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

Harteveld CL, Voskamp A, Phylipsen M, Akkermans N, den Dunnen JT, White SJ, Giordano PC

J Med Genet. 2005 Dec;42(12):922-31

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

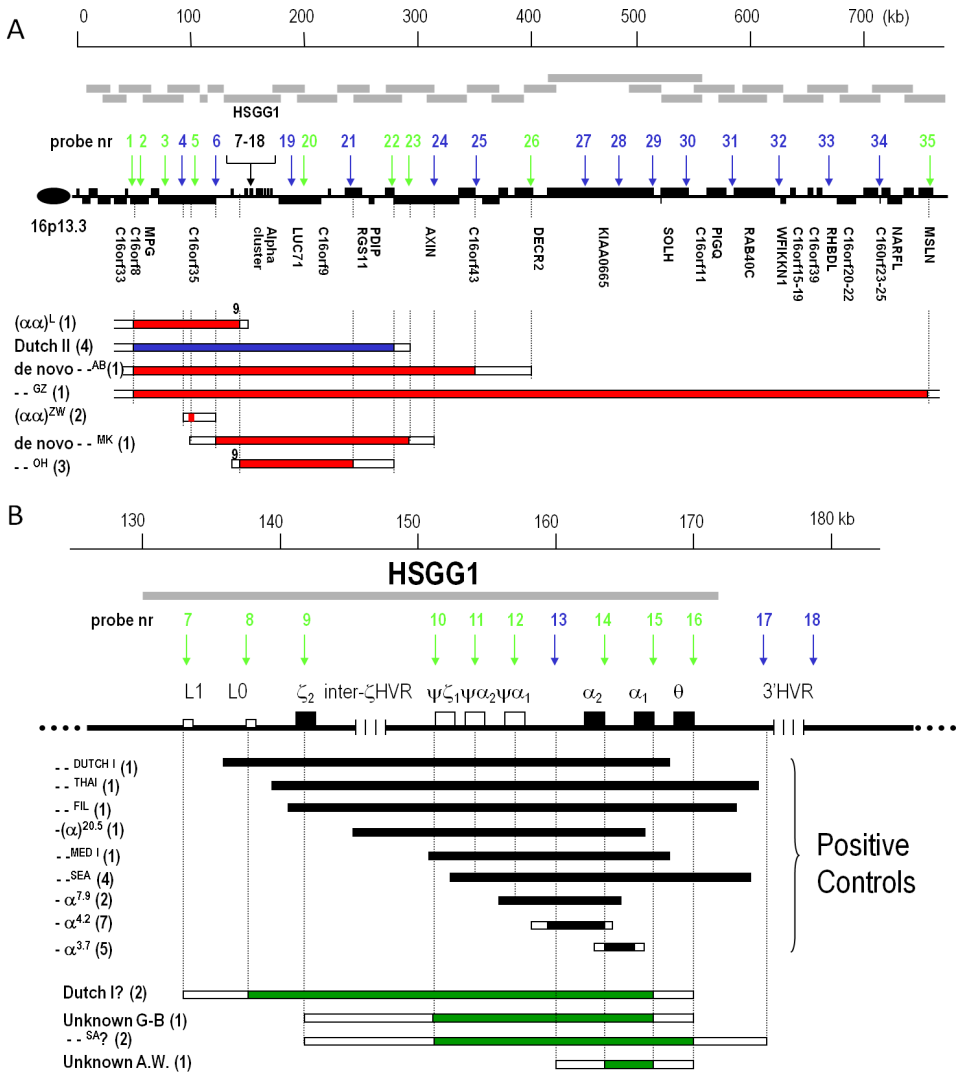

Figure 1 (A) Schematic representation of the short arm of chromosome 16 (16p13.3), showing a 700 kb region containing the α-globin gene cluster. Grey bars above the cluster indicate the minimal tiling path of clones covering this region. Oval shape denotes the telomeric repeat region and solid boxes 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.

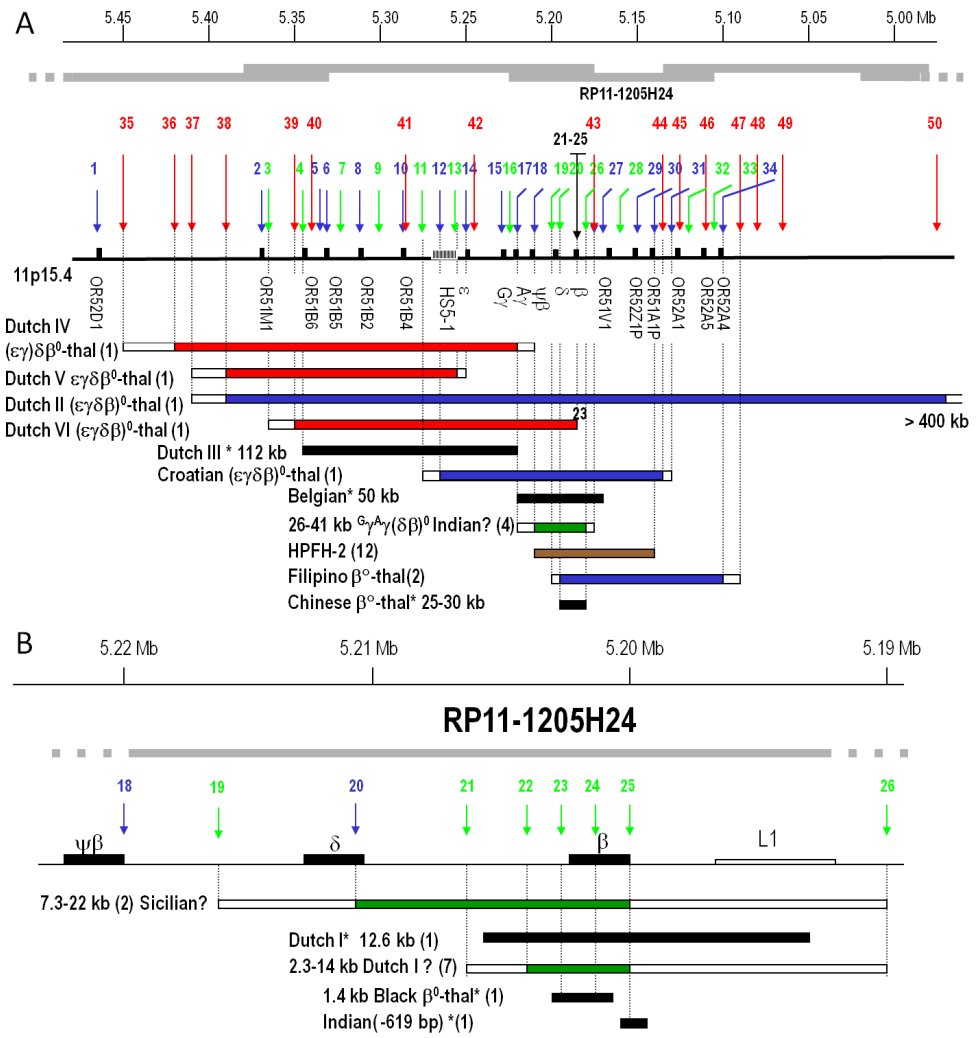

Figure 2 (A) Schematic representation of the short arm of chromosome 11 (11p15.4), showing a 500 kb region containing the β-globin gene cluster. The genes throughout the regions are indicated as solid boxes. The minimal tiling path of clones covering this region is indicated by grey bars above the cluster. 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].

Acknowledgements

We are very greatful to the patients and the referring physicians for their cooperation. We would like to thank Dr Hannes Frischknecht and Dr Lorenz Risch for sending us DNA of the Croatian deletion, and Dr Douglas R Higgs for FISH analysis of the Dutch II deletion.

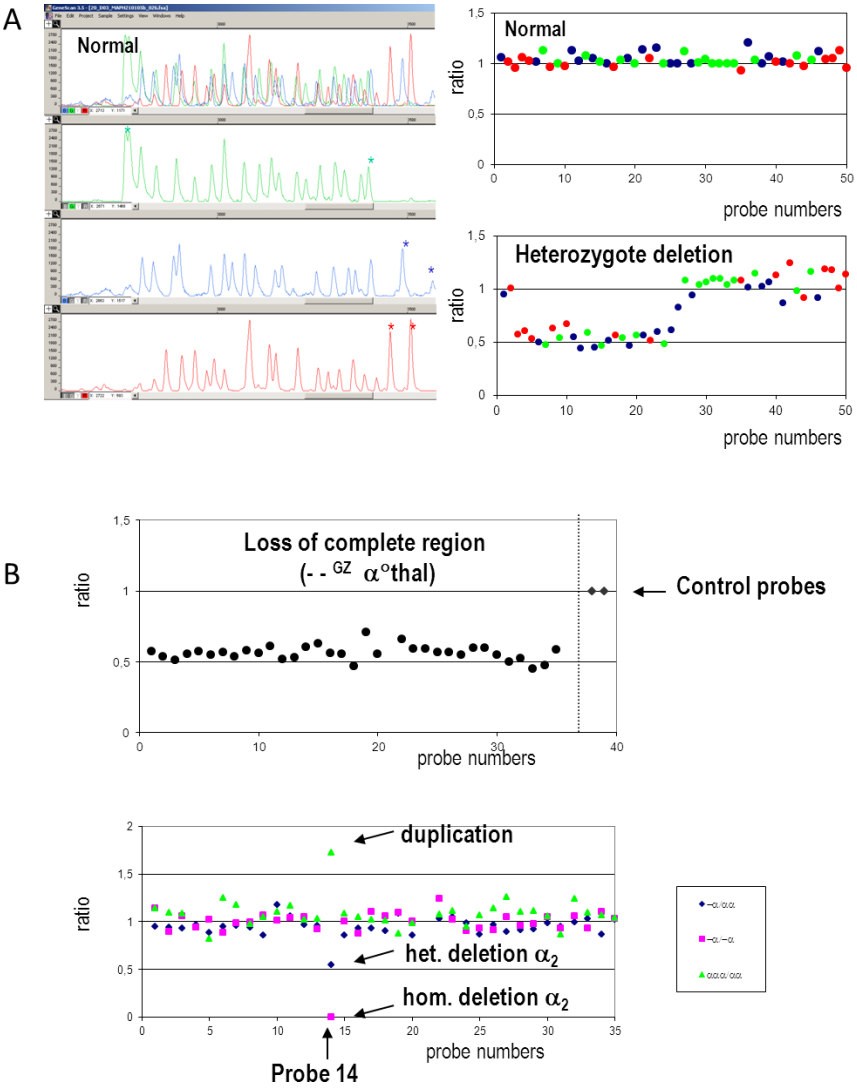

Figure 3. Peak patterns and scatterplots. **(A)** Peak patterns of an ABI 3700 fragment run of 50 β-specific probes labelled in three colours tested on a wildtype DNA sample. The upper window shows 17 probes in green, 17 probes in blue, and 16 probes in red, all between 80 and 125 bp in length. In the 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.

References

1. Weatherall DJ, Clegg JB ed. The Thalassaemia Syndromes. Oxford, London, Edinburgh, Boston, Melbourne Blackwell Scientific Publications 1981

2. Thein SL. β-Thalassaemia. In: Sickle Cell Disease and Thalassaemia. Rodgers GP, ed. Baillière's Clinical Haematology Vol 11 London: Baillière Tindall;1998:91 -126.

3. Bernini LF, Harteveld CL. α-Thalassaemia. In: Sickle Cell Disease and Thalassaemia. Rodgers GP ed. Baillière's Clinical Haematology Vol 11 London: Baillière Tindall; 1998: 53-90.

4. Higgs DR, Thein SL and Wood WG.The molecular pathology of the thalassaemias. In: Weatherall DJ, Clegg JB, eds. The Thalassaemia syndromes, 4th ed. Oxford: Blackwell Science, 2001: 133-192.

5. Silvestroni ID, Le Thalassemie, Istituto Italiano di medicina sociale, Rome 1998

6. Liu YT, Old JM, Miles K, Fisher CA, Weatherall DJ, Clegg JB. Rapid detection of α-thalassaemia deletions and α-globin gene triplications by multiplex polymerase chain reaction. Br J Haematol 2000;108:295-299.

7. Chong SS, Boehm CD, Higgs DR, Cutting GR. Single tube multiplex-PCR screen for common deletional determinants of α-thalassaemia. Blood 2000;95:360-362.

8. Craig JE, Barnetson RA, Prior J, Raven JL, Thein SL. Rapid detection of deletions causing delta beta thalassaemia and hereditary persistence of fetal haemoglobin by enzymatic amplification. Blood 1994;83:1673-1682.

9. Knight SJ, Horsley SW, Regan R, Lawrie NM, Maher EJ, Cardy DL, Flint J, Kearney L. Development and clinical application of an innovative fluorescence in situ hybridisation technique which detects submicroscopic rearrangements involving telomeres. Eur J Hum Genet 1997;5:1-8.

10. Horsley SW, Daniels RJ, Anguita E, Raynham HA, Peden JF, Villegas A, Vickers MA, Green S, Waye JS, Chui DH, Ayyub H, MacCarthy AB, Buckle VJ, Gibbons RJ, Kearney L, Higgs DR Monosomy for the most telomeric, gene-rich region of the short arm of human chromosome 16 causes minimal phenotypic effects. Eur J Hum Genet 2001;9:217-25.

11. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 2002;30:e57.

12. Taylor CF, Charlton RS, Burn J, Sheridan E, Taylor GR. Genomic deletions in MSH2 or MLH1 are a frequent cause of hereditary non-polyposis colorectal cancer: identification of novel and recurrent deletions by MLPA. Hum Mutat 2003;22:428-433

13. Sellner LN, Taylor GR. MLPA and MAPH: new techniques for detection of gene deletions. Hum Mutat. 2004;23:413-419.

14. Rooms L, Reyniers E, van Luijk R, Scheers S, Wauters J, Ceulemans B, Van Den Ende J, Van Bever Y, Kooy RF. Subtelomeric deletions detected in patients with idiopathic mental retardation using multiplex ligation-dependent probe amplification (MLPA). Hum Mutat. 2004;23:17-21.

15. White SJ, Vink GR, Kriek M, Wuyts W, Schouten J, Bakker B, Breuning MH and den Dunnen JT. Two-Color Multiplex Ligation-Dependent Probe Amplification: Detecting genomic rearrangements in Hereditary Multiple Exostoses. Human Mutat 2004;24:86-92.

16. Giordano PC, Harteveld CL, Heister AJGM, Batelaan D, van Delft P, Plug R, et al. The molecular spectrum of beta-thalassaemia and abnormal haemoglobins in the allochtonous and autochtonous Dutch population. Community Genet 1998;1:243-251.

17. Dacie JV, Lewis SM, ed. Practical Haematology. Edinburgh, U.K.: Churchill Livingstone 1991

18. Lin CK, Gau JP, Hsu HC and Jiang ML. Efficacy of a modified improved technique for detecting red cell haemoglobin H inclusions. Clin lab Haemat. 1990;12:409-415.

19. Harteveld CL, Losekoot M, Fodde R, Giordano PC, Bernini LF. The involvement of Alu repeats in recombination events at the α-globin gene cluster: characterization of two α^0-thalassaemia deletion breakpoints. Human Genetics 1997;99: 528-534.

20. Harteveld CL, van Delft P, Wijermans PW, Kappers-Klunne MC, Weegenaar J, Losekoot M, Giordano PC. A novel 7.9 kb deletion causing alpha$^+$-thalassaemia in two independent families of Indian origin. Br J Haematol 2003;120:364-366.

21. Harteveld CL, Osborne CS, Peters M, van der Werf S, Plug R, Fraser P, Giordano PC. Novel 112 kb (epsilonGgammaAgamma) deltabeta-thalassaemia deletion in a Dutch family. Br J Haematol 2003;122:855-858.

22. Losekoot M, Fodde R, Gerritsen EJ, van de Kuit I, Schreuder A, Giordano PC, Vossen JM, Bernini LF. Interaction of two different disorders in the beta-globin gene cluster associated with an increased haemoglobin F production: a novel deletion type of (G) gamma + ((A) gamma delta beta) (0)-thalassaemia and a delta(0)-hereditary persistence of fetal haemoglobin determinant. Blood 1991;77:861-867.

23. Dimovski AJ, Divoky V, Adekile AD, et al. A novel deletion of approximately 27 kb including the beta-globin gene and the locus control region 3'HS-1 regulatory sequence: beta zero-thalassaemia or hereditary persistence of fetal haemoglobin? Blood 1994;83:822-827.

24. Gilman JG. The 12.6 kilobase DNA deletion in Dutch beta zero-thalassaemia. Br J Haematol. 1987;67:369-372.

25. Orkin SH, Old JM, Weatherall DJ, Nathan DG. Partial deletion of beta-globin gene DNA in certain patients with beta 0-thalassaemia. Proc Natl Acad Sci USA. 1979;76:2400-2404.

26. White S, Kalf M, Liu Q, Villerius M, Engelsma D, Kriek M, Vollebregt E, Bakker B, van Ommen GJ, Breuning MH, den Dunnen JT. Comprehensive detection of genomic duplications and deletions in the DMD gene, by use of multiplex amplifiable probe hybridisation. Am J Hum Genet. 2002;71:365-374.

27. Kent WJ. BLAT-the Blast-like alignment tool. Genome Res 2002;12:656-664.

28. Wilkie AO, Buckle VJ, Harris PC, Lamb J, Barton NJ, Reeders ST, Lindenbaum RH, Nicholls RD, Barrow M, Bethlenfalvay NC, et al. Clinical features and molecular analysis of the alpha thalassaemia/ mental retardation syndromes. I. Cases due to deletions involving chromosome band 16p13.3. Am J Hum Genet. 1990;46:1112-1126.

29. Daniels RJ, Peden JF, Lloyd C, Horsley SW, Clark K, Tufarelli C, Kearney L, Buckle VJ, Doggett NA, Flint J, Higgs DR. Sequence, structure and pathology of the fully annotated terminal 2 Mb of the short arm of human chromosome 16. Hum Mol Genet. 2001;10:339-352.

30. Huisman THJ, Carver FMH, Efremov GD ed. A Syllabus of Human Haemoglobin Variants (2nd edition). Augusta GA, USA. The Sickle Cell Anemia Foundation 1998

31. Harteveld CL, Losekoot M, Heister AJ, van der Wielen M, Giordano PC, Bernini LF. Alpha-Thalassaemia in The Netherlands: a heterogeneous spectrum of both deletions and point mutations. Hum Genet. 1997;100:465-471.

32. Abels J, Michiels JJ, Giordano PC, Bernini LF, Baysal E, Smetanina NS, Kazanetz EG, Leonova JY, Huisman TH. A de novo deletion causing epsilon gamma delta beta-thalassaemia in a Dutch patient. Acta Haematol. 1996;96:108-109.

33. Henthorn PS, Smithies O, Mager DL. Molecular analysis of deletions in the human beta-globin gene cluster: deletion junctions and locations of breakpoints. Genomics 1990;6:226-237.

34. Craig JE, Barnetson R, Weatherall DJ, Thein SL. Rapid detection of a 13.4-kb deletion causing delta beta thalassaemia in an Egyptian family by polymerase chain reaction. Blood 1993;81:861-863.

35. Mishima N, Landman H, Huisman TH, Gilman JG. The DNA deletion in an Indian delta beta-thalassaemia begins one kilobase from the A gamma globin gene and ends in an L1 repetitive sequence. Br J Haematol. 1989;73:375-379.

36. Janssen B, Hartmann C, Scholz V, Jauch A, Zschocke J. MLPA analysis for the detection of deletions, duplications and complex rearrangements in the dystrophin gene: potential and pitfalls. Neurogenetics 2005;6:29-35.

37. Harteveld CL, Muglia M, Passarino G, Kielman MF, Bernini LF. Genetic polymorphism of the major regulatory element HS-40 upstream of the human alpha-globin gene cluster. Br J Haematol 2002;119:848-854.

38. Higgs DR, Sharpe JA and Wood WG. Understanding α globin gene expression: a step towards effective gene therapy. Sem Hematol. 1998;35:93-104.

39. Anguita E, Sharpe JA, Sloane-Stanley JA, Tufarelli C, Higgs DR, Wood WG. Deletion of the mouse α-globin regulatory element (HS-26) has an unexpectedly mild phenotype. Blood 2002;100:3450-3456.

40. Locke DP, Segraves R, Nicholls RD, Schwartz S, Pinkel D, Albertson DG, Eichler EE. BAC microarray analysis of 15q11-q13 rearrangements and the impact of segmental duplications. J Med Genet 2004;41:175-182.