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).

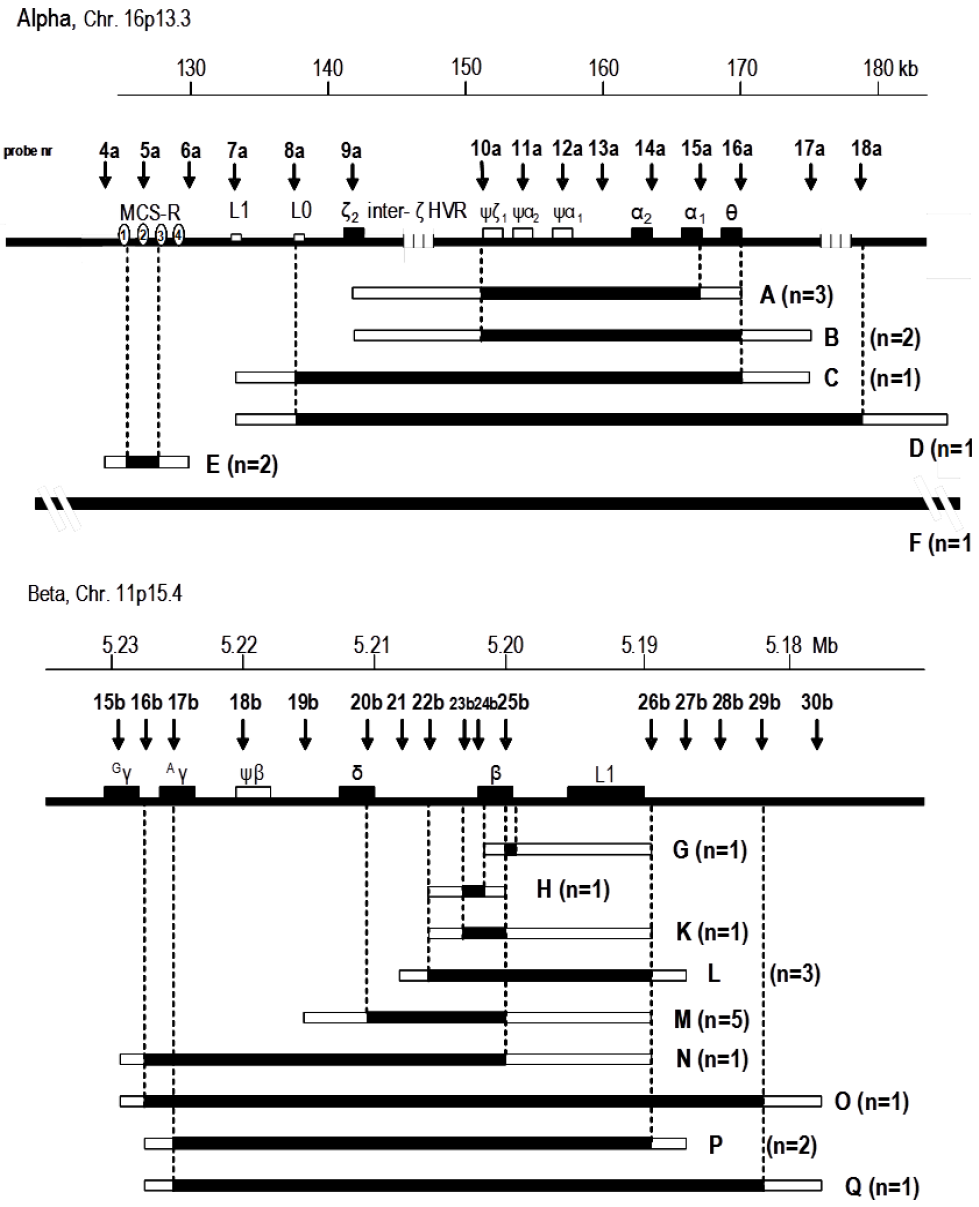

Figure 1. Schematic overview of the α- and β-globin gene clusters. The arrows and numbers indicate the locations of the probes. The deletions are indicated as solid bars, the open ends indicate the region where the breakpoint is located. (n = number of independent chromosomes).

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

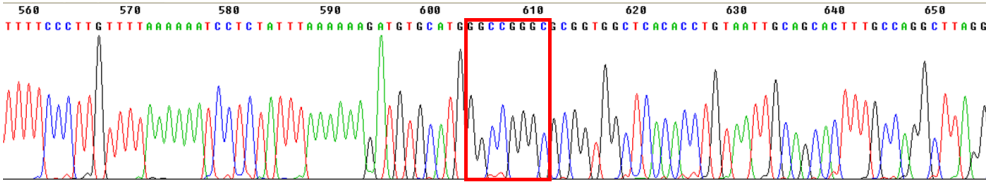

Figure 2 Sequence analysis of the --^{GB} fragment. The breakpoint area of 8 nt is indicated by the red square. The G's flanking the breakpoint region at the 5' and 3' ends are located at positions 151901 and 168681, respectively.

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.

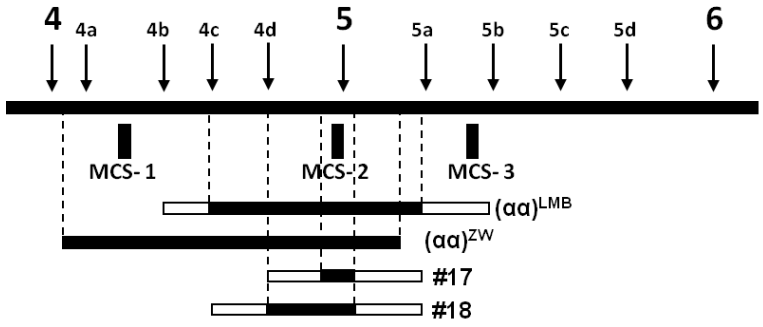

Figure 3 Schematic overview of the deletions in the multi-species conserved sequence region (MCS-R). Numbers and arrows indicate the location of the MLPA probes. Since all patients showed a deletion of MLPA probe 5 only, probes 4a-d and 5a-d were designed to check whether the deletions in these patients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