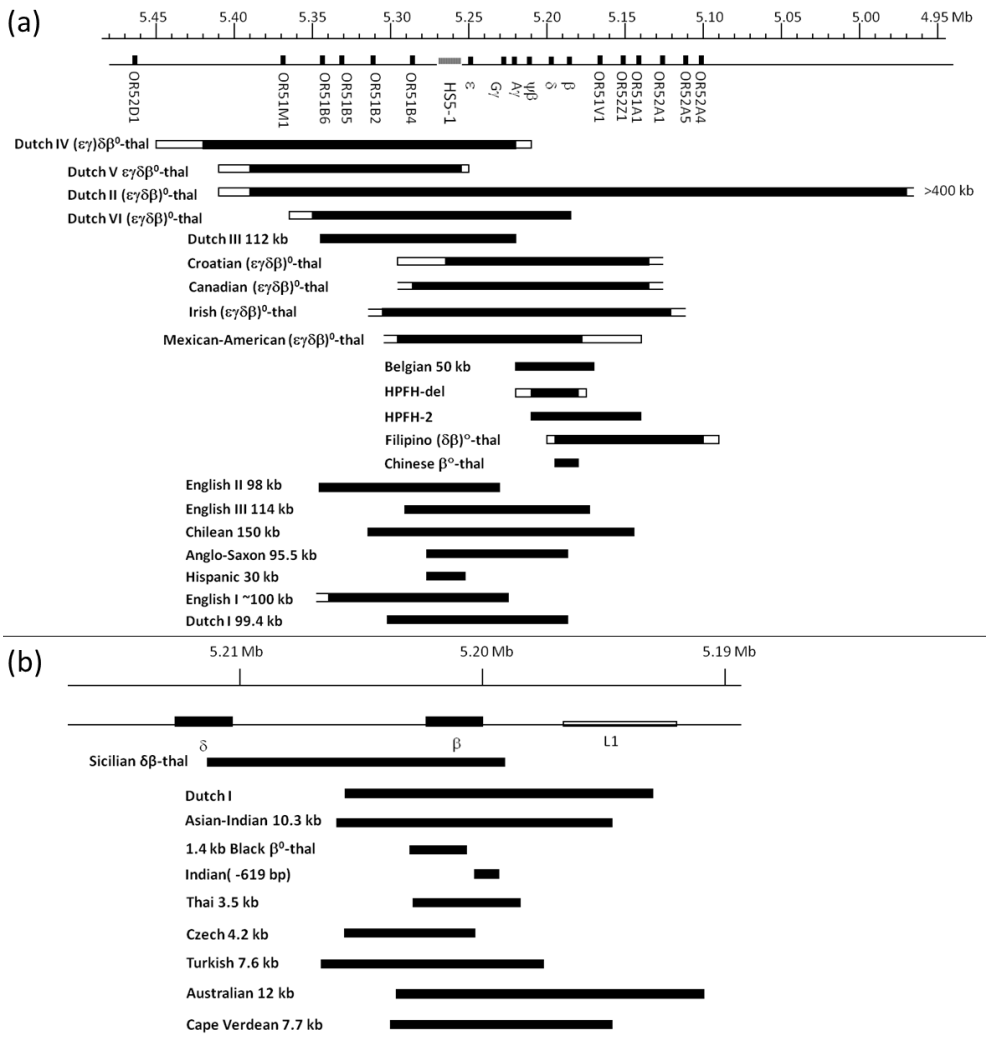


Figure 8 Deletions in the β-globin gene cluster. **(a)** Large deletions extending beyond the β-globin gene. The extent of each deletion is represented by a black bar. The thin bars at both ends of the thick bar denote the regions of uncertainty for the deletion breakpoints. **(b)** Deletions restricted to the β-globin gene. (Adapted from 'Disorders of hemoglobin, Genetics, pathophysiology and clinical management', 2nd edition, edited by M.H. Steinberg, B.G. Forget, D.R. Higgs and D.J. Weatherall, Cambridge University Press 2009)

Alpha-thalassemia

Each chromosome 16 carries two functioning α-genes, leading to a total of four genes in the normal situation. Therefore, individuals with α-thalassemia may have different levels of α-globin production, extending from none at all to very near normal levels. The latter is the case when only one of the α-genes is mutated or deleted (-α/αα). This is the most common condition worldwide, carriers are defined as heterozygous for α^+-thalassemia. They are slightly hypochromic and microcytic but barely anemic and usually asymptomatic and therefore called "silent carriers".

A defect in two of the α-genes (phenotypically defined α-thalassemia traits) can occur in two different ways. In one situation, both α-genes on the same chromosome are defective (--/αα, *in cis*). This condition, defined as heterozygous α^0-thalassemia, is mainly seen in individuals from Southeast Asian origin ($--^{SEA}$), but also in the Mediterranean area ($--^{MedI}$, $--^{MedII}$ and $-(\alpha)^{20.5}$). Alternatively, when only one normal α-gene on each chromosome is expressed (-α/-α, *in trans*), the condition is defined as homozygous α^+-thalassemia. Both homozygous α^+ and heterozygous α^0 phenotypes are relatively benign but associated with mild microcytic hypochromic anemia, and often confused with iron deficiency conditions.

A marked deficiency in α-globin production occurs when three of the α-genes are defective (-α/--). These cases occur in the progeny of healthy carriers of α^0- and α^+-thalassemia defects, mainly caused by deletions or frequent point mutations such as Hb Constant Spring. With only one active α-globin gene, the excess of unbound β-globin chains will form homotetramers (β_4). This non-functional fraction called HbH can be visualized as a fast Hb band or peak on electrophoresis or chromatography, respectively, and as precipitated erythrocytic inclusions after Brilliant Cresyl Blue staining. Depending on the kind of mutation, patients with HbH disease can experience a variety of symptoms, ranging from mild anemia to severe transfusion dependent hemolytic anemia. Due to the complications which may be caused by blood transfusions, the most severe forms of HbH disease can be lethal as a consequence of therapy. In addition, some viral infections, such as parvovirus B19, may be lethal for HbH patients when left untreated. The erythropoiesis, which is already maximized in HbH disease, cannot cope with the coexisting infection, leading to even more severe anemia (66).

When none of the α-genes is functioning (--/--), no fetal hemoglobin is synthesized *in utero* and γ_4 homotetramers will be formed (hemoglobin Bart's). This non-functional hemoglobin binds oxygen with high affinity but cannot release it to the tissues. The only functional embryonic tetramer, Hb Portland ($\zeta_2\gamma_2$), is synthesized in small amounts, which is not sufficient for the fetus to survive. Heart failure results because of the efforts of a poorly oxygenated heart to pump the small amount of dissolved oxygen in the blood to oxygen-starved tissues. As the heart fails, marked edema occurs (hydrops fetalis), and this situation is incompatible with life. Fetuses with a homozygous α^0-thalassemia usually die *in utero*, or, if born alive, die within a few hours after birth. Hydropic fetuses are profoundly anemic, even though the hemoglobin concentration might be relatively high owing to the presence of Hb Bart's and embryonic hemoglobins. The anemia during early development and organogenesis causes many other congenital defects, including a massively enlarged placenta. Common maternal complications, which usually arise in the third trimester of pregnancy, include anemia, hypertension, polyhydramnios, oligohydramnios, hemorrhage and the premature onset of labor. Complications after delivery include retained placenta, hemorrhage, eclampsia, sepsis and anemia. It has been suggested that, without

appropriate obstetric care, up to 50% of women carrying these affected fetuses would die as a result of pregnancy (67). It is therefore important to counsel couples at risk for homozygous α^0-thalassemia, both for prevention of a lethal condition and for the safety of the mother. Hydrops fetalis with Hb Bart's mainly occurs in individuals of Southeast Asian and Mediterranean origin, as this condition mainly arises when the α^0-thalassemia (--/) chromosome is inherited from both parents (1;30). An overview of deletional types of α-thalassemia is given in Figure 9. For a list of α-thalassemia causing point mutations is referred to the online database of human hemoglobin variants and thalassemias (http://globin.bx.psu.edu/cgi-bin/hbvar/).

Alpha-thalassemia and mental retardation

Two types of α-thalassemia are known to be associated with mental retardation, the ATR-X and ATR-16 syndromes. The ATR-X syndrome arises due to a mutation in the XH_2 gene, located on the X-chromosome. This gene encodes X-linked helicase-2, which is a putative regulator of gene expression. Since all the diverse XH_2 mutations are associated with an α^0 -thalassemia phenotype with rare inclusion bodies, it seems likely that this protein is normally necessary for correct regulation of α-gene expression. As the syndrome is X-linked, only males are affected. Obligate female carriers are physically and intellectually normal with cells positive for inclusion bodies at less than 1% of the frequency seen in affected boys, which appears to be the result of a highly skewed pattern of X-inactivation, with the disease-bearing X-chromosome being preferentially inactivated. Affected males show severe mental retardation, characteristic facial dysmorphology and a mild form of HbH disease (1;68;69).

ATR-16 is a continuous gene syndrome and is the result of large deletions on the short arm of chromosome 16 distal to the PKD1-TSC2 genes, including the complete α-globin cluster. Depending on the length of the deletion, the phenotype can vary from α-thalassemia with mild mental retardation to a broad spectrum of dysmorphic features and developmental abnormalities in carriers (68;70). The phenotype is determined by the extent of haplo-insufficiency of the area surrounding the α-globin gene cluster. For example, the tryptase genes appear to play an important role in the pathophysiology of asthma (71) and autosomal recessive epilepsy of infancy has been mapped to a region between markers D16S3024 and D16S423 (~1600 kb from the telomere) (72). Furthermore, cataracts with microphthalmia (CATM) maps to 16p13.3. The break point has not yet been refined, however, the SOLH-gene is a candidate because of its role in eye formation (73;74).

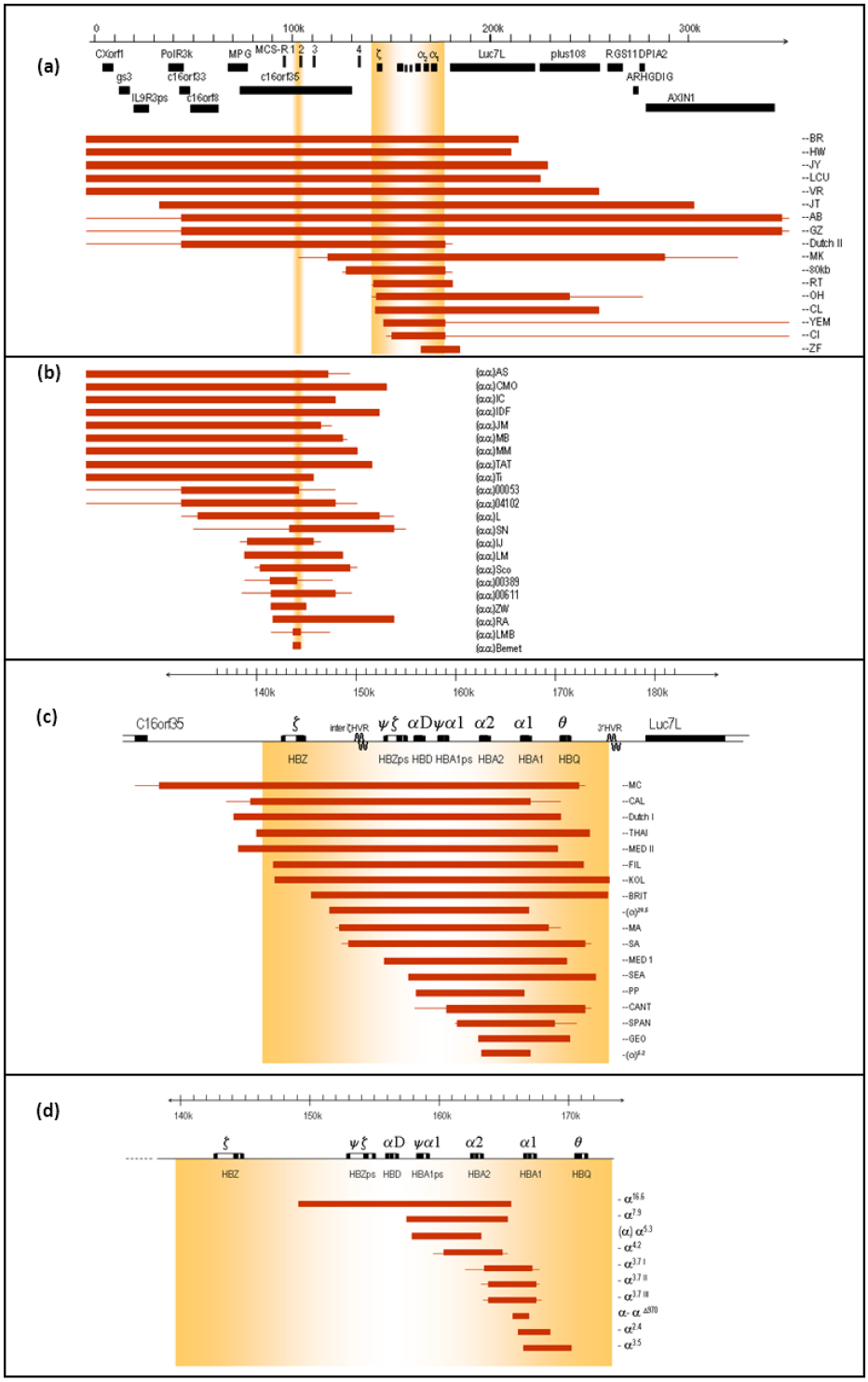
Until now, little is known about the critical genes involved in the deletions causing ATR-16. From a large deletion found in an earlier study it can be concluded that the critical region is located in the area of 960-1060 kb from the telomere. The SOX-8 gene (Sry-related transcription factor gene SOX-8) is located in this region and is involved in the regulation of embryonic development and in stem cell differentiation in mice (75). The encoded protein may act as a transcriptional activator after forming a protein complex with other proteins. This protein may be involved in brain development and function (76;77) and in development of the embryonic heart (78). Therefore, SOX-8 was considered to be a crucial gene in the occurrence of ATR-16 (79). However, DNA analysis in several members of a Brazilian family without mental retardation or dysmorphic features showed a deletion of the tip of chromosome 16p including the SOX-8 gene, which makes SOX-8 less likely as a candidate gene. More deletions should be studied to determine the effect of haploinsufficiency of other genes located on chromosome 16p13.3 (80).

Acquired α-thalassemia

In addition to the genetic cause of hemoglobinopathies, abnormalities of hemoglobin synthesis may also arise as a secondary manifestation of another disease. Acquired α-thalassemia is the best characterized of the acquired red blood cell disorders in patients with hematologic malignancy. The hematologic consequences in these patients may vary from minor changes in levels of HbF or HbA_2 (81-83) to an abnormal pattern of hemoglobin synthesis, which is often seen in patients with juvenile chronic myelocytic leukemia (84;85).

The majority of patients with acquired α-thalassemia have myelodysplastic syndrome and this condition is, therefore, referred to as 'alpha-thalassemia myelodysplastic syndrome' (ATMDS) (86). However, acquired α-thalassemia is not unique to MDS, it is also reported to be associated with acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) and myelofibrosis (87;88).

An acquired deletion of the telomeric region of chromosome 16p was found in one ATMDS patient with a complex karyotype. This deletion was limited to the neoplastic clone and leads to an acquired α-thalassemia genotype in these cells (89). In all other ATMDS patients described until now, no molecular defect in the α-globin genes was found, suggesting that the α-thalassemia phenotype is caused by a trans-acting mutation. In the myeloid cells of some of these patients, point mutations or splicing abnormalities were found in the ATRX-gene, located at chromosome Xq13.3 (90;91). Because inherited and acquired mutations in ATRX cause α-thalassemia, it is suggested that this gene plays a role in α-globin gene expression. However, the mechanism by which ATRX controls gene expression is still unclear. In a small number of ATMDS patients, the underlying molecular defect remains unexplained. They might be carriers of ATRX mutations which remain undetected by the current mutation detection techniques, or mutations in other genes than ATRX might be responsible for the α-thalassemia phenotype (88).


(a)
0
100k
200k
300k
CXorf1
PolR2k
MPG
MCS-R1
2
3
4
ζ
α2 α1
Luc7L
plus108
RGS11
DPIA2
gs3
C16orf33
c16orf35
ARHGDIG
IL9R3ps
C16orf8
AXIN1
--BR
--HW
--JY
--LCU
--VR
--JT
--AB
--GZ
--Dutch II
--MK
--80kb
--RT
--OH
--CL
--YEM
--CI
--ZF
(b)
(α.α.)AS
(α.α.)CMO
(α.α.)IC
(α.α.)IDF
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(α.α.)MB
(α.α.)MM
(α.α.)TAT
(α.α.)Ti
(α.α.)00053
(α.α.)04102
(α.α.)L
(α.α.)SN
(α.α.)IJ
(α.α.)LM
(α.α.)Sco
(α.α.)00389
(α.α.)00611
(α.α.)ZW
(α.α.)RA
(α.α.)LMB
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140k
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C16orf35
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ψζ αDψα1 α2 α1 θ
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HBZps HBD HBA1ps HBA2 HBA1 HBQ
--MC
--CAL
--Dutch I
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--MED II
--FIL
--KOL
--BRIT
--MA
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HBZ
HBZps HBD HBA1ps HBA2 HBA1 HBQ

Figure 9 Deletions in the α-globin gene cluster. **(a)** Rare, large deletions extending beyond the α-globin gene cluster that cause α-thalassemia but no other associated anomalies. The extent of each deletion is represented by a red bar. The thin bars at both ends of the thick bar denote the regions of uncertainty for the deletion breakpoints. To the right are the shorthand notations for each deletion. **(b)** Deletions that cause α-thalassemia but leaving the α-globin genes intact. **(c)** Deletions that cause α^0-thalassemia with breakpoints lying within the α-globin gene cluster. **(d)** Deletions that cause α^+-thalassemia. (Adapted from 'Disorders of hemoglobin, Genetics, pathophysiology and clinical management', 2nd edition, edited by M.H. Steinberg, B.G. Forget, D.R. Higgs and D.J. Weatherall, Cambridge University Press 2009)

Locus control regions

The α- and β-globin gene clusters are subject to several levels of regulation. They are expressed exclusively in the erythrocytes in a copy number-dependent manner and only during defined periods of development, which is perfectly programmed. Such control is dependent on regulatory regions of DNA located either in the proximity or at great distances from the globin genes. These regions are characterized by the presence of several DNAse I hypersensitive sites (HS) and known as the locus control region (LCR). The most prominent property of the LCRs is their strong transcription-enhancing activity. In addition, LCRs alter the chromatin configuration to modify the histones in the promoter region of the globin gene, leading to an open chromatin structure which is accessible for transcription factors such as GATA-1, EKLF and NF-E2 (92-94).

When a mutation is present in the LCR or when (part of) the LCR is deleted, the LCR cannot direct the correct expression of the globin genes. Patients carrying such a mutation or deletion present with thalassemia, although the globin genes are present and intact. Therefore, it is important to consider this type of mutations when no molecular defect is found in any of the globin genes in a thalassemia patient.

The β-globin LCR is located 6-20 kb upstream of the β-gene. It consists of five hypersensitive sites (HS1-5) which are necessary for a correct transcription of the β-globin gene cluster (95). The locus control region for the α-globin gene cluster consists of at least five hypersensitive sites (HS-48, -40, -33, -10 and -8), which are located in a multispecies conserved, noncoding regulatory sequence (MCS-R) 48, 40, 33, 10 and 8 kb upstream of the ζ-gene. Several studies indicate that the HS-40, or MCS-R2, is the most conserved element and is therefore considered as the major regulatory element. In addition, the HS-33, -10 and -8 cannot drive substantial levels of α-globin expression on their own (96-99). However, a patient with two deletions of variable extent of both regulatory regions has been described recently. Both deletions involve MCS-R2 and leave all four α-globin genes intact. This patient presented with HbH disease, which is less severe than expected from the predicted reduction of α-globin chain expression. From this study, it was concluded that loss of MCS-R2 on both alleles strongly down-regulates the expression of the α-globin genes, but is not associated with a complete absence of α-chain production (100).

B. Postnatal diagnosis of hemoglobinopathies

Hemoglobinopathies are possibly unique amongst all genetic disorders in that identification of carriers is possible by hematological and biochemical tests. Any at risk couple can then be offered reproductive choice and avoid the birth of an affected child by undergoing prenatal diagnosis, which involves mutation characterization and subsequent fetal DNA analysis. Therefore, genetic services for hemoglobinopathies require close collaboration between several specialties, most notably hematology and molecular genetics (101).

The first step in the diagnostic workflow for hemoglobinopathy diagnosis is to determine several hematological parameters, such as hemoglobin level (Hb), erythrocyte count (RBC), packed cell volume (PCV), mean cell volume (MCV) and mean corpuscular hemoglobin (MCH). Thalassemia carriers often present with mild microcytic hypochromic anemia and normal iron levels. Because of the phenotypic resemblance between thalassemia carriers and anemia due to iron deficiency, thalassemia patients are often unnecessarily treated with iron suppletion before they are referred for further diagnostics. Therefore, ferritin levels and erythrocyte count are important indicators in differential diagnosis. Ferritin level is normal or slightly increased in thalassemia carriers, and the number of erythrocytes is often increased under normal folic acid conditions due to the erythropoietic compensation for the chronic anemia (102).

Subsequently, biochemical analysis is performed by separating the different hemoglobins. This can be done by traditional electrophoresis or iso- electric focusing followed by manual estimation or using techniques such as high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). Normal Hb fractions and separable and stable hemoglobin variants can be visualized and measured, as well as elevated levels of hemoglobin A_2 and F, which are diagnostic parameters for β- or $\delta\beta$-thalassemia (102).

Carriers of hemoglobin S (HbS) are usually not anemic and will therefore present with normal hematological parameters, unless they carry a coexisting α-thalassemia. The HbS fraction is detected by HPLC and CE and will be visualized as an extra peak on the chromatogram. To confirm presence of HbS, a sickle cell test can be performed. In this test, a blood sample is mixed with a sodium-bisulphite solution and incubated on a microscope slide under a well-sealed cover glass. The sodium-bisulphite lowers the oxygen concentration and triggers polymerization of the HbS molecules. These polymers change the shape of the erythrocyte into the typical sickled form, which can be visualized under a microscope (103).

For a complete hemoglobinopathy diagnosis, molecular research has to be performed to confirm the hematological and biochemical results. In addition, molecular diagnosis is essential to be able to offer an informed reproductive choice to a couple at risk during counseling. It is required to know the exact molecular defect of both parents to be able to calculate the risk of having a severely affected child, as well as to be able to perform prenatal diagnosis. Several molecular techniques can be applied to come to a final diagnosis.

Gap-PCR

Approximately 80% of all α-thalassemias is caused by a large deletion in the α-globin gene cluster involving one (α^+) or both (α^0) α-globin genes. The gap-PCR method is used to detect the seven most common α-thalassemia causing deletions ($-\alpha^{3.7}$, $-\alpha^{4.2}$, $--^{SEA}$, $--^{MedI}$, $-(\alpha)^{20.5}$, $--^{FIL}$ and $--^{THAI}$) (104). In addition, the method is also suitable for several deletions causing

β-thalassemia (105). Specific oligonucleotide primers on both sides of the deletion are used to amplify the area across the deletion breakpoint revealing the size-specific fragments. To prevent false negative results, an additional primer close to one of the breakpoint primers is used to generate a normal control fragment. The gap-PCR is performed in presence of positive controls for each of the deletions, to be able to identify the deletion directly when fragments are identified as abnormal bands separated by agarose gel electrophoresis.

This method is relatively quick and easy to perform. In contrast to the Southern blotting method, little input DNA is needed and no radioactivity is used (106). Necessary equipment includes a thermal cycler and an agarose gel electrophoresis facility, which are available in most molecular laboratories in countries where hemoglobinopathies frequently occur. Although this method is suitable to detect the majority of the deletional α-thalassemias, uncharacterized rearrangements remain undetected using gap-PCR. Therefore, other methods to detect copy number variations (CNVs) have recently been investigated. These methods, including MLPA and aCGH (described below) are particularly useful in countries where a wide variety of deletion types is present and can be used to characterize deletions which may be common in other countries to develop gap-PCR assays.

In the scope of this thesis, a single-tube multiplex gap-PCR has been developed for rapid detection of eight different deletions in the β-globin gene cluster which commonly occur in the Southeast Asian population (*Chapter 2.1*). This PCR assay provides a fast, simple and cheap diagnostic test for deletions in the β-globin gene cluster which can be applied in every molecular diagnostic laboratory having standard PCR equipment.

Direct sequence analysis

The remaining 20% of the α-thalassemias and the majority of the β-thalassemias (~90%) is caused by point mutations in one of the globin genes. Mutations can be identified by applying direct sequencing for the whole globin gene, including the promoter, all introns, exons and the polyadenylation site. Sequencing of the entire globin genes is relatively easy when compared to other genes, because the globin genes are rather small. Two oligoprimer sets are used to amplify both α-globin genes separately. Subsequently, a second PCR with internal primers is performed to generate smaller fragments which are suitable to use for direct sequencing. This approach is necessary because of the high homology between both α-globin genes. For the β-globin gene, four different fragments are amplified which cover the whole gene. The direct sequencing method is able to detect all types of point mutations including small deletions and insertions and is therefore the method of choice for diagnosis of non-deletional types of α- and β-thalassemia (101).

Multiplex Ligation-dependent Probe Amplification

Traditional methods to detect CNVs with uncharacterized breakpoints include techniques such as Southern blotting and fluorescent in-situ hybridization (FISH) (107). However, Southern blot analysis is time consuming, technically demanding and success is very much dependent upon the hybridization probes available. The same is true for FISH analysis, which in addition involves laborious cell culturing to generate metaphase chromosome spreads. Furthermore, the resolution of FISH is low (>2 Mb).

Multiplex Ligation-dependent Probe Amplification (MLPA), is a relatively new technique

used to detect uncommon or unknown copy numbers of genomic sequences. This method is extremely sensitive and it requires only 20 ng of DNA. In MLPA, two oligonucleotide probes hybridize adjacently to a target sequence which is followed by a ligation reaction, which increases specificity. The target sequences are amplified in a multiplex PCR reaction with the use of only one pair of primers, which are complementary to universal 3'- and 5'-tags attached to each probe pair. This eliminates competition between primers and allows a quantitative PCR for all probes in the same reaction. The PCR products, previously chosen in increasing size, can be separated by their unique length using capillary electrophoresis, which allows up to 20 oligonucleotide probe pairs to be used simultaneously in a single reaction. The total amplicon length of oligonucleotide probes ranges from 80 to 130 nucleotides. As three differently labelled universal primer pairs can be used simultaneously, it is possible to use up to 60 probe pairs in a single reaction. The copy number of the target sequences is reflected in the relative intensities of the amplification products visualized by capillary electrophoresis (108-110) (Figure 10).

Many MLPA kits containing cosmid probes have already been designed, for instance for the detection of aneuploidy of chromosomes 13, 18, 21, X and Y (111). This technique has also been shown useful for the screening of large numbers of HNPCC patients (112). Furthermore, several rare mutations in the breast cancer gene in Dutch women have been discovered with the use of MLPA (113). These kits use probes consisting of two oligonucleotides, one synthetic and one derived from a M13 cloned cosmid. These latter probes contain the 25-43 nt target-specific sequence at the 5' phosphorylated end, a 36 nt sequence that contains the complement of the universal PCR primer, and a stuffer sequence of variable length in between. The length of the amplicons in a probeset containing cosmid probes varies from 130 to 410 nucleotides.

However, generating these cloned cosmid probes is laborious, time-consuming and therefore quite expensive. It has been shown that it is possible to perform an MLPA reaction with the use of two synthetic oligonucleotides in the detection of genomic rearrangements in hereditary multiple exostoses (114), for detection of duplications and deletions in the DMD gene (115) and for detecting copy number variation in Williams-Beuren patients (116).

After applying the standard diagnostic methods for hemoglobinopathies, a small group of patients remains with unknown molecular defects. To be able to unravel the cause of the thalassemia phenotype in this group of patients, development of new assays was required. In the scope of this thesis, an MLPA-based technique for detection of deletions and duplications in the globin gene clusters has been designed (*Chapter 2.2*). It was shown that all common, rare and novel forms of deletional thalassemia can be picked up by MLPA. Therefore, MLPA has now replaced the classical method of Southern blot analysis. *Chapter 2.3* describes the application of the MLPA technique in a cohort of Australian patients with a thalassemic phenotype, which remained unsolved after applying the standard diagnostic methods. This study showed that MLPA is a helpful method in routine diagnostics, especially in populations in which a large variety of deletion mutations occur. Furthermore, several rearrangements that were not described previously were detected during this study.

Array comparative genomic hybridization

As mentioned above, MLPA is a very useful technique in hemoglobinopathy diagnostics after point mutations and known deletions have been excluded by applying sequence and gap-PCR analysis. However, the exact breakpoints of deletions defined by MPLA will remain unknown because the distance between the MLPA probes varies from a few hundred base pairs up to >10 kb. Although not essential for demonstrating the presence of a deletion defect, knowledge of the exact breakpoint is important from a scientific point of view to unravel the mechanisms leading to these deletions. In addition, characterizing the breakpoints allows the design of a gap-PCR method specific for the new mutation. This can be of use for molecular diagnostics as an alternative to MLPA in populations where specific deletions occur at a significant frequency or for specific mutation detection in families.

The standard method to characterize such rearrangements is to randomly design primers in the breakpoint region and perform PCR across the breakpoint. The breakpoint region is determined by the most proximal and most distal MLPA probe still present and the first probe involved in the deletion. The distance between MLPA probes generally range between 3-10 kb which requires the use of long-range PCR and primer walking to sequence the breakpoint completely (117). Since this procedure is breakpoint specific and rather time consuming, other methods are needed to improve the resolution of the breakpoint position and to develop primers more closely located toward the breakpoint. In addition, it is necessary to develop techniques for quick and precise detection of deletions for diagnostic purposes.

The array comparative genomic hybridization (aCGH) technology (Figure 11) is a promising method in characterizing copy number variations. The development of aCGH in the past few years has facilitated the identification of the molecular basis of many genetic diseases (118-124). Originally, aCGH was developed as a research tool for the investigation of genomic imbalances in cancer (125) and has become an essential and routine diagnostic tool in genetics to search for copy number variation. The high resolution, simplicity, high reproducibility and precise mapping of imbalances are the most significant advantages of aCGH over traditional cytogenetic methods.

A fine-tiling oligonucleotide array was first used for breakpoint analysis of deletions causing neuroblastoma at sub-kilobase resolution on four different chromosomes (126). The same type of array was used to delineate the deletion breakpoints in chromosome 1p for patients with Wilms tumors (127). More recently, fine-tiling arrays have been used to unravel complex rearrangements involving the BRCA1 gene (128), the MECP2 gene (129), the GJB2 and GJB6 genes (130), the STK11 gene (131) and to characterize different balanced and unbalanced rearrangements in a group of 12 patients with phenotypic abnormalities (132). These studies not only confirmed the power of fine-tiling arrays to find breakpoint regions, but also underline the increasing importance of array technology as a follow up after MLPA for the characterization of deletions and breakpoints in common and rare rearrangements. Therefore, the aCGH technique was also used in the current study to improve diagnostics for hemoglobinopathies. *Chapter 2.4* describes the design and validation of a custom fine-tiling array for the detection of rearrangements in the globin gene clusters. Herewith we have shown that aCGH technology is suitable for high-resolution mapping of breakpoints and that it is a valuable tool for the design of simple gap-PCR assays. The latter might be useful as a quick screening method for the more local occurring deletions or in laboratories where aCGH or MLPA is not available.

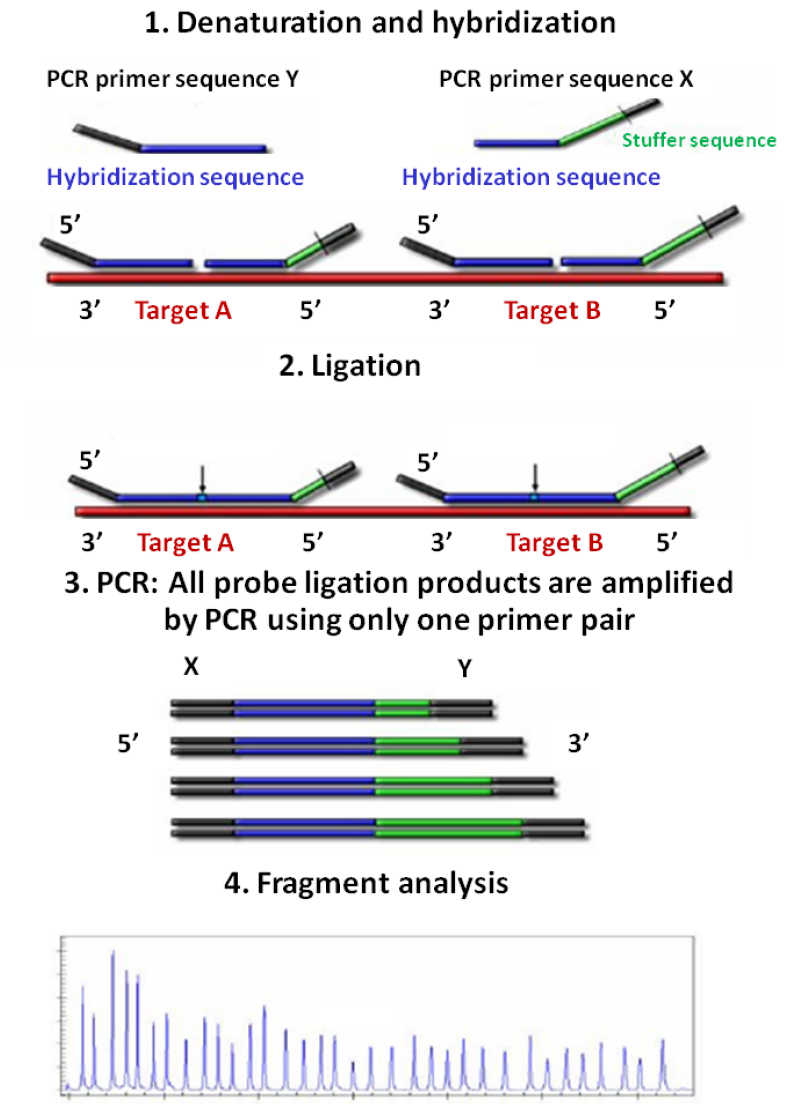

Figure 10 Outline of the MLPA technique. After hybridization to their target sequence in the sample DNA, the probe oligonucleotides are enzymatically ligated. One cosmid probe oligonucleotide contains a non-hybridizing stuffer sequence of variable length. In case of synthetic probes, the unique length of the product is determined by the length of the target sequence. Ligation products can be amplified using PCR primer sequences X and Y. Amplification products are separated by electrophoresis. Relative amounts of probe amplification products, as compared to a reference DNA sample, reflect the relative copy number of target sequences (Adapted from MRC Holland, http://www.mlpa.com).

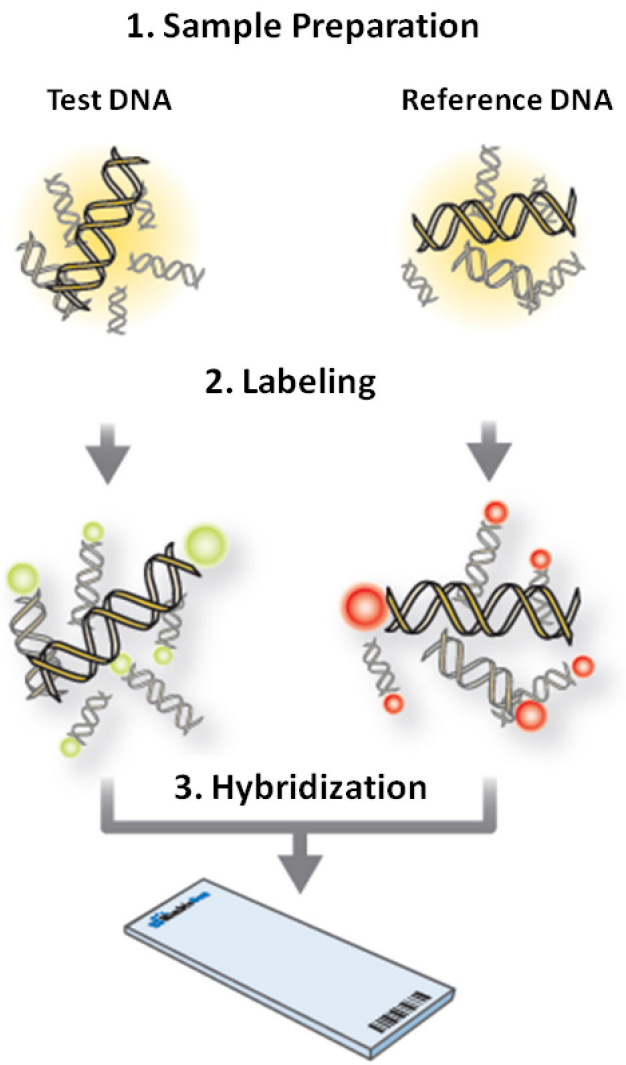

Figure 11 Outline of the array CGH technique. Test DNA and reference DNA samples are prepared separately. One sample is labeled with cyanine-5 fluorescent dye (red), and the other with cyanine-3 (green). Samples are then mixed and hybridized onto the array slide. By scanning the slide after ~72 hours of hybridization, the intensity of both fluorescent signals is measured. Differences in signal intensities, indicating copy number variations, between the two samples are visualized in a data plot (Adapted from Roche NimbleGen, http://www.nimblegen.com).

C. Prenatal diagnosis of hemoglobinopathies

As mentioned above, it is estimated that about 7% of the world population is a healthy carrier of a hemoglobinopathy. This results in approximately 350,000 severely affected children being born each year. Hemoglobinopathies occur mainly in areas where malaria is or has been endemic, because of the protective effect of the hemoglobinopathy traits against the lethal complications of malaria infection. However, the number of patients in north European countries is increasing due to recent migration (7;9;10;133).

In many endemic countries, prenatal diagnosis has been offered for several decades as part of a national primary prevention program by applying different techniques, including ultrasonography, amniocentesis and chorionic villus sampling. Except for the UK, primary prevention has not been included in the health care programs of most north European countries yet (10).

Ultrasonography

Ultrasonography or echography is a well established method used to visualize the embryo or fetus in the uterus of a pregnant woman. The method can be applied to determine the gestational age by measuring the crown to rump length of the fetus. In the Netherlands, the "echo" screening is usually performed around the 10^{th} week of gestation. Based on the gestational age, the expected date of delivery is predicted. Between the 11^{th} and 13^{th} week of gestation, a nuchal translucency (NT) test can be performed, by which the amount of fluid behind the neck of the fetus is measured. Fetuses with Down's syndrome (trisomy 21), Edwards Syndrome (trisomy 18) or Patau Syndrome (trisomy 13) tend to have a higher amount of fluid around the neck. The results of this test, together with screening of a maternal blood sample and maternal age, will yield a risk score for trisomy 21, 18 or 13 in the fetus.

As already mentioned, fetuses with severe β-globin gene defects are healthy *in utero* and at birth. Ultrasonography can be used only for detection of severe α-globin gene defects such as the Hb Bart's hydrops fetalis syndrome or for those rare deletions involving embryonic genes. Hb Bart's hydrops fetalis syndrome is a fatal condition with serious risk for the mother as well, but fetuses affected with embryonic gene deletions can be successfully treated with intrauterine transfusions.

Especially in areas with a high incidence of the α^0-thalassemia allele, such as Southeast Asian and Mediterranean countries, ultrasonography is playing a major role in early detection of this syndrome. In an affected pregnancy, the ultrasonographic measurements of fetal cardiothoracic ratio and placental thickness are higher because of severe fetal anemia. Screening of at risk pregnancies has a very high specificity and sensitivity, which leads to the avoidance of about 75% of invasive tests. However, invasive testing should be the definitive method to confirm the diagnosis (134-136).

Invasive prenatal diagnosis

Amniocentesis (AC) is usually performed between the 15^{th} and 20^{th} week of gestation. During the procedure, a small volume of amniotic fluid, containing fetal cells, is extracted by inserting a needle through the mother's abdominal wall. Chorionic villus sampling (CVS) can be performed earlier than AC, from the 10^{th} week of pregnancy onwards. The sample can be

obtained via the transcervical route, but the transabdominal procedure is preferred because of a lower reported miscarriage rate (137).

The fetal cells are separated from the sample and grown in a culture medium. The cells are then fixed and stained to be examined for chromosomal aberrations, such as trisomies, Turner syndrome (monosomy X) and fragile X syndrome. CVS and AC are most often used for karyotyping when a high risk of aneuploidy is suggested after the first trimester screening by ultrasound. The sample may also be used for fetal DNA analysis when both parents are known to be carriers of an identifiable gene mutation, such as hemoglobinopathies.

Non-invasive prenatal diagnosis

Screening by ultrasonography has the drawback that a definitive diagnosis cannot be given, only the risk of having an affected child can be calculated. Invasive procedures such as AC and CVS are necessary to obtain the genotype of the fetus. However, these tests carry a risk of miscarriage of approximately 0.5-1% (137;138). Therefore, there is a demand for non-invasive tests and a lot of research has been conducted in this field recently.

The discovery of free fetal DNA (ffDNA) circulating in the maternal blood during pregnancy has lead to the possibility to perform non-invasive prenatal diagnosis (NIPD) for many genetic disorders and for fetal sex determination (139;140). This fetal material enters the maternal blood circulation by apoptosis of fetal cells leaking across the placenta. NIPD has been successfully applied for RhD genotype (141;142) and trisomy 21 detection (143). FfDNA is detectable very early during pregnancy, and the average amount of fetal material is ~10% during the first and second trimester (144). However, a very specific technique is required to be able to detect this small amount of fetal material in the maternal plasma.

Allele-specific amplification by pyrophosphorolysis activated polymerization (PAP) is a technique that can be used for specific selection of fetal DNA from the maternal circulation (145). This PCR based method uses an oligonucleotide primer which contains a blocked dideoxy nucleotide at the 3' end. Removal of this block and extension can only occur when the primer matches exactly to the target DNA in the presence of pyrophosphate. This method has a very high specificity because of the serial coupling of pyrophosphorolysis and polymerization (146). In this way, it is possible to specifically detect the fetal material in maternal plasma by targeting mutations or polymorphisms inherited from father. The PAP technique has already been successfully applied for fetal sex determination (147).

In the Netherlands, most requests for prenatal diagnosis of hemoglobinopathies concern homozygosity or compound heterozygosities for the sickle cell mutation leading to sickle cell disease, or the detection of over 200 β-globin gene defects causing β-thalassemia major that may occur in the multi-ethnic Dutch immigrant population. It is therefore not always possible to use mutation-specific detection methods, such as PAP, for NIPD of hemoglobinopathies, because due to frequent homozygosity and consanguinity it is impossible to distinguish if the inherited mutated allele is of paternal or maternal origin. For this purpose, it would be more suitable to study paternal polymorphisms (SNPs) in linkage with the β-globin gene cluster. In this way, the number of assays to be designed can be limited to <20 polymorphisms, instead of designing assays for the >200 described β-thalassemia mutations. The melting curve analysis (MCA) technique can be used to screen family members for informative SNPs and to determine linkage to the normal or mutant allele. MCA is an assessment of the dissociation-characteristics of

double-stranded DNA during heating. A fluorescent dye is incorporated during the PCR, of which the extinction is measured during melting and visualized as a dissociation curve. Genotyping of SNPs can be performed by using probes covering the location of a SNP in the amplicon. The advantage of using MCA instead of direct sequencing analysis is that it is faster, simpler and less expensive (148-150). It is therefore an excellent screening method in a prenatal diagnosis setting. In combination with the PAP technique, NIPD for hemoglobinopathies would be possible (Figure 12). *Chapter 3.2* describes the application of the PAP-technique, in combination with melting curve analysis (MCA), for non-invasive prenatal diagnosis of β-thalassemia and sickle cell disease. PAP-primers were designed to detect SNPs in linkage with the paternal thalassemia-causing mutation, or with the normal allele. The MCA technique is used to determine which variants of the SNPs are in linkage with the normal and mutated paternal alleles. In this way, it is possible to perform NIPD for the majority of couples at risk with a limited number of PAP-assays. In principle, this approach would make half of the invasive procedures redundant (those in which the fetus inherited the wildtype allele from father), provided informative SNPs are present in the family and a previously born child or other family members are available to test the linkage of the SNPs.

Aim of this thesis

The main objective of this thesis was to improve post- and prenatal diagnostics of the hemoglobinopathies. Several molecular assays have been designed, tested and validated. In addition, a number of informative hemoglobinopathy cases have been studied in detail. *Chapter 2.1* describes the development of a single-tube multiplex gap-PCR for rapid detection of eight different deletions in the β-globin gene cluster. The design of an MLPA-based technique for detection of deletions and duplications in the globin gene clusters is outlined in *Chapter 2.2*. *Chapter 2.3* describes the application of the MLPA technique in a cohort of Australian patients with a thalassemic phenotype, which remained unsolved after applying the standard diagnostic methods. *Chapter 2.4* describes the design and validation of a custom fine-tiling array for the detection of rearrangements in the globin gene clusters. In addition, simple gap-PCR assays have been designed for 12 deletions. *Chapter 3.1* underlines the importance of screening for deletions in the globin gene clusters in couples at risk. Prenatal diagnosis might lead to a false adverse result, when the fetus has inherited a point mutation from one parent and a deletion allele with an intact – but inactive – β-globin gene from the other parent. MLPA showed to be an excellent method to quickly determine the extent of the deletion and is therefore also a valuable method in prenatal diagnosis of hemoglobinopathies. The application of PAP in combination with MCA as a non-invasive technique for prenatal diagnosis of hemoglobinopathies is presented in *Chapter 3.2*. *Chapter 4* is comprised of two case studies on the molecular characterization of novel deletions in the α-globin gene cluster. These studies stress the importance of MLPA as a diagnostic screening tool and the use of aCGH to obtain additional information about the exact breakpoints to be able to design a gap-PCR assay. It is important to be able to diagnose this kind of deletions; because of a 25% risk of Hb Bart's with hydrops fetalis in the offspring when in combination with another α^0-thalassemia allele. *Chapter 5* includes the conclusion and a discussion on the molecular techniques for hemoglobinopathy diagnosis presented in this thesis.

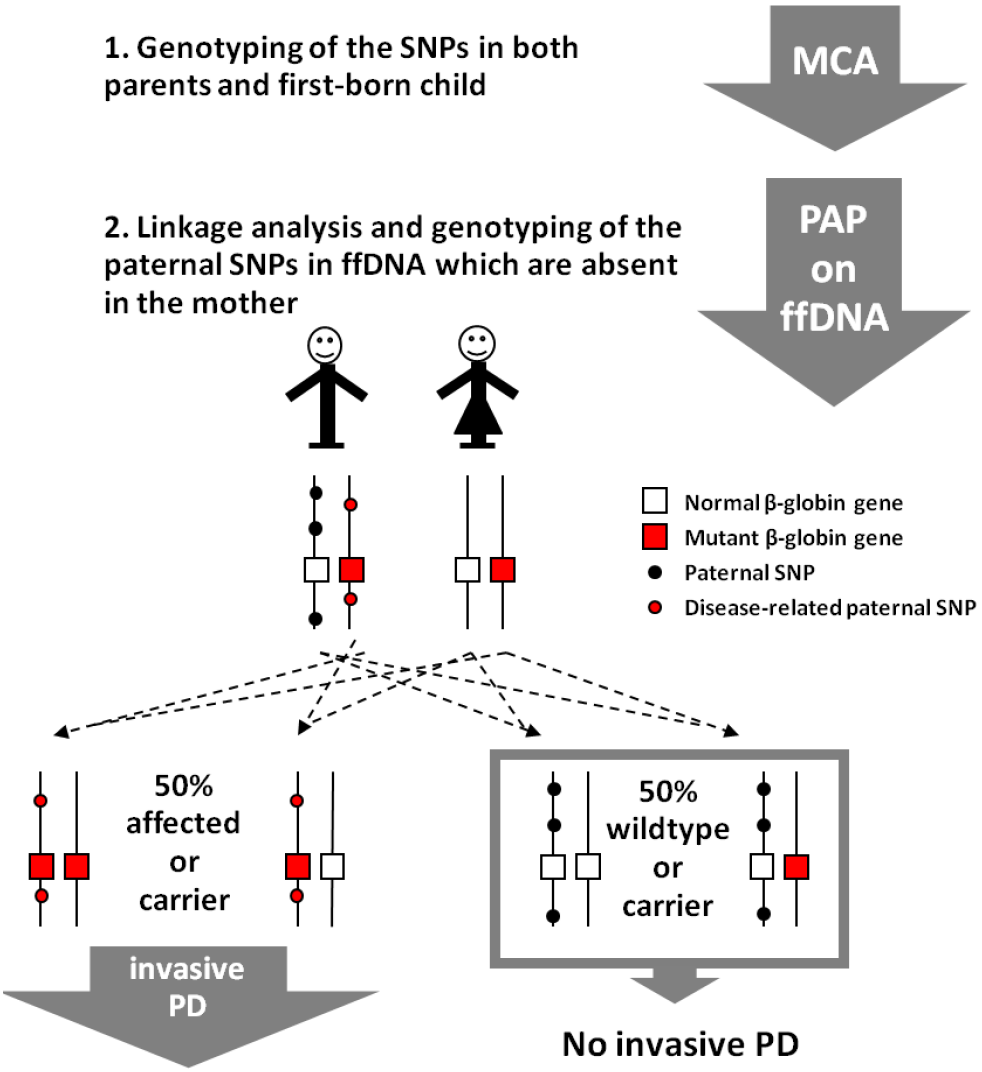

Figure 12 Principle of non-invasive prenatal diagnosis for β-thalassemia and sickle cell disease using SNPs. First, the SNPs are genotyped by MCA in father, mother and first-born child (or other family member) and linkage to the mutant paternal allele is determined. PAP is used to detect paternal SNPs in ffDNA isolated from maternal plasma. In this way, it can be determined which paternal allele is inherited by the fetus. In case the mutant allele is detected, an invasive prenatal diagnosis (PD) is still necessary to determine which maternal allele is inherited. If the paternal wildtype allele is detected, the fetus will be either wildtype or carrier of the maternal allele and further invasive PD is not necessary (courtesy of C.L. Harteveld).

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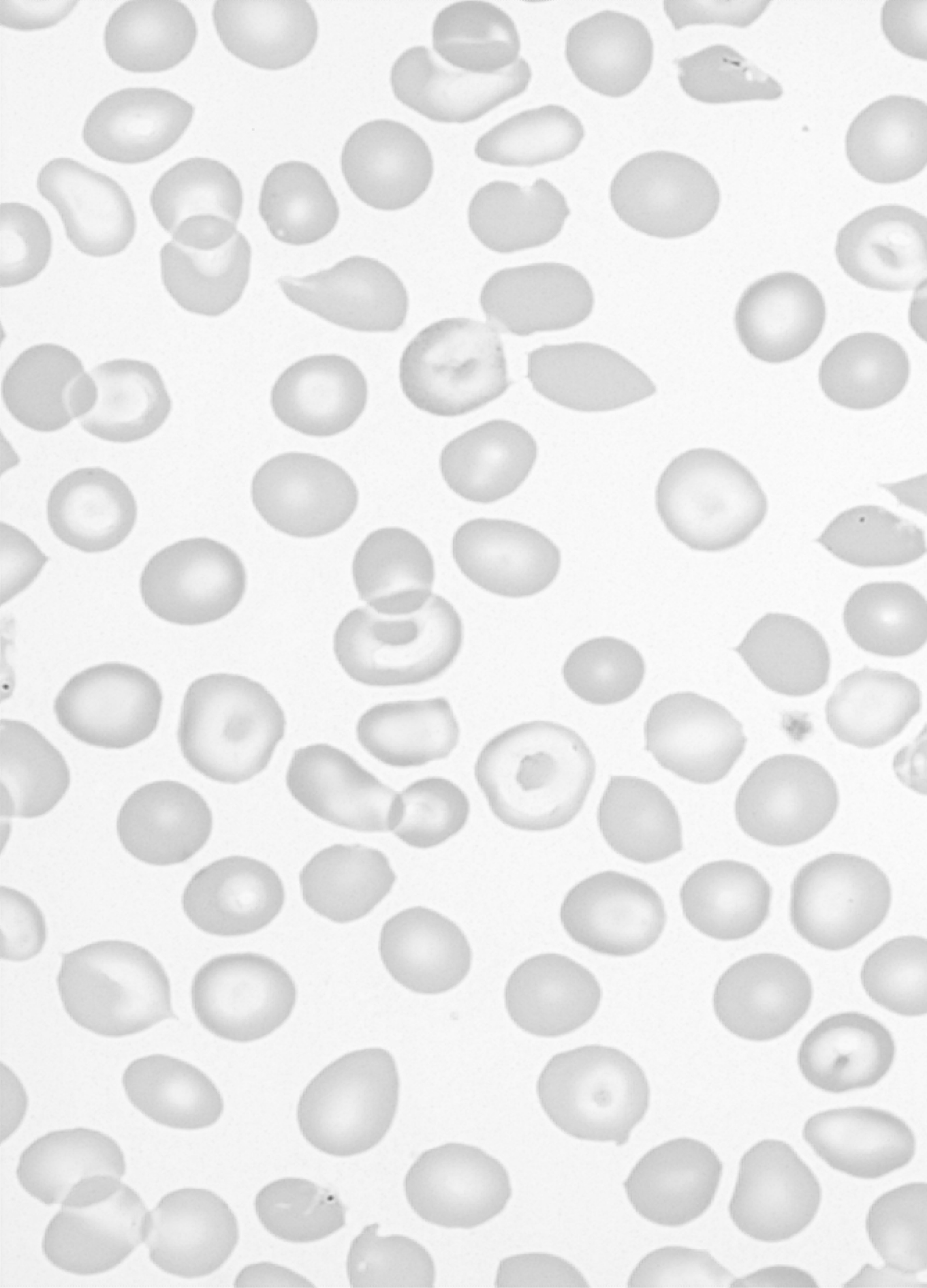

Chapter 2

Improving postnatal diagnostics for hemoglobinopathies

Chapter 2.1

A single-tube multiplex gap-PCR for the detection of eight β-globin gene cluster deletions common in Southeast Asia

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Abstract

Up to now, more than 200 different β-thalassemia (β-thal) mutations have been characterized. The majority are point mutations causing expression defects. Only approximately 10.0% of the defects are caused by large deletions involving the β-globin gene cluster causing β^0-thal, $(\delta\beta)^0$-thal, $^G\gamma(^A\gamma\delta\beta)^0$-thal and other conditions with or without persistence of fetal hemoglobin (Hb). For the prevention of severe forms of β-thal intermedia and β-thal major, it is important to identify carriers of point mutations as well as carriers of deletions.

β-Thalassemia and related disorders are most commonly present among populations from all Mediterranean countries as well as Southeast Asia, India, Africa, Central America and the Middle East. Twelve relatively frequently occurring deletion types have been described involving the β-globin gene cluster. These include the 105 bp β^0-thal deletion, the 619 bp deletion, the 3.5 kb deletion, the Southeast Asian (SEA) deletion, the Filipino deletion, Hb Lepore, the Thai $(\delta\beta)^0$-thal, the Siriraj I $^G\gamma(^A\gamma\delta\beta)^0$-thal, the Chinese $^G\gamma(^A\gamma\delta\beta)^0$-thal, the Asian Indian deletion-inversion $^G\gamma(^A\gamma\delta\beta)^0$-thal as well as the (hereditary persistence of fetal hemoglobin) HPFH-6 and HPFH-7 deletions.

To improve the rapid detection of the eight common β-globin cluster deletions in Southeast Asian countries, a simple molecular technique based on a single-tube multiplex gap-polymerase chain reaction (PCR) has been developed in this study. This technique provides a fast, simple and cost effective diagnostic test for deletion-types of β-thal that can be applied in every molecular diagnostic laboratory having standard PCR equipment.

Introduction

β-Thalassemia (β-thal) is a group of disorders resulting from a reduced or absent synthesis of β-globin chains [1]. In human, the β-globin gene cluster is approximately 50,000 base pairs long and consists of five functional genes (ε, $^A\gamma$, $^G\gamma$, δ and β) and one pseudogene (ψβ) on the short arm of chromosome 11 (11p15.5). The expression of these genes is regulated by the locus control region (LCR) that lies 20 kb upstream of the ε-globin gene. As normal individuals have two allelic β-globin genes, β-thal may exist in a heterozygous or homozygous state [2].

The World Health Organization has estimated that almost 70,000 infants with β-thal are born worldwide each year and 270 million people are carriers [3]. β-Thalassemia and related disorders are commonly present among populations in all Mediterranean countries as well as in Southeast Asia, India, Africa, Central America and the Middle East [1]. However, because of migration, the carrier rate of β-thal is increasing in countries that previously had low prevalence.

For the prevention of β-thal intermedia and β-thal major in a population, carriers of β-globin gene defects and couples at risk should be identified and referred to a genetic center for counseling, preferably before the first pregnancy. The most common reason for referral for couples at risk in Thailand is if both parents are carrier of Hb E [β26(B8)Glu>Lys, GAG>AAG] and β-thal or, less frequently, both are carrier of β-thal.

Up to the present time, more than 200 different β-thal [4] mutations have been characterized. Most of them result from point mutations causing a reduced expression of the β-globin gene [5]. Alternatively, deletions may involve the β-globin gene or regulatory elements, preventing the allelic expression of β-globin. Only approximately 10.0% are large deletions involving the β-globin gene cluster causing β^0-thal, $(\delta\beta)^0$-thal, $^G\gamma(^A\gamma\delta\beta)^0$-thal and other forms of hereditary persistence of fetal hemoglobin (Hb) (HPFH). Since these deletions can lead to mild,

intermediate or severe diseases in combination with a β-thal point mutation, it is important to include deletional types of β-thal in the molecular screening for couples at risk.

In Southeast Asian countries, approximately 12 types of deletions in the β-globin gene cluster have been described. They include the β^0-thal 105 bp deletion, the 619 bp deletion, the 3.5 kb deletion, the novel $^{G}\gamma(^{A}\gamma\delta\beta)^0$-thal; Siriraj I deletion (~118 kb) (Nipon Chalaow & Vip Viprakasit; unpublished data 2012), the Southeast Asian (SEA) deletion (~27 kb), the Filipino deletion (~45 kb), Hb Lepore (~7.4 kb deletion), the Thai $(\delta\beta)^0$-thal deletion (~12.5 kb), the Chinese $^{G}\gamma(^{A}\gamma\delta\beta)^0$-thal deletion (~100 kb) and the Asian Indian deletion-inversion $^{G}\gamma(^{A}\gamma\delta\beta)^0$-thal, as well as HPFH-6 and Vietnamese HPFH (known as HPFH-7 in HbVar) deletions. Their deletion breakpoints have been presented in Table 1 [3,4-24].

A number of polymerase chain reaction (PCR)-based techniques such as restriction endonuclease (RE), Southern blotting and multiplex ligation-dependent probe amplification (MLPA) have been applied for characterization of rare and unknown β-globin gene cluster deletions. However, the most widely technique used is gap-PCR that uses only two specific primers complementary to the sense and antisense strand in the DNA regions flanking the deletion. The importance of enzymatic amplification of deletions causing $\delta\beta)^0$-thal or HPFH was already demonstrated back in 1994 by Craig et al. [25] to facilitate the molecular diagnosis. Nine different deletions including a subset of Mediterranean, African and Asian deletions were performed in single-plex. To facilitate the use of gap-PCR in a diagnostic setting, multiplex-PCR assays containing a mixture of primers on each side of the breakpoints were designed for the detection of common thalassemia deletions in the α- and β-globin gene clusters [26-28].

To improve rapid diagnostics for detection of eight β-globin cluster deletions in Southeast Asian countries, a simple molecular technique based on a single-tube multiplex gap-PCR assay has been developed in this study. This technique provides a fast, simple and cost effective diagnostic test for β-thal and deletional types of HPFH which can be applied in every molecular diagnostic laboratory equipped with standard PCR apparatus.

Materials and methods

Details of all primers in the single-tube multiplex gap-PCR for the detection of eight β-globin gene cluster deletions in Southeast Asian countries are shown in Table 2 and Figure 1. The PCR reaction was performed in a total volume of 50 microliters (μL) and this multiplex PC reaction took about 2 hours 30 min. Each PC reaction contained 0.2 mM of each dNTPs, 1.2 mM $MgCl_2$, 1 unit of GoTaq DNA polymerase in supplied reaction buffer (Promega, Madison, WI, US), 100 ng of genomic DNA and 0.08-1 pmole of 15 different primers (Table 2).

The PCRs were performed on a Biometra TProfessional Thermocycler (Biometra, Göttingen, Germany). After an initial denaturation at 94°C for 1 min., 27 cycles with template denaturation at 94°C for 30 seconds, primer annealing at 62°C for 2 min. and 59°C for 1 min., DNA extension at 72°C for 1 min. and a final extension at 72°C for 10 min., were performed. Eight microliters of each amplified product was analyzed by electrophoresis on 1.2% agarose gel in 1X Tris Borate-EDTA (TBE) buffer at 120 volts for 3 hours After staining with ethidium bromide, the agarose gel was visualized under UV-light.

Among the 12 deletions described in Southeast Asian countries, eight deletions with available genomic DNA were selected for the development of the gap-PCR technology. Genomic DNA of carriers for the eight different β-thal deletions previously characterized by MLPA, by

Disorders	Types [References]	Locations on NCBI_ AC104389.8		Distribution
		5' Deletion breakpoint	3' Deletion breakpoint	
β^0-thalassemia	105 bp deletion [6]	29,436	29,331	Thailand
	619 bp deletion [7]	28,214	27,595	India Pakistan Thailand
	3.5 kb deletion [8,9]	29,539-30,221	26,128-26,881	Thailand
	SEA deletion (~27 kb deletion)	31,394	3,982	Cambodia Vietnam China
	Filipino deletion [11,12]	33,691-33,699	184,733-184,743[a]	Philippines Malaysia Indonesia
$\delta\beta$ hybrid	Hb Lepore [13-17]	36,324-36,666	28,910-29,254	Mediterranean Brazil Thailand
$(\delta\beta)^0$-thalassemia	Thai (~12.5 kb deletion) [18]	35,432-35,620	23,090	Thailand Laos PDR [21]
$^G\gamma(^A\gamma\delta\beta)^0$-thalassemia	Asian Indian deletion-inversion [20,21]	51,802	27,826	India Bangladesh Kuwait Thailand
	Chinese [22]	51,161	127,698[b]	Southern China
	Siriraj I (~118 kb deletion)	52,507	165,744[c]	Thailand
HPFH	HPFH-6 [23]	54,361	124,872[d]	Thailand
	HPFH-7 [24]	33,468	4,799	Vietnam

Table 1 Deletion breakpoints of β-globin gene cluster found in Southeast Asian countries [4-24]. [a]Data from HbVar ID 989 (HGVS name NG_000007.3), [b]Data from HbVar ID 1046 (HGVS name NG_000007.3), [c]Characterized by MLPA and direct sequence analysis, [d]Data from HbVar ID 1048 (HGVS name NG_000007.3).

Southern blotting or direct sequence analysis of breakpoint fragments were used as positive controls for optimizing the conditions of multiplex gap-PCR. In first instance, due to a limited availability of DNA of patients to be used as positive control for the detection of the Siriraj I $^G\gamma(^A\gamma\delta\beta)^0$-thal and the Southeast Asian β^0-thal deletions, reamplified PCR products were used to set up conditions together with genomic DNA of the other six rearrangements. To reduce the presence of non-specific background and primer dimers, the first PCR amplification was used at

a dilution of 1:1,000,000. To rule out amplification artifacts or failure as a consequence of PCR products used as positive controls, the experiment was repeated on genomic DNA for all eight deletions as positive controls, which confirmed the previously obtained results.

Results

A single-tube multiplex gap-PCR for detection of eight β-globin gene cluster deletions in Southeast Asian countries has been successfully optimized in this study. In this multiplex PCR system, the 304 and 1,889 bp fragments serve as an internal control of the PCR reaction. The 620, 688, 743, 836, 974, 1159, 1270 and 1447 bp fragments indicate the presence of the $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thal Siriraj I deletion, β^{0}-thal 3.5 kb deletion, Filipino β^{0}-thal, Southeast Asia (SEA) β^{0}-thal, HPFH-6, Hb Lepore, β^{0}-thal 619 bp deletion and Thai $(\delta\beta)^{0}$-thal, respectively (Table 2). The first and last lanes in Figure 2 contain a 100 bp ladder as a size standard.

Discussion

Literature for Thai $(\delta\beta)^{0}$-thal and Laotian $(\delta\beta)^{0}$-thal has been reviewed in this study [21, 22]. The results confirm that both deletional types of $(\delta\beta)^{0}$-thal are most likely the same as the deletion size and breakpoints are similar. The 5' breakpoint is located in the second intervening sequence (IVS-II) of the δ-globin gene and the 3' breakpoint lies within the L1 repetitive sequences at 4.7 kb 3'of the β-globin gene. As shown in Table 1, the deletion is approximately 12.5 kb in length.

In general, the profile of the PCR has three steps including template denaturation, primer annealing and DNA extension steps. In order to get a clearly positive band for Hb Lepore, the extra annealing step at 62^{0}C for 2 min. was added to the PCR program. This step is to support the binding of the Hb Lepore primers before the 619 bp primers, because of the higher melting temperature (Tm) of the Lepore primer pair as shown in Table 2. The double-step annealing multiplex PCR has been shown to work well with the high Tm primers [29].

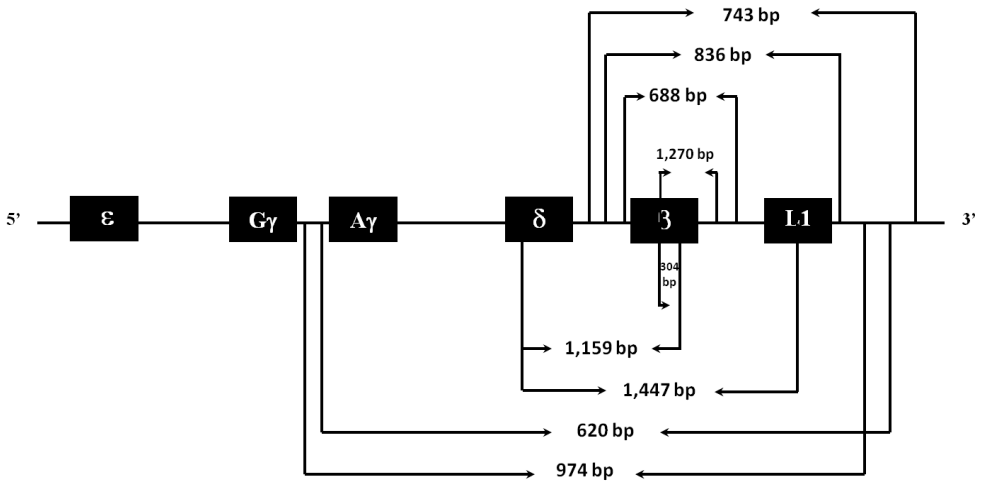

Figure 1 Schematic representation of the β-globin gene cluster, indicating the extent of the eight deletions and relative positions of the primer pairs.

Disorders	Primer name	Primer sequence (5'→3')	Primer concentration (μM)	Tm (°C)	Product size (bp)
$^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thal (Siriraj I deletion)	Siriraj-Fw Siriraj-Rev	GGTCACGAATTTGCTTGGTC GCGCTGAAACTGTGGCTTTA	0.08 0.08	60.50 61.46	620
β^{0}-thal (3.5 kb deletion)	3.5 kb-Fw 3.5 kb-Rev	CAGTCACGATGCTGTACATTAGA TGCAGATTAGTCCAGGCAGA	0.16 0.08	58.00 59.55	688
Filipino β^{0}-thal (~45 kb deletion)	Filipino-Fw Filipino-Rev	CCTTGAAGCTGGGTAGTGTGA GCAGAGCTACTCAGGGCATT	0.16 0.16	60.30 59.60	743
SEA β^{0}-thal (~27 kb deletion)	SEA-Fw SEA-Rev	TGCTTAGACATTTTCCCAAGG GGTGACAATTTCTGCCAATCA	0.4 0.4	59.21 60.89	836
HPFH-6	HPFH 6-Fw HPFH 6-Rev	CAGGATGGGGCTCAGAAATA AAGGAAATGAGCCAGCAGAA	1.0 1.0	60.03 59.96	974
Hb Lepore	Lepore-Fw Lepore-Rev	TGGTGCAAAGAGGCATGATA GGAGGACAGGACCAGCATAA	0.2 0.8	60.22 60.07	1,159
β^{0}-thal (619 bp deletion)	619 bp-Fw 619 bp-Rev	TTATGGTGTAAGACAAGGGTCTGA CACAGTCTGCCTAGTACAT	0.8 0.4	59.93 48.03	1,270
Thai $(\delta\beta)^{0}$- thal (~12.5 kb deletion)	Thai-Fw Lepore-Rev	TTCTCCCCATCACTTTCAGC GGAGGACAGGACCAGCATAA	0.16 0.8	60.20 60.07	1,447
Internal control	Lepore-Fw 619 bp-Rev	TGGTGCAAAGAGGCATGATA CACAGTCTGCCTAGTACAT	0.2 0.4	60.22 48.03	304
	619 bp-Fw 619 bp-Rev	TTATGGTGTAAGACAAGGGTCTGA CACAGTCTGCCTAGTACAT	0.8 0.4	59.93 48.03	1,889

Table 2 Primer details in a single-tube multiplex gap-PCR for detection of the eight β-globin gene cluster deletions in Southeast Asian countries (Tm is the melting temperature of each primer)

Several types of Hb Lepore have been described based on their deletion breakpoints. They include Hb Lepore-Hollandia (δ22Ala/β50Thr), Hb Lepore-Baltimore (δ50Ser/β86Ala or δ68Leu/β84Thr or δ59Lys/β86Ala) and Hb Lepore-Boston-Washington (δ87GIn/βVSII-8 or δ87GIn/β116His) [32-34]. The primer pair selected to amplify a fragment of 1159 bp suits all types of Hb Lepore. As three types are not clinically different, further direct genomic sequencing of the Hb Lepore deletion breakpoint will only be necessary for further anthropological studies to identify the origin of different subtypes of Hb Lepore. Practically, our simple multiplex-PCR should be sufficient for routine laboratory diagnosis for thalassaemia and Hb variants.

Even though PCR amplified DNA was used as positive control for the Siriraj I $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thal and the Southeast Asia β^{0}-thal deletions for the optimization of PCR conditions, the validation of the multiplex PCR had been successfully performed using genomic DNA as positive control for all 8 mutations. No DNA samples were available to be used as positive controls for the rare 105 bp β^{0}-thal deletion, the Vietnamese HPFH-7 deletion, the Chinese $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thal and Asian

Indian deletion-inversion $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thal. By adding four more set of primers to the mix (primer sequences and primer concentrations available on request), no interference of amplified PCR fragments from other known deletions was observed. However, the robustness and efficiency of these added primers on detecting four rare deletions remain elusive and warranted further analysis when these rare deletional control DNA samples become available.

So far, the actual prevalence of these mutations in the population is unknown. However, the 105 bp β^{0}-thal deletion, the 3.5 kb deletion, the Filipino deletion, the Thai $(\delta\beta)^{0}$-thal, HPFH-6 and Hb Lepore have been reported occasionally in Thailand and other Southeast Asian populations [4,6,9,30-34]. Siriraj I $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thal is a novel mutation. It has been found in two unrelated families from the southern part of Thailand, one from Hat Yai and another from Phuket (unpublished data). It can be assumed that there may be more if the detection method is available. The multiplex gap-PCR developed in this study is simple enough for any molecular laboratory to apply in routine practice.

In conclusion, a simple molecular technique based on a single-tube multiplex gap-PCR for the detection of eight β-globin cluster deletions in Southeast Asian countries has been successfully developed in this study. A single-tube multiplex gap-PCR will help diagnostic laboratories to perform diagnostics on β-thal quickly and accurately, using the presently described multiplex PCR assay. This screening assay is widely applicable as it only requires a PCR machine, which is available in most clinical molecular laboratories screening for hemoglobinopathies in Asia where the incidence of β-thal is high.

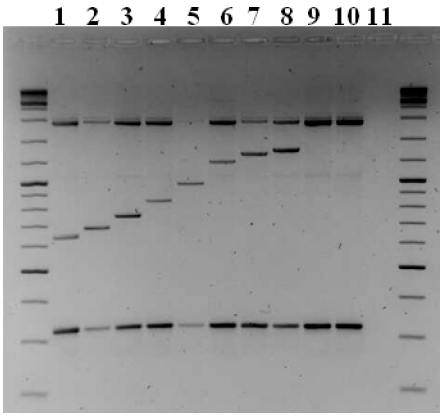

Figure 2 Representative 1.2% agarose gel electrophoresis of the PCR product. Lane 1: positive for $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thalassemia (Siriraj I deletion); lane 2: positive for β^{0}-thalassemia (3.5 kb deletion); lane 3: positive forβ^{0}-thalassemia (Filipino deletion); lane 4: positive for β^{0}-thalassemia (SEA deletion); lane 5: positive for HPFH-6;lane 6: positive for Hb Lepore; lane 7: positive for β^{0}-thalassemia (619 bp deletion); lane 8: positive for Thai $(\delta\beta)^{0}$-thalassemia; lanes 9 and 10: negative control; lane 11: blank.

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Declaration of Interest

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Chapter 2.2

Nine unknown rearrangements in 16p13.3 and 11p15.4 causing alpha- and beta-thalassaemia characterised by high resolution multiplex ligation-dependent probe amplification

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Abstract

Background: Approximately 80% of the α- and 10% of the β-thalassaemias are caused by genomic deletions involving the a- and β-globin gene clusters on chromosome 16p13.3 and 11p15.5 respectively. Gap-PCR, Southern blot analysis, and fluorescent in situ hybridisation are commonly used to identify these deletions; however, many deletions go undetected using conventional techniques.

Methods: Patient samples for which no abnormalities had been found using conventional DNA techniques were analysed by a three-color multiplex ligation-dependent probe amplification assay. Two sets of 35 and 50 probes, covering a region of 700 kb of the α- and 500 kb of the β-globin gene cluster, respectively, were designed to detect rearrangements in the α- and β-globin gene clusters.

Results: In 19 out of 38 patient samples, we found 11 different α-thalassaemia deletions, six of which were not previously described. Two novel deletions leaving the α-globin gene cluster intact were found to cause a complete downregulation of the downstream α-genes. Similarly, 31 out of 51 patient samples were found to carry 10 different deletions involving the β-globin gene cluster, three of which were not previously described. One involves the deletion of the locus control region leaving the β-globin gene cluster intact.

Conclusions: These deletions, which are not easily detected by conventional techniques, may have clinical implications during pregnancy ranging from mild to life threatening microcytic haemolytic anaemia in neonates. The approach as described here is a rapid and sensitive method for high resolution analysis of the globin gene clusters and for any region of the genome.

Introduction

Thalassaemias are inherited haemoglobin disorders characterised by a quantitative reduction of the α- or β-globin chains [1,2,3]. Genomic deletions involving the α-globin gene cluster on chromosome 16p13.3 are the most common molecular cause of α-thalassaemia (~80-90% of cases). Rearrangements in the β-globin gene cluster on 11p15.4 account for ~10% of all β-thalassaemia mutations and hereditary persistence of fetal haemoglobin (HPFH) syndromes. Besides the most common ones a large variety of less frequently occurring thalassaemia deletions have been found in different populations. At least 60 different deletions involving the β- and more than 50 involving the α-globin gene cluster have been described to date [4,5] (http://globin.cse.psu.edu/hbvar/menu.html).

The molecular tests commonly used to identify these deletions are gap-PCR, Southern blot analysis and fluorescent in situ hybridisation (FISH) analysis [6-10]. Gap-PCR can only be applied to known deletions, Southern blot analysis is time consuming and technically demanding and success is very much dependent upon the hybridisation probes available, and FISH analysis involves laborious cell culturing to generate metaphase chromosome spreads and has a low resolution (>20kb).

Recently, a simple technique suitable for rapid quantitative analysis, multiplex ligation-dependent probe amplification (MLPA), has been described [11]. This technique is based on the ligation and PCR amplification of two adjacently hybridising oligonucleotides. Each oligonucleotide pair is designed to give a product of a unique length, and by using common ends all probes can be amplified with one primer pair. Using a fluorescent label allows probe separation on a capillary sequencing system. This method has been applied successfully in a number of genes in which

deletions and duplications are common [12-14]. In the original description, the probes were generated by cloning into specially developed M13 vectors. Recently, we have simplified this method by using chemically synthesised oligonucleotides. Discrimination of probes based on chemically synthesized oligonucleotides (~40-60 nt) was doubled using two universal primer sets each labelled with a different fluorophore, allowing up to 40 probes to be used in a single reaction [15].

To simplify the detection of α- and β-thalassaemia deletions and increase the resolutions, we designed two probe sets for each cluster. For the α-cluster, two probe sets of 35 probe pairs in total were designed with an average distance of ~20 kb, covering a genomic region of ~700 kb. For the β-cluster, a total of three probe sets consisting of 50 probe pairs were designed covering a region of ~500 kb and an average distance of ~10 kb. Control DNA of known α- and β-thalassaemia deletion carriers was used and the deletion characterised by an independent method. Two groups of patient samples suspected of having a (large) deletion in either the α- or β-globin gene cluster, based on haematological findings, were analysed in this assay.

Materials and methods

Patients

Patients suspected of having haemoglobinopathies were referred to our laboratory for haematological, biochemical, and DNA analysis [16]. Based on this analysis, they were diagnosed as α- and/or β-thalassaemia carriers. The patients suspected for α-thalassaemia in whom no abnormalities were found by gap-PCR for the seven most common α-thalassaemia deletions and non-deletion types of α-thalassaemia were excluded by direct sequencing of the α-genes, were selected for MLPA. Some showed either an unbalanced α/β chain synthesis ratio (< 0.8) and/or inclusion bodies [17] indicative for a deletion of both α-genes on the same allele [18]. In addition, a few patients presented with haemoglobin H (HbH) disease, but analysis thus far only revealed one mutation, suggesting a deleted allele *in trans*. Some showed the presence of possible junction fragments by Southern blot, in which the deletion could not be characterised due to lack of probes in the region flanking the potential deletion. In total, 38 possible α-thalassaemia carriers were selected to be screened for rearrangements in 16p13.3. These samples were collected during a period of approximately 5 years.

Patients were selected who presented with a microcytic hypochromic anaemia in the presence of elevated HbA_2 levels, for which standard DNA analysis revealed no abnormalities in the β-globin gene sequence or the 5'and 3'UTR. These samples include patients showing a high HbF expression, indicative for HPFH, $(\delta\beta)^0$- or $^G\gamma(^A\gamma\delta\beta)^0$-thalassaemia, and patients showing normal HbA_2 and HbF levels with α/β chain synthesis ratios higher than 1.5, indicative for deletions involving the complete cluster and/or the regulatory elements. A total of 51 samples were analysed by MLPA.

As positive controls for MLPA of the α-globin gene cluster, we used seven deletions confirmed previously by gap-PCR (- - ^{SEA}, -$\alpha^{3.7}$, -$\alpha^{4.2}$, - - $^{Med\ I}$, - - ^{FIL}, - - ^{THAI} and – $(\alpha)^{20.5}$, indicated as black bars in Figure 1B). Two other deletions (33 kb - -$^{Dutch\ I}$ and the -$\alpha^{7.9}$) were previously characterised by Southern blot analysis and direct sequencing of the amplified break point fragments [19,20]. For MLPA of the β-cluster, the Dutch III $(\varepsilon^G\gamma^A\gamma)^0\delta\beta$-thalassaemia of 112 kb [21],

the 50 kb Belgian $(\gamma\delta\beta)^0$-thalassaemia [22], the 25-30 kb Chinese β^0-thalassaemia [23], the 12.6 kb Dutch I β^0-thalassaemia [24] and the Indian (-619 bp) β^0-thalassaemia deletions [25], all previously characterised by Southern blot analysis, were used as positive controls (Figure 2).

Probe design

In total 35 probe pairs were designed to detect rearrangements on 16p13.3, covering approximately 700 kb from the telomere to the MSLN gene (Table 3, Figure 1). For each probe pair, the common ends correspond to either the MLPA amplification primers (forward tag 5'-GGGTTCCCTAAGGGTTGGA-3'; reverse tag 5'-TCTAGATTGGATCTTGCTGGC-3') [11] or the multiplex amplifiable probe hybridisation (MAPH) primers (forward tag 5'-GGCCGCGGGAATTCGATT-3'; reverse tag 5'-CACTAGTGAATTCGCGGC-3') [26], which allows simultaneous amplification and detection of the separated fragments in different colours.

Similarly, 34 probe pairs to be analysed in two colours were designed to detect rearrangements in on 11p15.4 (Table 1, Figure 2). A third probe set, consisting of an additional 16 probe pairs, was designed for fine mapping some of the deletions found by MLPA (Table 2). In order to detect all 50 probe sets in the same fragment analysis sample run, a third common extension was used for the additional probe set, which allowed the use of a third colour (M13 forward tag 5'-GGCGATTAAGTTGGGTAAC-3'; M13 reverse tag 5'-GTTCACACAGGAAACAGC-3') .

Unique sequence was identified using the BLAT program (http://genome.ucsc.edu) [27], and care was taken that no known sequence variants were present in the primer annealing site. Probes within each set were designed to produce PCR products differing by 2 bp in length to allow separation in the size range from 80-125 bp using capillary electrophoresis on an ABI 3700 sequencer (Applied Biosystems, Foster City, CA). Primers have been designed using the RAW program (MRC-Holland, Amsterdam, The Netherlands) such that the melting temperature of the hybridising regions of each probe was at least 65°C with a GC percentage between 35% and 60%.

The oligonucleotides were from Illumina (San Diego, CA), synthesised in a salt-free environment (50 nmol scale) and used without further purification. The downstream primer of each probe pair was 5' phosphorylated to allow ligation. Separate probe mixes were prepared to allow the detection of deletions in either the α- or β-globin gene clusters, combining two sets of probes with MLPA and MAPH common ends at a final concentration of 4 fmol/µl. The α- and β-globin gene MLPA probe mixes are available on request (http://www. LGTC.nl).

MLPA Reaction

Reagents for MLPA and subsequent PCR amplification were purchased from MRC-Holland. All primers used for PCR amplification were purchased from Sigma-Genosys (Cambridge, UK). The MLPA reactions were performed as described by Schouten et al. [11] and White et al. [15]. In brief, approximately 200 ng of genomic DNA in a final volume of 5 µl was heated for 5 minutes at 98°C. After cooling to room temperature, 1.5 µl of the probe mix and 1.5 µl SALSA hybridisation buffer (MRC-Holland) were added to each sample, followed by heat denaturation (2 min at 95°C), hybridisation (16 hrs at 60°C). Ligation was performed by adding 32 µl of ligation mix at 54°C for 10 minutes and the reaction was stopped by incubating 5 minutes at 95°C. PCR amplification was carried out for 33 cycles in a final volume of 25 µl, adding both the MAPH-F and –R and the MLPA-F and –R primer sets to a final concentration of 100 and 200 nM, respectively, with MAPH-F being labelled with HEX and MLPA-F labelled with FAM. The third common primer set

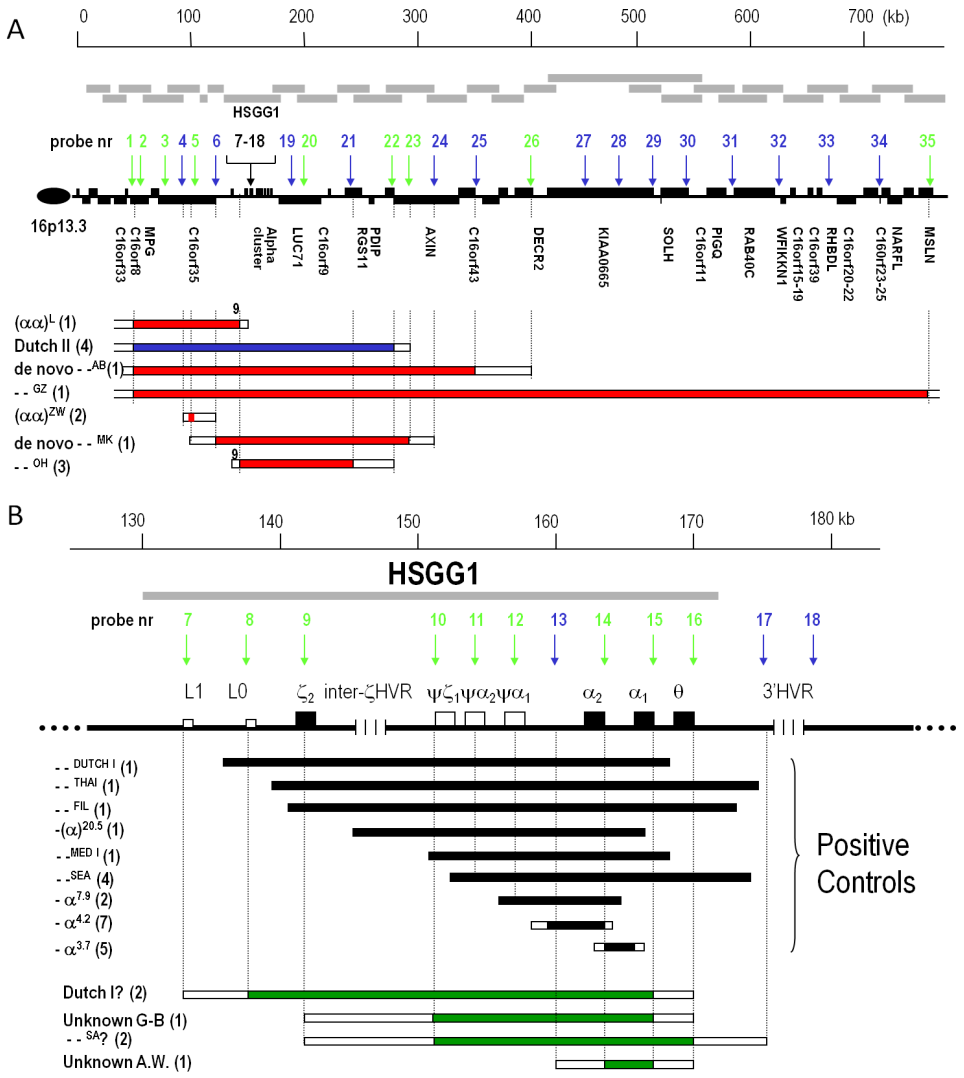

Figure 1 (A) Schematic representation of the short arm of chromosome 16 (16p13.3), showing a 700 kb region containing the α-globin gene cluster. Grey bars above the cluster indicate the minimal tiling path of clones covering this region. Oval shape denotes the telomeric repeat region and solid boxes the genes throughout the regions (adapted from Daniels et al. [29]). Vertical arrows show locations of the probe pairs; colours correspond to colour label used in MLPA reaction and probe numbers to numbers in Table 3. Bars below the figure indicate deletions found by MLPA, vertical lines marking the first and last probe deleted. Open boxes mark the region where deletion breakpoint should be located. Red indicates novel deletions found in this study. Blue indicates deletions previously described, but more accurately mapped by MLPA. The number of unrelated individuals found during analysis is indicated between brackets. **(B)** Schematic presentation of part of 16p13.3 showing the α-globin gene cluster. Black bars show deletions (all confirmed by gap-PCR or Southern blot analysis) used as positive controls to set up the assay. Green bars show deletions resembling described deletions in length and position of the breakpoints. The identity can only be determined by gap-PCR and direct sequencing.

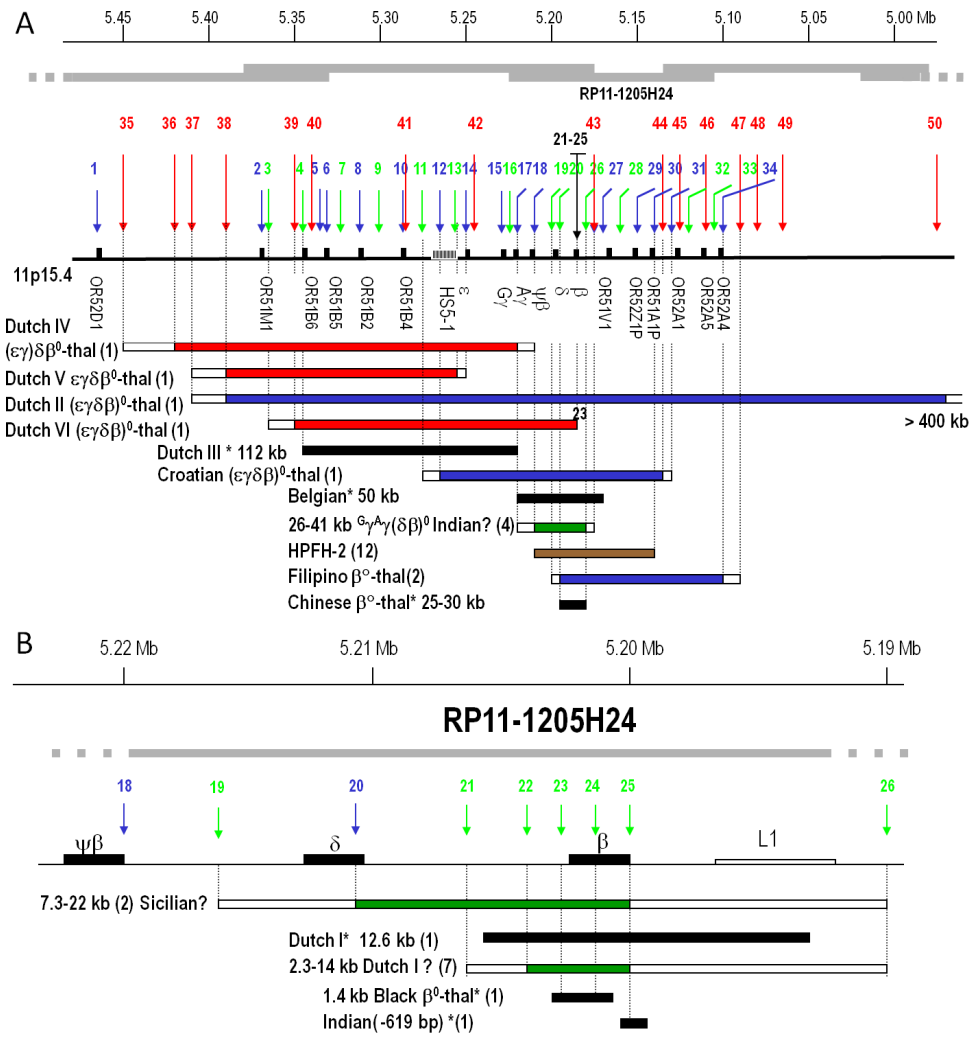

Figure 2 (A) Schematic representation of the short arm of chromosome 11 (11p15.4), showing a 500 kb region containing the β-globin gene cluster. The genes throughout the regions are indicated as solid boxes. The minimal tiling path of clones covering this region is indicated by grey bars above the cluster. Deletions found by MLPA are shown as bars below the figure; the colours of the bars are as indicated in Figure 1. The brown bars represent deletions found during this analysis and confirmed by gap-PCR. **(B)** Schematic presentation of the region surrounding the β-globin gene and deletions found during this analysis or used as positive controls.

No.	Name	Standard deviation (range)[1]	Upstream hybridising sequence	Downstream hybridising sequence	Positions chr. 11p15.4 UCSC Genome Browser (May 2004)
1b	OR52D1*	0.04 (0.93-1.09)	GGTGGATGCCTGGCCCAGATGTTT	TGTGTCCATTCTATCTATGCTCTGGAGTCC	5466733-5466857
2b	OR51M1*	0.03 (0.95-1.05)	CTGCTATTCATCTTCTTATGGCCAATGTCTACCTT	TTTGTGCCTCCCATGCTTAACCC	5368049-5368106
3b	Probe 79	0.04 (0.93-1.09)	CATGGGTCTCTGTCCTGGTTTTAGATCTGTATGCCCCGTTTTGGAGCTC	ATCCCCACACAACTCTAATCAACCATGCCC	5360654-5360732
4b	Probe 45	0.04 (0.92-1.05)	CCTTGTTTACAGGGTCCCCCCA	CCCTCTTCCTTCCTGAGCATGTG	5342559-5342603
5b	OR51B6*	0.06 (0.85-1.07)	CTTCCACCTTCCAGCTTACTGGCT	TCCCAGGCATGGAGAAGGCACATC	5329333-5329380
6b	OR51B5*	0.02 (0.95-1.03)	CTGATTCATCGTTTTGGAAAGCAGGTTCCACATATTGT	TCACCTCATTATGAGCTATGCCTATTTTCTGTTC	5320488-5320559
7b	OR51B5-OR51B2	0.06 (0.85-1.07)	CATTGCTTCCAGGGCCATTGGTGTGTTAATACAACACTTAGTAAAGTAA	GCAATGTTAGTGTTGGAGTGGGAGTAGAACCG	5311149-5311229
8b	OR51B2*	0.05 (0.91-1.12)	CTGGCTTGTGCTGACATAACTTTCAATAGACTTTACCCTGTAATT	TTGATCTCTTTAACAATCTTCCTAGACTGTCTG	5301474-5301551
9b	OR51B3P	0.07 (0.83-1.12)	GGGCCTGCTTCTTGCAGGTT	TACATTATCCACTCCCATTATCCACTTGC	5293186-5293243
10b	OR51B4*	0.03 (0.93-1.05)	CCACTGAGGTACAACTGCATTCTTACCAAT	TCCCGAGTGATGAACATAGGACTGGG	5279322-5279377
11b	OR51B4-HS4	0.05 (0.91-1.12)	GGGTGAGAAAGCTTAGATTCCATGAAGTATTACAGCATTTGGTAGT	CTTT TTGCACTCCAGGTCTTATTTTTACTGC	5272871-5272947
12b	HS-4*	0.05 (0.94-1.08)	CCGCAATGCTTCCTGCCCAT	TCAGGGCTCCAGCATGTAGAAATC	5263645-5263688
13b	HS-1	0.05 (0.83-1.07)	CACTGCCTTTAGCTAGGCCCCT	CCCTCATCACAGCTCAGCATAGTCC	5253777-5253823
14b	Hb ε*	0.05 (0.90-1.09)	GCTCTCAGGCCTGGCATCATGGTGCATT	TTACTGCTGAGGAGAAGGCTGCCG	5247663-5247714
15b	Hb γ G*	0.04 (0.88-1.04)	CGCCTAACACTTTGAGCAGATATAAGCCT	TACACAGGATTATGAAGTCTGAAAGGATTCC	5233901-5233960
16b	HbγG-HbγA	0.03 (0.93-1.09)	CCTTTTAGCCATCTGTATCAATGAGCAGATATAA	GCTTTACACAGGATCATGAAGGATGAAAG	5228976-5229038
17b	Hb γ A*	0.05 (0.89-1.10)	CCACAAAGGGTTTATATTGAGGGAAGTGTGTATGTGTAT	TTCTGCATGCCTGTTTGTGTTTG	5225968-5226029
18b	Hbψβ*	0.05 (0.84-1.09)	CATCTCCTTTAGATGGGGGAGGT	TGGGGAGAAGAGCAGTATCCTGC	5219835-5219934
19b	Hbψβ-Hbδ	0.03 (0.95-1.07)	CCCATACCATGTGGCTCATCCT	CCTTACATACATTTCCCATCTTTCACCCTAC	5215712-5215764
20b	Hb δ*	0.05 (0.91-1.14)	GGCTAATGCCCTGGCTCACAAGTACCATTGAGATCCTGGACTGTT	TCCTGATAACCATAAGAAGACCCTATTTCCC	5210725-5210800
21b	Hbβ(1b)	0.03 (0.95-1.08)	GTGTCATGATTTTCATGGAGGAAGTTAATATTCATCC	TCTAAGTATACCCAGACTAGGGCCATTCTG	5207650-5207716
22b	Hbβ(2)	0.03 (0.90-1.05)	CTGAGCCAAGTAGAAGACCTTTTCCCCTCCTACCCCTA	CT TTCTAAGTCACAGAGGCTTTTTGTTCCCC	5205743-5205811

Table 1 Names and sequences of probes used for MLPA for the β-globin gene cluster and flanking regions

No.	Name	Standard deviation (range)[1]	Upstream hybridising sequence	Downstream hybridising sequence	Positions chr. 11p15.4 UCSC Genome Browser (May 2004)
23b	Hbβ(3)	0.06 (0.91-1.14)	CAGAAGAGCCAAGGACAGGTACGGCTGTCAT	CACTTAGACCTCACCCTGTGGAGC	5204970-5205024
24b	Hbβ(4)	0.05 (0.94-1.11)	GGGTACAGTTTAGAATGGGAAACAGACGAA	TGATTGCATCAGTGTGGAAGTCTCAGG	5204244-5204300
25b	Hbβ(5)	0.03 (0.94-1.06)	GCTCGCTTTCTTGCTGTCCAATTTCTATTAAAGGTTCCTT	TGTTCCCTAAGTCCAACTACTAAACTGGGGG	5203333-5203403
26b	Hbβ(6)	0.03 (0.96-1.06)	GTTATCCTATTAAAACTGATCTCACACATCC	GTAGAGCCATTATCAAGTCTTTCTCTTTG	5193754-5193813
27b	OR51V1*	0.08 (0.76-1.11)	GCCTCACAATGGTGCACCGT	TTTGGCAAGCACCTTTCCCCCG	5177669-5177710
28b	OR52Z1P-OR51V1	0.05 (0.89-1.10)	CTAAAGTAATTCACAAACTCCACCTGGAAAGAAGAGTGGCTAT	TGCTCAAGCTATTCTTCAATGTGGCAGGGG	5166593-5166665
29b	OR52Z1P*	0.04 (0.92-1.10)	GCCTGGAATAAACTGTACATACGGCTCCTTTCT	TTCGCAATCACACTAATTCCCAGGATGTGTG	5156427-5156490
30b	OR51A1P*	0.05 (0.91-1.11)	GCCTTCCCACATTAGTTTAACTGCTTAGGTACTGAT	TCTCTTCTACTTCCTCAACCAACACTTTTAGAAC	5145679-5145748
31b	OR52A1*	0.06 (0.91-1.13)	CATTTACTTGCTGGTCCCTCCATTTCTCAATCCACT	TGTCTATGGTGCAAAGACCACACAGATTCG	5129268-5129333
32b	OR52A5-OR52A1	0.03 (0.89-1.10)	GGAGGTTACTGACAAGATGGAGTCACTGGCTCTTTATATGTAAAAAGAA	CAGGTCTCCTGAATAAGTCCAGACCC	5117804-5117878
33b	OR52A5-OR52A4	0.04 (0.94-1.07)	CAATAATGATAAGTTGAAGAATCTACTTTGGAAGATTGAGAT	GTTAAGGGCAGTTAGGAGCCTGC	5101566-5101630
34b	OR52A4*	0.02 (0.95-1.04)	GGCGTGCTATAGTATTCACTCGACAGCTAGTCACTTATAT	TGTAGTTGGAGTGACATTGCGGCCTGCCATTCTG	5098908-5098975

Table 1 (continued) Names and sequences of probes used for MLPA for the β-globin gene cluster and flanking regions
* with MLPA-F and –R common ends (fragments labelled in blue)
[1] calculated on 19 normal individuals

used for the beta-globin gene cluster is called M13-F and M13-R; the primers were labelled with ROX and added to a final concentration of 100 nM. A size standard (0.05 μl ROX 500, Applied Biosystems, www.appliedbiosystems.com) was added to each well and products were separated by capillary electrophoresis on the ABI 3700 sequencer (Figure 3).

Data analysis

For quantitative analysis, trace data from GeneScan (Applied Biosystems) were exported to Excel (Microsoft; www.microsoft.com) to calculate allelic loss in the patient samples tested [15]. In brief, two probes for unlinked loci were included per probe set as a reference in each sample. The height of each α- (or β-) globin cluster specific probe peak was divided by the sum of the heights of the two reference probe peaks to give a ratio. The median ratio for each probe across all samples was calculated and this value was used to normalise each probe to 1.0, which corresponds to a copy number of two. The upper threshold for deletions was set at 0.75 and the lower threshold for duplications at 1.25. The normalising factor for each sample was calculated as the mean value of the unaffected probes within a sample (defined as falling between 0.8 and 1.2) and dividing all values within that sample by this value.

All samples were tested at least twice. Detection of deletions is simplified by the fact that a series of flanking probes all generate a decreased signal. In cases of unlinked or single probe deletions, the region covering the MLPA probes is amplified and sequenced to rule out the presence of rare sequence variants under the ligation site.

Results

Design of the MLPA assay for α-thalassaemia rearrangements

Fragment analysis in the size range of 80-125 bp allows the simultaneous amplification of approximately 20 probes differing 2 bp in length. To maximise the number of loci that can be analysed in a single MLPA assay, we used a second primer set with common ends, to allow co-amplification of the two primer sets under the same PCR conditions. Probes were designed for each gene and pseudo-gene in the α-globin gene cluster, in the unique sequences L0 and L1, at the HS-40, the MPG gene, and more proximal at conserved sequences, respectively, 20 and 9 kb from the MPG gene (Figure 1). More distally, two probes were designed flanking the 3'HVR, known to be involved in many rearrangements of the α-cluster, and 15 probes at approximate intervals of 13-50 kb with the most proximal probe localised in the MSLN gene, known to be deleted in the alpha-thalassaemia mental retardation syndrome (ATR-16) [28,29]. The 35 probe pairs shown in Table 3 can detect all of the deletions described to date.

Of the 35 probes tested in triplicate on 14 healthy individuals, two gave a standard deviation of greater than 12% (Table 3, probes 17a and 21a). These probes were considered to be unreliable and were excluded from further calculations. To investigate the efficacy of the assay, DNA samples of nine carriers with known deletions were used as positive controls. All could be detected unequivocally and their extent could be confirmed (black bars in Figure 1B).

To demonstrate that duplications are also reliably detected, we tested a homozygote and heterozygote carrier for the common $-\alpha^{3.7}$ deletion, which results in the loss of the α_2-specific 3'UTR and a heterozygote for the so called α-triplication, which is characterised by a duplication of the α_2-specific 3'UTR. The results are summarised in Figure 3.

MLPA for β-thalassaemia rearrangements and HPFH

Similar to the α-cluster, 34 probes were designed for loci in the β-globin gene cluster and flanking regions. The region spans from the olfactory receptor gene OR52D1 to OR52A4 and covers an area of approximately 370 kb (Table 1, Figure 2). Most large deletions reported so far are located in this region and all should be detectable. In order to detect small deletions removing part or all of the β-gene [30], a subset of closely spaced probes (Figure 2B) surrounding the β-globin gene were selected. A third probe set was designed with different common ends (M13-F and –R) to allow amplification and detection with a third colour. Loci were selected in between some widely spaced probes and towards the centromere. Standard deviations for these probe sets were calculated on 19 healthy individuals; none showed standard deviations greater than 12%.

Positive controls (marked as black bars in Figure 2) were used to test the capacity of the MLPA assay to detect the deletions found by other methods in these patients. Probes covering deleted loci showed half the intensity of the surrounding probes, matching the positions and extensions of all the six known deletions.

Patient samples for α-thalassaemia

Our MLPA analysis revealed a large deletion involving the α-globin genes in 19 out of 38 patients. In the remaining 19 patients, 11 different deletions were detected, affecting either the α-globin genes or the regulatory elements known to be involved in globin gene expression. Six showed no resemblance to previously described deletions and were considered to be new ($--^{GZ}$, $--^{OH}$, $(\alpha\alpha)^{L}$, $(\alpha\alpha)^{ZW}$, $--^{AB}$, $--^{MK}$). One has been described (Dutch II α^0-thalassaemia) but the breakpoint position and deletion length could not be determined at the time [31]; FISH analysis performed in John Radcliff Hospital in Oxford revealed an approximate deletion length of 300 kb (Higgs, personal communication). Four deletions show similarity with previously described deletions (Figure 1B, last 4 deletions). One 14 year old Dutch girl showed haematological parameters typical for an α^0-thalassaemia carrier (MCV 65 fl, MCH 19.5 pg, RBC 5.79x1012 and positive HbH inclusion bodies test). The α-genes were structurally intact and we only detected the deletion of a single probe 5a (Figure 1A $(\alpha\alpha)^{ZW}$). The location of this probe coincides with one of the the cis-acting elements that regulate α-gene expression, known as the HS-40.

Patient samples for β-thalassaemia

Analysis of the 51 samples suspected for β-thalassaemic rearrangements or HPFH using MLPA revealed 10 different deletions in 31 out of patient 51 samples. In the remaining 20 samples a deletion of the probe sets tested could be excluded. In three cases, deletions were detected which do not match those described to date and are considered to be new. All three deletions, found in Dutch carriers, silence the expression of the complete globin gene locus and were named Dutch IV $(\varepsilon^{G}\gamma^{A}\gamma)\delta\beta^0$-, Dutch V $\varepsilon^{G}\gamma^{A}\gamma\delta\beta^0$- and Dutch VI $(\varepsilon^{G}\gamma^{A}\gamma\delta\beta)^0$-thalassaemia. One matched the HPFH-2 deletion and was confirmed by breakpoint PCR [8]. One sample belonged to a patient described in 1996 by Abels et al. [32] as a carrier of Dutch II $(\varepsilon^{G}\gamma^{A}\gamma\delta\beta)^0$-thalassaemia, however the deletion length was not determined at that time. Now the deletion length is estimated to be at least larger than 400 kb and the 5'breakpoint located between position 5408246 and 5387552 (UCSC Genome Browser, May 2004) (Figure 2A). Five deletions match the length and breakpoint locations of previously described deletions, two of which, the Croatian $(\varepsilon\gamma\delta\beta)^0$- (at least > 108 kb)

nr	Name	Standard deviation (range)[2]	Upstream hybridising sequence	Downstream hybridising sequence	Positions chr. 11p15.4 UCSC Genome Browser (May 2004)
35b	OR51A1OP **	0.05 (0.91-1.08)	CCCTCAACATCTTCAACCAAAATATCTGCAGAACC	TTCCTGAGGATCACCCAGCTTAGTATCATGCCTCT	5448616-5448685
36b	OR51I1**	0.04 (0.93-1.09)	CATGTCCCAATCCTTCCTCCAA	AGGAGCTTTTGGGGCCTTCAGCCTTC	5418327-5418374
37b	OR51K1P**	0.05 (0.88-1.05)	GGAAAAGGAAAATCCATTACTTCTGCCCCA	AAGTCACCATATCTGGTTAGAAGAGAAAGTCATGTA	5408181-5408246
38b	OR51Q1-OR51J1**	0.06 (0.91-1.11)	CAAGAGATAATGGTTGAATTCCCCTAAACTG	GTAAAACGCATCAATCCATATATCCAAGAAG	5387552-5387616
39b	79-45**	0.06 (0.88-1.09)	CAGTCCAGTAATTCTCAACAAGGCCACCAA	CACAATAGGGGAAGGATAGTCTCCTC	5350833-5350888
40b	45-OR51B6**	0.05 (0.93-1.09)	GTAGGGTCAAGGGGTATAGGGTAGCAAAAT	TTTCTTAAAGGATCAATGTAATGCCCTGCTCTGA	5333585-5333648
41b	OR51B3P-OR51B4**	0.09 (0.84-1.09)	GTTTTGAGATAAGAGTTTAGGGTAGAGTTGTGTATCT	GCACGATCTTAGACTACTTAAATAGAAACTCAGCA	5284744-5284815
42b	Hb ε- Hb γG**	0.06 (0.86-1.14)	GGAGAGAGACAAATAATTATTGTAGTTGCAGGCTATCAACAA	TACTGGTCTCTCTGAGCCTTATAACCTTTCAA	5244693-5244766
43b	Hbβ (6)- OR51V1**	0.06 (0.93-1.16)	GATCCCCATCCTTATAGCAATGGG	AGGGCTCATAGGCAAGTCATGTCATG	5184855-5184904
44b	OR51A1P- OR52A1**	0.05 (0.86-1.06)	GGGAAACCCTCCAGGGCATT	CATTGATTTGGGCAAATACTGTCTATGAGACTTC	5135864-5135917
45b	OR52A1-75**	0.11 (0.89-1.19)	GCAGGTAAGTACAGCACCTCCCCACA	CCAGAGACTCAACATCATGGTTTACAAGTTAC	5124990-5125047
46b	OR52A5**	0.09 (0.86-1.21)	GCCGACATTCAATGGCTCAGTCTT	CATGCCCTCTGCGTTTATACTAATTGGG	5110395-5110446
47b	OR52A4-OR52J1P**	0.09 (0.79-1.15)	CCACTATTATACTGGAACCCTGCTTGCATGGTGGCAAGG	TATGGCAGAAGGAAAGCTCTCAATAATCTTCCAATTA	5088458-5088533
48b	OR52J1P-OR52E3P**	0.07 (0.90-1.14)	GATTTTAGTTGTCTTATTTCCATCCGAATCATCTGTCAGTG	TCTTCCTCCTGCTGCCTTCCTAAACACATTCAGATTA	5078734-5078811
49b	OR52E3P-OR52S1P**	0.09 (0.86-1.17)	GTTGCCTGTGTTGTTTCAATAGGCAAAGGCTTTATGAGGGCACCA	CTCCTTGCCTGAAAAGAGCTTTCAATAGAAAATCCCC	5066851-5066932
50b	OR51L1**	0.05 (0.94-1.13)	GTGTCAGAACAAAGCAGATTCGTCTAGGAATTCTCCACAAGTT	TGTCCTAAGGAGGAGGTTTTAAGTAACCTCTGTCCTC	4977672-4977751

Table 2 Names and sequences of third probe set for the β-globin gene cluster designed to map the large deletions.
** with MLPA-2-F and –R common ends (fragments labelled in red)
[2] calculated on 16 normal individuals

and the Filipino β^0-thalassaemia (at least > 45 kb), were incompletely mapped. More accurate length estimations were obtained by MLPA, being between 128-143 kb and 109-122 kb, respectively. The other three showed similarity to the Dutch I 12.6 kb β^0-thalassaemia deletion (in seven independent patients of Dutch origin), the 13.4 kb Sicilian $(\delta\beta)^0$-thalassaemia deletion, which are also frequently found in the Mediterranean basin [33,34] and the 32.6 kb Indian $^G\gamma^A\gamma(\delta\beta)^0$-thalassaemia [35], found in four independent chromosomes from Surinam-Hindustani subjects (Figure 2B.).

Discussion

We describe the application of MLPA for high resolution mapping of deletions causing α- and β-thalassaemia. Using synthetic oligonucleotides, 35 loci along a genomic region of 700 kb from the tip of the short arm of chromosome 16, containing the alpha-globin gene cluster, could be analysed in two colours in a single reaction. More loci could be analysed simultaneously by using a third pair of amplification primers, labelled with a third fluorophore. This increased the number of probes to 50 loci spanning a genomic region of 500 kb on 11p15.4 and used to detect rearrangements causing β-thalassaemia or HPFH. Although slightly better results can be obtained when performing the PCR with the three sets of labelled universal primers separately, the ligation of all 50 probes was done in a single tube reaction. The fragment analysis was performed on a single sample of the three pooled PCR products per patient, which allowed the simultaneous analysis of 86 patient samples along with 10 normal controls in a 96 wells format fragment analysis run on the ABI 3700.

The use of chemically synthesised oligonucleotides instead of cloning the half-probes into M13 vectors, as originally described for MLPA [11], allows cheap and rapid probe development, which increases the flexibility of MLPA for characterising genomic rearrangements. Only 2 out of 85 probes (2%) were excluded from further calculations due to standard deviations higher than 12% when tested on a validation set of 12 wild type controls. The majority showed standard deviations between 0.05 and 0.08. Although these deviations seem significant, please note that due to the probe density rearrangements are mostly detected using a series of flanking probes (>2).

The ability to detect rearrangements in both regions was tested using positive controls, heterozygous for the seven most common α-thalassaemia deletions confirmed by gap-PCR, and for two less frequent mutations, Dutch I and $-\alpha^{7.9}$, confirmed by Southern blot analysis. By selecting 12 probes closely distributed along the 40 kb α-globin gene cluster, all of the common deletion types (except for the $--^{FIL}$ and $--^{THAI}$) could be distinguished from each other by MLPA. In our eyes, the simplicity, work-load, and cost make MLPA a superior alternative to Southern blot analysis when a single technique is preferred for the detection of deletions causing α-thalassaemia in a research setting. When desired, gap-PCR can be used for independent confirmation. Similarly, six positive controls were selected, based on confirmation by different methods (Southern blotting and/or direct sequencing of break point fragments) and tested for the beta-cluster probe set. All of the probes expected to be deleted were confirmed in the heterozygotes tested.

In 19 and 20 samples large rearrangements involving the α- and β-globin genes, respectively, could be excluded. Point mutations or micro-deletions affecting expression and located in between the probes, would not be picked up by MLPA. However, since iron levels were not known for some patients and anaemia due to iron deficiency could easily be mistaken for α- or

nr	Name	Standard deviation (range)[3]	Upstream hybridising sequence	Downstream hybridising sequence	Positions chr. 11p15.4 UCSC Genome Browser (May 2004)
1a	c16orf33	0.05 (0.93-1.10)	CACAGTCCTGGACCTGAAGAAGGCCATC	CAGAGATACGTGCAGCTCAAGCAGGAGCGT	45799-45856
2a	c16orf8/RHBDF1	0.05 (0.90-1.08)	CACGAGCAGCCTGCAGCGCAAGAA	GCCACCCTGGCTAAAGCTGGACATTCCCTC	54937-54990
3a	MPG	0.06 (0.84-1.07)	CAAGAGCTTTGACCAGAGGGAC	CTGGCACAGGATGAAGCTGTATGGCT	75563-75610
4a	c16orf35 (2) *	0.04 (0.97-1.14)	CTAACCATGCACACAGATCAGAAAACACC	CTGCTCAATGGTGCTGATTCCCCTGCT	90548-90603
5a	c16orf35 (HS-40)	0.09 (0.86-1.17)	GTGAATGGTACTGCTGATTACAACCTCTGGTGCTGCCTCCCCCTC	CTGTTTATCTGAGAGGGAAGGCCATGCCCAAAGTG	103695-103774
6a	c16orf35 (3) *	0.11 (0.84-1.18)	GTAAGCCGCGTAGCAGATACGCTGC	CAGCAACACGGGCGACCATGCTGAT	120541-120590
7a	L1	0.07 (0.86-1.12)	CCTGGACAATGAAGCACCAGGGCCAAC	CTCCATTTGCTACAGGGGACATCCT	132952-133003
8a	L0	0.04 (0.93-1.09)	GTGACCAGGGAGGGCCAGTTCATCTCGGTCTGAAAGAAGC	CCCAGATGAGCAAAGGATACACTGGCCTCCTG	139157-139228
9a	HBZ	0.05 (0.90-1.09)	CAGATCCAGTACATCTCCCCTCAGCGCTGGGTGGACCTAAC	CCTTGCTTTCTTGGAGGAAACCCAGGAATCCAG	142344-142417
10a	HBZP	0.05 (0.89-1.09)	CTCTAGGTCACCCTGTCATCACAGGGACAGGGAG	GTCAAGGACAGTCACTCCTGAGGCCA	152736-152795
11a	HBAP2	0.09 (0.92-1.21)	GTGCAAATGCAAGCGGCGTGGGACAAGTTC	CTGACTGGTGTGGCCGTGGTGCTGACCGAAAA	156636-156679
12a	HBAP1	0.04 (0.92-1.07)	GACTCAGAAATAAGCTGCCGTGGTGCTGTCTC	CTGAGGACAAGGCTAACACCAAGGCGGTCTGGGAGA	158659-158726
13a	ψα1-α2 *	0.09 (0.87-1.16)	CATCCCATGCTGAGGGAACACAG	CTACATCTACAACTACTGCCACAGGCTCTCT	160969-161022
14a	HBA2	0.11 (0.84-1.15)	GAAGATCCAACGGGGGAAGCATTG	CTAAGCTGGTCGGAGCTACTTCCTTC	165128-165176
15a	HBA1	0.04 (0.88-1.03)	GTGCCAAGAACTGGCTGGCTTTCTGCCTG	GGACGTCACTGGTTTCCCAGAGGTCCT	167825-167880
16a	HBQ	0.09 (0.71-1.05)	CTGGACAAGTTCCTGAGCCAC	GTTATCTCGGCGCTGGTTTCCGAGT	171054-171099
17a	3'HVR (tel) *	0.15 (0.78-1.26)	GATGGCTCTGAGGGTGACGCTGTCTGCTTAAGGC	CCAGGGAAACCCAGGTGCAAACTCACACTC	175806-175869
18a	3'HVR (cen) *	0.06 (0.89-1.09)	CTCAAGGGTGGCATGTGTACC	CCTGCAGAAACAGAGCGGATGAGGA	177548-177599
19a	LUC7L (2) *	0.06 (0.88-1.08)	GTCTGTTCAGCCTACCTTGGTCTCCATGACAATGACCGTCGC	CTGGCAGACCACTTCGGTGGCAAGTTACACTTGGGG	189203-189080
20a	LUC7L	0.05 (0.91-1.10)	GCTGAATGTGATCGGAGAACTGAGCTCGCCAAGAAGCG	GCTGGCAGAAACACAGGAGGAAATCAGTGCGG	198092-198161
21a	c16orf9 *	0.21 (0.81-1.37)	CTTGCGAAGGCACAGATTCCCCGTCCACAGCTCAC	GACCAGATGCACCAGCAGGAGTCCACATCGAGGAC	239560-239629
22a	PDIP	0.08 (0.77-1.11)	GAGCTGGCTGAGGAGTTTGGTGTGACGGAGTAC	CCTACGCTCAAGTTCTTCCGCAATGGGAACCGC	274507-274572

Table 3 Names and sequences of probes used for MLPA for the α-globin gene cluster and flanking regions

nr	Name	Standard deviation (range)[3]	Upstream hybridising sequence	Downstream hybridising sequence	Positions chr. 11p15.4 UCSC Genome Browser (May 2004)
23a	AXIN1	0.04 (0.92-1.09)	GGATGCACACGAGGAGAACCCTGAGAGCATCCTG	GACGAGCACGTACAGCGTGTGCTGAGGACA	288064-288127
24a	AXIN1 (2) *	0.06 (0.89-1.13)	CTCAGAGCCTCCTCAGCAGAAGTCTGAATACATCACTTACC	CAGAACCAGGTTGCCGGCTTACAGTCATGGT	321023-321094
25a	c16orf43 *	0.04 (0.95-1.07)	GGAGAGTCCATTGACAAGCTCTTAATCGCAGCTGCCCTTGCAGGGC	CTGTCCCTGAAGTCCCGAGTAATGGGATTT	350871-350946
26a	DECR2	0.09 (0.81-1.16)	CTTGTCCTTCAACGCCTTCAAGACCGTGATGGACATCGATAC	CAGCGGCACCTTCAATGTGTCTCGTGTGCTCTATGA	400278-400355
27a	KIAA0665 (1) *	0.06 (0.91-1.08)	GGACTTAACTAAGTACTTGGATCCCAGTG	GGCTCGGCGTGATCAGCTTTGAAGACTTC	451410-451467
28a	KIAA0665 (3) *	0.08 (0.86-1.13)	GAACGGATGCTTCTGTCTCTCTAGCACTGACCCTCTTGCCGCAAAG	CTGCACAGCATCCTCACTGATGAGGCGTTTGAGT	481111-481190
29a	KIAA0665 (2) *	0.08 (0.85-1.14)	CGCAACCTGAAGGAGCAGAACGAGGAGCTGAACG	GGCAGATCATTACCCTCAGCATCCAGGGCGCCAA	510466-510533
30a	SOLH *	0.08 (0.76-1.09)	GTCAAGAAGTTCGTCAGCTGCGACGTCATGCTGGAGCCTGGC	GAGTACGCTGTGGTGTGCTGCGCCTTCAACCA	542420-542493
31a	RAB40C *	0.05 (0.92-1.09)	CAGACAGGTGTGTGAGCGTACATTGCCCTCTCCAG	CTTCACAAACAAATGAGTCATCGAAGTGACC	583598-583663
32a	WFIKKN1 *	0.06 (0.85-1.11)	CGAGGACGTGCTCAAGGATGACAAGATGGGC	CTCAAGTTCTTGGGCACCAAGTACCTGGAGG	623788-623849
33a	RHBDL1 *	0.07 (0.86-1.10)	GATGAGCCAGGCCTAGGTGTCTACAAG	CGGTTTGTGCGTTACGTGGCCTACGAGATCCTG	666751-666810
34a	c16orf25 *	0.05 (0.89-1.09)	GTGCTGGTCCACGTACTCCTGTAG	CTCAGAAAGTTGCTCTTCAGCCATCGTG	712920-712982
35a	MSLN (ATR-16)	0.07 (0.85-1.14)	CTGAGGACATTCGCAAGTGGAATGTGACGTCCCTGGAGAC	CCTGAAGGCTTTGCTTGAAGTCAACAAAGGGCACGA	756403-756478

Table 3 (continued) Names and sequences of probes used for MLPA for the α-globin gene cluster and flanking regions
* with MLPA-F and –R common ends (fragments labelled in blue)
[3] calculated on 12 normal individuals

normal HbA_2 β-thalassaemia, we believe that negative samples may fall into this category.

Polymorphisms in the genome, interfering with probe annealing and ligation of the two probe pairs, may cause the loss of probe signal leading to a false positive MLPA result [11,14,36]. During the screening of patient samples suspected for α-thalassaemia one case showed repeatedly the deletion of a single probe 5a (in Figure 2A), named the $(\alpha\alpha)^{ZW}$ deletion found in an adopted child. This probe was selected in a highly conserved region of the HS-40, not containing the polymorphic sites known to be present in human populations [37]. Deletion of this regulatory element is expected to give a severe down regulation of α-gene expression of the affected chromosome. Even though nothing can be said about the extent of deletion, the fact that the HbH inclusion bodies test was positive and that no other rearrangements involving the α-genes were found, is strongly in favour of a deletion involving the HS-40. Whether or not this deletion, which is at maximum 30 kb in length, involves also HS-33 as found by Higgs et al. [38] needs further analysis. These types of deletions in human carriers may contribute to understand the mechanisms involved in regulation of downstream α-gene expression [39] and will be studied further.

In conclusion, MLPA is an attractive alternative for FISH analysis for screening large deletions, for example in ATR-16 syndrome [9,10]. The tiling paths of cloned probes currently available for cytogenetic analysis of the 16p13.3 and 11p15.4 are shown in Figure 1 and 2. The distribution of synthetic probes coincide with the available cosmids, and allows a higher resolution of mapping than the available BAC or PAC probes. In contrast to in situ hybridisation, no laborious cell culture to generate metaphase spreads is necessary. MLPA can be performed directly on (stored) DNA samples.

MLPA uses standard technology only, that is, hybridisation, ligation, PCR, and capillary electrophoresis. Since most diagnostic laboratories have these technologies operational, implementation of MLPA should be straightforward. The robustness, simplicity, and intrinsic redundancy (probe density) of this approach, and the additional specificity offered by the ligation step, make MLPA an attractive technique for the detection and characterisation of copy number variaton (deletions/duplications) in any region of the genome, particularly for high resolution analysis, and those regions not amenable to analysis by array comparative genomic hybridisation (array CGH) [40].

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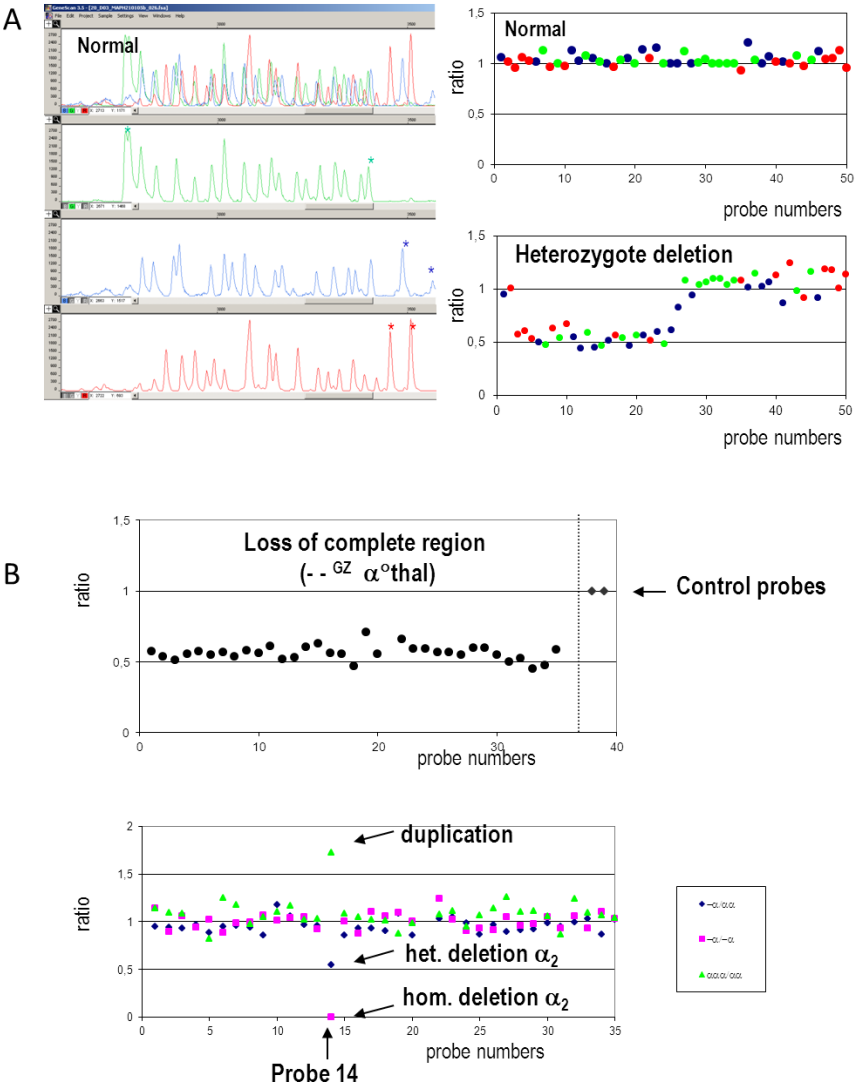

Figure 3. Peak patterns and scatterplots. **(A)** Peak patterns of an ABI 3700 fragment run of 50 β-specific probes labelled in three colours tested on a wildtype DNA sample. The upper window shows 17 probes in green, 17 probes in blue, and 16 probes in red, all between 80 and 125 bp in length. In the lower three windows, the peaks are split by colour. Within each probe set, two probes for unlinked loci were included as a reference (marked by an asterisk). At the right are scatter plots are shown of a wildtype and a heterozygote for the Dutch IV (εγ)δβ^0-thalassaemia deletion. The corresponding probes in different colours are ordered according to their position along the β-globin gene cluster on the x-axis, the y-axis showing the ratios calculated for each probe. **(B)** The first scatter plot represents the deletion of all the α-cluster specific probes on one allele as found in the - - ^{GZ} α^0-thalassaemia deletion; the two independent control probes are indicated on the right. The second scatter plot shows heterozygosity for a duplication, a deletion, and homozygosity for a deletion of probe 14, located in the 3'UTR of the α_2-globin gene, which demonstrates the capacity of the assay to detect copy number changes.

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Chapter 2.3

Thalassemia in Western Australia: 11 novel deletions characterized by Multiplex Ligation-dependent Probe Amplification

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Abstract

The number of immigrants in Western-Australia from many different areas where hemoglobinopathies are endemic has increased dramatically since the 1970s. Therefore, many different thalassemia mutations have been introduced in the country, which add a technological diagnostic problem to the serious burden of hemoglobinopathy management and to public health care.

Recently, we have developed a rapid and simple technique based on Multiplex Ligation-dependent Probe Amplification to detect deletions causing α- and β-thalassemia, δβ-thalassemia and Hereditary Persistence of Fetal Hemoglobin. A screening for (unknown) deletions was performed in a cohort of patients of different ethnic backgrounds preselected for their thalassemia phenotype, in which common deletions and point mutations were excluded.

Out of 37 cases suspected to carry a deletion, 27 were found to carry 17 different deletion types of which 6 causing α-thalassemia and 5 causing β-thalassemia were novel. For 3 of the deletions we have been able to characterize the exact breakpoint sequences by long-range PCR and direct sequencing.

These results show that MLPA is a suitable technology to detect unknown and uncommon deletions. These could represent a diagnostic problem when offering prevention to couples at risk presenting with unclear phenotypes and might result in a serious fetal problem when the deletion involves embryonic genes.

Introduction

Thalassemias are common hereditary disorders of hemoglobin characterized by a mild microcytic hypochromic anemia in the carrier. These are caused by quantitative reduction in gene expression and reduced amount of α- or β-globin chains in the erythrocytes [1-3]. Genomic deletions involving the α-globin gene cluster on chromosome 16p13.3 are the most common molecular cause of α-thalassemia. Rearrangements in the β-globin gene cluster on chromosome 11p15.4 account for approximately 10% of all β-thalassemias. In addition, deletions in the β-globin gene cluster are responsible for the majority of the δβ-thalassemias and Hereditary Persistence of Fetal Hemoglobin (HPFH) syndromes. Until now, more than 150 different deletions involving the globin gene clusters have been described (http://globin.cse.psu.edu/hbvar/menu.html).

The present Western Australian population consists for more than 30% of immigrants, mainly of northwest European extraction. However, since the 1970s an increasing number of immigrants are from Southeast Asia and Sub-Saharan Africa, where hemoglobin disorders are prevalent. Many thalassemia mutations have been introduced due to this migration profile and hemoglobinopathy is becoming an increasing health problem [4, 5]. Therefore, it is important to identify thalassemia carriers at the molecular level. This will enable the clinical geneticist to inform couples at risk and to offer prenatal diagnosis for prevention.

At present, gap-PCR, Southern blot or Fluorescent In Situ Hybridization (FISH) analysis [6-9] are the molecular tests commonly used to identify deletion types of thalassemia. However, the applicability of gap-PCR requires the definition of the breakpoints and is thus limited to known and well defined deletions. Southern blot is time consuming, technically demanding and requires available hybridization probes. Similarly, specific probes are needed for FISH analysis, which additionally requires laborious cell cultures to generate metaphase chromosome spreads. Furthermore, the resolution of FISH is low (>2 Mb).

Recently, we have developed an assay based on Multiplex Ligation-dependent Probe Amplification (MLPA)[10] to perform high resolution screening for unknown rearrangements on chromosome 11p15.4 and on the telomeric region of chromosome 16p, causing β- and α-thalassemia, respectively [11]. We have introduced the MLPA assay in our standard diagnostic protocol to be used for those thalassemic cases that remain uncharacterized after sequence analysis and gap-PCR for the common deletions.

In the current study, we screened a hematological well-diagnosed subset of thalassemia carriers of different ethnic backgrounds living in Western Australia for deletions in the globin gene clusters by using MLPA.

Materials and methods

Patients

Thalassemia patients of different ethnic backgrounds were collected for screening of copy number variations in the globin gene clusters. These patients were preselected for their thalassemia phenotype and common deletions and mutations were excluded (see Table 1).

Hematologic data were obtained by standard methodology using a Beckman-Coulter LH750 analyzer (Beckman-Coulter, Sydney, NSW, Australia). Serum ferritin was assayed on the Roche Modular E170 using electrochemiluminescence technology. Hemoglobin separation by High Performance Liquid Chromatography (HPLC) was performed using the β Thalassemia Short Programme on the Variant I™, Bio-Rad Laboratories, Hercules, CA, USA. HbH preparations were processed by incubating an aliquot of whole blood for 1 hour at 37oC with 1% Brilliant Cresyl Blue (Fronine, Riverstone, NSW, Australia) in buffered saline.

MLPA reaction

The MLPA reactions were performed according to a protocol based on the methods described before [10, 12], and conditions were adjusted for detection of copy number variation in the α- and β-globin gene clusters [11]. Products were separated by capillary electrophoresis on the ABI 3130 (Applied Biosystems) and data analyzed using GeneMarker (SoftGenetics). Threshold ratios for deletion and duplication were set at <0.75 and >1.3, respectively.

Breakpoint analysis

The exact breakpoint sequences of several deletions in the α-globin gene cluster were characterized by multiplex long-range PCR. A set of unique primers was designed for each case, consisting of 3 to 5 primers in the breakpoint area on each side of the deletion, which was determined by MLPA (Table 2). Breakpoint areas are defined as the region between the position of the last MLPA probe present and the first probe deleted. GC percentage, melting temperature and length were similar for all primer pairs. All primers were ordered at Biolegio, Nijmegen, The Netherlands. For the PCR reaction, the TaKaRa LA Taq Kit Ver.2.1 (Cat.# RR013A;TaKaRa Bio Inc. Japan.) was used and reactions were performed according to the manufacturer's instructions, applying the following PCR conditions: hotstart of 2 min at 94 °C, 30 cycli of denaturation for 20 s at 94 °C and elongation for 5 min at 68 °C followed by a final elongation step of 10 min at 68 °C. Specific PCR products containing the breakpoint fragment were used for further analysis. After fine-mapping of the breakpoints using restriction enzyme analysis, sequence primers (Table 2)

No.	Ethnic group	Sex	Age	Hb	MCV	MCH	HbA_2 %	HbF %	Ferritin	IB	Deletion of probe #	Mutation	Fig.1
1	Chinese	F	52	12,1	68	22	5,2	0,5	282	-	None	None	
2	Caucasian	M	37	14,8	74	25	5,4	0,5	274	-	None	None	
3	unknown	F	52	12	77	26	5,9	0,4	17	ND	None	None	
4	Sudan	F	6	11	74	26	2,5	9,6	47	ND	dup. 16b		
5	Asian	F	44	14	71	22	2,7	0.7	40	-	None	None	
6	Italian	F	48	12,4	71	22	2,2	<1.0	88	-	None	None	
7	unknown	F	17	11,1	72	22	2,6	<1.0	47	-	None	HBA2:c.301-24delGinsCTCGGCCC	
8	unknown	F	8	7,6	62	19	2,7	<1.0	101	-	None	None	
9	Spanish	F	19	11,5	68	22	2,5	0.4	50	-	None	None	
10	unknown	F	17	11,6	67	21	2,3	<1.0	20	-	10a-15a		A*
11	Arab	F	23	11,9	66	21	2,6	0.2	31	-	10a-15a		A*
12	Indonesian	F	16	12,9	65	20	2,5	<0.5	36	+	10a-15a		A*
13	Indian	M	46	12,3	66	21	2,5	0.3	100	-	10a-16a		B
14	unknown	M	8	11,7	63	20	2,5	<1.0	30	+	10a-16a		B
15	unknown	M	46	14,7	66	21	2,6	<0.5	106	+	8a-16a		C
16	unknown	M	45	13,2	69	22	2,6	<0.2	119	-	8a-18a		D
17	Caucasian	M	46	14	73	24	2,7	0.5	139	-	5a		E*
18	Chinese	M	24	13,3	69	23	2,9	7.4	114	-	5a		E*
19	Mediterranean	F	36	12,9	66	21	2,5	<1.0	31	-	1a-20a		F
20	Arab	F	3	12,3	69	23	2,7	0.9	55	-	25b		G
21	African	F	15	14,3	65	21	6,9	7	54	ND	23b-24b		H
22	Sierra Leone	F	28	10,6	64	21	7,3	8,2	70	ND	23b-24b		H
23	unknown	F	40	11,3	70	22	7,2	5,9	53	ND	23b-25b		K
24	Chinese	F	29	11,8	70	22	3,6	19,5	87	-	22b-26b		L
25	Chinese	M	58	13,1	78	25	4,4	19,5	441	-	22b-26b		L
26	Chinese	M	58	13	75	24	5,4	14,7	112	-	22b-26b		L
27	Mediterranean	M	73	9,7	69	22	2,8	8,5	97	-	20b-25b	13.4 kb Sicilian	M
28	unknown	M	23	14,3	67	22	2,8	11,2	88	-	20b-25b	13.4 kb Sicilian	M
29	Italian	F	57	11,5	72	23	2,6	11,1	ND	-	20b-25b	13.4 kb Sicilian	M
30	Italian	F	29	11,5	63	20	2,9	9,6	78	-	20b-25b	13.4 kb Sicilian	M
31	Italian	M	46	12.9	65	21	4.9	0.7	105	-	20b-25b		M
32	Caucasian	M	23	13,5	59	19	3	1.8	82	-	16b-25b		N
33	Thai	?	38	12,1	74	25	1,9	21,6	12	-	16-b29b		O
34	unknown	M	71	14,3	73	24	2,4	9,5	70	-	17b-26b		P
35	Mediterranean	F	42	11,1	68	21	2,4	11,4	13	-	17b-26b		P
36	Chinese	F	43	13,8	75	23	2,6	12	231	ND	17b-29b		Q
37	South Europe	F	50	12,3	64	20	3,1	<1.0	50	-	None	HBB:c.444+112A>G	

Table 1 Overview of ethnicity and hematological parameters of the patients suspected of having a deletion in one of the globin gene clusters. (Hb: hemoglobin concentration in g/dL, MCV: mean corpuscular volume in fL, MCH: mean corpuscular hemoglobin, ferritin in ng/mL, +/- = positive/negative for Inclusion Bodies (IB) test, showing presence of HbH, ND: not determined, *: breakpoint characterized).

were designed as close to the breakpoint as possible, in order to bridge the breakpoint in a single sequence reaction. Sequencing reactions were performed in a Tetrad PCR Machine and separation was performed on an ABI 3730 DNA Analyzer (Applied Biosystems) and results were analyzed using Chromas (Technelysium) and SeqScape (Applied Biosystems).

Results

In most diagnostic laboratories, a subset of thalassemia phenotypes in which no molecular defect has been found by the common techniques remains uncharacterized. However, these cases remain suspected of a possible thalassemia based on persisting microcytic hypochromic anemia in spite of normal iron status. In this study, a cohort of 37 patients that remained uncharacterized at the Hematology and Molecular Department of the Sir Charles Gairdner Hospital in Nedlands, Australia, were evaluated by MLPA. The cohort consisted of two groups. The first group of 16 patients showed moderate anemia and clear microcytic hypochromic parameters, normal HbA_2 and HbF fractions and normal iron levels, indicative for α-thalassemia or mild β^+-thalassemia. The second group of 21 patients presented with normal or elevated HbA_2 and/or HbF levels, compatible with β^0-thalassemia traits, $\delta\beta$-thalassemia or Hereditary Persistence of Fetal Hemoglobin (HPFH) syndrome.

At first, all 37 samples (Table 1) were screened for the 7 most common deletions of the α-globin gene cluster ($-\alpha^{3.7}$, $-\alpha^{4.2}$, $-(\alpha)^{20.5}$, $--^{MedI}$, $--^{SEA}$, $--^{FIL}$ and $--^{THAI}$) by gap-PCR [6]. Subsequently, point mutation analysis of the α- or β-globin gene was performed by direct sequencing. Sequence analysis revealed a mutation in the poly-A-signal of the β-gene (HBB:c.444+112A>G) in case 37. Sequence analysis of the δ-gene showed no abnormalities.

MLPA

All patients were screened for deletions in the α- and/or β-globin gene clusters by MLPA. A total number of 27 patients was found to carry 17 different deletions. A schematic overview of all deletions is shown in Figure 1.

In 9 patients no mutation was found which could explain the hematological parameters, including a γ-globin triplication (#14) and heterozygosity for the African polymorphism (HBA2:c.301-24delGinsCTCGGCCC) (#23).

Three individuals (#10, #11 and #12) showed the same type of deletion in the α-globin gene cluster, ranging from probe 10a to probe 15a (Fig. 1A), thereby deleting both α-genes. The exact breakpoint of this deletion was determined by long-range PCR, restriction enzyme analysis and direct sequencing (described below in detail). Two cases (#13 and #14) showed a slightly larger deletion in the α-globin gene cluster, ranging from probe 10a to 16a (Fig. 1B). Three different deletions involving the whole α-globin gene cluster were found in case #15, #16 and #19, ranging from probes 8a -16a, 8a -18a and 1a -20a, respectively (Fig. 1C, D and F). Two patients (#17 and #18) showed a deletion limited to the single probe 5a (Fig. 1E), which is located in the multi-species conserved sequence region (MCS-R). This region contains the major regulatory element for the α-globin genes. Breakpoint analysis of these cases is described below.

Deletions involving the β-gene alone were found in 7 cases. One patient (#20) with normal HbA_2 showed no abnormalities in the α-globin gene cluster, but a deletion involving probe 25b in the β-globin gene (Fig.1G). The -619 bp deletion [13] gives the same MLPA output, however, PCR for this specific deletion as described by Baysal et al. [14] was negative. Two cases

Primer	5' > 3' sequence	location on chromosome 16
10-15F	GTGGAGTAGGCTTTGTGGGGAACTT	149486-149510
10-15R	CAGGTGTTTCTTCAGGGCAGTGAAC	169762-169786
10-15seq-F	GTCCTGTGCGTCCTTTCAAT	151167-151186
10-15seq-R	CTCAACCTCCCGAGTAGCTG	169136-169155
MCS-F-A	GCTGGCCCATAAGAAGGAGGTTAATAAGCACACCC	100477-100511
MCS-F-B	CTCAGAATAAGGGAACAATGTCCAAGGAA	97892-97920
MCS-R	GATTCTTTTTAATGTGGTGTTTGCACTGAGG	107300-107330
MCSseq-F-A	CCCGCCAACATCTGTATCAT	102507-102526
MCSseq-F-B	TGCATGCTACTCAGCAAAGG	99624-99643
MCSseq-R-B	TGCTTCAGTGGCATCTGGTA	107077-107096

Table 2 Sequences and locations of the primers used for long-range PCR and sequencing analysis. Locations are according to the UCSC Genome Browser.

(#21 and #22) showed a deletion of probes 23b and 24b (Fig. 1H). A second deletion was found in case #23, ranging from probe 23b to 25b (Fig. 1K). Three unrelated individuals of Chinese origin (#24, #25 and #26) showed the same type of deletion in the β-gene, from probe 22b to probe 26b (Fig. 1L).

A deletion of probes 20b-25b, compatible with the 13.4 kb Sicilian deletion (Fig. 1M), was found in five cases (#27 to #31). Gap-PCR to detect this deletion [15, 16] was performed as described by Craig et al.[17] in the presence of a positive control and giving the corresponding band of 1150 bp resulted positive for four of the samples (#27 to #30) , but negative for case #31.

Four different deletions involving the $^{A}\gamma$-, δ- and β-globin genes were observed in 5 cases. The first was found in one of the cases who was initially suspected of α-thalassemia (#32), and revealed a deletion involving probes 16b-25b (Fig 1N). The largest $^{A}\gamma\delta\beta$-deletion in case #33 involves probes 16b-29b (Fig. 1O). In two unrelated individuals the deletion ranged from probe 17b to 26b (Fig. 1P, #34 and #35). A larger deletion was found in the third patient (#36), ranging from probe 17b to 29b (Fig. 1Q).

Breakpoint analysis

Three unrelated individuals (#10, #11 and #12) of different ethnic origin showed a deletion of probes 10a-15a in the α-globin gene cluster (Fig. 1A), indicating the deletion breakpoint region between probes 9a and 10a at the 5' end and between probes 15a and 16a at the 3'end. A similar deletion was previously found in a Dutch individual of mixed ethnic backgrounds, described as $--^{GB}$ by Harteveld et al. [11]. A 3 kb breakpoint fragment was obtained by PCR with primers 10-15F and 10-15R (Table 2) for the three Australian and the Dutch case. Primers 10-15seq-F and 10-15seq-R (Table 2) were used for direct sequencing. Analysis revealed that all three unrelated Australian cases and the Dutch $--^{GB}$ deletion have exactly the same breakpoint sequence. The breakpoint is located in an 8 nt overlapping sequence which is between position 151902 and 151909 at the 5' end and between position 168673 and 168680 at the 3' end, indicating a deletion length of 16771 bp (Figure 2).

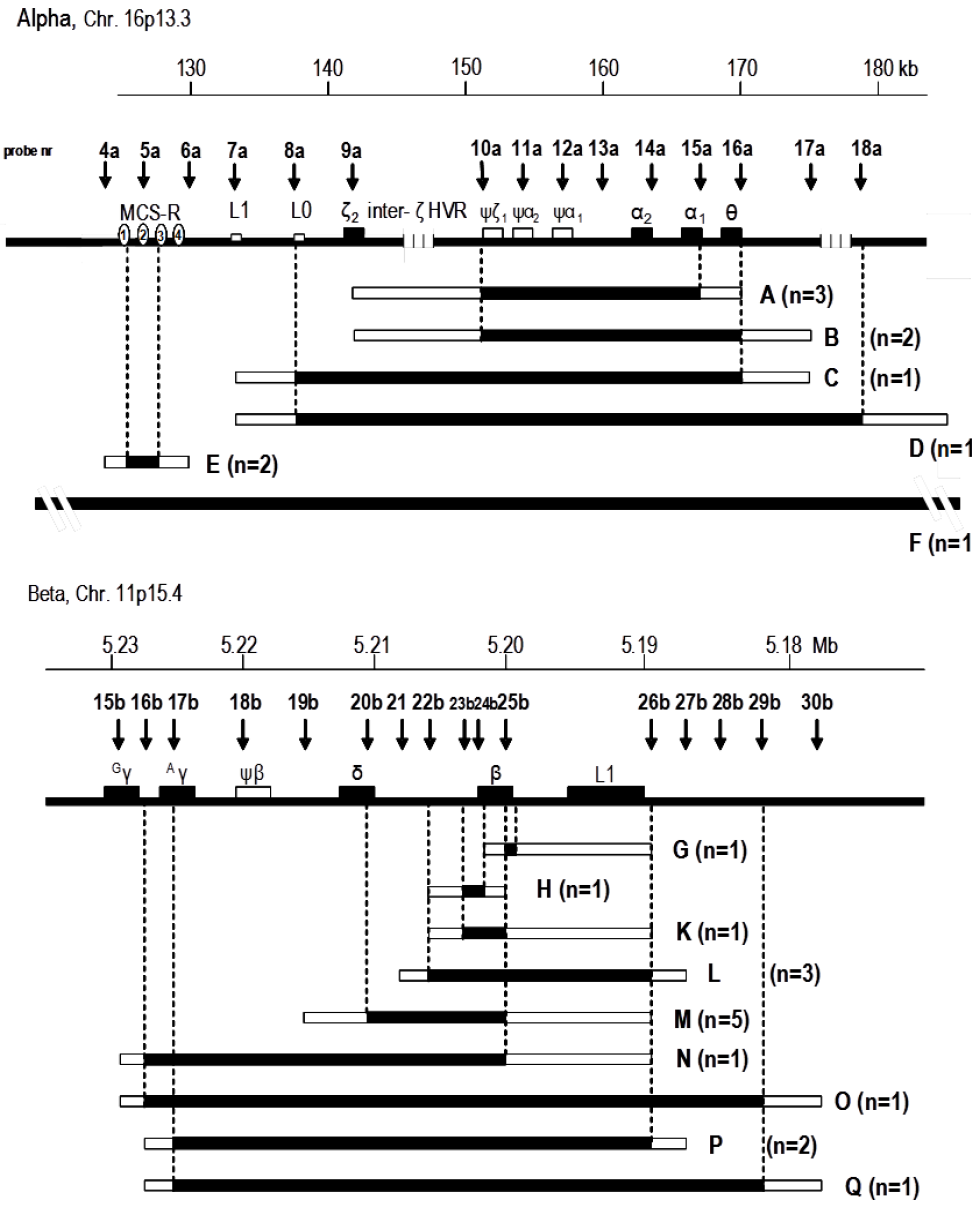

Figure 1. Schematic overview of the α- and β-globin gene clusters. The arrows and numbers indicate the locations of the probes. The deletions are indicated as solid bars, the open ends indicate the region where the breakpoint is located. (n = number of independent chromosomes).

A deletion of probe 5a only was found in two patients (Fig. 1E), indicating the involvement of the HS-40 site, which is part of the multi-species conserved region (MCS-R) of the α-globin gene cluster. By designing more MLPA probes in the breakpoint area, it was shown that the length of the deletion in the two Australian cases was different from the similar $(\alpha\alpha)^{LMB}$ [18] and $(\alpha\alpha)^{ZW}$ [19] (Figure 3). Primers MCS-F-A, MCS-F-B, and MCS-R (Table 2) were designed in the breakpoint regions of both cases #17 (primer A) and #18 (primer B). Two breakpoint PCR fragments of 3.5 and 2.5 kb, respectively, were obtained. Sequencing analysis with primer MCSseq-F-A for case #17 showed that the deletion ranged from position 103192 to 106554 (3362 bp deleted). For case #18, primers MCSseq-F-B and MCSseq-R-B were designed for sequencing analysis. Results showed that the deletion of 6710 bp ranged from position 99993 to 106703 (for all primer sequences see Table 2).

Discussion

Sequence analysis

Case #37 was initially suspected to have α-thalassemia, because of normal HbA_2 level. However, no mutation or deletion was found in the α-globin gene cluster. Large deletions involving the δ- and β-genes were excluded as well. Sequence analysis of the β-globin gene revealed a mutation in the poly-A signal: HBB:c.444+122A>G. Since the HbA_2 level was normal, and β-thalassemia carriers with normal HbA_2 level because of a coexisting δ-thalassemia are not that uncommon [20], sequencing of the δ-gene was also performed, but no δ-gene mutations were found. Because this mutation has been described only once in a case in combination with HbE heterozygosity, it is unknown whether the poly-A mutation alone causes a β^+-thalassemia with normal HbA_2 level [21]. Other types of mutations in the poly-A signal of the β-globin gene are associated with elevated or borderline-elevated HbA_2 level, which could therefore be misdiagnosed as α-thalassemia. It is therefore important to perform extensive molecular research of both the α- and β-globin gene clusters in these particular cases.

MLPA

All deletions found in the α-globin gene cluster are α^0-thalassemia deletions, with complete loss of function of both α-globin genes, and have not been described before, except for the three cases with the $--^{GB}$ deletion (#10, #11 and #12, as described below). Both the deletions ranging from probe 10a-16a (#13 and #14) and the $--^{GB}$ deletion leave the ζ-globin gene intact, indicating that they are not lethal in early embryonic life and are potentially at risk for Hb Bart's Hydrops Fetalis syndrome in combination with another α^0-thalassemia allele. As can be seen in Table 1, HbH inclusions were not found in all α^0-thalassemia cases. Finding HbH inclusions is time consuming and dependent on freshness of the blood sample and skills of the technician. Therefore, HbH inclusions might be missed in some cases.

Four types of $^A\gamma\delta\beta$-deletions were observed in 5 unrelated individuals (#32 to #36) of different ethnic origin. All of them had normal HbA_2 levels and four had elevated HbF levels. In contrast to the 4 other cases, HbF was 1.8% for #32, which may indicate that the deletion in this case disrupts the function of the $^G\gamma$-globin gene as well. Several deletions in this region have been described (e.g. Malay, Chinese, Yunnanese). However, only breakpoint sequence analysis can prove whether these deletions identified by MLPA correspond to known deletions. From a clinical

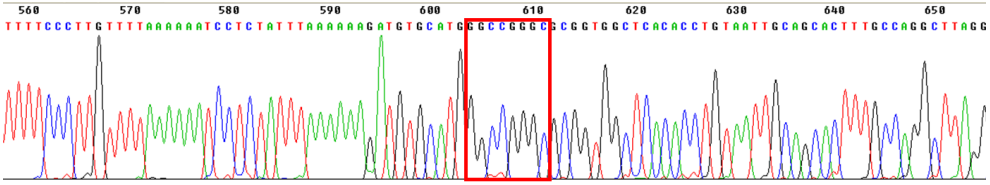

Figure 2 Sequence analysis of the --^{GB} fragment. The breakpoint area of 8 nt is indicated by the red square. The G's flanking the breakpoint region at the 5' and 3' ends are located at positions 151901 and 168681, respectively.

point of view, it is important to be able to screen for this type of deletions, as they might cause complications for the fetus during pregnancy when they extend to the embryonic ε-globin gene [22, 23].

A deletion of probes 20b to 25b in the β-globin gene cluster was found in five individuals. Four of them presented with microcytic hypochromic anemia, normal HbA_2 and elevated levels of HbF, characteristic for a δβ-thalassemia. The 13.4 kb Sicilian type δβ-deletion was confirmed by gap-PCR. Three carriers were of Mediterranean origin, the ethnicity of the other carriers was unknown. The fifth case, of Italian origin, presented with elevated HbA_2 and normal HbF level, in contrast to the other four cases. Although MLPA results were similar to the other patients, the 13.4 kb Sicilian deletion could not be confirmed in this case. It is likely that the deletion in case #31 is smaller than the 13.4 kb Sicilian type, thereby leaving the δ-globin gene intact, which would explain the elevated HbA_2 level.

A single-probe (25b) deletion in the β-gene was found in one of the suspected α-thalassemia carriers (#20). A known deletion of 619 bp maps to this location, however, gap-PCR showed the presence of the normal allele only. This indicates that the deletion in this patient is larger, with a maximum of 10.5 kb (distance between MLPA probes 24b to 26b). A large L1-repeat area (~6 kb) is located between the positions of probe 25b and 26b, which makes it difficult to design an appropriate MLPA or PCR assay to determine the breakpoints of this deletion.

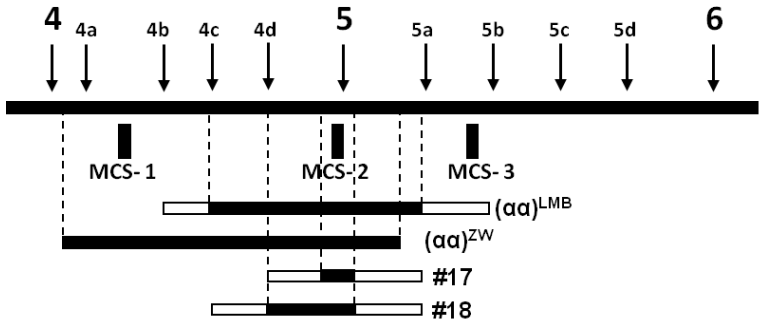

Figure 3 Schematic overview of the deletions in the multi-species conserved sequence region (MCS-R). Numbers and arrows indicate the location of the MLPA probes. Since all patients showed a deletion of MLPA probe 5 only, probes 4a-d and 5a-d were designed to check whether the deletions in these patients were similar. The solid bars indicate the deletions, the open ends represent the breakpoint areas.

Breakpoint analysis

The 16.7 kb --^{GB} deletion (Fig. 1A), previously reported [11] and now characterized at the molecular level, was found in 3 unrelated individuals living in Australia and in 1 Dutch patient, taking away both α-genes as well as the $\psi\zeta$, $\psi\alpha_2$ and $\psi\alpha_1$ genes. Since they all have exactly the same breakpoint sequence, it is likely that this is a single molecular event passed on by a common ancestor. This suggestion is supported by the fact that sequence analysis showed presence of a known SNP (rs 3760053, T>G) and a novel base substitution (position 151791, C>T) upstream of the 5' end breakpoint of the deletion found in all deletion carriers. As both breakpoint ends are located within and surrounded by Alu repeat sequences, the deletion may have occurred due to an unequal crossover event between partially homologous regions.

Two new types of MCS-R deletions were found in cases #17 and #18. In the ~3.5 kb PCR product obtained from case #17, a 39 bp 'orphan sequence' was found, which is not of human origin or any other species described in the UCSC Genome Browser and NCBI databases. The 3' breakpoint is located within an Alu repeat area; however, the 5' breakpoint and the region surrounding the breakpoint are not located in a repetitive sequence. Furthermore, alignment of both sequences show poor similarity, indicating that this deletion event might have occurred due to a non-homologous break and ligation. Both breakpoint ends of the deletion in case #18 are located within an Alu repeat sequence. The areas surrounding the breakpoint ends contain numerous repeated sequences, thus it is likely that this type of deletion arose due to recombination between the repeats. Both deletions involve only the MCS-2 sequence, suggesting that this is sufficient to cause an α-thalassemia phenotype. However, a patient with a homozygous deletion of the MCS-R has been recently described. This patient presented with HbH disease, suggesting that absence of the regulatory element does not completely silence the expression of the α-globin genes [24]. More cases with deletions in the MCS-R need to be studied for a better understanding of the mechanisms underlying the role of this element.

No mutations detected

In 9 out of 37 cases, the molecular findings could still not explain the phenotype. The thalassemic parameters in these patients might be caused by a mutation in the regulatory element or locus control region, which have not been screened for in this study. All patients were screened for large deletions in the α- and β-globin gene cluster, because the majority of thalassemias is caused by mutations and rearrangements within these regions. Deletions outside the globin gene clusters have not been screened for. Unfortunately, family members of the patients were not available for this study, thus it could not be determined whether the phenotype is hereditary. An increase in copy number of the γ-genes was found in case #4. This patient presented with microcytic hypochromic anemia, normal HbA_2 and HbF 9.6%. However, duplication of the γ-genes does not necessarily explain 9.6% HbF in adults [25]. Sequence analysis of the promoter region of the γ-genes showed no abnormalities which are indicative for a non-deletional type of HPFH. The increased HbF level might be caused by mutations elsewhere in the genome, which are not linked to the β-globin gene cluster (i.e. to 6q23 [26]), or by the presence of bone marrow malignancies [27] or erythropoietic stress [28]. In three cases (#1 to #3) with normal HbF and elevated HbA_2 levels, the phenotypes might be explained by acquired causes of increased levels of HbA_2. These include hyperthyroidism and side effects of HIV treatment [29]. However, these conditions are not usually associated with microcytosis. Furthermore, β-thalassemia caused

by mutations in unlinked erythroid specific transcription factors [30] might lead to increased HbA_2 levels. In five patients (#5 to #9) suspected to have α-thalassemia, no deletions or pathogenic mutations were found in the α-globin gene cluster and large deletions in the β-globin gene cluster were excluded as well. Although the reason for the microcytic hypochromic anemia remains unknown for these cases, iron deficiency seems the most likely alternative.

Concluding remarks

In this study, identifying 6 novel α- and 5 novel β-thalassemia deletions, we have shown that MLPA is a suitable method to detect unknown and uncommon deletions and in particular to characterize those cases which remain unsolved after performing standard diagnostics. Many deletions which cannot be detected by other methods (PCR, Southern blot or FISH) can easily be overlooked if no elevated HbF fraction is present. It is therefore important to define thalassemia traits at the molecular level, especially in areas where, due to multi ethnic migration, hemoglobinopathies are becoming a health problem.

By education, carrier diagnostics and molecular characterization, it is possible to offer an informed reproductive choice to couples at risk who may request prevention by prenatal diagnosis. In addition, it is important to characterize deletions which involve the embryonic ε- and ζ-globin genes because these are clinically relevant in the heterozygous state, as they can cause severe complications during the pregnancy.

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Chapter 2.4

Fine-tiling array CGH to improve diagnostics for α- and β-thalassemia rearrangements

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Abstract

Implementation of multiplex ligation-dependent probe amplification (MLPA) for thalassemia causing deletions has lead to the detection of new rearrangements. Knowledge of the exact breakpoint sequences should give more insight into the molecular mechanisms underlying these rearrangements, and would facilitate the design of gap-PCRs.

We have designed a custom fine-tiling array with oligonucleotides covering the complete globin gene clusters. We hybridized 27 DNA samples containing newly identified deletions and nine positive controls. We designed specific primers to amplify relatively short fragments containing the breakpoint sequence and analyzed these by direct sequencing.

Results from nine positive controls showed that array comparative genomic hybridization (aCGH) is suitable to detect small and large rearrangements. We were able to locate all breakpoints to a region of approximately 2 kb. We designed breakpoint primers for 22 cases and amplification was successful in 19 cases. For 12 of these, the exact locations of the breakpoints were determined. Seven of these deletions have not been reported before.

aCGH is a valuable tool for high-resolution breakpoint characterization. The combination of MLPA and aCGH has lead to relatively cheap and easy to perform PCR assays, which might be of use for laboratories as an alternative for MLPA in populations where only a limited number of specific deletions occur with high frequency.

Introduction

Thalassemias are hereditary microcytic hypochromic anemias characterized by abnormalities in hemoglobin production due to absent or reduced expression of either the β-globin gene (HBB, MIM# 141900), leading to β-thalassemia, or the α-globin genes (HBA1, MIM# 141800 and HBA2, MIM# 141850), giving rise to α-thalassemia. The majority of all α-thalassemias (~80-90% of cases) is caused by genomic deletions involving the α-globin gene cluster on chromosome 16p13.3, whereas rearrangements in the β-globin gene cluster on chromosome 11p15.4 account for approximately 10% of all β-thalassemias and Hereditary Persistence of Fetal Hemoglobin (HPFH) syndromes. Until now, more than 70 different deletions in the α- and more than 60 involving the β-globin gene cluster have been described (Harteveld and Higgs, 2010; Higgs, 2009; Thein and Wood, 2009).

Historically, diagnostic methods used to detect these deletions include Southern blot, fluorescent in situ hybridization (FISH) and gap-PCR. However, Southern blot is dependent on the availability of hybridization probes, such as FISH, which in addition is time consuming, laborious and has a low resolution (>2 Mb). Therefore, these methods are less suitable for a routine application in a diagnostic setting for hemoglobinopathies. Gap-PCR is used to amplify a sequence across a deletion breakpoint in presence of a positive control sample. Products are analyzed by gel electrophoresis and presence of the correct mutant band is indicative for carriership of the particular deletion. Yet, this method can only be applied to deletions with known breakpoints.

Recently, an assay based on multiplex ligation-dependent probe amplification (MLPA) (Schouten et al., 2002) was developed to screen for unknown rearrangements on chromosome 11p15.4 and on the telomeric region of chromosome 16p (Harteveld et al., 2005). We have introduced the MLPA assay in our standard diagnostic routine for those cases that remain negative for mutation detection after gap-PCR and sequencing analysis. Due to the implementation of MLPA for the detection of deletions and duplications in the α- and β-globin gene clusters, a range of new

rearrangements have been identified (Babashah et al., 2009; Gallienne et al., 2009; Harteveld et al., 2005; Lee et al., 2010; Liu et al., 2008; Phylipsen et al., 2010a; So et al., 2009). However, the exact breakpoints of these deletions remain unknown. Characterizing the breakpoints would facilitate elaboration of the gap-PCR method, which might be of use for laboratories as an alternative for MLPA as diagnostic method in populations where only a limited number of specific deletions occur with high frequency.

The standard method to characterize such rearrangements is to randomly design primers in the breakpoint region and perform PCR across the breakpoint. The breakpoint region is determined by the most proximal and most distal MLPA probe still present and the first probe involved in the deletion. The distance between MLPA probes generally range between 3 and 10 kb that requires the use of long-range PCR and primer walking to sequence the breakpoint completely (Chinault and Carbon, 1979). Since this procedure is breakpoint specific and rather time consuming, other methods are needed to improve the resolution of the breakpoint position and to develop primers more closely located toward the breakpoint. In addition, it is necessary to develop techniques for quick and precise detection of deletions for diagnostic purposes.

The array comparative genomic hybridization (aCGH) technology is a promising method in characterizing rearrangements. The development of aCGH in the past few years has facilitated the identification of the molecular basis of many genetic diseases (Shinawi and Cheung, 2008). Originally, aCGH was developed as a research tool for the investigation of genomic imbalances in cancer (Pinkel et al., 1998) and has become an essential and routine diagnostic tool in genetics to search for copy number variation. The high resolution, simplicity, high reproducibility, and precise mapping of imbalances are the most significant advantages of aCGH over traditional cytogenetic methods.

A fine-tiling oligonucleotide array was first used for breakpoint analysis of deletions causing neuroblastoma at sub-kilobase resolution on four different chromosomes (Selzer et al., 2005). The same type of array was used to delineate the deletion breakpoints in chromosome 1p for patients with Wilms tumors (Natrajan et al., 2007). More recently, fine-tiling arrays have been used to unravel complex rearrangements involving the BRCA1 gene (Rouleau et al., 2007), the MECP2 gene (Carvalho et al., 2009), the GJB2 and GJB6 genes (Wilch et al., 2010), the STK11 gene (Resta et al., 2010) and to characterize different balanced and unbalanced rearrangements in a group of 12 patients with phenotypic abnormalities (Lindstrand et al., 2010). These studies not only confirmed the power of the fine-tiling arrays to find breakpoint regions, but also underline the increasing importance of fine-tiling array technology as a follow-up after MLPA for the delineation of deletions and breakpoints in common and rare rearrangements.

For the current research, we designed a custom fine-tiling array for high-resolution determination of deletion breakpoints in the α- and β-globin gene clusters. First, we evaluated the applicability of the fine-tiling aCGH technology by testing nine DNA samples from patients with known deletions. In addition, DNA samples of 27 thalassemia patients who were prescreened by MLPA were hybridized to the array. PCR and sequencing were used to determine the exact deletion breakpoints in patients. This knowledge is important to unravel the mechanisms leading to these deletions, and facilitates the design of a gap-PCR assay for diagnostic applications as well. The latter might be of use in laboratories where MLPA is not available or for the locally occurring deletions, reaching high frequencies in the population.

Materials and methods

The patient cohort consisted of 36 selected thalassemia patients of different ethnic backgrounds. They were referred to our laboratory for hematological, biochemical, and molecular analysis. Hematologic parameters were determined on Micros 60 (SBX Diagnostics, Montpellier, France). Biochemical analysis was performed on HPLC VARIANT II (Bio-Rad Laboratories, Hercules, CA) using the β-Thalassaemia Short Programme, and on the Capillarys capillary electrophoresis (CE) device (Sebia, Evry, France). Presence of cellular inclusions of hemoglobin H (Hb H) was determined after incubation with brilliant cresyl blue (Lin et al., 1990) in cases suspected of having α^0-thalassemia. Genomic DNA was isolated using the Qiagen AutoPure LS Genomic DNA Purification System (Gentra Systems, Minneapolis, MN). Gap-PCR was applied to detect the 7 most common α-thalassemia deletions ($-\alpha^{3.7}$, $-\alpha^{4.2}$, $--^{SEA}$, $-(\alpha)^{20.5}$, $--^{MedI}$, $--^{THAI}$, $--^{FIL}$) (Liu et al., 2000). The α- and β-globin genes were amplified by PCR as described previously (Dode et al., 1990; Losekoot et al., 1990) and direct sequencing for point mutation analysis was performed on an ABI PRISM™ 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

MLPA was applied to detect unknown and uncommon rearrangements in both α- and β-globin gene clusters as described elsewhere (Harteveld et al., 2005, 2009). For analysis of the α-globin gene cluster, the P140B2 probe set (MRC Holland) and the HBA probe set (Leiden Genome Technology Center (LGTC), Leiden, The Netherlands) were used. The P102B (MRC Holland) and HBB probe set (LGTC) were used for analysis of the β-globin gene cluster (Harteveld et al., 2005).

For this research, we designed and ordered a custom fine tiling array covering the α- and β-globin gene clusters and surrounding areas (Roche NimbleGen, Madison, WI). Design of the array was based on NCBI Build 36.1 (hg18). Sample preparation, array hybridization and analysis of the results was performed according to the manufacturer's instructions (NimbleGen Arrays User's Guide: CGH Analysis v4.0). In brief, 500 ng of sonicated DNA was denatured in presence of 1 OD_{260} (optical density) of either Cy3 (test samples) or Cy5 (reference samples) dye-labeled random nonamers (TriLink Biotechnologies, San Diego, CA). The denatured sample was chilled on ice, and then incubated with 10 mM dNTPs (each) and 100 U Klenow Fragment 3'->5'exo^- (New England Biolabs, Ipswich, MA), for 2 hr at 37 °C. After washing, each test sample and corresponding reference sample were mixed and dried in a SpeedVac (Savant, Thermo Fisher Scientific, Waltham, MA). Pellets were resuspended in Sample Tracking Controls (NimbleGen). After mixing the resuspended sample with the hybridization solution (containing Hybridization Buffer, Hybridization Component A and an Alignment Oligo, all from NimbleGen), the samples were loaded onto the array. Samples were hybridized at 42 °C in the Hybridization System (NimbleGen, Cat. No. 05223687001) for approximately 72 hr. Arrays were washed by using the NimbleGen Wash Buffer Kit and scanned with the Agilent G2565BA microarray scanner (Agilent Technologies, Santa Clara, CA). Data analysis was performed with NimbleScan v2.5 and SignalMap v1.9 software (NimbleGen).

All 36 samples were hybridized to the array. DNA from a healthy individual in whom rearrangements in the α- and β-globin gene clusters were excluded by MLPA, was used as reference sample for all experiments. A second healthy control sample was used as a negative control for each array.

According to the array results, primers for PCR were designed in the breakpoint region. The PCR was performed using the TaKaRa LA Taq™ kit (TaKaRa Bio Inc, Otsu, Shiga, Japan) according

to the manufacturer's instructions. Instead of the recommended 50 ng of template DNA, 200 ng was used. The reaction was performed in a T-Professional thermal cycler (Biometra, Göttingen, Germany). Direct sequencing of the breakpoint fragment was performed on the ABI PRISM™ 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

Results

Array design

The custom designed fine-tiling array consists of 12 identical sub-arrays with 135,000 probes each (12 x 135 K format). From a diagnostic point of view, we chose to use the 12-plex design for a more efficient use of the array. In addition, each array can be reused so 24 samples can be analyzed per array, which further reduces the costs of the experiments. The probes cover the telomeric 2 Mb of chromosome 16p, including the α-globin gene cluster (position 1-2,000,000, according to UCSC Genome Browser, March 2006, hg18), and the region surrounding the β-globin gene cluster on chromosome 11p (position 4,900,000-5,500,000) with an average spacing of 20 bp. The probe oligonucleotides have a length of 60-80 bp, indicating an overlap between probes and approximately 3 x coverage of the region of interest.

The α- and β-globin gene clusters contain nonunique sequences,for example the duplicated α- and γ-globin genes and the Alu- and LINE-repeat regions. The probes on the array are all unique, in order to increase specificity and to prevent false positive results. Therefore, these nonunique sequences of the globin gene clusters are not covered by the probes. The largest repeat unit in our region of interest is an approximately 7 kb LINE-repeat in the β-globin gene cluster. Thus, in case the breakpoint is located within one of these repetitive sequences, determination of the breakpoint by the array can differ maximally 7 kb from the actual breakpoint. A schematic overview of the coverage is given in Figure 1.

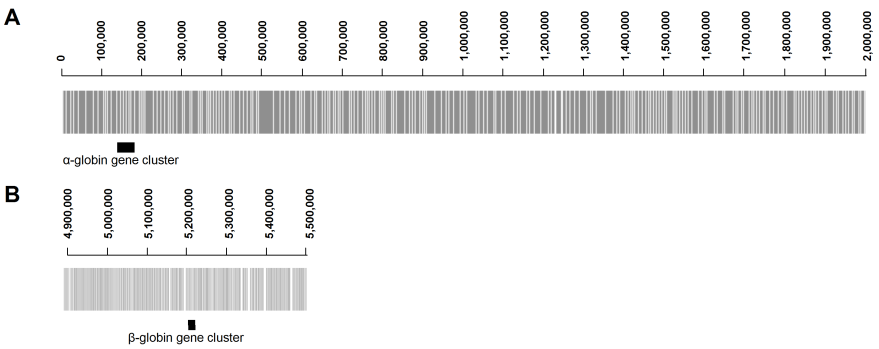

Figure 1 Schematic overview of the coverage of the probes. The vertical grey lines indicate where probes are located, uncovered areas are left in white. The array covers the telomeric 2 Mb of chromosome 16p including the α-globin gene cluster **(A)** and a 600 kb region including the β-globin gene cluster on chromosome 11p **(B)**. The black bars below the figures represent the location of the globin gene clusters. Positions are according to the UCSC Genome Browser (March 2006, hg18).

Samples

All 36 patients (nine positive controls and 27 with unknown rearrangements) showed clear microcytic hypochromic anemia and normal iron levels. Sequence analysis of the globin genes revealed no abnormalities. All patients were carrier of a deletion in the α- or β-globin gene cluster, identified by MLPA. Among the 17 α-thalassemia carriers, nine different types of deletions were identified, and the 19 β-thalassemia patients contained 13 different deletions. The patient samples were selected from a previous study (Harteveld et al., 2005) or identified during routine diagnostic MLPA screening. The cohort of patients was chosen in such a way to have a high variety of deletion types. The group included patients with relatively small deletions as well as patients carrying larger rearrangements. In addition, some patients carrying upstream deletions were included in the study. An overview of the MLPA results is given in Table 1.

Performance of the array

To test the performance of the fine-tiling array, nine patients with known deletions were analyzed. These included the $-\alpha^{3.7}$ deletion, the 18.8 kb $--^{SEA}$ deletion and the 8.2 kb $--^{AW}$ deletion (Phylipsen et al., 2010b) for the α-globin gene cluster and the Corfu δ-globin gene deletion, the 13.4 kb Sicilian δβ-thalassemia deletion and the HPFH-2 deletion for the β-globin gene cluster. The locations of the breakpoints differ among these deletions along the α- and β-globin gene clusters and breakpoint sequences are known. Two patients carrying very large deletions of 1.5-2 Mb in the α-globin gene cluster causing the ATR-16 syndrome ($--^{NL}$ and $--^{HN}$) (Harteveld et al., 2007) were also tested.

Analysis of the array data was performed by determining the average signal difference between test and reference sample using different reading frames. The software calculates these signal differences in blocks of 100, 250 or 500 base pairs along the region of interest. We decided to use the 100 bp frame for analysis of the unknown deletions, because determination of the breakpoint location in the positive controls was shown to be most accurate by applying this frame.

According to the array results, the $-\alpha^{3.7}$ deletion ranged from position 161999 to 167199, thereby differing 1301 bp (5' end) and 96 bp (3' end) from the actual breakpoint. The large difference on the 5' end of the breakpoint is due to the presence of a nonunique sequence, which is not covered by the probes (Fig. 2A). The difference for the $--^{SEA}$ deletion was 699 bp on the 5' end and 549 bp on the 3' end. Two family members carrying the $--^{AW}$ deletion were tested. Detection of this deletion differed 80 bp on the 5' end and 96 bp on the 3' end of the deletion in both cases. The deletions in the two ATR-16 patients run into the telomeric end of chromosome 16, making it impossible to design a unique primer at the 5' end of the deletion. Therefore, the exact breakpoint cannot be determined by breakpoint PCR. However, the array results for the two patients with ATR-16 were in concordance with the results obtained with MLPA and the 3' end breakpoint could be determined more accurately (Fig. 2B). For the Corfu type δ-globin gene deletion, the distance between the actual breakpoint and the deletion breakpoint defined by the array was 355 bp at the 5' breakpoint and 438 bp at the 3' breakpoint. The array results for the HPFH-2 deletion showed a difference of 405 and 1094 bp at the 5' and 3' end of the breakpoint, respectively (Fig. 2C). The difference at the 5' end breakpoint region of the 13.4 kb Sicilian deletion, which falls within the approximately 7 kb LINE-repeat region, was 3365 bp compared to the actual breakpoint sequence. The 3' end breakpoint showed a difference of only 37 bp (Fig. 2D).

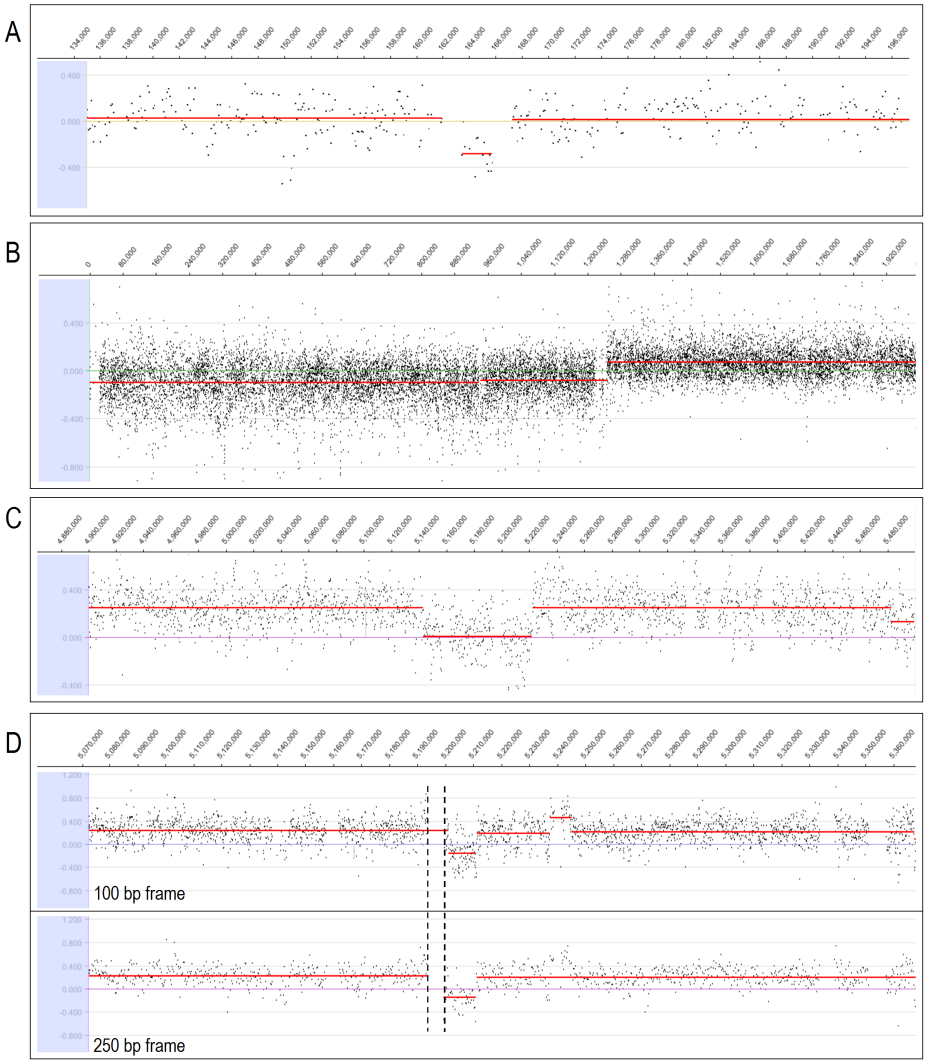

Figure 2 Array results of the positive controls. The probe locations are mentioned at the top of each graph (according to UCSC Genome Browser, March 2006). The horizontal line indicates the average signal difference between reference and patient in blocks of 100 bp along the region of interest. **A** and **B** show results of the controls in the α-globin gene cluster (chromosome 16) and **C** and **D** are the controls with deletions in the β-globin gene cluster. A: $-\alpha^{3.7}$ deletion; B: $--^{NL}$ deletion, causing ATR-16 syndrome; C: HPFH-2 deletion; D: Sicilian δβ-deletion. This picture also shows the difference between using the 100 bp and 250 bp reading frames. An approximately 6 kb LINE repeat is located at the 5' end of the deletion, which is not covered by the probes (area between dashed lines). The LINE repeat appears to be involved in the deletion when using the 250 bp frame, in contrast to the 100 bp frame. The actual deletion breakpoint is located within the repeat, thus only part of the stretch is involved in the deletion. Furthermore, a duplication appears to be present when using the 100 bp frame. This result is not seen in the analysis with the 250 bp frame, indicating that the noise increases when applying higher resolution analysis.

On array, the -$\alpha^{3.7}$ allele showed a deletion which was approximately 250 bp different at the 5' end of the deletion than the MLPA result. The array contains only unique probes, therefore, the duplicated homology boxes containing the α-globin genes and other non-unique sequences such as Alu-repeats are not covered by the array. They appear as gaps on the array output and these areas are not included in the calculation of the deletion length. Therefore, it is important to take the maximum length of the deletion when designing primers for gap-PCR. The maximum length of the deletion is defined as the distance between the last probe present at the 5' end of the deletion and the first probe present at the 3' end of the deletion.

These results show that the fine-tiling aCGH technology is suitable to detect small and large rearrangements (from ~4 kb up to 2 Mb) in the α- and β-globin gene clusters with high resolution. With the information provided by the array, it is possible to design primers to amplify a relatively short product containing the breakpoint sequence. This product can then be easily analyzed by direct sequencing.

Patients

The array results of all 36 deletions were in concordance with MLPA results, that is the deletion length was within the limits as determined by MLPA (Table 1). Primers for gap-PCR were designed for the cases with deletions not including the telomere. Primers were designed to amplify a product of maximum 2,000 bp, based on the maximum deletion found on the array. Of the 27 cases tested, primers could be designed for 22 cases. Gap-PCR was successful in 19 cases, and for 12 of them we were able to determine the exact breakpoint location by sequence analysis. In the other seven cases, sequence analysis failed due to presence of long stretches of repetitive sequences, suggesting the involvement of these repeats in the generation of the deletions. Sequences of the primers used in this study can be found in Table 2.

Alpha-thalassemia patients

In total, 11 cases carrying an unknown type of α-thalassemia deletion were tested. The deletions in cases 7, 8 and 9 involve the multi-species conserved sequence region (MCS-R) of the α-globin gene cluster, but leave all globin genes intact. Breakpoint PCR could not be performed, because the 5' end of the deletion runs into the telomeric end of chromosome 16. This telomeric region contains a lot of repetitive sequences and it is therefore not possible to design a unique primer pair (Lamb et al., 1993). The deletion types in these patients have not been described before. The deletion in case 7 was called $(\alpha\alpha)^{UD}$, and the deletion in patient 8 and 9, who are related, was called $(\alpha\alpha)^{BJ}$. Cases 10 and 11 showed a 31.5 kb deletion of the ζ- and both α-globin genes. Sequence analysis of the breakpoint fragment revealed that these patients carry the $--^{Dutch\ I}$ deletion (Harteveld et al., 1997). A similar but slightly different deletion of 31 kb was found in cases 12 and 13. Direct sequencing of the breakpoint fragment confirmed presence of the $--^{MedII}$ deletion(Kutlar et al., 1989). A rearrangement of 28,073 bp was found in case 14. Sequence analysis showed a 23 bp overlap between the 5' end and 3' end breakpoint. The 5' end breakpoint is located at position 145374 and the 3' end at position 173447. This type of rearrangement involves both α-globin genes and was not described before. We named this deletion $--^{JB}$, after the initials of the patient (NC_000016.8:g.145374-173447del). For cases 15, 16 and 17, an approximately 1400 bp breakpoint fragment was obtained, but the exact breakpoint sequence could not be determined because of a stretch of T-nucleotides located approximately 400 bp

#	maximum deletion defined by MLPA	Deletion on array	5' breakpoint	3' breakpoint	difference 5'	difference 3'	Remarks
1	chr16: 162254-167584	chr16: 161999-167199	163300	167103	1301	96	$-\alpha^{3.7}$
2	chr16: 152769-175806	chr16: 156099-175249	155400	174700	699	549	$--^{SEA}$
3	chr16: 160380-169762	chr16: 161199-169549	161279	169453	80	96	$--^{AW}$ (Phylipsen et al., 2010b)
4	chr16: 160380-169762	chr16: 161199-169549	161279	169453	80	96	$--^{AW}$
5	chr16: 1-1304272	chr16: 1-1246849	ND				$--^{NL}$ (unpublished)
6	chr16: 1-1928982	chr16: 1-1920449	ND				$--^{HN}$ (Harteveld et al., 2007)
7	chr16: 1-139157	chr16: 1-136449	ND				$(\alpha\alpha)^{UD}$ (this study)
8	chr16: 1-132952	chr16: 1-128349	ND				$(\alpha\alpha)^{BJ}$ (this study)
9	chr16: 1-132952	chr16: 1-128249	ND				$(\alpha\alpha)^{BJ}$ (this study)
10	chr16: 133676-169762	chr16: 137499-169249	137661-137669	169199-169207	162	42	$--^{Dutch\ I}$ (Harteveld et al., 1997)
11	chr16: 133676-169762	chr16: 138099-168549	137661-137669	169199-169207	438	650	$--^{Dutch\ I}$
12	chr16: 133676-169762	chr16: 137249-168649	137924-137935	169132-169143	675	483	$--^{MedII}$ (Kutlar et al., 1989)
13	chr16: 133676-169762	chr16: 138199-168649	137924-137935	169132-169143	275	494	$--^{MedII}$
14	chr16: 142384-175806	chr16: 145299-173749	145374-145396	173447-173469	75	302	$--^{JB}$ (this study)
15	chr16: 142384-175806	chr16: 148699-172949	ND				$--^{SA*}$(Vandenplas et al., 1987)
16	chr16: 142384-175806	chr16: 143099-172899	ND				$--^{SA*}$
17	chr16: 142384-175806	chr16: 149799-173049	ND				$--^{SA*}$
18	chr11: 5210827-5219830	chr11: 5210624-5218624	5210979	5218186	355	438	Corfu δ-thalassemia deletion (Galanello et al., 1990)
19	chr11: 5193754-5212264	chr11: 5201049-5211049	5197684	5211086	3365	37	Sicilian $\delta\beta$-thalassemia deletion (Ottolenghi et al., 1977)
20	chr11: 5129268-5225968	chr11:5136849-5220649	5136244	5220555	605	94	HPFH-2 (Ringelhann et al., 1977)
21	chr11: 5253777- > 5272927	chr11: 5257549-5295849	5257863	5294280	314	1569	Dutch VII (this study)

Table 1 Overview of the results obtained by MLPA and array.

#	maximum deletion defined by MLPA	Deletion on array	5' breakpoint	3' breakpoint	difference 5'	difference 3'	Remarks
22	chr11: 5129268-5360732	chr11: 5137649-5346849	ND				Turkish 209 kb $\varepsilon\gamma\delta\beta$-deletion (this study)
23	chr11:5219835-5466733	chr11: 5219749-5430749	5220166	5430759	417	10	Dutch IV (Harteveld et al., 2005)
24	chr11: 5145679-5229038	chr11: 5147849-5226849	5147699-5147700	5226628-5226631	150	221	Chinese $^{A}\gamma\delta\beta$-deletion (Mager et al., 1985)
25	chr11: 5145679-5229038	chr11: 5147649-5226849	5147699-5147700	5226628-5226631	150	221	Chinese $^{A}\gamma\delta\beta$-deletion
26	chr11:5177669-5225968	chr11: 5192499-5225374	5192267-5192269	5224930-5224932	232	25	Indian 32.6 kb $\delta\beta$-deletion (Gilman et al., 1992)
27	chr11: 5166593-5226029	chr11: 5171249-5222249	5172260	5222030	1011	219	HPFH-3 (Kutlar et al., 1984)
28	chr11:5166593-5225968	chr11: 5171549-5221749	5172260	5222030	711	281	HPFH-3
29	chr11:5202740-5212264	chr11: 5203249-5210949	ND				Turkish 7.7 kb $\delta\beta$-deletion (this study)
30	chr11:5193754-5207650	chr11: 5193749-5207349	ND				Dutch 12.6 kb β-deletion* (Gilman, 1987)
31	chr11:5193754-5207650	chr11: 5201149-5207549	ND				Dutch 12.6 kb β-deletion*
32	chr11:5258519-5342559	chr11: 5262049-5338949	ND				Dutch VIII (this study)
33	chr11: <5202740-5207688	chr11: 5201049-5207349	ND				Dutch 12.6 kb β-deletion*
34	chr11: <5202740-5207688	chr11: 5193749-5207349	ND				Dutch 12.6 kb β-deletion*
35	chr11:5193754-5210725	chr11: 5201149-5209449	ND				Double 7.6 kb Turkish β-deletion*(Oner et al., 1995)
		chr11: 5216349-5223549					
36	chr11:5193754-5210725	chr11: 5201649-5209449	ND				Double 7.6 kb Turkish β-deletion*
		chr11: 5216349-5223949					

Table 1 Overview of the results obtained by MLPA and array (continued). The first column gives the sample number, the second column indicates the maximum deletion as defined by MLPA, that is from the position of the last probe present at the 5' end of the deletion to the first probe present at the 3' end (locations refer to the UCSC Genome Browser, March 2006, hg18). The third column shows the location of the deletion determined by the fine-tiling array using the 100-bp reading frame. For the cases with successful breakpoint PCR and sequence analysis, the location of the breakpoint is indicated, as well as the number of bp difference between the actual breakpoint and the breakpoint characterized by array on either side of the deletion. *Suggested deletion type based on results, but not confirmed yet, ND, not determined.

downstream of the position of the forward primer. However, the analysis so far suggests that these patients are carriers of the $--^{SA}$ deletion (Mathew et al., 1983).

Beta-thalassemia patients

A total of 16 unknown β-thalassemia deletion carriers were hybridized to the array. The 36415 bp deletion in case 21 appeared to be *de novo* and involves the Locus Control Region (LCR) and the ε- and both γ-globin genes, but leaving the δ- and β-genes intact. The 5' end breakpoint was located at position 5294279 and the 3' end breakpoint at position 5257864 of chromosome 11. An insertion of two T-nucleotides was present at the breakpoint. This $(\varepsilon\gamma)\delta\beta^0$-thalassemia deletion is not described before and was called Dutch VII (NC_000011.8:g.5257864-5294279delinsTT). For case 22, we were not able to obtain a specific breakpoint fragment by PCR. According to the array results, the deletion was approximately 209 kb in length, thereby deleting the complete β-globin gene cluster. Such a rearrangement has not been described before and we named it Turkish 209 kb $\varepsilon\gamma\delta\beta^0$-thalassemia deletion. Patient 23 was known to be carrier of the previously described Dutch IV-$\varepsilon\gamma(\delta\beta)^0$ thalassemia deletion (Harteveld et al., 2005). During this study, the exact breakpoint sequence was determined. This rearrangement has its 5' end breakpoint at position 5430759 and the 3' end breakpoint at 5220166 (NC_000011.8:g.5220166-5430759del). Patients 24 and 25 showed a deletion of 79 kb, involving the γ-, δ- and β-globin genes. Sequence analysis of the obtained product confirmed carriership of the previously described Chinese type γδβ-deletion (Mager et al., 1985). Case 26 showed a deletion of 32.6 kb, involving the δ- and β-globin genes, but leaving the γ-genes intact. Sequence analysis revealed presence of the Indian 32.6 kb δβ-deletion. Another type of δβ-thalassemia deletion was found in case 27 and 28, which was confirmed to be the HPFH-3 deletion after sequencing. Patient 29 was carrier of an approximately 7.7 kb deletion. The extent of this deletion resembles the 13.4 kb Sicilian or the Turkish inversion-deletion type, but PCR for these specific deletions was negative. This new rearrangement at this location was called Turkish 7.7 kb δβ-deletion. For cases 30, 31, 33 and 34, an approximately 3,500 bp breakpoint fragment was obtained, but the exact breakpoint sequence could not be located because of a stretch of CA repeats in the fragment.

	Upstream primer 5' > 3'	Downstream primer 5' > 3'	Product size (bp)
Dutch I	GCCAAGATCGCATCACTGTA	TTGGTGTCCGGCTTCTTTTA	~550
Med II	GCCAAGATCGCATCACTGTA	TTGGTGTCCGGCTTCTTTTA	~900
$--^{JB}$	CACCTCCCTGATAGGCAAAA	TCACCACCACCTGTGTAGGA	~2500
$--^{SA}$	GCTGCGAATTTAACCGTATCA	TCTCGCCAAAGATGGCTACT	~1400
Dutch VII	CATGTCAGGAACAGCAAGGA	TGTGCTGGAATACGTGGCTA	~3000
Dutch IV	TGAATCAGCAGGCAGGATAC	TTACTATATCCTACCTCTGGTATCAA	~3000
Chinese	CCACATCCCTAACACAACAAAAT	CTAATCCACAGTACCTGCCAAAG	~2200
Indian	AATCATATTGTCCAGGGCTTTTT	CTTGTGGATGCCTAAGAAAGTGA	~1200
HPFH-3	CTTTGCAATTGGATTGTTTGTTT	CCAGCATCCTGAACCTCTAAGTA	~2500
12.6 kb Dutch	TCCTGTGCGTCCTTTCAATA	CTGGCCTGGAGTAAGTTGGA	~1400

Table 2 Sequences of the primers used for PCR and sequence analysis.

Based on the length and position of this deletion, this might be the previously described 12.6 kb β-deletion (Gilman, 1987). Patient 32 showed a deletion of approximately 77 kb involving the LCR, but leaving all the globin genes intact. We were not able to generate a specific breakpoint fragment by PCR. A deletion of such extent in the LCR has never been described. We named this new rearrangement Dutch VIII. Patients 35 and 36 are closely related and showed the exact same results on array. Interestingly, the array results revealed a second deletion in both patients, in addition to the one found earlier by MLPA. The second deletion is too small to be detected by MLPA. Although it has not been confirmed yet, the results so far strongly suggest carriership of the previously described double 7.6 kb Turkish β-deletion (Oner et al., 1995).

All data on the newly characterized deletions were submitted to the globin gene variant database (http://globin.cse.psu.edu/hbvar/menu.html).

Discussion

Array-based CGH represents a powerful and effective tool for the detection of copy number variations CNVs in the genome. Nowadays, it is possible to have >1 million targets per array, leading to very high resolution and accurate determination of rearrangements.

For this study, we developed a fine-tiling array to detect rearrangements in the α- and β-globin gene clusters and surrounding regions. These areas are covered with ultra-high density, with a 60-80 bp oligonucleotide probe every 20 bp, indicating an overlap between probes. Nine positive control samples and 27 patients' DNA samples were analyzed. All rearrangements, previously detected by MLPA, were successfully detected by the array. Moreover, a further refinement of the breakpoints was obtained by the results of the array. Furthermore, we were able to determine the exact breakpoint location in 12 cases, of whom two were carrier of novel types of deletions. In addition, five novel types of deletions were found of which the exact breakpoint could not be determined due to the presence of repetitive sequences impairing sequence analysis and proper primer design.

Performance and applicability of the array

For data analysis, we applied three different reading frames of 100, 250 and 500 bp to determine the deletion locations. Use of the 500-bp frame resulted in the lowest background signal, but a larger distance to the actual breakpoint compared to the smaller frames. Although the noise level increased when using the 100-bp frame, noise and real signal could still easily be discriminated. Therefore, we decided to use these results which are more accurate than the 250 or 500-bp frames and thus provide the best information for designing the primers.

In five cases, the presence of repetitive sequences close to the predicted breakpoint prevented proper primer design. Since these regions are not covered by probes on the array, it is not possible to determine the breakpoint position and to design unique primers. In seven other cases, presence of a repeat stretch in the obtained breakpoint fragment impaired sequencing analysis. The exact location of the breakpoint could therefore not be determined. However, the fact that the breakpoint could be allocated to this repeat unit gives the required information to speculate about the mechanism and to design primers for gap-PCR.

During data analysis, we noticed that several probes do not give consistent and reproducible results. This problem might be mended by excluding them from data analysis or by filtering these unreliable probes and remove them from future manufacture of the array slides. However,

the software calculates an average difference between signal in reference and patient sample along a region, which largely compensates for outlier probe signals. In addition, removing probes from the array design might result in larger gaps and thus in less precise breakpoint determination.

In some samples (including healthy controls) we found additional rearrangements on chromosome 11p15.4 besides those detected by MLPA. A duplication of approximately 5 kb was shown starting at position 5055700, and an approximately 7.5 kb deletion was found starting at position 5136700. These areas contain no genes and since the healthy controls used in this study do not present with a thalassemic phenotype, we considered these CNVs as not relevant and they were therefore not further investigated. Other additional rearrangements found in the patients were reported in the database of genomic variants and were considered clinically irrelevant as well.

The array used in this study is suitable to accurately detect small and large rearrangements in the globin gene clusters, as well as deletions in the upstream regulatory elements. All results were in concordance with the MLPA results. However, the array results provide far more information because it reduces the breakpoint area to <1,400 bp. As shown in this study, it is relatively easy to cover the final gap by PCR and direct sequence analysis. By designing simple PCR assays for these deletions, family members or other patients who appear to carry a similar type of deletion detected by MLPA can quickly be screened. Furthermore, complex rearrangements such as double deletions and combinations of α- and β-thalassemia deletions can be detected in the same experiment.

Mechanisms leading to rearrangements in the α- and β-globin gene clusters

Lots of rearrangement breakpoints across the human genome have been reported, which provides pivotal information to study the mechanisms underlying genomic recombination events. This knowledge is necessary to improve the design of methods to detect these rearrangements.

The $--^{\text{Dutch I}}$ deletion was found in cases 10 and 11. Both patients are of Dutch origin, but it is yet unknown whether they are related. Since this particular type of deletion is found in only one family until now, it is likely that they are both related to this family. The deletion found in patients 12 and 13 appeared to be similar based on MLPA results. However, the array showed slightly different breakpoint areas, which was confirmed by subsequent analysis of the breakpoint fragment. These patients are both of Turkish origin and carry the $--^{\text{MedII}}$ deletion. The 3' breakpoints of both deletions are located in Alu-repeat areas, which are known to be involved in homologous recombination in the α-globin gene cluster (Harteveld et al., 1997).

Results of the α-thalassemia patients in this study indicate a hotspot for deletion breakpoints between MLPA probes 15a and 16a. Patients 14, 15, 16 and 17 appeared to carry the same deletion based on MLPA results. However, the specific breakpoint fragment for the $--^{\text{JB}}$ rearrangement (case 14) could not be amplified in the other three patients, indicating a different breakpoint. This finding was supported by the array results, which showed an approximately 800 bp difference at the 3' end of the deletion. Based on the results so far, it is suggested that patients 15, 16 and 17 carry the $--^{\text{SA}}$ deletion. This deletion has its 3' breakpoints in the same Alu-repetitive sequence as the DutchI and MedII deletions, thus it is likely that these rearrangements arose due to a similar nonallelic homologous recombination (NAHR) event. NAHR is a type of double-stranded DNA break repair which can result in deletion, inversion, duplication or translocation due to misalignment of homologous sequences such as Alu-repeats (Chen et al, 2010).

In contrast to the α-globin gene cluster rearrangements, the deletions in the β-globin gene cluster appear to arise by non-homologous end joining (NHEJ). Areas surrounding the breakpoints were extensively studied, but no homology was found in any of the β-thalassemia rearrangements. In addition, in the Dutch VII deletion an insertion of two nucleotides was found at the join point, which is a typical characteristic of an NHEJ event (Mladenov and Iliakis, 2011).

Concluding remarks

For now, diagnostic application of the aCGH technology is too expensive, it costs approximately € 90,- per patient versus approximately € 12,- per patient for MLPA (reagents only, excluding purchase of the required instruments). However, our results suggest that this fine-tiling aCGH technology is a valuable tool for high resolution breakpoint characterization in α- and β-globin gene cluster rearrangements. The delineation of breakpoints will further improve our understanding of the mechanisms causing the rearrangements. Furthermore, the information provided by the aCGH led to the design of simple PCR-based tests to detect the variant alleles. These assays are useful to laboratories in areas where specific deletions reach high frequency in the local population. In addition, gap-PCRs are relatively easy and cheap to perform, rendering it a good alternative for techniques such as MLPA and Southern blotting.

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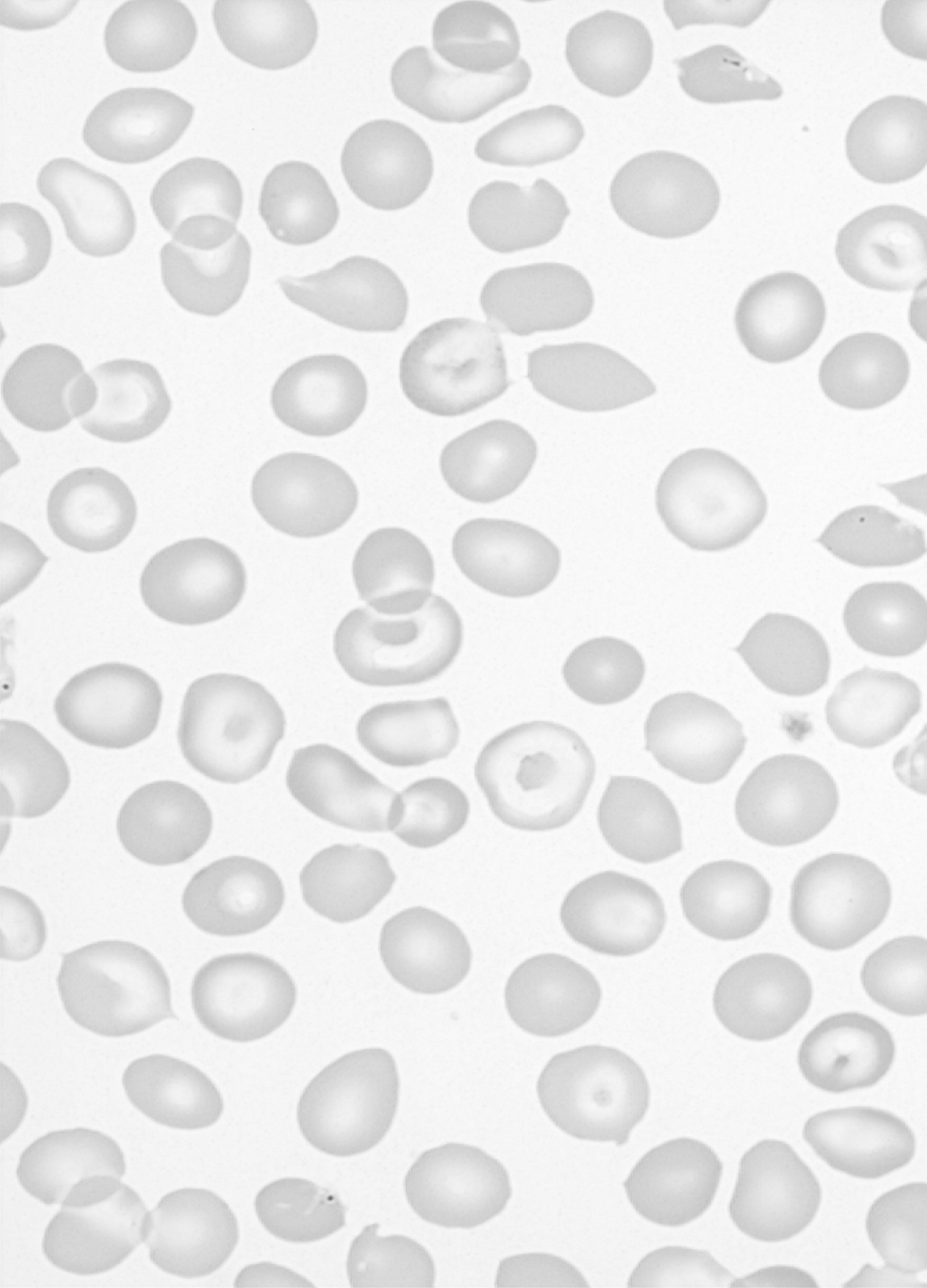

Chapter 3

Improving prenatal diagnostics for hemoglobinopathies

Chapter 3.1

Two new beta-thalassemia deletions compromising prenatal diagnosis in an Italian and a Turkish couple seeking prevention

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Abstract

When the molecular background of couples requesting prevention is unclear, family analysis and tools to define rare mutations are essential. We report two novel deletion defects observed in an Italian and in a Turkish couple. The first proband presented with microcytic hypochromic parameters without iron deficiency, a normal HbA_2 and an elevated HbF (10.6%). His father presented with a similar phenotype and his wife was heterozygous for the common Mediterranean codon 39 (HBB:c.118C>T) mutation. Having excluded point mutations and common deletions, Multiplex Ligation-dependent Probe Amplification was performed revealing an unknown $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thalassemia defect spanning from the $^{A}\gamma$ gene to downstream of the β-globin gene provisionally named *Leiden 69.5 kb deletion*. In the second case, the wife presented with a mild thalassemic picture, normal HbA_2, elevated HbF (18.5%) and a β/α globin chain synthesis ratio of 0.62, without iron deficiency or any known β-thalassemia defect, while the husband was a simple carrier of the common Mediterranean IVS1-110 (HBB:c.93-21G>A) mutation. A new large deletion involving the β-gene and part of the δ-gene was identified by Multiplex Ligation-dependent Probe Amplification, provisionally named *Leiden 7.4 kb*.

Introduction

Assessment of couples for the risk of transmitting β-thalassemia major is usually achieved through basic hematology diagnostics and molecular analysis. As the majority of β-thalassemias is caused by point mutations, direct sequencing analysis is the first method to be applied, and if negative, gap-PCR can be used to investigate the presence of known deletions. Rare cases with large deletions in the β-globin gene cluster have been associated with microcytic hypochromic parameters, along with normal HbA_2 and elevated or normal HbF, and such cases may be difficult to define at the molecular level or even be overlooked. The interaction of β^0-thalassemia point mutations with *elevated HbF* β-globin gene deletions might result in mild or intermediate phenotypes. However, combinations with deletions involving both γ-globin genes may result in severe compound heterozygosity due to the absence of the compensatory effect of fetal hemoglobin. Prenatal diagnosis might lead to a false adverse result, when the fetus has inherited a point mutation from one parent and a deletion allele with an intact – but inactive - β-globin gene from the other parent. We report 2 cases of novel deletions identified by MLPA in at risk couples requesting prevention, which were studied in collaboration with different reference centers.

Design and methods

Propositus A

An Italian male who was requesting prevention for β-thalassemia due to a diagnosis of β-thalassemia minor codon 39 (HBB:c.118C>T) mutation in his wife (Figure 1A).

Propositus B

A Turkish woman requesting prenatal diagnosis, with a history of an inconclusive result from a previous pregnancy which was consequently terminated. Her husband was diagnosed as a carrier of the IVS-I-110 (HBB:c.93-21G>A) β-thalassemia point mutation (Figure 1B). In both probands, who presented with elevated HbF level, no point mutations or known deletions were found. Case A was studied in Rome, Genoa and Leiden; case B in Istanbul, Cagliari, Athens and Leiden.

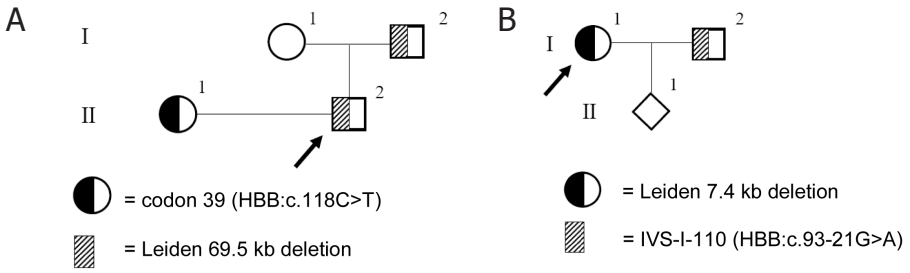

Figure 1 Pedigrees of the Italian **(1A)** and Turkish **(1B)** families. Both probands carry an unknown deletion in the β-globin gene cluster, while their partners are diagnosed with a known point mutation.

Hematologic data were obtained on several different occasions using the locally available standard protocols. Separation of the Hb fractions and measurement of the HbA_2 and HbF levels were obtained from different automatic high performance liquid chromatographic (HPLC) devices (1). Globin chain synthesis was performed in Cagliari using a modified procedure based upon Kan *et al.* (2). Genomic DNA was isolated from peripheral leukocytes using either automatic or manual high salt extraction methods based on Miller *et al*. (3). Molecular analysis for common deletions and the α-triplication was performed at different centers using multiplex PCR methods (4,5). Point mutation analysis of the β-globin gene cluster was performed by direct sequencing in different locations and on different apparatuses. Multiplex Ligation-dependent Probe Amplification (MLPA) was carried out in Leiden as previously described (6,7).

Results

Case A

Propositus (II-2), presented with microcytic hypochromic red cell parameters (Hb 12.4 g/dL; RBC 6.07 $x10^{12}$/L; MCV 63 fL; MCH 20.5 pg) without iron deficiency (ferritin 154 ng/mL), with normal HbA_2 (3.2%) and elevated HbF (10.6%) levels, compatible with a δβ-thalassemia deletion. His father presented with a similar phenotype but they were both negative for the common δβ-deletions such as the Black, Sicilian, Macedonian-Turkish, Asian-Indian, Filipino, Chinese, HPFH-I or HPFH-V). Sequencing of the β-globin genes revealed no point mutations. The ^{A}γ-globin gene promoter sequence showed heterozygosity for the common 4 nt deletion (HBG2:c.-278_-275_ delAGCA), a mutation described in a mild Spanish codon 39 (HBB:c.118C>T) homozygous case (8). Finally MLPA of the β-globin gene cluster revealed a deletion of at least 69.5 kb spanning from the ^{A}γ gene to ~37 kb downstream of the β-globin gene. We have provisionally named this event *Leiden 69.5 kb* deletion (Figure 2A left and 2B). His wife was carrier of the codon 39 (HBB:c.118C>T) mutation. Her parents were not available for analysis.

Case B

The propositus (I-1) presented with mild anemic hematologic parameters, normal HbA_2, elevated HbF (18.5%) and a β/α-globin chain ratio of 0.62, with low ferritin and normal ZPP. No point mutations or known deletions of the β-globin gene were found. The same phenotype was

present in her mother (data not shown). The 13.4 kb Sicilian δβ-deletion and the Turkish δβ-inversion/deletion were excluded by gap-PCR. A new large deletion involving the β-globin gene and part of the δ-globin gene was detected by MLPA. The deletion is at least 7.4 kb in length, with a maximum of 22 kb. The 5' break point is located within or upstream of the δ-globin gene and the 3' break point is located around a ~6.1 kb L1-repeat at the 3' end of the β-globin gene cluster. We have provisionally named the new deletion *Leiden 7.4 kb* (Figure 2A right and Figure 2B). The husband was found to be a carrier of the common IVS1-110 (HBB:c.93-21G>A) mutation and presented with the expected red cell parameters and elevated HbA_2 level. All hematologic, biochemical and molecular data are summarized in Table 1.

Individuals	I-1	I-2	II-1	II-2*	I-1**	I-2
Age/Gender	60/F	68/M	32/F	34/M	30/F	44/M
Hb (g/dL)	13.6	13.3	10.8	12.4	12.4	13.1
PCV (l/L)	0.41	0.43	0.34	0.38	0.37	0.39
RBC ($x10^{12}$/L)	4.68	6.47	5.22	6.07	5.21	6.43
MCV (fL)	88	67	64	63	70.8	64.4
MCH (pg)	29.2	20.6	20.6	20.5	23.8	20.41
Ferritin (ng/mL)	n.d	202	56	154	5.52	n.d.
ZPP (mmol/mol)	n.d.	n.d.	n.d.	n.d.	88	n.d.
HPLC	A-A2	A-F-A2	A-A2	A-F-A2	A-F-A2	A-A2
HbA_2 %	2.7	2.9	6.1	3.2	2.7	5.2
HbF %	0.5	4.2	0.5	10.6	18.5	<1
β/α ratio	n.d.	n.d.	n.d.	n.d.	0.62	n.d.
DNA β	n.d.	69.5 kb del	Cd 39 (C>T)	69.5 kb del	7.4 kb del	IVS-I-110 (G>A)

Table 1 Overview of the hematologic, biochemical and molecular data. * = casus propositus A; ** = casus propositus B; n.d.: not determined.

Discussion

Deletion defects affecting the β-globin gene cluster, associated with a high HbF expression in adult life, are usually subdivided in two categories. This distinction is purely clinical and based on the observation of the hematologic indices of the specific cases. Those cases with (near) normal hematological indices are defined as HPFH, those presenting with abnormal indices as δβ-thalassemia.

In fact, all deletion defects involving the β-globin gene should be considered as β-thalassemia determinants with a variable phenotype. The variability depends upon the *rescue effect* from the β-thalassemia minor phenotype associated with the presence of elevated HbF production. However, this normalization has to be evaluated with caution. The normocytic or macrocytic F-cells contribute to a false impression of normalized red cell indices and furthermore, since HbF is a poor carrier of oxygen to the tissues, chronic hypoxia remains. Some erythrocytosis is usually triggered, generating a so called *normal* (non-anemic, normocytic-normochromic) state but still in absence of β-globin gene expression. Moreover, polymorphisms on the promoters of the $^{G}\gamma$- and $^{A}\gamma$-globin genes, the only conditions to be appropriately categorized as HPFH, may be

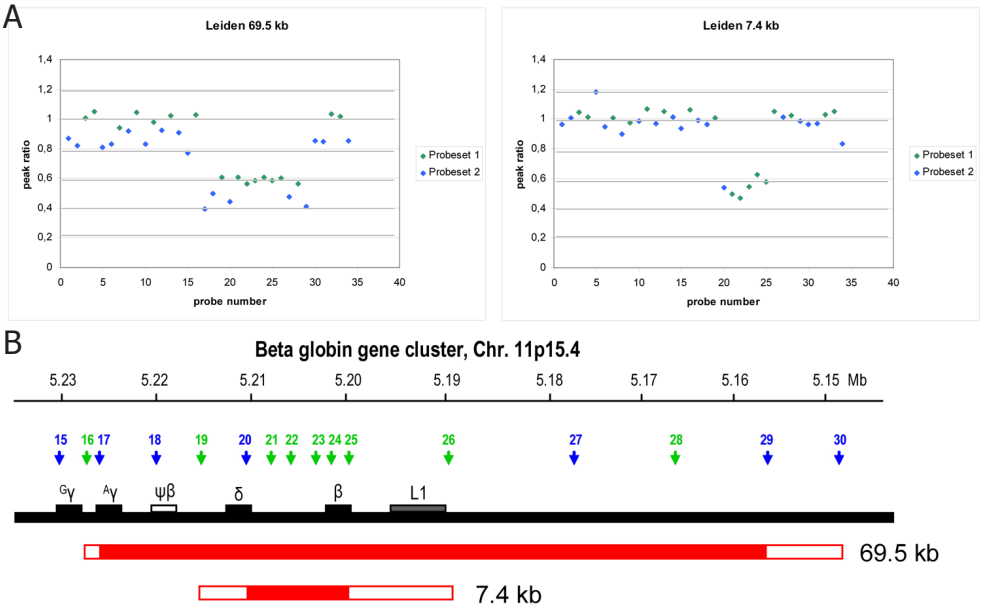

Figure 2 (A) Peak ratio of the specific MLPA products (~1.0 or ~0.5) indicating the presence or absence of a particular segment of the β-globin gene cluster. Left the 69.5 kb deletion, right the 7.5 kb deletion. Data were obtained using the GeneScan Analysis program (Applied Biosystems) and Microsoft Excel. **(B)** Overview of the locations of the MLPA probes in the β-globin gene cluster. The red bars represent the new Leiden 69.5 kb and Leiden 7.4 kb deletions. For probe numbers, sequences and exact locations see Harteveld *et al*. (7).

present and modulate the HbF expression of the partially deleted allele, introducing more phenotype variability. In the end it will be the β/α and γ/α synthetic ratio that will differentiate thalassemia from HPFH.

In case A, MLPA showed that the 5' break point is located in an area between the two γ-globin genes, which is common to several HPFH and $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thalassemia deletion defects, such as the Indian (32.6 kb) and the Turkish (30 kb) HPFH, as well as the Indian (8.5 kb), the Black (35.7 kb), the Belgian (50 kb) and the Italian (52kb) $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thalassemia defects.

The new deletion was associated with the expected microcytic hypochromic parameters of a $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thalassemia defect because the HbF expression, in the absence of the $^{A}\gamma$ -globin gene, was limited to 10.6% in the proband, and is unexpectedly even lower (4.2%) in his father. The heterozygous state for the 4 nt deletion polymorphism in the $^{A}\gamma$-globin gene promoter (HBG2:c.-278_-275_delAGCA) indicates that this region of the $^{A}\gamma$ gene is still present. This polymorphism has been described in association with a mild β^{0}/β^{0} patient with high HbF expression (8), but cannot account for a higher HbF expression of the deleted allele, since the $^{A}\gamma$-globin gene is partially deleted. Since the Xmn-I polymorphism was absent, the 10% HbF expression could be directly associated with the deletion, or alternatively be increased by some $^{A}\gamma$-globin gene expression in trans if the -4 nt deletion polymorphism is not on the deleted allele. An attempt to justify the difference in HbF expression between father and son by the absence of

this polymorphism in the father was unsuccessful, since the proband and both his parents were all carriers of the -4 nt deletion.

In conclusion, heterozygosity for the -4 nt deletion polymorphism indicates the $^{A}\gamma$-globin gene promoter as break point boundary. None of the known deletions matches the 3' break point at approximately 69.5 kb downstream. We are in the process of narrowing down the gaps to define the break points precisely in order to design specific primers. For the time being, we may assume with sufficient confidence that we are dealing with a new $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thalassemia deletion defect, that in combination with a β^{0}-thalassemia defect, as in this couple with the codon 39 (HBB:c.118C>T), will probably cause a thalassemia intermedia phenotype with a limited transfusion dependency.

In case B, the deletion extends from at least the 5' end of the 3rd exon of the δ-globin gene to the 3' end of the 3rd exon of the β-globin gene and is possibly extending to the L1 repeat area. The deletion reduces the level of the HbA_2 to normal and elevates the HbF expression of both $^{G}\gamma$ and $^{A}\gamma$ genes *in cis*, as one would expect from a classic $\delta\beta$-thalassemia deletion, as confirmed by the 0.60 β/α globin chain synthesis ratio. However, this deletion does not match with any of those previously reported.

In this couple, analysis of a prenatal diagnosis could distinguish between affected and unaffected. If heterozygosity for the point mutation of the father is found in the fetus, then this implicates inheritance of the normal allele form the mother and thus a carrier status. Conversely, IVS-I-110 homozygosity (hemizygosity) would mean an affected fetus compound heterozygous for deletion and point mutation. Unfortunately, since the extent of the deletion was not known at the time of a first prenatal diagnosis, this couple chose precautional abortion since the fetus was defined as having the IVS-I-110 mutation.

Unless rare and new deletions can be identified by break point gap-PCR (including the two described in this report), the important issue when performing prenatal diagnosis for combinations of point mutation and unknown deletions, is to define whether the β-globin gene is absent by using MLPA, or present but non-functional by using MLPA and globin chain synthesis. In the first case, the only unfavorable result would be homozygosity (in fact hemizygosity) for the point mutation. In the second case, an apparent favorable heterozygosity could cause a misdiagnosis. Finally, the prediction of phenotype remains a complex matter. It cannot be guaranteed that a child with a combination β^{0} / $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$ will be healthy and able to survive without hematologic complications and without blood transfusions.

Acknowledgements

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Chapter 3.2

Non-invasive prenatal diagnosis of beta-thalassemia and sickle-cell disease using pyrophosphorolysis-activated polymerization and melting curve analysis

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Abstract

Objective: The aim of this study was to develop a pyrophosphorolysis-activated polymerization (PAP) assay for non-invasive prenatal diagnosis (NIPD) of β-thalassemia major and sickle-cell disease (SCD). PAP is able to detect mutations in free fetal DNA in a highly contaminating environment of maternal plasma DNA.

Methods: Pyrophosphorolysis-activated polymerization primers were designed for 12 informative SNPs, genotyped by melting curve analysis (MCA) in both parents. The PAP assay was tested in a series of 13 plasma DNA samples collected from pregnant women. A retrospective NIPD was performed in a couple at risk for SCD.

Results: All PAP reactions were optimized and able to detect <3% target gDNA in a background of >97% wildtype gDNA. In all 13 cases, the paternal allele was detected by PAP in maternal plasma at 10 to 18 weeks of gestation. For the couple at risk, PAP showed presence of the normal paternal SNP allele in the maternal plasma, which was confirmed by results of the chorionic villus sampling analysis.

Conclusions: In contrast to other methods used for NIPD, the combined PAP and MCA analysis detecting the normal paternal allele is also applicable for couples at risk carrying the same mutation, provided that a previously born child is available for testing to determine the linkage to the paternal SNPs.

Introduction

Hemoglobinopathies are hereditary microcytic hypochromic anemias, characterized by a reduced, altered or absent synthesis of either the α- globin or β-globin chains and represent the most common autosomal recessive disorders worldwide. Approximately 7% of the world population is a healthy carrier of a hemoglobinopathy, resulting in ~350 000 severely affected newborns annually (1). Hemoglobinopathies are common in areas where malaria is or has been endemic, but the burden on health care in north Europe is increasing due to recent migration (2) and absence of effective prevention strategies.

Because of the severity of the disease and the lack of treatment options, prenatal diagnosis is offered in many countries as part of a national prevention program (3;4) by invasive tests using chorionic villus sampling (CVS) or amniocentesis (AC). However, these tests carry a risk of fetal loss of approximately 0.5-1% (5;6). Furthermore, the test is costly because it requires hospitalization and expert personnel to perform the procedure.

The discovery of cell-free DNA from the fetus in the maternal blood circulation has led to the possibility of non-invasive prenatal diagnosis (NIPD) (7). This has been applied successfully for fetal sex determination, RhD genotype and trisomy 21 detection (8-12). Free fetal DNA (ffDNA) is detectable very early during pregnancy and the average amount of cell ffDNA during the first and second trimester is ~10% of the total amount of cell-free DNA (13).

Recently, the pyrophosphorolysis-activated polymerization (PAP) technique was successfully applied in fetal sex determination (14). Because of the serial coupling of pyrophosphorolyis and polymerization, this reaction has a very high specificity (one mutated allele can be detected in 10^9 wildtype alleles) (15;16). PAP uses oligonucleotides with a blocked 3' end, which can only be removed (and thus permitting extension) when they anneal to specific sequences of target DNA. It has been shown that if a mismatch occurs within 16 nt from the 3'-terminus of the PAP-primer, amplification is still inhibited (17). In this way, it is possible to

specifically amplify DNA inherited from father by using paternally specific mutations or polymorphisms that are absent in mother.

The aim of the current study was to develop a PAP assay for NIPD of β-thalassemia major and sickle-cell disease (SCD). In the Netherlands, most requests for prenatal diagnosis concern homozygosity for the sickle-cell mutation or compound heterozygosities causing SCD, or a variety of β-thalassemia causing mutations that commonly occur in the Dutch immigrant population (18). This makes mutation-specific detection using ffDNA impossible as the paternally and maternally inherited alleles are indistinguishable. Therefore, we decided to use paternal-specific SNPs in linkage to the normal or mutant allele to determine the risk of having an affected fetus. This procedure is suitable for the >200 β-thalassemia mutations (19) without the design of separate assays for each individual molecular defect. The advantage of this approach is that it reduces theamount of reactions to be designed and optimized to the limited number of informative SNPs. Furthermore, the use of SNPs also makes it possible to perform NIPD when both parents carry the same mutation, as is often the case in populations with a high incidence of a small number of mutant alleles. Mutation-specific assays can only detect the disease-causing allele, whereas SNPs can be a target for the normal allele as well. Melting curve analysis (MCA) was used to quickly screen family members for the informative SNP markers and to determine linkage to the normal or mutant paternal allele. In this way, it is possible to pre-select the SNPs that need to be tested with the PAP technique for at risk pregnancies. An invasive procedure is unnecessary when the normal paternal allele is detected in the maternal plasma.

Methods

Samples

Twenty-four SNPs along the β-globin gene cluster were selected on the basis of previous studies (20;21) (Table 1). DNA samples from healthy individuals of seven subsets of different populations were genotyped by MCA to test their informativity. These included 50 Dutch, 50 Czech, 20 Turks, 20 Moroccans, 20 Greek, 20 Cypriots and 30 Surinamese individuals. Thirteen pregnant couples referred to our laboratory for carrier diagnostics were randomly selected and used to optimize and test the PAP reactions. In one family, only the mother was available for testing. A retrospective NIPD was performed in a Turkish couple at risk for SCD, referred to our clinic for CVS analysis. Their previously born unaffected child was also included in the study to determine the linkage of informative SNPs with the paternal wildtype allele. Prior to the CVS, a blood sample was drawn from mother at 18 weeks of gestation, to be used for the non-invasive test.

All individuals of whom blood and/or DNA was used in this study consented to use their material in scientific research.

Isolation of DNA

Genomic DNA was isolated from leukocytes using the Autopure LS robotic workstation (Gentra Systems, Minneapolis, MN, USA). Maternal blood samples were centrifuged to separate the plasma from the leukocytes, erythrocytes and platelets within 48 h after blood drawing. Plasma samples were stored at -80 °C until they were used for testing. Extraction of free DNA from the plasma was performed with the EZ1 Advanced workstation and the included EZ1 Virus Mini Kit v2.0 protocol (Qiagen, Venlo, The Netherlands).

Melting curve analysis (MCA)

With the use of Primer3 (22) and Beacon Designer software (Bio-Rad Laboratories Inc, Hercules, CA, USA), primers were designed to amplify 12 different fragments along the β-globin gene cluster covering all 24 selected SNPs (Figure 1). In addition, 20 MCA probes were designed to cover SNPs specifically. Design of the primers and probes was based on the UCSC Genome Browser, February 2009 assembly (hg19). One of the probes covered three SNPs, and two probes covered two SNPs (Table 1). Because of the presence of highly homologous regions in the β-globin gene cluster, the PreG frame, XmnI site, F2 and F3 fragments were amplified in a first external reaction to obtain a specific polymerase chain reaction (PCR) product. This product was subsequently used as template for a second nested amplification to generate a shorter fragment, suitable to perform MCA. The other eight fragments were suitable for direct use for MCA without nested PCR. For the healthy control population, PCR products were also analyzed by direct sequencing to confirm the results and to validate the MCA technique.

The external PCR reaction was performed in a 20 μL volume containing 10x PCR buffer with $MgCl_2$ (Roche, Basel, Switzerland), 0.2 mM dNTPs (Roche), 6 μM of each primer (Biolegio, Nijmegen, The Netherlands), 1 unit GoTaq polymerase (Promega, Madison, WI, USA) and 20 ng genomic DNA. The reaction was initiated by 3 min denaturation at 94 °C, followed by 33 cycles of 30 s denaturation, 1 min annealing at optimal annealing temperature (Table 1) and 90 s extension at 72 °C. Final extension occurred for 6 min at 72 °C.

The PCR for MCA was performed asymmetrically to generate an excess of one of the strands for the probe to hybridize. The reaction was performed in 10x PCR buffer with $MgCl_2$ (Roche), 0.2 mM dNTPs (Roche), 1 μM forward primer, 10 μM reverse primer, 5 μM probe (Biolegio), 1 μL LCGreen® Plus dye (Idaho Technology Inc., Salt Lake City, UT, USA), 1 unit FastStart Taq polymerase (Roche) and 20 ng genomic DNA. In case of the nested PCR, 1:100 diluted external PCR product was used instead of genomic DNA. The PCR was performed in a 4Titude Framestar 96 wells plate (Bioké, Leiden, The Netherlands) and 15 μL mineral oil (Sigma) was added to prevent evaporation. The PCR was initiated with a 10 min hold at 95 °C. Thermal cycling was performed for 40 cycles of 20 s denaturation at 95 °C, 30 s annealing at optimal annealing temperature (Table 1) and 40 s extension at 72 °C. Finally, the reaction was elongated at 72 °C for 5 min and heteroduplexes were generated in an additional step at 95 °C for 1 min. Subsequently, samples were melted in the LightScanner HR96 (Idaho Technology Inc.) by using a melt range of 55 °C to 98 °C at a hold temperature of 50 °C. Data analysis of the melt curves was performed using the Call IT 1.5 software (Idaho Technology Inc.).

Pyrophosphorolysis-activated polymerization (PAP)

The PAP reaction was performed to detect the paternal allele in DNA isolated from maternal plasma. Primer pairs were designed for 12 SNPs, containing a blocked dideoxynucleotide at the 3' terminus (ddC or ddA) of either the forward or reverse primer. Primers were designed in such a way that the blocked nucleotide is complementary to the position of the SNP, or maximally 1 nt downstream of the SNP. Amplicon lengths ranged from 79 to 214 bp, which is suitable to amplify the fragmented ffDNA. The blocked primers were ordered from Fidelity Systems Inc. (Gaithersburg, MD, USA), the unblocked contra-primers were ordered from Biolegio. An overview of all the primers is given in Table 2. The PAP reaction was performed in PCR buffer (250 mM Tris-HCl pH 7.8, 80 mM $(NH_4)_2SO_4$, 17.5 mM $MgCl_2$, 125 μM dNTPs (each) and 450 μM Na_4PPi), 2.5 U Klentaq

Fragment	SNP (position*)	Upstream primer (5' > 3')	Downstream primer (5' > 3')	Probe (5' > 3'-phos)	Ta (°C)
External reactions					
preGframe		TTTCTTTCCTTTCTTATTCAAC	TGACATATTGGCCACTTAAC		58
XmnI-site		GAAACTGTTGCTTTATAGGAT	GCTTGTGATAGTAGCCTTGTC		62
F2		CCAGTGACTAGTGCTGCAAGAA	AAGTGTGGAGTGTGCACATGA		63
F3		AATCTCAGGCTTTGAGGGAAGTTAAC	TAAATGAGGAGCATGCACACAC		63
MCA reactions					
F1	rs113040651 (5291564)	TCCCACTGTGGACTACTTGCT	TTGCCTTAAAGGTGGTGACA	GCCCTGTTTTTGTCAACTGTCACCACCTTT	63
preGframe SNP 1	rs2855121 (5277291)	GCCTTACACAGGATTATGAAGTCTGAAAG	AGAAACTCTGGAAATCTGGCTTATTGG	TAATTCCTATCAACCTGATAAGTTAGGG	63
preGframe SNP 2	rs2855122 (5277236)	GCCTTACACAGGATTATGAAGTCTGAAAG	GGCAGGGACTGTTTTATTGACTAATAG	CCAGAGTTTCTGACGTCATAATCTACCAA	63
preGframe SNP 3	rs112035597 (5277116)	CCAATAAGCCAGATTTCCAGAGTTTC	GGCAGGGACTGTTTTATTGACTAATAG	GAATACTTCCTGCCATGTTAAGTGG	63
XmnI-site	rs7482144 (5276169)	TGAATCGGAACAAGGCAAAGGC	CCTCACTGGATACTCTAAGACTATTGG	AAATATCTGTCTGAAACGGTTCCTGGC	65
F2 SNP 1	rs113425530 (5274720)	TGGGAAAGCAAAATCTCAGG	GAATTCTTTGCCGAAATGGA	TTCTGGGTGGAAGCTTGGTGTGTAGTTA	63
F2 SNP 2	rs2070972 (5274717)	TGGGAAAGCAAAATCTCAGG	GAATTCTTTGCCGAAATGGA	TTCTGGGTGGAAGCTTGGTGTGTAGTTA	63
F2 SNP 3	rs113047906 (5274708)	TGGGAAAGCAAAATCTCAGG	GAATTCTTTGCCGAAATGGA	TTCTGGGTGGAAGCTTGGTGTGTAGTTA	63
F2 SNP 4	rs60097179 (5274452)	TAGGCTTGATTCTGGGTGGA	GAACTGAAGACAACCATGTGTGA	GGCTTTATTCTGCAAGCAATACAAATAATA	63
F3 SNP 1	rs28379094 (5269806)	TCTCAGGCTTTGAGGGAAGT	CATGTGTGATCTCTCAGCAGAA	TCTGGGTGGAAGCTGGGTGTGTAGTTATCT	63
F3 SNP 2	rs28440105 (5269799)	TCTCAGGCTTTGAGGGAAGT	CATGTGTGATCTCTCAGCAGAA	TCTGGGTGGAAGCTGGGTGTGTAGTTATCT	63
F3 SNP 3	rs5789383 (5269586)	TCTCAGGCTTTGAGGGAAGT	CATGTGTGATCTCTCAGCAGAA	AGATACCACTGAGCCTCTTGCCCATGATT	63
F3 SNP 4	rs3841756 (5269534)	TCTCAGGCTTTGAGGGAAGT	CATGTGTGATCTCTCAGCAGAA	GGCTTTATTCTGCAAGCAATACAAATAAT	64
F4	rs10128556 (5263683)	TCACAATATGTTGGGGTAGTGA	GCACCTCCCATTGTGTCCTA	GGGGTAGTGAGTTGGCATAGCAAGTAAGA	61
F5	rs968857 (5260458)	CCTAAACTGAGGAACCTTTGG	TGATGTGAATAAATGCATGACAC	GTTTGATTAGTCAGCAAGCATGTGTCA	61
F6	rs16911905 (5249290)	CGATCACGTTGGGAAGCTAT	TAGGGGTAGGAGGGGAAAAG	TAGGAACTTGAATCAAGGAAATGATTT	61
voBRsa1 SNP 1	rs10742584 (5248770)	GCACAGAGCACATTGATTTGTATTTATTC	CAGAATATGCAAAATACTTACAGGACAGAATG	GGAGAAGATATGCTTAGAACTGAGGTAGAG	61

Table 1 Sequences of the primers and probes used for MCA.

Fragment	SNP (position*)	Upstream primer (5' > 3')	Downstream primer (5' > 3')	Probe (5' > 3'-phos)	Ta (°C)
voBRsa1 SNP 2	rs10742583 (5248641)	GCAGGAAGAGATCCATCTACATATC	CGTAATATTTGGAATCACAGCTTGG	TTTAGTGCATCAATTTCTTATTTGT	67
voBRsa1 SNP 3	rs16911894 (5248614)	GCAGGAAGAGATCCATCTACATATC	CGTAATATTTGGAATCACAGCTTGG	TAATAAGAAAATTGGGAAAACGATCTTC	65
β frame SNP 1	rs10768683 (5247791)	GCACGTGGATCCTGAGAACT	TCATTCGTCTGTTTCCCATTC	GGTGAGTCTATGGGACCCTTGATGTTTTC	65
β frame SNP 2	rs7480526 (5247733)	GCACGTGGATCCTGAGAACT	TCATTCGTCTGTTTCCCATTC	TAGGAAGGGGAGAAGTAACAGGGTAC	65
β frame SNP 3	rs7946748 (5247726)	GCACGTGGATCCTGAGAACT	TCATTCGTCTGTTTCCCATTC	TAGGAAGGGGAGAAGTAACAGGGTAC	65
F7	rs10837631 (5246356)	CCCATTTGCTTATCCTGCAT	TTCAGGGGAAAGGTGGTATC	ATCTCTCAGCCTTGACTCCACTCAGTTCT	65
naBHpa1 SNP 1	rs4426157 (5240771)	GGCAAAGGGATCTATTCAAGAAG	GCTTGGTGCAGAGCTGAGTC	CAACGAGACAGAAAGTTAACAAGGA	65

Table 1 (continued) Sequences of the primers for the four external polymerase chain reactions, and primers and probes covering the 24 SNPs used for MCA. The optimal annealing temperature (Ta) for each reaction is indicated in the last column. MCA, melting curve analysis. *Positions are according to the UCSC Genome Browser, February 2009 (hg19).

SNP name	Position*	RS-nomen-clature	WT	mutant	Upstream primer (5' > 3')	Downstream primer (5' > 3')
preGframe SNP2	5277236	rs2855122	G	A	GTAGAGCTCTCCTCCAATAAGCCAGATTTCCAGA TCTCCTCCAATAAGCCAGATTTCCAGAGTTTCTGAC[dd**A**]	CTCTGAACTCGATCCATGACCTTGGTAGATTATGA[dd**C**] CTTTTGTTTTTTCTCTGAACTCGATCCATGACCTTGGTAG
F2 SNP2	5274717	rs2070972	T	G	GGCCAGTGACTAGTGCTGCAAGAAGAAC	AGCCTGGCCTCCAGATAACTACACACC[dd**C**]
F2 SNP4	5274452-5274453	rs60097179	-	ins T	CTTTCAAGGATAGGCTTTATTCTGCAAGCAAT[dd**C**]	GACAACCATGTGTGATCTCTTAGCAGAATAGA
F3 SNP3	5269586	rs5789383	A	del A or G	CCCTGTCCTCCAGATACCACTGAGC[dd**C**]	CAAACAGGCATGCAGAAATACACATACACACTTCCC
F3 SNP4	5269534	rs3841756	A	del A	TCAGAGCTTTCAAGGATAGGCTTTATTCTGCAAGCAAT[dd**A**]	GCAGAAATACACATACACACTTCCCTCAATATAAACCC
F5	5260458	rs968857	A	G	TTAGTATTTATAGGTCATGAGGTTCCTTCCTCTGC CCTTCCTCTGCCTCTGCTATAGTTTGATTAGTCA**A**[ddC]	CTGATGTGAATAAATGCATGACACATGCTTG[dd**C**] GGTTGAGTAACTGATAATGGTTTGTCCTTTCCTTGATGG
F6	5249290	rs16911905	G	C	GGAGGTTTAAACAAACAAAATATAAAGAGAAATAGGAACTTGAAT[dd**C**] GTCCACTAAGAATACTGCGTTTTAAAATCATTTCCTT[dd**C**]	AGGGGAAAAGGTCTTCTACTTGGCTCAGATTAT TCACGTTGGGAAGCTATAGAGAAAGAAGAGTA
voBRSal SNP2	5248641	rs10742583	G	A	GAAGATCGTTTTCCCAATTTTCTTATTACACAAATAAGAA[dd**A**] GGTAGACAAAACTCTTCCACTTTTAGTGCATCAA[dd**C**]	CGCAGGAAGAGATCCATCTACATATCC GGAATCACAGCTTGGTAAGCATATTGAAGATCGTT
β-frame SNP1	5247791	rs10768683	C	G	TGAGAACTTCAGGGTGAGTCTATGGGAC[dd**C**] CCATAGAAAAGAAGGGGAAAGAAAACATCAAG[dd**C**]	CTGTACCCTGTTACTTATCCCCTTCCTATGAC TTTGCCACACTGAGTGAGCTGCACTGT
β-frame SNP2	5247733	rs7480526	A	C	GGTGAGTCTATGGGACGCTTGATGTTTTCTTTCC GGTGAGTCTATGGGACGCTTGATGTTTTCTTTCC	CGTCTGTTTCCCATTCTAAACTGTACCCTGTTACTT[dd**A**] CGTCTGTTTCCCATTCTAAACTGTACCCTGTTACTT[dd**C**]
β-frame SNP3	5247726	rs7946748	G	A	TAAGTTCATGTCATAGGAAGGGGAGAAGTAA[dd**C**] ATTCGTCTGTTTCCCATTCTAAACTGTACCCT[dd**A**]	TAAAACGATCCTGAGACTTCCACACTGATGC CTTCCCCTTCTTTTCTATGGTTAAGTTCATGTC
F7	5246356	rs10837631	T	A	TACCCATTTGCTTATCCTGCATCTCTCAGCCTTG[dd**A**]	GCCGTAAAACATGGAAGGAACACTTCAGGGGAA

Table 2 Sequences of all primers used for PAP. The position of the SNP complementary to the target strand is indicated in bold. We were able to design pyrophosphorolysis-activated polymerization (PAP) assays for 12 of the 17 SNPs that appeared to be informative in the healthy control populations. For seven SNPs, two PAP primer pairs were designed to detect both the wildtype (WT) and the mutant. *Positions are according to the UCSC Genome Browser, February 2009 (hg19).

	F1 rs113040651	preGframe SNP1 rs2855121	preGframe SNP2 rs2855122	XmnI-site rs7482144	F2 SNP2 rs2070972	F2 SNP4 rs60097179	F3 SNP1 rs28379094	F3 SNP3 rs5789383	F3 SNP4 rs3841756	F4 rs10128556	F5 rs968857	F6 rs16911905	voBRsa1 SNP2 rs10742583	β frame SNP1 rs10768683	β frame SNP2 rs7480526	β frame SNP3 rs7946748	F7 rs10837631
Turks (n=40)	57.5	70.0	42.1	75.0	42.5	70.0	70.0	27.5	12.5	26.5	55.0	5.0	12.5	12.5	40.0	10.0	27.5
Moroccans (n=40)	50.0	80.0	58.3	77.5	50.0	0.0	71.1	18.4	10.5	20.0	45.0	17.5	12.5	15.0	40.0	7.5	17.5
Czechs (n=100)	54.2	68.0	44.7	68.0	44.0	66.0	67.0	23.0	14.0	33.0	54.0	4.0	17.7	17.0	57.0	15.0	37.0
Surinamese (n=60)	33.3	86.7	73.3	86.7	63.8	83.3	73.3	10.0	21.7	13.3	38.3	13.0	40.0	36.7	63.3	18.3	14.3
Cypriots (n=40)	40.0	77.5	ND	78.9	62.5	ND	77.8	13.2	23.7	22.5	32.4	2.5	10.0	10.0	40.0	15.0	25.0
Greek (n=40)	47.5	69.4	ND	70.0	52.5	ND	71.1	21.1	18.4	27.5	47.4	10.5	18.4	11.1	62.5	20.0	47.4
Dutch (n=100)	59.2	57.1	43.6	58.2	37.0	59.0	45.9	15.0	11.2	42.7	58.2	10.2	18.4	18.4	63.3	12.2	32.3
Total (n=420)	50.2	70.5	50.9	71.2	48.1	59.6	64.9	18.1	15.3	29.0	49.5	8.5	19.4	18.4	55.0	14.1	29.8

Table 3 Overview of the frequencies of the informative SNPs in each of the control populations. Out of the 24 selected SNPs, 17 appeared informative (frequency >5% and <95%) and were used to design the pyrophosphorolysis-activated polymerization (PAP) assay. The numbers indicate the percentage of alleles in the population containing the SNP. ND, not determined, n = number of alleles tested.

(ScienTech Corp., Chesterfield, MO, USA) and 10 μL DNA isolated from maternal plasma. Thermal cycling consisted of 45 cycles of 15 s denaturation at 94 °C, a stepwise annealing for 2 min (information on the optimal conditions for each primer pair is available upon request) and extension for 40 s at 72 °C. Amplified products were visualized by electrophoresis on a 3.5% Tris/Borate/EDTA (TBE) agarose gel.

Results

Screening of healthy control populations

Along the β-globin gene cluster, 24 SNPs previously described were selected for which MCA primers were designed (20;21). Seventeen SNPs occurred with a frequency between 5% and 95% in the seven different populations and were therefore considered informative (Table 3).

Optimization of the PAP reactions

Pyrophosphorolysis-activated polymerization reactions were initially tested in genomic DNA samples with known genotype to test the sensitivity of the PAP and to determine the optimal conditions for each primer pair. Optimization of the PAP reactions included testing at different annealing temperatures and annealing times. Most reactions worked properly after trying three to four different conditions. For three SNPs, we designed new primers to obtain better results. After optimization, we tested the PAP reactions in dilution series of genomic DNA, in which we mixed a wildtype sample with a homozygous SNP genotype. For all 12 SNPs, we were able to detect <1% target gDNA mixed in > 99% wildtype gDNA isolated from whole blood. The next step included testing in cell-free DNA isolated from plasma samples from healthy control individuals. This was also performed in a dilution series by mixing a wildtype and a homozygous SNP plasma DNA sample. In all cases, ~3% of the SNP plasma DNA sample was detectable in ~97% wildtype DNA, indicating that our method was suitable to use for DNA isolated from blood plasma.

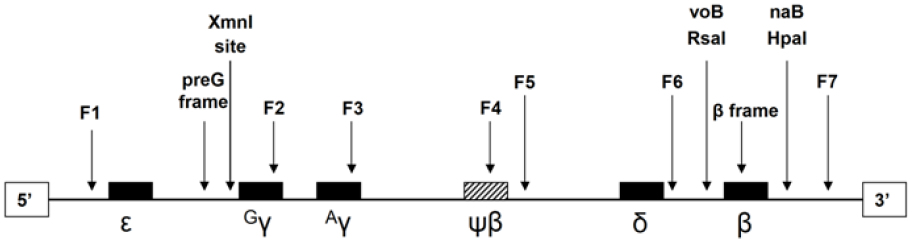

Figure 1 Overview of the positions of the 12 different fragments along the β-globin gene cluster, which are amplified by melting curve analysis (MCA). Each arrow represents one fragment covering one, two or three SNPs. Eight fragments were of suitable length (up to 400 bp) to perform MCA directly. The PreG frame, XmnI site, F2 and F3 fragments were first amplified in an external polymerase chain reaction (PCR) to obtain a specific PCR product, which was then used as template for a second nested MCA reaction.

Test cases: 13 pregnant couples

Thirteen pregnant couples were referred to our laboratory for carrier diagnostics. In all cases, only one of the parents was a carrier of a hemoglobinopathy, so the couples were not at risk of having an affected child and prenatal diagnosis was not indicated. PAP reactions were tested in plasma DNA from these pregnant women at 10 to 18 weeks of gestation to test the performance of the PAP assay in maternal plasma. The MCA technique was used to genotype the SNPs prior to the PAP test to determine which SNPs were absent in the mother but present in the father and, when detected by PAP in maternal plasma, should be of fetal origin. In all 13 couples, at least one SNP, and in eight families (~60% of the cases), two or more were informative (Table 4). Obviously, we could not determine the linkage of the SNP to the paternal mutations in these cases, as no other family members were available. However, in all cases, we were able to detect a paternal allele in maternal plasma. In ten families, genomic DNA of the baby (after birth) was available for testing, and PAP results were confirmed by direct sequencing analysis. All PAP reactions performed in this group of test cases showed the correct results. These results indicated that our method is suitable to perform NIPD for β-thalassemia major and SCD, provided that a previously born child or other family member is available for testing to determine the linkage to the paternal SNPs.

Proof of principle: retrospective NIPD

A Turkish couple at risk for SCD was referred to our clinic for prenatal diagnosis for their second pregnancy. Their first child was born unaffectedly. We performed MCA for both parents and the first child to determine the informative SNPs, as well as the linkage to the paternal alleles. One of the SNPs in the β-globin gene, rs7480526 (wildtype A, variant C), was informative in this family. The mother had a wildtype genotype (A/A), and both the father and the child were heterozygous (A/C). From this data, it could be concluded that the paternal A allele is linked to the hemoglobin S (HbS) mutation and C is linked to the paternal wildtype allele, because the first child inherited the C allele but not the HbS mutation from the father (Figure 2).

As a proof of principle, PAP was performed for the informative SNP on free plasma DNA isolated from maternal blood. Results showed the presence of the A and C allele in the reaction with the oligonucleotide specific for the A and C nucleotide, respectively. As the mother does not carry the C allele, it can be deduced that the fetus inherited the C allele from the father, which is linked to the normal allele (Figure 2). The fetus did not inherit the mutated allele from the father and will be either a carrier of HbS or normal and thus not affected with SCD. The result was confirmed by performing MCA for this SNP on the fetal material obtained by CVS, showing heterozygosity for the SNP (A/C). In addition, results from the CVS showed that the fetus was a carrier of HbS, indicating inheritance of the maternal mutation.

Discussion

The discovery of circulating ffDNA in maternal plasma during pregnancy has provided the possibility to perform non-invasive DNA tests on the fetus. In this study, we used a strategy which combined the use of the MCA and PAP technique for retrospective NIPD of β-thalassemia major and SCD. The PAP method is sensitive enough to detect the small amount of ffDNA in cell-free DNA isolated from maternal plasma. In this study we tested the PAP assay in 13 test couples and in 1 couple at risk. Our results show that this approach is suitable to detect the paternal allele in

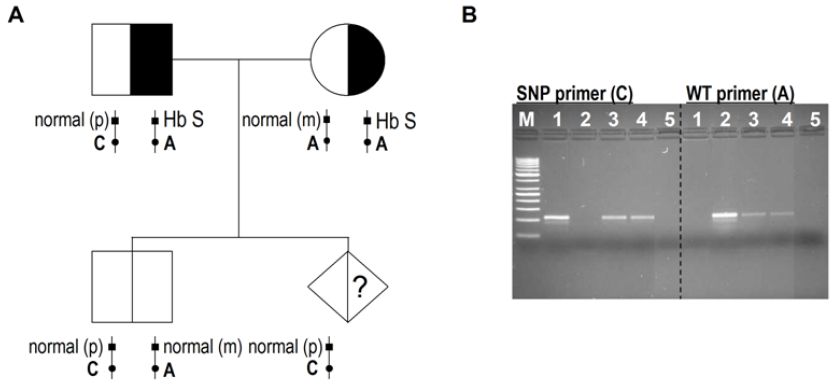

Figure 2 (A) Pedigree of the test family. The first child inherited the C allele from the father but not the HbS mutation. Therefore, it can be concluded that the C is linked to the normal allele [normal (p)]. The C allele was detected in the maternal plasma, indicating that the second child also inherited the normal paternal allele and will thus not be affected with sickle-cell disease. **(B)** Results of gel electrophoresis of the pyrophosphorolysis-activated polymerization products. The left panel shows the results from the reaction of the primer pair to detect the variant (C), the right panel depicts the results from the wildtype (A) primer pair. The A allele is obviously present, as the mother carries this allele. However, the C allele is also detected, which must be of fetal origin. (M: Gene Ruler 100 bp marker, lane 1: control sample C/C, lane 2: control sample A/A, lanes 3 and 4: DNA sample isolated from maternal plasma, lane 5: blanc)

plasma from pregnant women as we were able to detect the paternal allele in all cases. The major advantage of this kind of prenatal testing is its early and non-invasive approach. There is no increased risk for fetal loss due to the invasive procedure and this will make prenatal diagnosis more safe for pregnant women. Admission to the hospital is not necessary, which reduces the costs compared with invasive testing. Furthermore, ffDNA can be detected in maternal blood from the 6th week of gestation, which enables early prenatal diagnosis (23).

Melting curve analysis is a helpful screening method to identify which of the SNPs are informative in the family. Analysis of both parents and selection of the informative SNPs can be performed within 1 day, which is a major advantage compared to any sequence-based technology. It is highly robust, costs are relatively low and results are obtained with high sensitivity and specificity. Furthermore, SNPs that do not create or eliminate restriction sites can also be screened for with this method. The MCA method is high throughput, 96 reactions can be performed and analyzed simultaneously. MCA can be used as a quick screening method to select the informative markers, and the sensitivity of the PAP assay is necessary for detection of the ffDNA. The PAP assay can be performed in 1 day, so the complete NIPD can be performed within 2 days.

A limitation of using linkage for NIPD is the requirement of a previously born child or other family member to determine the phase of the paternal mutation in combination with the SNP. However, the number of requests for prenatal diagnosis is increasing due to the implementation of hemoglobinopathy in the newborn screening in the Netherlands as of January 2007 (24). When the first-born in a family appears to be carrier of or affected with a hemoglobinopathy, parents will be tested. If it concerns a couple at risk, prenatal diagnosis will be offered for any following pregnancies.

It is well known that the human β-globin gene cluster contains a hotspot for meiotic recombination between the δ-globin and β-globin genes (20). In previous studies, the rate of recombination in the β-globin gene cluster is estimated to be 3 to 30 times (25) and 30 to 50 times (26) greater than the genome-wide average. The presence of a recombinant β-globin haplotype in a family has significant implications when linkage is used for prenatal diagnosis (27). Therefore, it is important to perform a complete haplotype analysis and, whenever possible, use SNPs located at both the 3' and 5' end of the recombination hotspot. In the couple at risk tested in the current study, only one informative SNP was present. However, this particular SNP is located in the β-globin gene itself, which eliminates the risk of having a false positive or negative result due to meiotic recombination. If none of the SNPs within the β-globin gene are informative, it is required to test at least two SNPs, one at each side of the recombination hotspot, to obtain a reliable diagnosis.

It might occur that none of the 12 SNPs is informative in some families. To be able to perform NIPD by PAP, it is required that the father has a heterozygote genotype for one of the SNPs whereas the mother is wildtype or homozygous. Therefore, we will continue developing and optimizing PAP assays for the other five informative SNPs which will enable us to test for both the wildtype and the mutant alleles in all 17 informative SNPs. This will increase the chances of finding an informative SNP within a family and will make the test applicable to more couples at risk.

The purpose of this study was to design PAP assays for the 17 SNPs that appeared to be informative from the screening of the healthy control populations by MCA. We were able to design PAP primers for 12 of the SNPs, and two primer sets were designed for seven of these SNPs, one to detect the wildtype and one for the mutant allele. Because of the stringencies for the PAP primer design (e.g. GC content, T_m, location of the SNP), assays for the other five SNPs are not optimized yet. However, the 12 SNPs provided sufficient information to perform the current study. In the near future, we will perform the NIPD by PAP in parallel to the standard CVS and AC procedures. After a period of validation, the PAP technique will be introduced as a standard diagnostic method for prenatal diagnosis.

Other studies describing NIPD for paternally inherited β-thalassemia mutations include detection of the codon 41/42 (-CTTT) mutation by using real-time PCR (28) and hemoglobin E detection by restriction enzyme analysis of PCR products (29). However, these approaches are suitable for a single mutation and are not applicable when both parents carry the same mutation. The allele-specific arrayed primer extension (AS-APEX) method combines detection of mutations and genotyping of SNPs (30), similar to the current study. Although this approach showed to be very useful for the southern Chinese population, only four mutations can be detected by this AS-APEX assay, which would not be sufficient to apply in north European countries. Another possibility is to detect the paternal allele by the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (31). In addition, this method, in combination with multiplex PCR of single-copy DNA (M1-PCR), enables direct haplotyping without the use of pedigree data. This would make the NIPD method described in this study applicable to more pregnant couples at risk (32). These mass spectrometry methods showed high specificity and sensitivity, but the use of sophisticated and expensive equipment limits the applicability in diagnostic laboratories. PAP in combination with MCA is relatively cheap and quick to perform. In principle, this approach is suitable to perform NIPD for all β-thalassemia causing mutations and the sickle-cell mutation, even when both parents carry the same mutation.

		F2 SNP2		F2 SNP4		F5		F6		β frame SNP1		β frame SNP2		F7	
		rs2070972		rs60097179		rs968857		rs16911905		rs10768683		rs7480526		rs10837631	
Family	Member	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2
1	Mother					G	G					C	C		
	Father					G	A					C	A		
2	Mother					G	G					C	C		
	Father					G	A					C	A		
3	Mother					G	G	C	C					T	T
	Father					G	A	C	G					A	A
	Child					G	A	C	G					T	A
4	Mother	T	T			A	A								
	Father	T	G			G	A								
	Child	T	G			G	A								
5	Mother			-	-										
	Father			ins T	ins T										
	Child			-	ins T										
6	Mother											A	A		
	Father											C	A		
	Child											C	A		
7	Mother	T	T			A	A					C	C		
	Father	T	G			G	A					C	A		
8	Mother											C	C		
	Child											C	A		

Table 4 Overview of the informative SNPs in each of the families.

		F2 SNP2		F2 SNP4		F5		F6		β frame SNP1		β frame SNP2		F7	
		rs2070972		rs60097179		rs968857		rs16911905		rs10768683		rs7480526		rs10837631	
Family	Member	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2
9	Mother							C	C	C	C	A	A		
	Father							C	G	C	G	C	A		
	Child							C	G	C	G	C	A		
10	Mother							C	del	C	del	C	del		
	Father							C	G	C	G	C	A		
	Child							del	G	del	G	del	A		
11	Mother					G	G	C	C			C	C		
	Father					A	A	C	G			C	A		
	Child					G	A	C	G			C	A		
12	Mother	T	T												
	Father	G	G												
	Child	T	G												
13	Mother											C	C		
	Father											A	A		
	Child											C	A		
testcase	Mother											A	A		
	Father											A	C		
	Fetus (CVS)											A	C		

Table 4 (continued) Overview of the informative SNPs in each of the families. All parents were screened by melting curve analysis for the 12 SNPs for which pyrophosphorolysis-activated polymerization primers were designed. DNA of the babies (if available) was analyzed by direct sequencing to confirm the results. Only the SNPs that were informative in each family are shown in the table. CVS = material obtained from chorionic villus sampling.

In conclusion, this study has shown that the PAP assay, in combination with the MCA method, can be used for paternal allele detection in maternal plasma. This enables NIPD of β-thalassemia major and SCD, provided that a previously born child or other family member is available for testing to determine the linkage to the paternal SNPs. If the SNP linked to the paternal mutation is detected, an invasive procedure will still be necessary to check whether the fetus also inherited the maternal mutation. However, NIPD methods will make half of the invasive procedures redundant when informative SNPs are present. The use of SNPs instead of mutations makes it possible to perform NIPD in couples carrying the same mutation. In addition, this approach might also be applicable for other genetic disorders as linkage to informative SNPs can be used for most disease genes.

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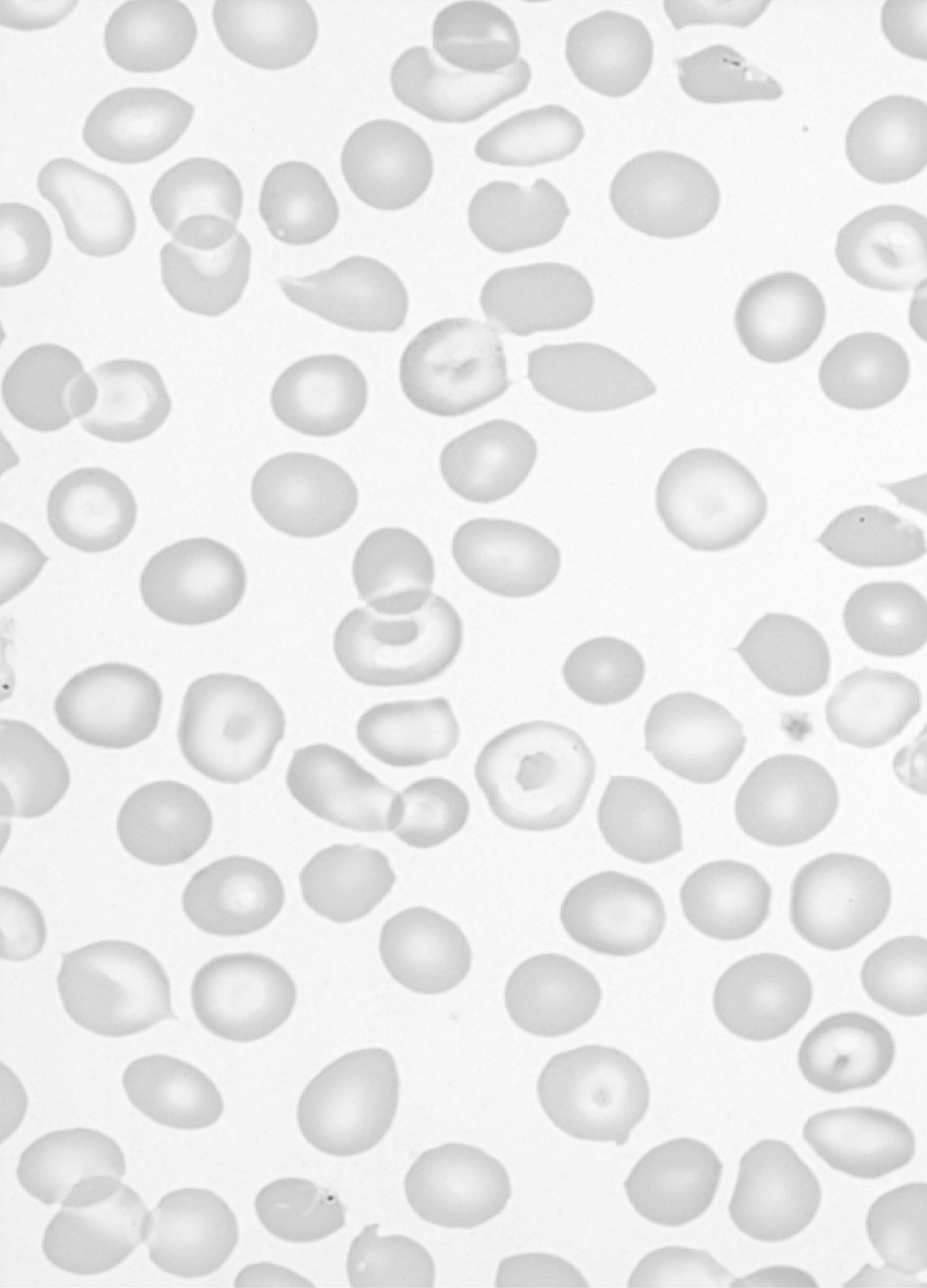


Chapter 4

Case studies

Chapter 4.1

A new α^0-thalassemia deletion found in a Dutch family ($--^{AW}$)

Phylipsen M, Vogelaar IP, Schaap RA, Arkesteijn SG, Boxma GL, van Helden WC, Wildschut IC, de Bruin-Roest AC, Giordano PC, Harteveld CL

Blood Cells Mol Dis. 2010 Aug 15;45(2):133-5

Abstract

Alpha-thalassemia is an inherited hemoglobin disorder characterized by a microcytic hypochromic anemia caused by a quantitative reduction of the α-globin chain. The majority of the α-thalassemias is caused by deletions in the α-globin gene cluster. A deletion in the α-globin gene cluster, which was found in a Dutch family, was characterized by MLPA, long-range PCR and direct sequencing. We describe the molecular characterization of a novel 8.2 kb deletion ($--^{AW}$), involving both α-globin genes *in cis*. The deletion is caused by a non-homologous recombination event between an Alu and an L1-repeat sequence. This deletion is the third example of a non-homologous recombination event involving an Alu and an L1 repeat, and the first described in the human α-globin gene cluster. Because of a 25% risk of Hb Bart's with hydrops foetalis in the offspring when in combination with another α^0-thalassemia allele, it is important to diagnose this deletion.

Introduction

Thalassemias are inherited hemoglobin disorders characterized by a quantitative reduction of the α- or β-globin chains. Approximately 80-90% of all α-thalassemias is caused by genomic deletions involving the α-globin gene cluster on chromosome 16p13.3. Besides the most common deletions, a large variety of less occurring thalassemia deletions have been found in different populations. More than 50 different types have been described until now (HbVar). Gap-PCR, Southern blot analysis and fluorescent in situ hybridization (FISH) are commonly used to identify these deletions; however, many deletions remain unknown using conventional techniques.

Multiplex Ligation-dependent Probe Amplification (MLPA) (1) is a relatively simple technique for rapid detection of genomic rearrangements in the α-globin gene cluster. It was previously shown that this assay is suitable to detect known and unknown deletions in a cohort of cases that remained unsolved after performing the standard diagnostic techniques (2). Here we describe the molecular characterization of the $--^{AW}$ deletion which was found in a Dutch family. It is important to diagnose this type of deletion, because of a 25% risk of Hb Bart's with hydrops fetalis in the offspring when in combination with another α^0-thalassemia allele.

Materials and methods

Fresh blood samples of 6 members of a Dutch family (Fig. 1) were analyzed using standard methods. All members agreed to participate in this study by written informed consent. Hematological parameters were determined on Horiba ABX Micros 60 Hematology Analyzer. Biochemical analyses were performed on HPLC Variant II (Bio-Rad Laboratories, Hercules, CA, USA) using the β-Thalassemia Short Program and on Capillarys (Sebia, Evry, France). DNA was extracted using the salting out procedure (3). Molecular analyses included gap-PCR for the seven most common α-thalassemia deletion defects (4;5), sequence analysis of the α-globin genes based on procedures previously described (6) and MLPA to detect deletions in the α-globin gene cluster. Two different probe mixes were used, HBA (2) (Service XS, Leiden, The Netherlands) and P140B2 (MRC Holland, Amsterdam, The Netherlands). Furthermore, long range PCR (TaKaRa LA Taq™ kit, TaKaRa Bio Inc, Otsu, Shiga, Japan) was performed according to the manufacturer's instructions applying the following PCR conditions: hotstart of 2 min at 94 °C, 30 cycles of denaturation for 20 s at 94 °C and elongation for 5 min at 68 °C, followed by a final elongation step of 10 min at 68 °C. Direct sequencing of the PCR product containing the breakpoint fragment was performed on the ABI PRISM™ 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

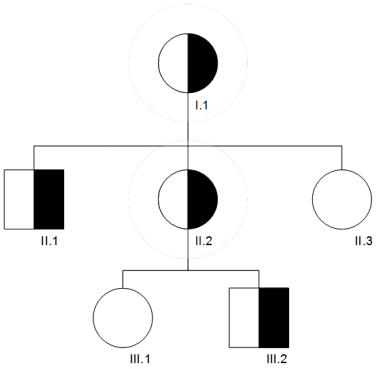


Figure 1 Pedigree of the Dutch family.

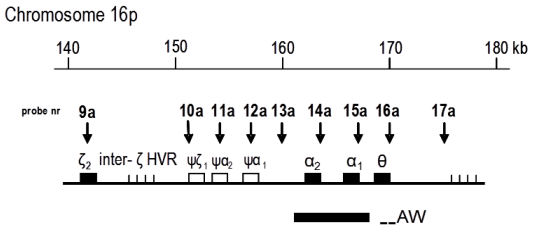


Figure 2 Schematic overview of the MLPA result. The probe numbers refer to the HBA probe mix (Service XS). The black box below the figure represents the deleted area.

Results

An overview of the hematological, biochemical and molecular data is given in Table 1. Four family members presented with microcytic hypochromic parameters, borderline-low HbA_2 levels and presence of HbH inclusions (not performed for I.1), which are indicative for an α^0-thalassemia carriership. However, gap-PCR for the seven most common α-thalassemia deletions and sequence analysis of both α-genes showed no abnormalities. Concentrations of haptoglobin and zinc protoporphyrin (ZPP) were normal.

After performing MLPA with the HBA probe mix, the four patients appeared to carry a deletion in the α-globin gene cluster, involving both α-genes. Signal ratios of ~0.5 were found for probes 14a and 15a (for probe numbers and locations, see Harteveld et al. 2005), indicating that the deletion breakpoints are located between the locations of probe 13a and 14a (between position 160991 and 165128) on the 5' end, and between 15a and 16a (between position 167853 and 171054, all positions mentioned are according to UCSC Genome Browser, March 2006) on the 3' end (Fig. 2). Primers for long-range PCR were designed in these regions. MLPA results for the P140B2 probe mix were in concordance with the HBA probe mix. By using primers AW-F and AW-R (Table 2) in a long-range PCR reaction a ~700 bp breakpoint fragment was obtained in all cases. Restriction-enzyme mapping allowed a more accurate localization of the exact breakpoint, which allowed the design of sequencing primer AW-Rseq close to the breakpoint. Sequence analysis showed the 5' breakpoint at position 161279 and the 3' end breakpoint at position 169453, indicating a deletion of 8174 bp. However, the breakpoint fragment contained a 14 bp orphan sequence (Fig. 3), which maps to exon 3 of the α_2- and α_1-globin genes (position 163542-163555 for α_2 and position 167353-167366 for α_1). In addition, an unknown mutation was found in the breakpoint fragment 5' of the breakpoint at position 161273 (G>A).

The other two family members (II.3 and III.1) presented with normocytic normochromic parameters, indicating that they are not carrier of α-thalassemia. Therefore, the inclusion bodies test has not been performed for these individuals. Sequence analysis of the α-genes and MLPA results were normal.

Parameters	Reference range	I.1	II.1	II.2	II.3	III.1	III.2
Age-sex		82-F	61-M	57-F	55-F	34-F	29-M
Hb (g/dL)	M: 13.7-17.7 F: 12.1-16.1	8.5	14.7	13.0	13.2	13.5	15.3
RBC (10^{12}/L)	M: 4.6-6.2 F: 4.2-5.4	4.33	6.88	5.94	4.58	4.55	7.02
MCV (fL)	76-100	69	71	72	89	89	73
MCH (pg)	27.4-33.8	19.8	21.4	22.1	28.7	29.8	21.7
HbA_2 (%)		2.2	2.4	2.1	2.4	2.5	2.2
HbF (%)		1.3	0.6	0.7	0.5	0.6	0.4
HbH		N.D.	+	+	N.D.	N.D.	+
ZPP (μmol ZP/mol heme)	< 100	122	45	62	74	46	56
Haptoglobin (mg/100 mL)	50-180	133	110	110	68.1	78	93.6
Hematocrit	M: 0.40-0.53 F: 0.36-0.48	0.298	0.488	0.425	0.409	0.403	0.512
Gap-PCR α		Normal	Normal	Normal	Normal	Normal	Normal
DNA α-genes		Normal	Normal	Normal	Normal	Normal	Normal
MLPA		Deletion α_2 and α_1 genes	Deletion α_2 and α_1 genes	Deletion α_2 and α_1 genes	Normal	Normal	Deletion α_2 and α_1 genes

Table 1 Hematological, biochemical and molecular data of the 6 family members. (Hb: hemoglobin, RBC: red blood cell count, MCV: mean corpuscular volume, MCH: mean cell hemoglobin, ZPP: zinc protoporphyrin, M: male, F: female, N.D.: not determined)

Primer	5' > 3' sequence
AW-F	GCTGAGGGAACACAGCTACA
AW-R	CAGGTGTTTCTTCAGGGCAGTGAAC
AW-Rseq	ATGCAGCACTCACTCTGCTG

Table 2 Sequences of the primers used for long-range PCR and sequencing of the breakpoint fragment.

Discussion

We describe a new 8.2 kb deletion in the α-globin gene cluster, involving both α-genes in 4 members of a Dutch family. The breakpoint at the 3' end of the deletion is located in an Alu-repetitive sequence, while the 5' end breakpoint is located in an L1-repeat. Patient I.1 presented with low RBC, Hb, hematocrit and elevated zinc protoporphyrin levels compared to the other $--^{AW}$ carriers in this family. The elevated zinc protoporphyrin is indicative for iron deficiency. Only one wild type allele is present in the $--^{AW}$ deletion carriers, which explains the normal results for gap-PCR and sequence analysis.

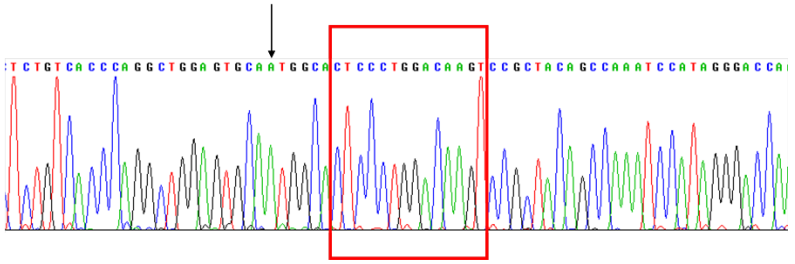
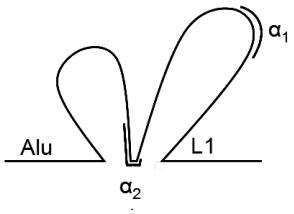


Figure 3 Breakpoint fragment of the --^{AW} deletion. The 14 bp orphan sequence is indicated by the red square. The first (C) and last (T) nucleotide of the orphan sequence overlap with the 5' and 3' breakpoint sequence, respectively. The unknown base pair substitution (G>A) is indicated by the arrow.

Alu and L1 sequences, which are present at more than 500,000 locations of the human genome, are the two most common repeat elements identified, representing more than 5% and 10% of the human genome, respectively (7). Non-homologous recombination events between an Alu and L1 repeat have been described before in the dystrophin (DMD) gene (8) and in thedihydrolipoyl transacylase (E2) gene (9). To our knowledge, this deletion is the third example of a non-homologous recombination event involving an Alu and an L1 repeat, and the first described in the human α-globin gene cluster.

It has been proposed that the 5' and 3' deletion breakpoint sequences are physically closely located in the wild-type situation because of looping of the chromatin during the replication cycle. It has been suggested that four different types of deletions in the human β-globin gene cluster arose due to losses of complete loops by a breakage event at the replication fork (10). Interestingly, a 14-bp orphan sequence which maps to the α_2- or α_1-globin gene is still present in the --^{AW} allele. A similar phenomenon was found in the --^{Med1} deletion, which contains an 134 bp insert at the recombination junction, mapping to a region upstream the α-globin gene cluster (11). We suggest that this deletion arose due to a double loop formation during replication in which both loops were eliminated by breakage at the replication fork and reunion to a replication fork nearby. This event results in a joint of an Alu and an L1 repeat, with a 14-bp orphan sequence in between (Fig. 4).

It is important to detect this type of deletion as there is a 25% risk of having a child with Hb Bart's hydrops fetalis if the partner is carrier of an α^0-thalassemia allele (i.e. --^{SEA}, --^{MedI} or -$(\alpha)^{20.5}$). Furthermore, a combination of the --^{AW} allele and an α^+-thalassemia allele, for example the common Dutch IVS-1-116 in the α_2-gene, might result in a severe HbH disease.



CATTCAGCTCTGCGCCATTCCACAGTCTCACTGATTATTACTATGTTTCC
ATCATGATCCCCCCAAAAAATCATGACTTTATTTTTTTATTTTTATTATT
ATTATTTTTTTTTTTTTTTTTGTGACGGAGTCTCGCTCTGTCACCCAGGC
TGGAGTGCA**a**TGGCA**CTCCCTGGACAAGT**CCGCTACAGCCAAATCCATAG
GGACCAAAAGCGGATTAGTGGCTGCCGGGGCCAGAGTTACTGTTAATGAG
TACCGAGGTGGCGTTTGGGATGATGAAAAAGTTCT

Figure 4 Schematic representation of the double loop formation hypothesis. The two loops are deleted by breakage, resulting in a joint of the Alu and L1 repeat sequences with a 14-bp orphan sequence in between. Next to the figure is the sequence of the --^{AW} allele, with the orphan sequence in bold and underlined, and the upstream base pair substitution represented as bold 'a'.

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Chapter 4.2

A novel α^0 -thalassemia deletion in a Greek patient with HbH disease and β-thalassemia trait

Phylipsen M, Traeger-Synodinos J, van der Kraan M, van Delft P, Bakker G, Geerts M, Kanavakis E, Stamoulakatou A, Karagiorga M, Giordano PC, Harteveld CL

Eur J Haematol. 2012 Apr;88(4):356-62

Abstract

Objectives: To determine the molecular basis in a Greek child suspected of having HbH disease and β-thalassemia trait.

Methods: Standard hematology, Hb electrophoresis, and HPLC. Multiplex ligation-dependent probe amplification (MLPA), direct sequencing, and breakpoint characterization by NimbleGen fine-tiling array analysis.

Results: The index patient showed a moderate microcytic hypochromic anemia with normal ZPP and elevated HbA_2, indicative for β-thalassemia trait. However, the moderate microcytic hypochromic anemia along with the observation of HbH inclusions in occasional red blood cells suggested a coexisting α-thalassemia. Molecular analysis indicated that the propositus inherited the β^+-thalassemia mutation IVS2-745 (c>g) and a novel α^0-thalassemia deletion from the mother, and the common non-deletion α-thalassemia allele α_2(-5nt)/α from the father. The α^0-thalassemia deletion, named $--^{BGS}$, is approximately 131.6 kb in length. It removes the major regulatory elements along with the functional α-globin genes but leaves the theta-gene intact.

Conclusions: The compound interaction of a β-thalassemia defect along with a single functional α-globin gene is quite rare. Although patients with HbH/β-thal and simple HbH disease have comparable levels of Hb, the absence of free β-globin chains and thus detectable non-functional HbH means that in HbH/β-thal, the levels of functional Hb are higher, resulting in a better compensated functional anemia. Rare large deletions as the one described here remain undetected by gap-PCR in routine molecular screening. The introduction of MLPA as a diagnostic screening tool may improve laboratory diagnostics for these defects. The use of NimbleGen fine-tiling arrays may give additional information about the precise location of breakpoints.

Introduction

Hemoglobin H disease is the severest form of α-thalassemia compatible with postnatal life. HbH disease resulting from the combination of the deletion of two structural genes (α^0-thal) with non-deletion types of α-thalassemia affecting the α_2 gene ($--/\alpha^T\alpha$) has a more severe clinical picture in children or adults as compared to HbH disease resulting from a combination of deletion types of α-thalassemia (-α/--) and non-deletion types affecting α_1 ($--/\alpha\alpha^T$) (1). However, other unlinked modifying factors, such as coinheritance of high HbF expression or β-thalassemia, may ameliorate (or modify) the disease phenotype (2-6). The study of modifiers and knowing their influence is crucial in predicting the disease severity and risk assessment in couples at risk for hemoglobinopathy in their offspring.

A Greek child with a moderate microcytic hypochromic anemia suspected to have HbH disease and β-thalassemia trait was studied. Molecular analysis using multiplex ligation-dependent probe amplification (MLPA) and NimbleGen fine-tiling array revealed HbH disease as a result from a common α^+-thalassemia mutation inherited from the father and a novel deletion which would go undetected during routine molecular analysis using standard gap-PCR and direct sequencing.

This study shows the benefit of combining hematological analysis with advanced molecular techniques like MLPA and array comparative genome hybridization (aCGH) to detect telomeric deletions involving the α-globin gene cluster, determining a novel deletion and estimating length and position of breakpoints.

Materials and Methods

Patient history

The propositus, a child of Greek origin, presented at 2.5 yr old with a moderate microcytic hypochromic anemia following a routine checkup by his pediatrician (Hb 8.6 g/dL, hematocrit 35%). His liver was just palpable, but all other clinical investigations were negative, including absence of splenomegaly and bone changes. His height and weight were at the lowest normal range (3rd-10th and 3rd centile, respectively)

Hematological Analysis

The hematological indices were obtained on an automatic blood counter, in Athens with the Advia 120 (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) and in Leiden with the Micros 60 (ABX Diagnostics, Montpellier, France). HPLC separation was achieved using the Variant-II (Bio-Rad Laboratories, Hercules, CA, USA) with the β-thalassemia short program. Capillary electrophoresis was performed on the Capillarys 2 apparatus (Sebia, Lisses, France).

Gap-PCR and Direct sequencing

Genomic DNA was purified from leucocytes using the Qiagen AutoPure LS™ Genomic DNA Purification System (Gentra Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. Modified gap-PCR was used to screen for the seven common α-thalassemia deletions ($-\alpha^{3.7}$, $-\alpha^{4.2}$, $--^{MED-I}$, $-(\alpha)^{20.5}$, $--^{SEA}$, $--^{THAI}$, $--^{FIL}$) (7;8).

For point mutation analysis of the α genes, PCR was performed on a T-Professional thermal cycler (Biometra, Göttingen, Germany) using the Qiagen® Multiplex PCR kit (cat.no.206143). For sequence analysis, the α-globin genes were amplified using α_2- and α_1-specific primers (S13F 5'-tgtaaaacgacggccagtcgccagccaatgagcgcc-3' and S6R 5'-caggaaacagctatgacctccattgttggcacattccg-3' for the α_2-globin gene; S13F and S8R 5'-caggaaacagctatgacctgtccacgcccatgctggcac-3' for the α_1-globin gene). The first amplification was followed by a second using the universal forward (F: 5'-tgtaaaacgacggccagt-3') and α_2 specific internal primer S18R (5'-caggaaacagctatgacccgttgggcatgtcgtccac-3') (α_2 fragment A) and universal reverse (R: 5'-caggaaacagctatgacc-3') and S3F (5'- tgtaaaacgacggccagtcacgg-caagaaggtggccgac-3') (α_2 fragment B), respectively. Similarly, the first α_1 amplification was followed by a nested PCR (α_1 fragment A and B; respectively F and S18R, S3F and R). Amplification is performed on a T-Professional thermal cycler (Biometra) in an end volume of 25 μL using 200 ng DNA template, 12.5 μL Mastermix MM , 2.5 μL Q solution (Qiagen® Multiplex PCR kit cat.no.206143), one unit AmpliTaq Polymerase (Promega, Madison, WI, USA) and 25 pmol primers. The following conditions were used: 1 cycle 10 min 97°C, 30 cycles 30 s 97°C, 1 min 65°C, 2 min 72°C, followed by 5 cycles 30 s 97°C, 1 min 65°C, 3 min 72°C ending with 1 cycle of 10 min at 72°C. Booster PCR was performed on 1 μL of the first PCR in an end volume of 25 μL, using 2.5 μL of a 10x PCR buffer (Promega) and 25 pmol of primers at the following conditions: 30 cycles 30 s 97°C, 1 min 55°C, 2 min 72°C. (9).

Sequence analysis of the α_1- and α_2-genes was performed using universal F and R primers and the ABI PRISM® Big Dye Terminators v2.0 Cycle Sequencing Kit on an ABI PRISM™ 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), according to the instructions of the manufacturer.

MLPA and Array CGH

Multiplex ligation-dependent probe amplification (MLPA) was applied to detect unknown and uncommon rearrangements in both α- and β-globin gene clusters as described elsewhere (10-14). For analysis of the α-globin gene cluster, the P140B2 probe set (MRC Holland) and the HBA probe set (Leiden Genome Technology Center (LGTC), Leiden, The Netherlands) were used.

A custom fine tiling array covering the α- and β-globin gene clusters and surrounding areas was designed (Roche NimbleGen, Madison, WI, USA) (19). Sample preparation, array hybridization, and analysis of the results was performed according to the manufacturer's instructions (NimbleGen Arrays User's Guide: CGH Analysis v4.0). In brief, 500 ng of sonicated DNA was denatured in presence of 1 OD_{260} of either Cy3 (test samples) or Cy5 (reference samples) dye-labeled random nonamers (TriLink Biotechnologies, San Diego, CA, USA). The denatured sample was chilled on ice, and then incubated with 10 mM dNTPs (each) and 100 U Klenow Fragment 3'->5'exo^- (New England Biolabs, Ipswich, MA, USA), for 2 h at 37°C. After washing, each test sample and corresponding reference sample were mixed and dried in a SpeedVac. Pellets were resuspended in Sample Tracking Controls (NimbleGen). After mixing the resuspended sample with the hybridization solution (containing Hybridization Buffer, Hybridization Component A and an Alignment Oligo, all from NimbleGen), the samples were loaded onto the array. Samples were hybridized at 42°C in the Hybridization System (NimbleGen, Cat. No. 05223687001) for approximately 72 h. Arrays were washed by using the NimbleGen Wash Buffer Kit and scanned with the Agilent G2565BA microarray scanner (Agilent Technologies, Santa Clara, CA, USA). Data analysis was performed with NimbleScan v2.5 and SignalMap v1.9 software (NimbleGen).

Primer design and breakpoint PCR

The database used for defining the position of primers and breakpoint locations is the UCSC Genome Browser on Human Mar. 2006 (NCBI36/hg18) Assembly (http://genome.ucsc.edu). According to the array results, primers for PCR were designed flanking the breakpoint region (forward primer 169695-169714 5'- ATGCAGCACTCACTCTGCTG-3', reverse primer 37551-37570 5'-CACGACTCTGGCAATGAGAA -3'). PCR was performed using the TaKaRa LA Taq™ kit (TaKaRa Bio Inc, Otsu, Shiga, Japan) according to the manufacturer's instructions. Instead of the recommended 50 ng of template DNA, 200 ng was used. The reaction was performed in a T-Professional thermal cycler (Biometra). Direct sequencing of the breakpoint fragment was performed on the ABI PRISM™ 3730 DNA Analyzer (Applied Biosystems) according to manufacturer's instructions. Oligo-primers used for sequencing from the 5' and 3' end of the fragment are identical to the PCR primers. An internal sequencing primer was designed in the orphan sequence to close the gap flanking the orphan sequence (5'-GGTTAGAAAGTTTATGTGAACGGGT-3'), however, sequencing failed repeatedly owing to the presence of an Alu repeat related poly-A stretch impeding sequence analysis.

Results

The propositus showed a moderate microcytic hypochromic anemia (Hb 8.6 g/dL, PCV 0.33, RBC $6.93*10^{12}$/L, MCV 52.3 fl, MCH 13.7 pg) with normal ZPP (73 μmol/mol heme) without signs of hemolysis (Hp 93.6 mg/dL). The elevated HbA_2 was indicative for β-thalassemia trait; however, the moderate microcytic hypochromic anemia, along with HbH inclusions in occasional red blood cells, suggested a coexisting α-thalassemia, although HbH was not detected by chromatography or electrophoresis.

Patient	Mother	Father	Propositus	Normal range
age			2.5 yr	
Hb (g/dL)	11.9	15	8.6	m: 14-18, f: 12-16
Hct (%)	41.8	47	33	m: 42-52, f: 37-47
MCV (fL)	76.5	87.9	52.3	80-97
MCH (pg)	21.8	28.1	13.7	27-33.8
HbA_2 (%)	5.6	2.6	4.6	2.2-3.2
HbF (%)	0	0	1.8	<0.5
RDW	15.5	14.5	18.5	11-14
Retics (%)	n.d.	n.d.	0.8	0-2
I.B.	+	-	++	-
DNA α-genes	$\alpha\alpha/--^{BGS}$	$\alpha^{(-5nt)}\alpha/\alpha\alpha^{1}$	$\alpha^{(-5nt)}\alpha/--^{BGS}$	$\alpha\alpha/\alpha\alpha$
DNA β-genes	IVS2-745/N[2]	N/N	IVS2-745/N	N/N

Table 1 Hematological and molecular data of all family members. N, normal; n.d., not done; "-" , "+", negative, positive; m, male; f, female; Hct, hematocrit; HbA2, hemoglobin A2; HbF, fetal hemoglobin; RDW, red cell distribution width; I.B., inclusion bodies; MCH, mean corpuscular hemoglobin. HGVS nomenclature: [1]HBA2c.95+2_95+6delTGAGG; [2]HBBc.316-106C>G.

Molecular analysis indicated that the propositus inherited the β^{+}-thalassemia mutation IVS2-745 (C>G) (HBBc.316-106C>G) and a novel α^{0}-thalassemia deletion from the mother, and the common non-deletion α-thalassemia allele α_2 IVS1(-5nt) (HBA2c.95+2_95+6delTGAGG) appearing as hemizygous, from the father. The α^{0}-thalassemia deletion, investigated by MLPA, is approximately 122-171 kb in length. It removes the major regulatory elements involved in regulation of α-gene expression along with the functional α-globin genes but leaves the θ-gene intact. This novel deletion was named $--^{BGS}$, after the initials of the propositus. Hematological and molecular data all family members is summarized in Table 1.

Fine mapping using a fine-tiling array of overlapping primers covering the region of unique sequences from the telomere of chromosome 16 to approximately 2 Mb from the telomere suggests an interstitional deletion leaving the telomeric region intact. The 5' deletion breakpoint is located within the POLR3K gene, close to the telomere (Fig. 1), while the 3' breakpoint maps between the α_1- and θ-genes (HBA1 and HBQ1, respectively), a region known to be rich in Alu repeats. Primer design as close to the breakpoint as possible was hampered by the lack of unique sequences due to stretches of Alu repeats. The best option rendered a breakpoint PCR fragment of approximately 1200 bp. Sequence analysis from the 3' end showed 514 bp located from position 169679- 169165 (according to the UCSC Genome Browser on Human Mar. 2006 (NCBI36/hg 18) Assembly database) between the α_1-globine gene and θ-gene and 56 bp orphan sequence located between position 90554-90498 located in an intron of C16orf35, about 5 kb upstream of HS-48, the most distal of the hypersensitive sites involved in α-globin gene expression. Owing to a poly-A stretch at the end of the sequence, analysis of the 5' breakpoint fragment was only feasible for approximately 300 bp, from position 37871 to 37593 which coincides with part of the first intron of the POLR3K gene. Judging from the length of the

breakpoint fragment and the mapping of sequences in BLAT to the genomic sequence, the remaining 300 bp coincide with the Alu repeat flanking the 5' breakpoint sequence at position 37871, where the poly-A stretch hampers further sequence analysis.

The presence and location of the orphan sequence allows an impression of the mechanism which might have resulted in the observed deletion. The orphan sequence, which is in the same orientation as in the wild-type genomic context, maps to an exonic sequence of C16orf35, upstream of the multispecies conserved sequence (MCS 1-4) (15;16) and may have formed the bridge between two loops of respectively 52.6 kb and 78.6 kb (Fig. 2). The crossover between both stem loops may explain the eventual deletion of approximately 131 kb taking away the complete α-globin gene region, but leaving the telomere intact.

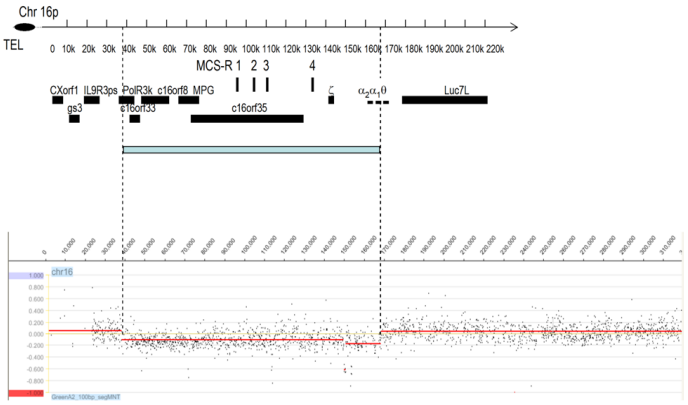

Figure 1 NimbleGen fine-tiling array results analyzed by SignalMap software, 100 bp reading frame. The deletion is approximately 131.6 Kb in length from position 37749 to 169374 (UCSC Genome Browser version 2006) . From the normal ratio of fine-tiling primers at the telomeric site of the deletion, it became clear that the deletion does not involve the telomere.

Discussion

Rare large deletions as the novel one described here will for obvious reasons remain undetected by routine molecular screening by gap-PCR for the most common α-thalassemia deletions, which subscribes the importance for interpretation of molecular results in the context of the patient's phenotype. In the presently described case, the hematological phenotype could not be explained by homozygosity for the observed α_2(-5nt) mutation. The introduction of MLPA as a diagnostic screening tool has improved laboratory diagnostics for unknown deletions (10;17;18). Alternatively, characterization of the breakpoints is necessary to design primers for gap-PCR as a simple and fast screening tool for the molecular diagnosis of known deletions. The design of new deletion-specific oligo-primers for gap-PCR and breakpoint sequence characterization is facilitated by a more refined mapping of the breakpoint junction by aCGH. The use of a NimbleGen fine-tiling array of overlapping oligo probes covering the α-globin gene cluster region is a recently described strategy for fine mapping (19) novel α- and β-thalassemia deletion breakpoints. Once the approximate position of breakpoint is determined by looking at the ratio between normal control DNA labeled in green (Cy5) and the patient DNA labeled in red (Cy3) within a specific range of 100 bp, a unique forward and reverse oligo-primer is designed to

bridge the breakpoint junction (20-25). Direct sequencing of the amplified breakpoint fragment allows the identification of the exact locations of the breakpoints. The gap-PCR assay can be used to screen for carriers in the family, to confirm the diagnosis of α-thalassemia trait, or can be used at the population level if the frequency in which these deletions occur is elevated for certain isolated geographic regions (7;8). Recently, a similar strategy has been used to identify deletion breakpoints of two other α-thalassemia deletions, using CGH-array analysis with oligonucleotides (8 x 60K Agilent Technologies) and SNP genotyping (26).

The propositus in this study was characterized by DNA analysis as having HbH disease genotype interacting with β-thalassemia trait. Compound interaction of a β-thalassemia defect with a single functional α-globin gene is rare. Hematological findings were comparable to those previously reported for this rare genotype interaction (2-4;27-30). HbH disease is usually characterized by the detection of HbH (β_4) in the peripheral blood; however, in patients with HbH/β-thal trait, the interaction of a β-thalassemia mutation reduces the β-globin chain excess to levels insufficient to form electrophoretically or chromatographically detectable HbH. In comparison with simple β-thalassemia carriers, patients with HbH/β-thal trait have lower levels of hemoglobin and more pronounced microcytic hypochromic parameters. The association of HbH and β-thalassemia trait does not lead to a reduction of HbA_2 in such a way that laboratory diagnosis of β-thalassemia is altered. On the other hand, the hematological findings in the propositus carrying the HbH/β-thal trait genotype are almost indistinguishable from those found in iron-deficient β-thalassemia heterozygotes and may confound the diagnosis when parents are not available (31).

The absence of free β-globin chains and thus detectable non-functional HbH means that in HbH/β-thal, the levels of functional Hb are higher, resulting in a better compensated anemia. Overall hematologic findings in HbH/β-thalassemia trait are consistent with the modified globin chain imbalance and hemoglobin synthesis caused by the complex genotype interaction, to such an extent that these features form an intermediate clinical phenotype in between HbH and β-thal carrier. The patient's functional anemia is compensated relative to their hemoglobin levels, which has implications toward the prognosis of clinical severity in genetic counseling.

As shown in previous studies, breakpoints of deletions from the α-globin cluster are not randomly distributed. Out of 79 α-thalassemia deletions presently known (15;26), 28 show a breakpoint located between position 167,500 and 176,000 (α_1-globin gene and the 3' HVR or Hyper Variable Region, UCSC Genome Browser on Human Mar. 2006). From these, at least 12 break between α_1- and θ-globin gene. This segment is therefore considered a breakpoint cluster region (32). An unusual feature of the described novel deletion is the presence of an orphan sequence flanking the 3' breakpoint, mapping to a sequence at position 90554 and 90498 in between the 5' and 3' breakpoints. A similar orphan sequence segment of 134 bp in the recombination junction of the $--^{MED-1}$ deletion was suggested to illustrate the recombination mechanism to involve two similarly sized chromatin loops at a base point closely apposed during replication. Nicks or gaps at replication forks could provide the initial template for replication. The orientation of the orphan sequence is predicted to be inverted when coming from up- or downstream of the chromatin loops involved in the deletion, while it would be in the same orientation when located in between both loops, as found in the presently described deletion. This subscribes to the mechanism as hypothesized by Nicholls *et al.* (1987), (32) compatible with the chromatin loop model (33), giving deletions spanning a single or a double loop. However, the length of the loops involved appears to be variable in size.

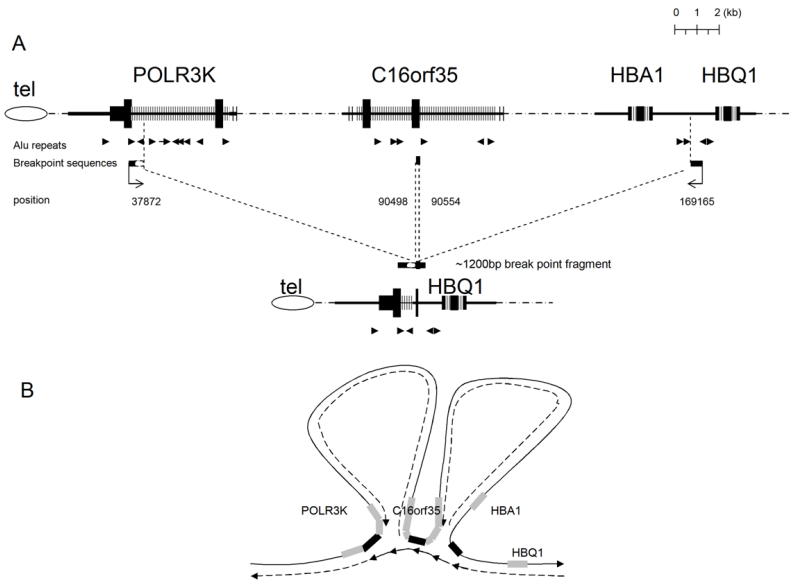

Figure 2 (A) Chromosomal location of the breakpoint fragments. Bars indicate the chromosomal regions, exons are indicated as filled blocks, vertical lines mark intronic sequences, stippled line interconnecting the bars indicates the distance between the chromosomal regions, and the telomere is indicated as an oval. The arrows indicate Alu repeats, and the breakpoint fragment obtained by gap-PCR is indicated as small filled bars below the figure with positions of breakpoints. The open bar indicates the region of uncertainty as sequence is hampered by the presence of poly-A stretches, and the length of this region is determined by combining sequence results and length of the breakpoint fragment. Breakpoint fragment analysis revealed the involvement of an orphan sequence (57 bp) in an intron of C16orf35, upstream of the highly conserved regulatory elements MCS 1-4, which suggests that the recombination occurred when both loops were geographically close during replication in meiosis. Regions of high homology due to the presence of Alu repeats may have facilitated strand switch and subsequent non-homologous recombination. **(B)** Double-stranded DNA is shown arranged in two chromosomal loops. Black bars indicate sequence homology with the breakpoint fragment. The orientation of replication is shown as a thin stippled line, and arrows indicate the discontinuous DNA replication strand around the breakpoint junctions. According to this model, two germ cells will be formed during meiosis, one with and the other without the deletion of both loops.

Acknowledgements

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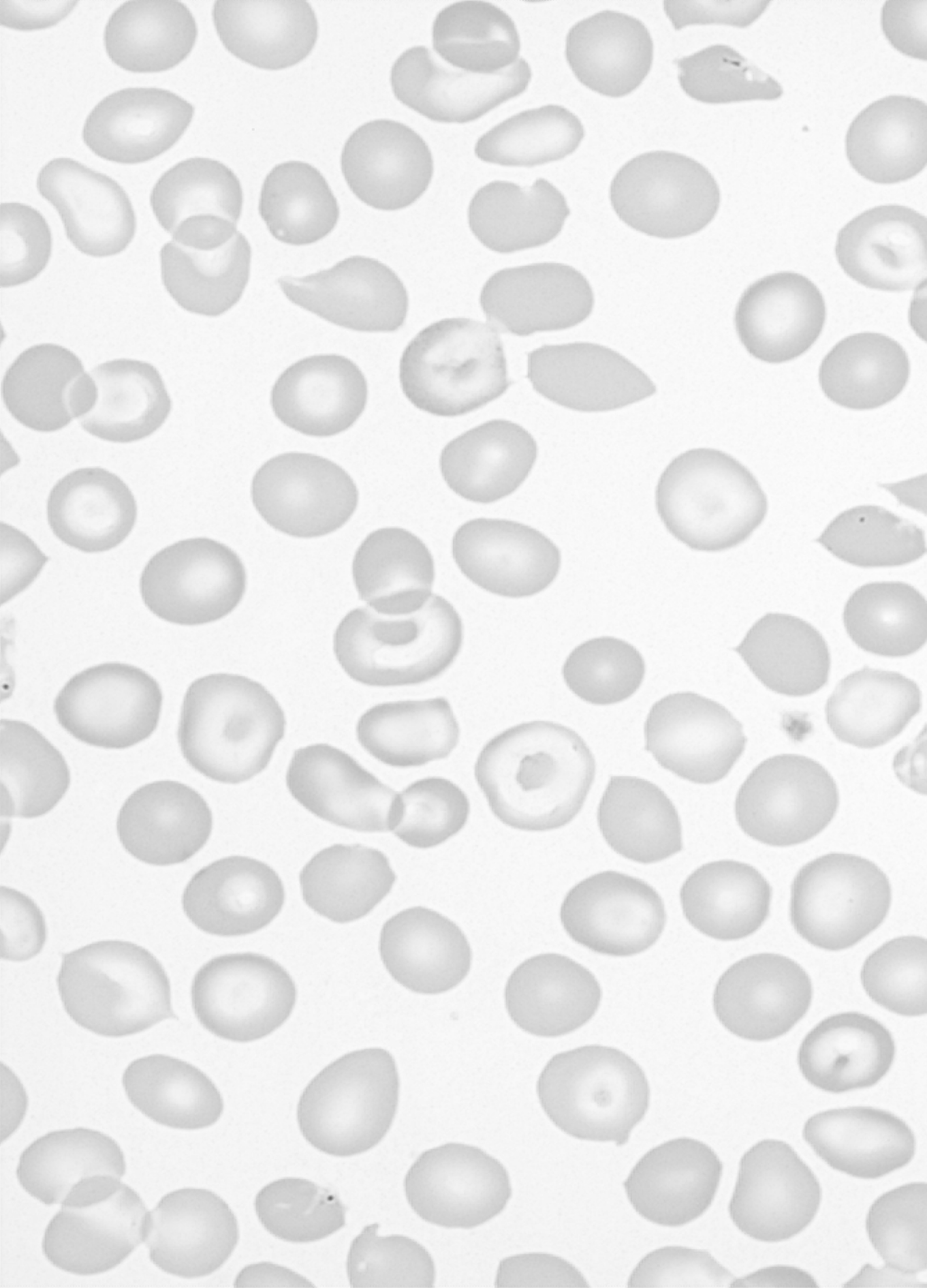

Chapter 5

Discussion

Improvement of molecular diagnosis of hemoglobinopathies

Molecular detection of hemoglobinopathies

Hemoglobinopathies constitute the commonest recessive monogenic disorders worldwide. They are caused by mutations which affect the genes that direct synthesis of the globin chains of hemoglobin, and may result in reduced synthesis (thalassemia syndromes) or structural changes (hemoglobin mutants) (1). More than 280 mutations causing β-thalassemia have been described, the majority of which are point mutations, and more than 100 α-thalassemia mutations have been reported, most of which involve deletions within the α-gene cluster. In addition, more than 1150 mutations causing structural variants have been characterized. (Summarized in HbVar http://globin.bx.psu.edu/hbvar/menu.html).

The most commonly used procedures for known mutations include the reverse dot blot analysis with allele specific oligonucleotide probes, the amplification refractory mutation system (ARMS) and restriction enzyme digestion of amplified fragments. In reverse dot blot hybridization, a panel of oligonucleotides specific for a subset of mutations and their wildtype counterpart are immobilized on a solid support and subsequently hybridized with an amplified fragment from the patient as a probe. This method has been described for the detection of both α- and β-thalassemia mutations (2-6). ARMS detects known point mutations by allele specific amplification of genomic DNA (7;8). Both ARMS and reverse dot blot hybridization are simple and rapid to perform and relatively inexpensive. However, availability of DNA sample controls to validate the tests and to standardize new mutations can be limited. Restriction enzyme analysis of amplified β-globin gene product is used to detect mutations which create or abolish restriction enzyme cleavage sites. This method is fast, simple and cheap, but the number of mutations that can be detected is very limited (9-11). Currently, mutation spectrums within geographical regions have become much broader due to the trend of global migration. Therefore, the more generic method of direct Sanger sequencing has become more appropriate for detecting point mutations (12). Although the investment of implementing automated direct sequencing may be relatively expensive, this method is rapid and easy to perform and able to directly characterize mutations.

Deletions causing thalassemia can be detected by Southern blot and fluorescent in situ hybridization (FISH). These techniques use radioactively or fluorescently labeled probes to confirm presence of a large deletion in the globin gene cluster. Southern blot analysis is time consuming, technically demanding and success is very much dependent upon the hybridization probes available. FISH analysis involves laborious cell culturing to generate metaphase chromosome spreads and has a low resolution (>20 kb). Therefore, an increasing number of laboratories is replacing these techniques by gap-PCR and MLPA nowadays. Gap-PCR is used to screen for the most common α-thalassemia deletions ($-\alpha^{3.7}$, $-\alpha^{4.2}$, $--^{SEA}$, $-(\alpha)^{20.5}$, $--^{THAI}$, $--^{FIL}$ and $--^{MedI}$) and the α-globin gene triplication (13). This technique provides a quick diagnostic test for α^{+}- and α^{0}-thalassemia deletion mutations. It is simple, non-radioactive and little starting material is required. In addition, gap-PCR is able to identify ~80% of all α-thalassemia carriers. However, deletions for which no primers are available, especially previously uncharacterized rearrangements, will be missed by this procedure.

Multiplex ligation-dependent probe amplification

The multiplex ligation-dependent probe amplification (MLPA) method is now a major diagnostic tool for the detection of disease causing deletions and duplications in many clinical genetic and research laboratories. MRC-Holland (www.mlpa.com) developed many commercially

available MLPA probe sets, which facilitated the widespread use of this technique. These kits contain MLPA probes consisting of one short synthetic oligonucleotide and one long probe oligonucleotide, which are generated by cloning target-specific sequences into M13 vectors containing different lengths of stuffer sequences. The major disadvantage of this approach is the complex, expensive and time-consuming probe generation procedure (14;15).

Another possibility is to generate both half-probes through chemical synthesis. This strategy has been successfully applied for detection of copy number variations (CNVs) in many genetic disorders (16-18). An advantage of this approach is that multi-color labeling of the probes can increase the number of target sequences up to ~60 in one probe set. In 2005, chemically synthesized probe sets, HBA and HBB, were designed for detection of rearrangements in the α- and β-globin gene clusters, respectively (Chapter 2.2). Shortly thereafter, MRC Holland probe sets for both globin gene clusters became commercially available. The main difference between the HBA and HBB and the MRC Holland probe sets is that the latter has target sequences mainly in the globin genes, whereas the HBA and HBB probe sets also cover the region surrounding the globin gene clusters. This makes the HBA and HBB probe sets more suitable for the detection of larger rearrangements, such as the majority of the α-thalassemia deletions (19), εγδβ-, γδβ- and δβ-thalassemia deletions (20) and the ATR-16 causing deletions (21).

Introducing the MLPA technique as a standard diagnostic tool has revolutionized the detection of CNVs in human disease genes, including hemoglobinopathies. MLPA has made the classic Southern blot and FISH procedures redundant for detection of thalassemia causing CNVs; all CNVs in the α- and β-globin gene clusters can be detected by MLPA. Therefore, cases which remained uncharacterized after applying standard techniques like FISH and Southern blot, can now be diagnosed at the molecular level. This has led to the detection of a variety of uncommon and novel deletions (Chapter 2.3, 4.1 and 4.2). From a diagnostic point of view, MLPA gives enough information, as it is sufficient to know which genes are deleted or duplicated to be able to give a diagnosis.

The MLPA technique has also shown its use in prenatal diagnosis, mainly for aneuploidies of chromosomes 13,18, 21 and the sex chromosomes (22). Although the yield of DNA from a chorionic villus or amniotic fluid sample is relatively low, MLPA results are very reliable and reproducible. Therefore, the laborious and time-consuming conventional karyotyping can be replaced by rapid tests such as MLPA in cases suspected of common chromosome aneuploidies (23). In addition, MLPA can be used as a screening method for pregnant couples at risk for β-thalassemia major due to a deletion in the β-globin gene cluster in one of the parents. The β-globin gene might be present but inactive due to an upstream deletion of the γ- and δ-globin genes and the LCR. MLPA is a useful technique to determine quickly which genes are involved in the deletion and thereby preventing misdiagnoses (see Chapter 3.1). However, this diagnostic technique does not provide detailed information on the breakpoints, therefore, similar deletions involving the same probes cannot be distinguished by a specific amplification, nor can insight be acquired into the developmental mechanism of these gross rearrangements.

Array comparative genomic hybridization

The fine-tiling array comparative genomic hybridization (aCGH) has been shown to be a powerful tool in fine-mapping of CNV breakpoints in many genetic disorders, including MUTYH-associated polyposis (MAP) (24), multiple osteochondromas (MO) (25), 17p13.1 microdeletion syndromes (26) and α- and β-thalassemia (see Chapter 2.4). Delineation of the exact breakpoints

of several thalassemia-causing deletions gives more insight in the mechanisms underlying these rearrangements. Furthermore, simple gap-PCR assays can be designed with the knowledge of exact deletion breakpoints. These assays can be used as a quick screening method, especially in laboratories where MLPA is not available or for the locally occurring deletions, reaching high frequencies in the population. In this way, the aCGH technology is also an important and useful tool in diagnostics (See Chapter 2.1, 2.4 and 4.2). Even though array results might deviate up to 1 kb from the actual breakpoint, valuable information on the breakpoints can be obtained, especially on the deletions causing the ATR-16 syndrome. Because many genes are located at chromosome 16p13.3, loss of this region may have significant clinical consequences such as dysmorphic features and developmental delay. It is yet unknown which genes are responsible for the mental retardation phenotype in these patients (21;27-29). Therefore, it is important to study this kind of deletions to gain more insight in the genotype-phenotype correlations.

The α-globin gene cluster contains many Alu-repetitive sequences, which belong to the retrotransposon family of Short Interspersed Elements (SINE). SINEs do not encode a functional protein and rely on other elements for transposition. Alu-repeats are ~350 bp in length and are known to be involved in non-allelic homologous recombination (NAHR) events (30). NAHR can occur when two highly homologous sequences misalign during meiosis, resulting in duplications, deletions, inversions or translocations. Several different deletions in the α-globin gene cluster, including --^{DutchI} (31), --^{MedII} (32), --^{JB} (Chapter 2.4), --^{SA} (33), --^{CAL} (34) and --^{TG} (unpublished) have their 3' end breakpoint in the same Alu-repeat, indicating a hotspot for recombination at this location. Another hotspot of recombination is formed by the hypervariable region located downstream of the α-globin gene cluster (3' HVR). This region consists of an array of 70-450 copies of 17 bp tandem repeats (35;36). It is suggested that the unusual chromatin structure predisposes the hypomethylated, highly repetitive region between the α_1-globin gene and the 3' HVR to recombination.

In contrast, the β-globin gene cluster is very poor in Alu-repeats, repetitive sequences in this area are mainly of the L1-type. L1 repeats belong to another family of retrotransposons: the Long Interspersed Elements (LINE), which are generally over 5 kb in length. LINEs code for the reverse transcriptase enzyme or endonucleases and are able to move across the genome by copying themselves (37;38). It is likely that rearrangements in the β-globin gene cluster arise due to other mechanisms than NAHR. The non-homologous end joining (NHEJ) pathway ligates double-strand DNA breaks directly without the need of an homologous template, as is the case in NAHR. Because the breakpoint regions of all β-thalassemia causing deletions studies showed no homology at all, it is suggested that NHEJ plays an important role in rearrangements in the β-globin gene cluster (39;40).

As discussed above, several molecular techniques have been developed in the recent years to improve postnatal diagnosis of hemoglobinopathies. Until now, no cure is available for severe forms of hemoglobinopathy, such as β-thalassemia major and sickle cell disease, except for a complex bone marrow transplantation which is only available for patients with full HLA-matching donors. These diseases form an increasing burden on health care, also in non-endemic countries, and it is therefore important to develop national programs to identify carriers of hemoglobinopathy to facilitate primary prevention. Couples at risk can then be offered an informed reproductive choice and termination of severely affected pregnancies. Therefore, it is important to focus on the improvement of molecular techniques for prenatal diagnosis of hemoglobinopathies as well.

Prenatal diagnosis of hemoglobinopathies

Prenatal diagnosis is an important part of obstetric care. Conventionally, sampling of fetal genetic material is performed by invasive techniques including amniocentesis (AC) and chorionic villus sampling (CVS). These methods are associated with a small risk of miscarriage (41) and with a relevant physical and psychological discomfort. Therefore, new developments are directed towards improving the safety and the burden of the procedures. The well-established presence of fetal cells (42) and free fetal DNA (ffDNA) (43) in the maternal circulation has promoted much research effort towards approaches for non-invasive prenatal diagnosis (NIPD). To date, fetal sex (44;45) and rhesus D status determination (46;47) by ffDNA analysis is performed in many genetic centers.

Research groups have used both fetal cells and ffDNA as target to develop approaches for NIPD through maternal blood analysis. However, fetal cells in maternal blood are rare, only 1 or 2 occur per ml blood, making it technically challenging to isolate these cells. Furthermore, fetal lymphocytes can remain in the mother's body for several years after the pregnancy, which may render any following prenatal diagnoses unreliable (48;49). In contrast, ffDNA has a half-life of ~16 minutes and is cleared from maternal plasma very rapidly (50). The average amount of cell ffDNA during the first and second trimester is ~10% of the total amount of cell-free DNA in a pregnant woman (51). Furthermore, ffDNA can be detected in maternal blood from the 6th week of gestation, which enables very early prenatal diagnosis (52). Therefore, ffDNA in maternal plasma is a more promising source of fetal genetic material then fetal cells for the development of NIPD assays (53;54).

The high frequency and severity of hemoglobin disorders lead to the application of population wide or targeted carrier screening, mainly through preconception or antenatal screening. In addition, some countries have introduced newborn screening for sickle cell disease (55;56). It is important to note that hemoglobinopathies differ from other genetic disorders in that identification of carriers is possible by simple hematological and biochemical tests, rather than DNA analysis. Once a carrier couple has been identified based on their hematology, the underlying mutations must be characterized to be able to counsel them on the risk of a hemoglobin disorder and to perform prenatal diagnosis if indicated.

Prenatal diagnosis using ffDNA in maternal plasma was first applied to the hemoglobinopathies in 2002. This study described a real-time PCR assay to detect paternal inheritance of the HBB:c.126_129delCTTT mutation, which is the most common β-thalassemia mutation in China (57). Several other investigators have studied paternal inheritance of other mutations by combining allele-specific or single-allele extension reactions and mass spectrometry (58;59). The major disadvantage of these approaches is that it can only be applied to a limited number of mutations, and cannot be used in couples at risk carrying the same mutation.

It has been shown that β-thalassemia major can be excluded by detecting a paternally inherited single nucleotide polymorphism (SNP) that is linked to the non-mutant β-globin gene of the father (58). The allele-specific arrayed primer extension (AS-APEX) method combines detection of mutations and genotyping of SNPs (60). Although this approach showed to be very useful for the southern Chinese population, only four mutations can be detected by this AS-APEX assay, which would make the method not applicable in other geographic areas.

The most significant difficulty with the detection of ffDNA in maternal plasma is the excess of maternally derived DNA molecules. Digital PCR is a single-molecule analysis approach,

in which many PCRs are performed in parallel. In this way, it is possible to detect the small allelic imbalance in mutant and wild-type alleles, when the fetus is homozygous for the mutant or wild-type allele. This digital counting approach has been successfully applied for NIPD of several β-thalassemia causing mutations (61). The drawback of this technique is that it is very laborious and time consuming to perform the many PCR reactions required to obtain a statistically significant result.

In the scope of this thesis, the pyrophosphorolysis-activated polymerization (PAP) technique, in combination with melting curve analysis (MCA), was used for NIPD of β-thalassemia and sickle cell disease. As described in Chapter 3.2, this approach is used for the detection of paternal SNPs in linkage with the normal or mutant allele. The use of SNPs instead of mutations makes it possible to perform NIPD in couples carrying the same mutation. In addition, this approach might also be applicable for other genetic disorders as linkage to informative SNPs can be used for most disease genes. The method showed to be reliable, relatively easy, quick and cheap to perform. This enables NIPD of β-thalassemia major and SCD provided a previously born child or other family member is available for testing to determine the linkage to the paternal SNPs. If the SNP linked to the paternal mutation is detected, an invasive procedure will still be necessary to check whether the fetus also inherited the maternal mutation. However, NIPD methods will make half of the invasive procedures redundant when informative SNPs are present.

Concluding remarks

Diagnostics for hemoglobinopathies is strongly improved over the recent years. In particular the implementation of the MLPA technique made it possible to detect deletions and duplications in the globin gene clusters in patients who remain undiagnosed by applying the conventional techniques. The aCGH technology was developed in order to characterize the breakpoints of novel deletions detected by MLPA more precisely. This has led to the design of several relatively simple gap-PCR assays, which are useful in laboratories where MLPA and aCGH is not available. In addition, gap-PCR can be used for quick screening for the more locally occurring deletions or in family studies. A non-invasive prenatal diagnosis assay for hemoglobinopathies was developed by combining the PAP and MCA techniques. This method will be implemented in the current flow for prenatal diagnosis and will eventually make 50% of the invasive procedures redundant.

The emerging technologies, including next generation sequencing (NGS) and whole genome sequencing (WGS) are very promising for molecular diagnostics (62). These techniques are able to sequence the complete human genome; which enables to obtain a lot of information from one experiment. Nowadays, the possibilities to use NGS for non-invasive prenatal diagnosis are investigated. For example, detection of fetal trisomies in maternal plasma by massive parallel sequencing was shown to be successful (63). Massive parallel sequencing has also been performed for prenatal diagnosis of β-thalassemia and it was shown to be able to detect paternally inherited mutations in maternal plasma (64). These new technologies will become very important in the near future, both in pre- and postnatal diagnostics. They will replace the current molecular techniques including gap-PCR, sequencing analysis, MLPA and aCGH. However, these highly sophisticated and expensive technologies will not be available in all laboratories performing hemoglobinopathy diagnosis. In addition, it is possible to identify hemoglobinopathy carriers by hematological and biochemical tests only, which are very accurate and relatively cheap and quick to perform. Therefore, determining the hematological parameters and separation of hemoglobin fractions will still be the first large scale methods of choice in hemoglobinopathy diagnosis.

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Summary

Summary

Hemoglobinopathies (HbP) are recessive hereditary disorders of hemoglobin, characterized by microcytic hypochromic anemia. Carriers of HbP are usually (nearly) asymptomatic, but children of parents who are both carrier have a 25% chance of being severely affected. HbPs are caused by mutations in the genes encoding the subunits of the hemoglobin molecule, the α- and β-globin genes. These mutations may either lead to a change in the primary structure of the gene product, which generates abnormal hemoglobins, or disrupt the expression of a specific globin gene which causes thalassemia.

Because carriers of a HbP are protected against de severe consequences of malaria tropica, HbPs mainly occur in areas where malaria is or has been endemic, such as the Mediterranean area, the Middle East and Southeast Asia. However, due to recent migration, HbPs are also becoming a health risk in the northern European countries. It is estimated that approximately 7% of the world population is a healthy carrier of a HbP, resulting in ~350.000 severely affected newborns each year. In the Netherlands, approximately 60 babies affected with thalassemia major or sickle cell disease are born annually. Until now, no cure is available for these children, except for a complex bone marrow transplantation. Nowadays, national programs to identify carriers of HbP have been implemented in many endemic countries to facilitate primary prevention. Couples at risk can be offered an informed reproductive choice, including termination of pregnancy. However, such a national prevention program for primary prevention has not been implemented in the Dutch health care system yet.

HbP diagnostics encompasses three specialties: hematological, biochemical and molecular testing. Results of all tests together form the complete diagnosis. The focus of this thesis will be the development and optimization of molecular techniques for post- and prenatal diagnosis, in order to improve HbP diagnostics.

Chapter 2.1 describes a single-tube multiplex gap-PCR for the detection of eight β-globin gene cluster deletions that commonly occur in Southeast Asia. This technique provides a fast, simple and cheap diagnostic test for deletion-types of β-thalassemia which can be applied in every molecular diagnostic laboratory having standard PCR equipment. The application of multiplex ligation-dependent probe amplification (MLPA) technique for the detection of deletions in the α- and β-globin gene clusters is described in Chapter 2.2. In contrast to conventional techniques such as Southern blotting and fluorescence in-situ hybridization (FISH), MLPA is quick, cheap and able to detect known and unknown deletions and duplications with high resolution. This study shows that the MLPA method is highly suitable to detect deletions in the α- and β-globin gene clusters; deletions with known breakpoints, less frequently occurring deletions and nine deletions which were not previously described were detected. The applicability of MLPA is further delineated in Chapter 2.3. A cohort of 37 possible thalassemia carriers from Australia, in whom the underlying molecular cause could not be found by applying the conventional techniques, was screened with MLPA. Deletions were detected in the α- or β-globin gene cluster in 27 cases. In addition, 11 not previously described deletions were found. This study shows that the MLPA technique is a valuable tool in HbP diagnostics. Although MLPA can determine deletion lengths relatively precise, exact breakpoints remain unknown. From a scientific point of view, it is interesting to determine the breakpoints. Knowledge of the exact breakpoint sequences should give more insight into the molecular mechanisms underlying these rearrangements, and would facilitate

the design of gap-PCRs. Chapter 2.4 describes the development and application of the fine-tiling array comparative genomic hybridization (aCGH) method for HbP. This study shows that aCGH can determine breakpoints accurately in all 36 patients. With this knowledge, relatively simple gap-PCR assays were designed for 10 deletions. Furthermore, this study shows that most deletions in the α-globin gene cluster appear to arise due to the non-allelic homologous recombination (NAHR) mechanism, whereas non-homologous end joining (NHEJ) seems to be the preferred mechanism in the β-globin gene cluster.

The $--^{AW}$ deletion is described in Chapter 4.1. This deletion, found in a Dutch family, is 8.2 kb in length and involves both α-globin genes. Sequencing analysis of the breakpoint fragment showed that this deletion probably arose due to non-homologous recombination between an Alu- and a L1-repeat. This type of recombination is very rare and was not described before in the α-globin gene cluster. Chapter 4.2 describes a patient with HbH disease and β-thalassemia as a result of a combination of a point mutation in the α- and β-globin gene and an unknown deletion in the α-globin gene cluster. Breakpoints of this $--^{BGS}$ deletion were characterized by using MLPA, aCGH, PCR and sequencing analysis. The 131 kb deletion involves the regulatory element and both α-globin genes, but leaves the θ-gene intact. The breakpoint fragment contained a 57 bp orphan sequence, indicating that this deletion is the result of a double loop formation because of recombination between Alu-repetitive sequences on both alleles. These studies underline the usefulness of MLPA, aCGH and breakpoint analysis in HbP diagnostics. Furthermore, it is important to be able to detect this kind of α^0-thalassemia deletions, because of a 25% risk of Hb Bart's with hydrops fetalis in the offspring when in combination with another α^0-thalassemia allele (such as $--^{SEA}$, $--^{MedI}$, $--^{MedII}$ en $--^{DutchII}$).

MLPA is also a valuable tool in prenatal diagnosis of HbP, as described in chapter 3.1. Two couples at risk, in which one partner was carrier of a point mutation in the β-globin gene and the molecular cause of β-thalassemia was not found in the other partner, were tested with MLPA. A deletion of the upstream regulatory element may lead to a β-thalassemia phenotype, although the β-globin gene itself is present and intact. In this case, the present (but inactive) β-globin gene might lead to a misdiagnosis: the fetus will be characterized as carrier of the mutation, but has in fact no functional β-globin genes. Analysis by MLPA showed a novel deletion involving the β-globin gene in both couples at risk.

A pyrophosphorolysis-activated polymerization (PAP) assay was developed in order to improve prenatal diagnosis of HbP (Chapter 3.2). This technique is able to detect free fetal DNA, which is present in the blood circulation of a pregnant woman. Because couples at risk often carry the same mutation, this study was designed for 12 SNPs in linkage to the β-globin gene, instead for the mutations itself. Genotyping of the SNPs is done by melting curve analysis (MCA), a quick and accurate method. Subsequently, PAP is performed for the SNPs which are present in father but absent in mother, to be able to determine which paternal allele was inherited by the fetus. The combination of PAP and MCA forms a reliable method for non-invasive prenatal diagnosis of β-thalassemia and sickle cell disease, provided a previously born child or other family member is available for testing to determine the linkage to the paternal SNPs. If informative markers are present in father (and absent in mother), in principle, this method will make 50% of the invasive procedures, which have a risk of miscarriage, redundant.

Diagnostics for HbP is strongly improved over the recent years, mainly due to implementation of MLPA. This has led to application of aCGH to determine the breakpoint with

higher resolution, which resulted in characterization of several deletions and duplications in the globin gene clusters. With this knowledge, relatively simple PCR assays were designed to expand the current gap-PCR method. Development of MLPA, PAP and MCA also improved the prenatal diagnosis. In particular, the non-invasive prenatal diagnosis will have its impact in the near future, because of the minimal risk of miscarriage. The next generation technologies, which are able to sequence the whole genome, will be very important in the future, both in pre- and postnatal molecular diagnostics.

Samenvatting

Samenvatting

Hemoglobinopathieën (Hb-pathieën) zijn recessieve erfelijke vormen van bloedarmoede, gekenmerkt door microcytaire hypochrome anemie. Dragers van Hb-pathie zijn meestal asymptomatisch, maar kinderen van ouders die beiden drager zijn hebben een risico van 25% op een ernstige vorm van Hb-pathie. Hb-pathieën worden veroorzaakt door mutaties in de genen die de sub-eenheden aanmaken om het hemoglobine (Hb) molecuul te vormen, de α- en β-globine genen. Deze mutaties kunnen enerzijds leiden tot een verandering in de primaire structuur van het eiwit waardoor een abnormaal Hb ontstaat. Anderzijds kan een mutatie zorgen voor een verminderde aanmaak van Hb, dit veroorzaakt een thalassemie.

Doordat dragers van een Hb-pathie beschermd zijn tegen de ernstige gevolgen van malaria tropica, komen Hb-pathieën het meest voor in gebieden waar malaria heerst of geheerst heeft, zoals de Mediterrane landen, het Midden Oosten en Zuidoost Azië. Echter, door immigratie uit landen met een hoge incidentie komen Hb-pathieën nu ook steeds vaker voor in noord Europese landen. Ongeveer 7% van de totale wereldbevolking is gezonde drager van een Hb-pathie; dit leidt jaarlijks tot de geboorte van ongeveer 350000 kinderen met een ernstige vorm van Hb-pathie. In Nederland worden elk jaar ongeveer 60 kinderen geboren die aangedaan zijn met thalassemie major of sikkelcelziekte. Tot nu toe is er, behalve beenmergtransplantatie, nog geen genezing mogelijk voor deze patiënten. Tegenwoordig worden er in endemische landen nationale programma's geïmplementeerd om dragers van een Hb-pathie te identificeren om primaire preventie te faciliteren. Men kan risicoparen dan een geïnformeerde reproductieve keuze aanbieden, waaronder afbreken van de zwangerschap. Zo'n nationaal programma ten behoeve van preventie is echter nog niet geïmplementeerd in de Nederlandse zorg.

De diagnostiek voor Hb-pathie onderzoek bestaat uit drie verschillende onderdelen, namelijk hematologische, biochemische en moleculaire onderzoeken. De uitslagen van alle testen samen vormen de complete diagnose. In dit proefschrift wordt ingegaan op het ontwikkelen en optimaliseren van moleculaire technieken, zowel voor post- als prenataal onderzoek, ten behoeve van verbetering van de diagnostiek voor Hb-pathieën.

Hoofdstuk 2.1 beschrijft een diagnostische test, gebaseerd op de gap-PCR techniek, om acht in Zuidoost Azië veel voorkomende deleties in het β-globine gencluster te detecteren. Deze techniek is snel, eenvoudig, goedkoop en kan in elk moleculair diagnostisch laboratorium toegapast worden. In hoofdstuk 2.2 beschrijven wij de toepassing van de multiplex ligation-dependent probe amplification (MLPA) techniek voor de detectie van deleties in de α- en β-globine genclusters. In tegenstelling tot conventionele methoden zoals Southern blotting en fluorescente in-situ hybridisatie (FISH), is MLPA in staat om op een snelle en goedkope manier bekende en onbekende deleties en duplicaties te detecteren met een zeer hoge resolutie. Dit onderzoek laat zien dat de MLPA methode zeer geschikt is om deleties in de α- en β-globine genclusters te detecteren; zowel deleties met bekende breekpunten, minder frequent voorkomende en negen nog niet eerder beschreven deleties werden gevonden. De toepasbaarheid van MLPA wordt verder toegelicht in hoofdstuk 2.3. In deze studie is een cohort van 37 Australische patiënten verdacht van thalassemie dragerschap, waarvan de onderliggende moleculaire oorzaak met de conventionele technieken nog niet gevonden was, gescreend met MLPA. Uiteindelijk konden 27 patiënten gediagnosticeerd worden als thalassemie patiënt vanwege een deletie in het α- of β-globine gencluster. Daarbij werden in deze studie 11

niet eerder beschreven deleties gevonden. Hieruit blijkt dat de MLPA techniek een waardevolle aanvulling is op de huidige technieken die gebruikt worden voor Hb-pathie diagnostiek. Hoewel met behulp van MLPA de lengte van deleties relatief nauwkeurig te bepalen is, blijven de exacte breekpunten onbekend. Vanuit wetenschappelijk oogpunt is het interessant om de breekpunten te bepalen, omdat zo meer kennis verkregen zou kunnen worden over mechanismes die ten grondslag liggen aan het ontstaan van deze deleties.

Hoofdstuk 2.4 beschrijft de ontwikkeling en toepassing van de fine-tiling array comparative genomic hybridization (aCGH) methode voor Hb-pathieën. Deze studie laat zien dat het met gebruik van deze techniek mogelijk is om breekpunten zeer nauwkeurig te bepalen in alle 36 patiënten die getest zijn. Met deze kennis was het relatief eenvoudig om simpele gap-PCR methoden te ontwikkelen voor 10 deleties. Verder toont deze studie aan dat deleties in het α-globine gen cluster meestal lijken te ontstaan door het 'nonallelic homologous recombination' (NAHR) mechanisme, terwijl er in het β-globine gencluster vaker sprake is van het 'nonhomologous end joining' (NHEJ) mechanisme.

In hoofdstuk 4.1 wordt de $--^{AW}$ deletie beschreven. Deze deletie, gevonden in een Nederlandse familie, heeft een lengte van 8.2 kb en neemt beide α-globine genen weg. Na sequentie analyse van het breekpuntfragment kon worden geconcludeerd dat deze deletie waarschijnlijk is ontstaan door een niet-homologe recombinatie tussen een Alu- en een L1-repeat. Dit type recombinatie is zeer zeldzaam en nog niet eerder beschreven in het α-globine gencluster. Hoofdstuk 4.2 beschrijft een patiënt met HbH ziekte en β-thalassemie, als gevolg van gecombineerd dragerschap van een mutatie in het $\alpha-$ en β-globine gen en een onbekende deletie in het α-globine gencluster. Deze deletie, $--^{BGS}$ genaamd, is met behulp van MLPA, aCGH, PCR en sequentie analyse nader gekarakteriseerd. De deletie neemt 131 kb van het α-globine gencluster weg; het regulatoire element en beide α-globine genen zijn gedeleteerd maar het θ-gen is nog intact. Omdat er een orphan sequentie van 57 bp in het breekpuntfragment werd gevonden, kan worden geconcludeerd dat deze deletie waarschijnlijk is ontstaan door vorming van twee loops vanwege recombinatie tussen verschillende Alu-repeats op beide allelen. Deze studies tonen het belang aan van MLPA, aCGH en breekpuntanalyse binnen de diagnostiek voor Hb-pathieën. Het is van belang om dergelijke α^0-thalassemie deleties te kunnen detecteren, omdat er in combinatie met andere α^0-thalassemieën (bijvoorbeeld $--^{SEA}$, $--^{MedI}$, $--^{MedII}$ en $--^{DutchII}$) een risico van 25% op het letale HbBart's hydrops foetalis syndroom in het nageslacht bestaat.

De MLPA methode is ook zeer waardevol in de prenatale diagnostiek van Hb-pathieën, zoals wordt beschreven in hoofdstuk 3.1. Twee risicoparen, waarvan een partner drager is van een bekende puntmutatie in het β-globine gen en de ander van een β-thalassemie waarvan de moleculaire oorzaak niet gevonden kon worden, werden onderzocht met behulp van MLPA. Een deletie van het regulatoire element, dat upstream van het β-globine gen ligt, veroorzaakt namelijk ook een β-thalassemie terwijl het β-globine gen zelf intact is. In dit geval zou het intacte (maar inactieve) gen kunnen leiden tot een misdiagnose: de foetus wordt ten onrechte gekarakteriseerd als een gezonde drager van β-thalassemie, maar heeft geen functionele β-globine genen, wat leidt tot thalassemie major. Dit was echter niet het geval in de onderzochte risicoparen; na analyse met behulp van MLPA bleek er sprake te zijn van een niet eerder beschreven deletie die het β-globine gen wegneemt.

Ten behoeve van verbetering van de prenatale diagnostiek voor Hb-pathieën is een pyrophosphorolysis-activated polymerization (PAP) assay opgezet (hoofdstuk 3.2). Deze techniek

is in staat om het vrij foetaal DNA, dat circuleert in de bloedbaan van een zwangere vrouw, te detecteren. Omdat het vaak voorkomt dat beide ouders drager zijn van dezelfde mutatie, waarbij geen onderscheid gemaakt kan worden tussen foetale en maternale mutaties, is besloten om deze studie op te zetten voor 12 SNPs in linkage met het ziekte gen, in plaats van voor de mutatie zelf. Genotypering van deze SNPs gebeurt met behulp van melting curve analysis (MCA), een zeer snelle en nauwkeurige methode. PAP wordt vervolgens uitgevoerd voor de SNPs die vader wel heeft en moeder niet, om zo te kunnen bepalen welk paternale allel de foetus heeft geërfd. De combinatie van PAP en MCA vormt een betrouwbare methode voor niet-invasieve prenatale diagnose van β-thalassemie en sikkelcelziekte, op voorwaarde dat een eerder geboren kind of ander familielid beschikbaar is om linkage met de paternale SNPs te bepalen. Indien er informatieve SNPs aanwezig zijn in vader (en afwezig in moeder), maakt deze methode in principe 50% van de huidige invasieve ingrepen, die een risico op een miskraam met zich meebrengen, overbodig.

De diagnostiek voor Hb-pathieën is de laatste jaren sterk verbeterd, voornamelijk door de implementatie van de MLPA techniek. Naar aanleiding hiervan is de arrayCGH technologie toegepast om de breekpunten met een hogere resolutie te bepalen. Dit heeft geleid tot karakterisatie van verschillende deleties en duplicaties in de globine genclusters. Met deze kennis konden relatief eenvoudige PCR assays opgezet worden om de huidige gap-PCR uit te breiden. De ontwikkeling van de MLPA, PAP en MCA technieken heeft ook geleid tot verbetering van de prenatale diagnostiek. Vooral de niet-invasieve prenatale diagnostiek zal een aanzienlijke verandering teweeg brengen, vanwege het minimale risico op een miskraam. In de toekomst zal de nieuwste generatie technieken die het mogelijk maken om het gehele genoom te sequencen een belangrijke rol gaan spelen, zowel in post- als (niet-invasieve) prenatale moleculaire diagnostiek.

Curriculum Vitae

Curriculum Vitae

Marion Phylipsen werd geboren op 11 mei 1983 te Rotterdam. In 2001 behaalde zij het gymnasium diploma aan de christelijke scholengemeenschap Johannes Calvijn in Rotterdam. Aansluitend volgde zij de opleiding Biomedische Wetenschappen aan de Universiteit Leiden, waarvan het bachelor diploma behaald werd in 2005. De eerste stage werd uitgevoerd op de afdeling Parasitologie van het Leids Universitair Medisch Centrum (LUMC), onder leiding van Dr. Elly van Riet en Prof. Dr. Maria Yazdanbakhsh. De afstudeeropdracht werd verricht bij het Hemoglobinopathieën Laboratorium op de afdeling Klinische Genetica in het LUMC, onder leiding van Dr. Piero Giordano en Dr. Kees Harteveld. In 2007 behaalde ze haar Master of Science titel.

In januari 2007 is zij begonnen als onderzoeker in opleiding op de afdeling Klinische Genetica van het LUMC, onder leiding van Prof. Dr. Bert Bakker en Dr. Kees Harteveld, met als onderwerp ontwikkeling en verbetering van technieken voor post- en prenatale diagnose van hemoglobinopathieën.

Sinds mei 2012 is Marion Phylipsen werkzaam als assistent staflid moleculaire genetica op de afdeling Klinische Genetica van het Leids Universitair Medisch Centrum.

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List of publications

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