Characterization of Deletions of the *HBA* and *HBB* Loci by Array Comparative Genomic Hybridization

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Thalassemia is among the most common genetic diseases worldwide. α-Thalassemia is usually caused by deletion of one or more of the duplicated *HBA* genes on chromosome 16. In contrast, most β-thalassemia results from point mutations that decrease or eliminate expression of the *HBB* gene on chromosome 11. Deletions within the *HBB* locus result in thalassemia or hereditary persistence of fetal Hb. Although routine diagnostic testing cannot distinguish thalassemia deletions from point mutations, deletional hereditary persistence of fetal Hb is notable for having an elevated HbF level with a normal mean corpuscular volume. A small number of deletions accounts for most α-thalassemias; in contrast, there are no predominant *HBB* deletions causing β-thalassemia. To facilitate the identification and characterization of deletions of the *HBA* and *HBB* globin loci, we performed array-based comparative genomic hybridization using a custom oligo-nucleotide microarray. We accurately mapped the breakpoints of known and previously uncharacterized *HBB* deletions defining previously uncharacterized deletion breakpoints by PCR amplification and sequencing. The array also successfully identified the common *HBA* deletions −[SEA] and −[FIL]. In summary, comparative genomic hybridization can be used to characterize deletions of the *HBA* and *HBB* loci, allowing high-resolution characterization of novel deletions that are not readily detected by PCR-based methods. *(J Mol Diagn 2016, 18: 92−99; http://dx.doi.org/10.1016/j.jmoldx.2015.07.011)*

Thalassemia is one of the most common genetic diseases and is responsible for significant morbidity worldwide. α-Thalassemia is most commonly caused by deletion of one or more of the duplicated *HBA* genes on chromosome 16. In contrast, most β-thalassemia results from point mutations that cripple or completely inactivate expression from the *HBB* gene on chromosome 11. These mutations commonly result in a decrease in HBB and a relative increase in HBD protein expression, resulting in an elevated HbA_2 level. There is frequently a relative increase in HBG1/2 expression as well, with a corresponding increase in HbF. Deletions within the *HBB* locus occur and can eliminate HBB expression. If the *HBD* and *HBG* genes are deleted along with the *HBB* gene, the characteristic increases in HbA_2 and HbF will not be seen, making it impossible to distinguish from α-thalassemia on the basis of HbA_2 quantification alone. Deletions resulting in levels of HBD and HBG polypeptide production insufficient to balance that from the *HBA* locus result in δβ- or γδβ-thalassemia with a decreased mean corpuscular volume (MCV). In contrast, some deletions result in elevated levels of HBG sufficient to balance that from the *HBA* locus, resulting in hereditary persistence of fetal Hb (HPFH), the hallmark of which is a normal MCV. In both of these cases, an *HBB* locus deletion is assumed. *HBB* deletions causing thalassemia may be more common than generally thought because they may not be recognized during routine thalassemia diagnostic procedures, especially in the rare deletions that do not result in HbF elevation.

Table 1 PCR and Sequencing Primers Used in This Study

Subject No.	Forward PCR primer	Reverse PCR primer	Additional sequencing primers, if used
4	5′-TAGATCCCTTTGCCATTATG-3′	5′-TTGGGTTTCTGATAGGCACTG-3′	
5	5′-AGCCTCATGGTAGCAGAATC-3′	5′-TGGTATCTGCAGCAGTTGCC-3′	
7*	5′-CCTTTTTCTTGTGGTAAATG-CTT-3′	5′-TTTCCTTGTGTTTGAAAGTGCT-3′	F: 5′-ACCCTTTGAGTAATAGTTTCCTGA-3′ R: 5′-GCAAATAAGCACACATATATTCCAA-3′
	5′-ATGCCAGTGCTCTCCACAAT-3′	5′-ATAATAAGCCTGCGCCCTTC-3′	R: 5′-AGTTTCTTGTTTACTCTGGA-3′ R: 5′-GGCCATATAGGGTAACTTTCTGAC-3′ R: 5′-AAAAGTGTGCCATGGTTTTAATG-3′
8	5′-CATCCACCACTTTCTGATAGG-3′	5′-TAGCATGCATGAGCAAATTAAGA-3′	
9	5′-GCCACATGGTATGGGAGGTA-3′	5′-TGTTACTTGTCTGGTGTGGCTAA-3′	

*Upper line shows sequences for telomeric breakpoint; lower line shows sequences for centromeric breakpoint.
F, forward; R, reverse.

Several well-characterized deletions account for most α-thalassemia; thus, gap-PCR—based methods are routinely used to identify *HBA* gene deletions.[1] In contrast, *HBB* deletions, when they occur, are diverse, so routine PCR-based methods are not efficient or routinely available. This is especially true in the United States, where no set of mutations predominates because of the ethnic diversity of this population. To provide a rapid inexpensive and efficient platform to identify common, previously described, and unique deletions of either the *HBA* or *HBB* loci, we developed a microarray-based comparative genomic hybridization (CGH) assay. This method can be used to genotype patients with deletional forms of α- or β-thalassemia, HPFH, or complex mixed *HBA* and *HBB* globin deletions, whose genotype is not apparent from protein-based studies, such as electrophoresis and high-performance liquid chromatography (HPLC). We used this array to characterize both previously characterized and novel deletions of the *HBB* locus. This array has been used in patients with apparent thalassemia trait but no evidence of α-thalassemia and no increase in HbA_2, providing diagnostic information that allows accurate genetic counseling.

Materials and Methods

Patient Specimens and Diagnostic Studies

Patient specimens were sent to the University of Washington Red Cell Disorders laboratory for routine clinical testing. Clinical testing at the University of Washington included qualitative Hb analysis by isoelectric focusing (Perkin-Elmer Multiphor II; Perkin-Elmer, Waltham MA) and HPLC (Bio-Rad Variant II; Bio-Rad, Hercules, CA). HbF and HbA_2 were quantified by HPLC. Detection of HbH inclusions was performed by brilliant cresyl blue staining as previously described.[2] Iron deficiency was assessed by determining the zinc protoporphyrin/heme ratio[3]; serum iron, ferritin, total iron binding capacity, and transferrin saturation were determined in a subset of cases. For samples collected at the University of Washington, DNA was isolated using a Corbett automated DNA extractor (Qiagen, Valencia, CA). PCR to detect *HBA* deletions was performed as previously described.[4] Additional specimens were obtained from Boston Children's Hospital. All studies were approved by the University of Washington Human Subjects Division.

Microarray Design

Custom arrays were designed essentially as described, including the software used for probe design and repetitive sequence masking.[5] Genome coordinates used throughout this article are from the human hg38 assembly (December 2013). For chromosome 11, probes were tiled at a median density of 60 bp across an approximately 450-kbp region surrounding the *HBB* locus (coordinates chr11:5,022,271-5,472,133), and at a median density of 57 to 62 kbp on either side of this region for the remainder of chromosome 11 (coordinates chr11:218,364-5,022,271 and chr11:5,472,133-134,998,453). For chromosome 16, probes were tiled at a median density of 64 bp across an approximately 400-kbp region surrounding the *HBA* loci (coordinates chr16:11,177-414,936) and a median density of 56,261 bp for the remainder of chromosome 16 (chr16:429,413-90,081,925). An additional 883 probes representing the other chromosomes (median spacing of 1.8 Mbp) were included for the purpose of signal normalization. The array design is accessible in the National Center for Biotechnology Information Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo*; accession number GPL20136).

Array Hybridization, Scanning, and Data Analysis

Subject genomic DNA was labeled with Cy5-dUTP, and normal human genomic DNA (Promega, Madison, WI) was labeled with Cy3-dUTP using the Agilent Genomic DNA Labeling kit PLUS (Agilent Technologies, Santa Clara, CA). Array hybridization, scanning, and data analysis were performed as described previously.[5] The raw data are accessible at the National Center for Biotechnology Information Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo*; accession number GSE68587).

Table 2 Clinical Data for Patients with *HBB* Deletions

Subject no.	Age	Sex	MCV, fL	HbA$_2$, %	HbF, %	Hb, g/dL	Hct, %	Electrophoresis	Ethnicity
1	2 months	F	93	0.9	91.1	11.3		HbF, HbS	African
2	20 months	F	76	3	34.1	11.6		HbS, HbF	African
3	26 years	F	82	16.5	11.7	13.1		HbA, Hb Kenya	African
4	25 years	F	69	ND	44.2		35	HbE, HbF	Cambodian
5	25 years	F	79	4.3	20.6		35	HbA, HbF, HbA$_2$	Vietnamese
6	36 years	M	83	1.9	40.8	16.6	50.6	HbA, HbF, HbA$_2$	Sri Lankan
7	30 years	F	65	8.2	1.2		34		Kurdish
8	29 years	F	72	7	9.5	10.2		HbA, HbF, HbA$_2$	Irish/Romanian
9	12 years	M	60.5	3	ND	11.6		HbA, HbA$_2$	Belgian
10	4.5 years	F	69.1	2.6	16.8	12	36.8	HbA, HbF, HbA$_2$	Sri Lankan
11	2 months	F	66	2.1	26.3	7.4		HbA, HbF, HbA$_2$	White

F, female; M, male; Hct, hematocrit; MCV, mean corpuscular volume; ND, not determined.

Confirmation of Deletion Breakpoints

PCR primers flanking breakpoints identified by CGH were designed using Primer 3 software version 1.1.0 (*http://bioinfo.ut.ee/primer3*, last accessed June 1, 2009). DNA sequencing was performed using standard BigDye Terminator reagents (Applied Biosystems, Foster City, CA) using the same PCR primers (Table 1).

Results

Deletions within the *HBB* Locus

Subjects at the University of Washington were suspected of having a deletional form of β-thalassemia if they were microcytic without evidence of iron deficiency, had normal levels of HbA$_2$, and had no evidence of α-thalassemia trait (eg, negative HbH inclusion body assay). Other subjects were identified because of unexplained elevations of HbF or unusually high HbA$_2$. Hematologic findings are detailed in Table 2.

For array CGH, Cy5-labeled subject DNA was combined with Cy3-labeled reference DNA, and the labeled DNA was hybridized to a microarray containing oligonucleotide probes spanning the *HBA* and *HBB* loci. The density of probes varied (see *Materials and Methods* for genomic coordinates), with a high density of probes spanning structural genes of the loci and their upstream regulatory elements (approximately one probe per 60 to 64 bp) and a low density of probes in the flanking regions of the loci (approximately one probe per 56 to 57 kbp). Additional probes from the other chromosomes were included as normalization controls.

Figure 1 shows the results of array CGH for four DNA specimens from patients (subjects 1 to 4) with previously characterized *HBB* locus deletions (a generous gift of Dr. David H.K. Chui, Boston University, Boston, MA), including HPFH-1 (subject 1) (Figure 1A), HPFH-2 (subject 2) (Figure 1B), and Hb Kenya (subject 3) (Figure 1C). The HPFH-1 deletion is shown as a region of Cy5:Cy3 ratio of log$_2$ −1 extending from chromosome 11 coordinate chr11:5,153,221 within a cluster of olfactory receptor genes at the telomeric end of the locus to

coordinate chr11:5,238,138 between the *HBD* and *HBG1* genes (Figure 1A), resulting in deletion of the *HBB* and *HBD* genes. This deletion is most commonly found in populations of African ancestry and results in very high HbF levels (25% to 30%) in heterozygotes.[6] The HPFH-2 deletion is very similar to the HFPH-1 deletion, with breakpoints just centromeric to the HPFH-1 breakpoints at both ends (telomeric breakpoint at chr11:5,158,438, centromeric breakpoint at chr11:5,242,749). The HPFH-2 deletion is also common in individuals of African ancestry, with HbF levels similar to those seen with HPFH-1.[6] Hb Kenya (Figure 1C) is a deletion that excises sequences between the *HBB* and *HBG2* genes (telomeric breakpoint chr11:5,226,631 to centromeric breakpoint at chr11:5,249,422), resulting in a *HBG1-HBB* fusion gene and protein.[7] The abnormal fusion protein co-elutes with HbA$_2$ on HPLC, explaining the elevated level of HbA$_2$ in subject 3; the intact *HBG2* gene in *cis* is expressed at an elevated level as well.
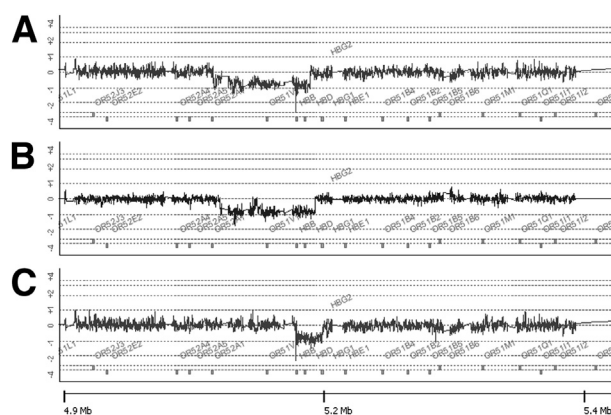

Figure 1 Examples of array comparative genomic hybridization showing previously characterized *HBB* deletions. Deletions identified include hereditary persistence of fetal Hb (HPFH)-1 (subject 1, **A**), HPFH-2 (subject 2, **B**), and Hb Kenya (subject 3, **C**). Numbers at the bottom of the figure represent distance from the chromosome 11p telomere. The ratio of Cy5 to Cy3 is 1 in the absence of copy number change, reflected in a log$_2$ of 0. Heterozygous deletions are identified by a 50% loss (log$_2$ change of −1) of Cy5 signal in a group of contiguous probes. Homozygous deletions result in a log$_2$ change of −∞.
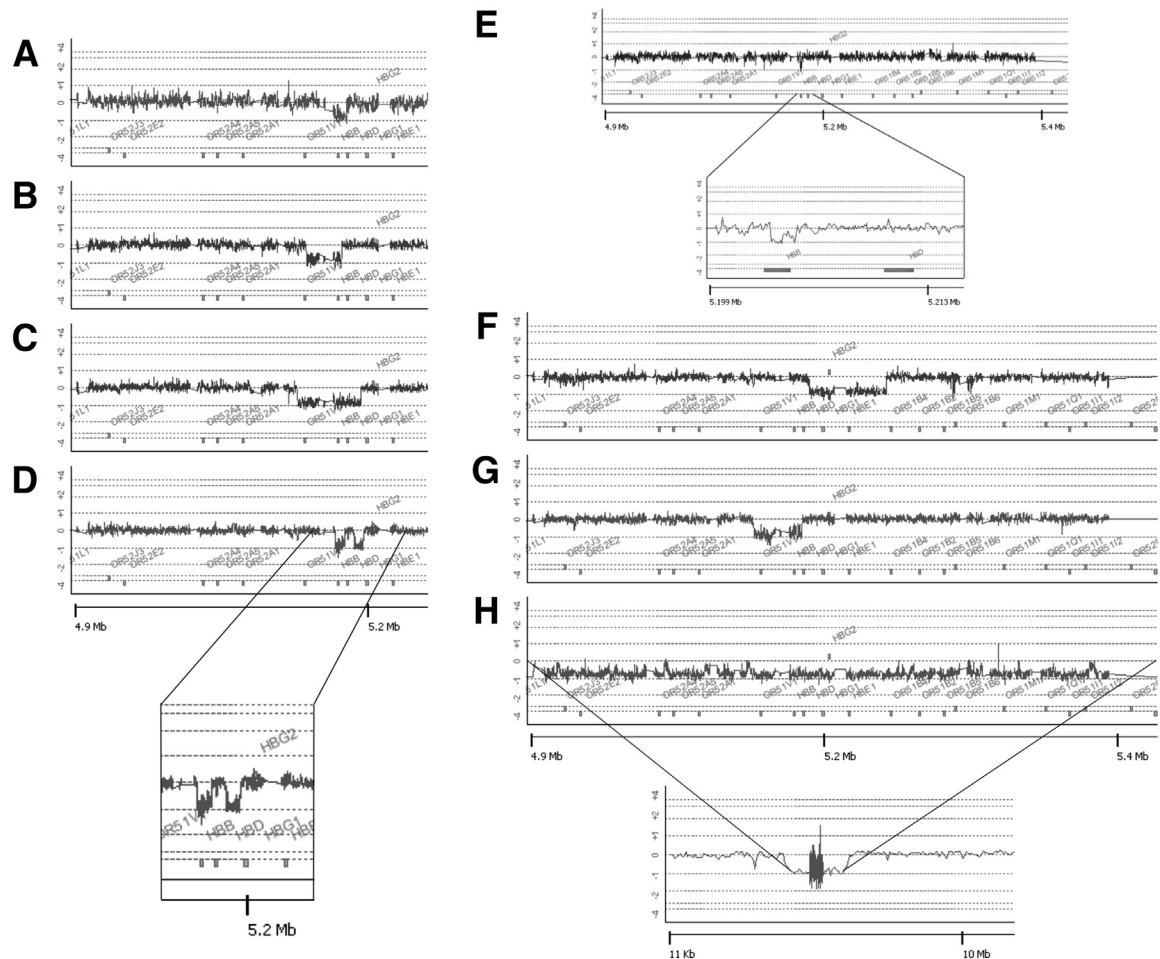
Figure 2 Array comparative genomic hybridization used to characterize deletions in patient samples that had not been defined molecularly. These samples include a deletional hereditary persistence of fetal Hb co-inherited with HbE trait in a Cambodian subject (subject 4, **A**), an *HBB* deletion in a Vietnamese subject (subject 5, **B**), a deletion causing HPFH in a Sri Lankan individual that is identical to HPFH-3 (subject 6, **C**), a noncontiguous deletion removing the *HBBP1* and *HBB* genes while sparing the intervening *HBD* gene in a Kurdish individual (subject 7, **D**, expanded in **inset**), a novel deletion causing δβ-thalassemia in an individual of Irish/Romanian ancestry (subject 8, **E**, expanded in **inset**), a deletion causing β-thalassemia that includes the β-globin locus control region and the *HBE1*, *HBG1/2*, and *HBD* genes that spares the linked *HBB* gene (subject 9, **F**), a deletion causing δβ-thalassemia in a Sri Lankan individual (subject 10, **G**), and a very large (>2 Mbp) deletion causing β⁰-thalassemia with an **inset** showing an overview of the 11-Mb region containing the deletion (subject 11, **H**). Numbers at the bottom of the figure represent distance from the chromosome 11p telomere. Note that the **inset** for **H** shows a sequence span of approximately 10 Mb.

Figure 2 shows the results obtained from subjects with HPFH or deletional β-thalassemia whose *HBB* locus deletions had not been characterized molecularly (subjects 4 to 11). All these deletion breakpoints were sequenced by designing PCR primers that flanked the breakpoints and determining the sequence of the resulting PCR products using Sanger sequencing other than subject 11, whose breakpoints occurred in sequences tiled at low density. Breakpoint sequence data for deletions not shown elsewhere can be found in Supplemental Figure S1 and Supplemental Table S1. A diagram showing the extent of the deletions with respect to the globin genes on chromosome 11 is shown in Figure 3. Figure 2A is a DNA specimen from an adult Cambodian patient with HbE, markedly elevated HbF, and no HbA, suggesting compound heterozygosity for HbE and a *HBB* gene

deletion (subject 4). The lack of anemia, very high HbF level, and MCV consistent with HbE trait suggested a deletional HPFH rather than β⁰-thalassemia trait on the non-E allele. This patient was found to have a 12.6-kbp deletion from telomeric breakpoint chr11:5,220,623 to centromeric breakpoint chr11:5,233,234, resulting in deletion of the *HBB* gene and a small segment of the 3′ end of the *HBD* gene. This deletion has been termed the Thai/Vietnamese (δβ)⁰ deletion.[8] Figure 2B shows a 27.4-kbp deletion (telomeric breakpoint chr11:5,201,647 to centromeric breakpoint chr11:5,229,059) in a Vietnamese patient with an elevated HbF level, slightly elevated HbA₂ level, and mild microcytosis (subject 5) having the SEA HPFH deletion.[9] The *HBB* gene is deleted, but the *HBD* and *HBG* genes are intact, resulting in a relative increase in both HBD and HBG expression. Figure 2C shows a 49.8-kbp *HBB-HBD* deletion (telomeric breakpoint
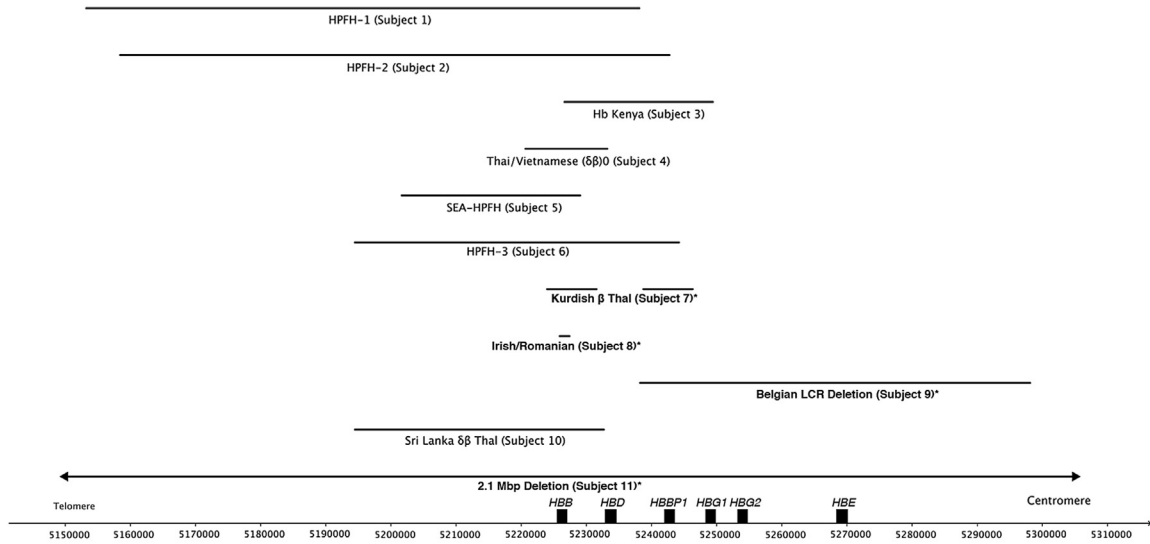
Figure 3 Comparison of deletions for subjects with *HBB* locus deletions. Coordinates of chromosome 11 are shown at the bottom. Deletions not previously described in the literature are indicated by **asterisks**.

chr11:5,194,453 to centromeric breakpoint chr11:5,244,225) consistent with HPFH-3[10,11] in a patient from Sri Lanka (subject 6). Figure 2D shows a complex deletion in a Kurdish family that was previously described but only partially characterized[12] (subject 7). This is a discontinuous deletion that removes the *HBB* (deleted nucleotides chr11:5,223,960-5,231,570) and *HBBP1* (deleted nucleotides chr11:5,238,759-5,246,317) genes but spares the *HBD* gene and results in microcytosis consistent with β-thalassemia trait but with more elevated HbA$_2$ and normal HbF levels. Figure 2E shows the results from a patient of Irish/Romanian ancestry (subject 8) with a previously unreported 1518-bp deletion of the *HBB* promoter region (telomeric breakpoint chr11:5,225,894 to centromeric breakpoint chr11:5,227,412), resulting in a β-thalassemic phenotype with relatively high levels of both HBG and HBD. Figure 2F shows a 59.9-kbp deletion (telomeric breakpoint chr11:5,238,245 to centromeric breakpoint chr11:5,298,126) in a Belgian male with microcytosis and normal HbF level (subject 9). This deletion spares the *HBB* gene but removes the remaining globin genes and the locus control region. Figure 2G shows a 38.2-kbp deletion (telomeric breakpoint chr11:5,194,461 to centromeric breakpoint chr11:5,232,686) in a Sri Lankan patient, causing δβ-thalassemia with increased HbF (subject 10). Of note, this deletion shares a common breakpoint downstream of the locus with the HPFH-3 deletion but differs in the breakpoint within the locus, located immediately telomeric to the *HBD* gene and has been termed Sri Lankan (δβ)^0 thalassemia.[10] Finally, Figure 2H shows a deletion of >2 Mbp in a 2-month-old girl with microcytosis (subject 11). Because the resolution of the array is lower at large distances from the structural *HBB* genes, we were not able to precisely map the breakpoints of this deletion. However, the telomeric breakpoint

is between chr11:4,109,643-4,188,151, and the centromeric breakpoint is between chr11:6,384,408-6,422,730. Of interest, this patient had early neurologic developmental abnormalities and white matter changes, raising the
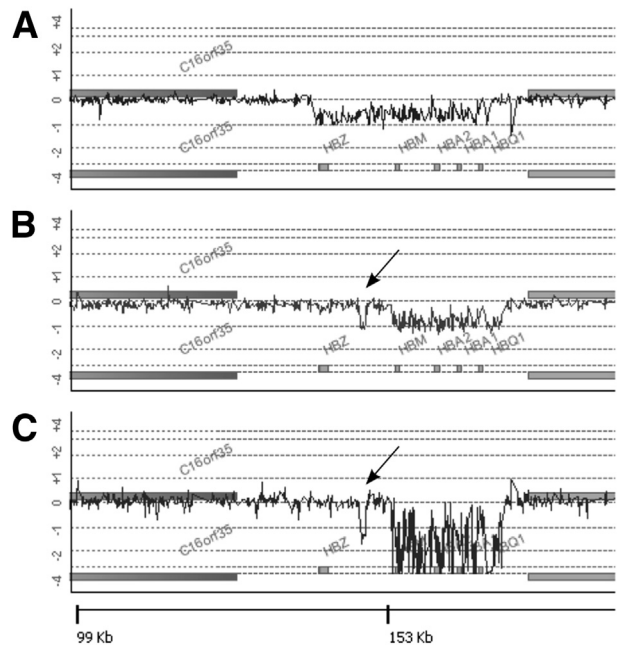

Figure 4 Array comparative genomic hybridization characterization of *HBA* two-gene deletions in *cis*-causing α-thalassemia. These deletions include heterozygosity for the common Filipino αα/–^{FIL} deletion (**A**), heterozygosity for the common Southeast Asian deletion αα/–^{SEA} (**B**), and homozygosity for the –^{SEA} deletion (**C**). The **arrows** in **B** and **C** indicate the inter-ζ hypervariable region, which is shorter in individuals with the –^{SEA} deletion than in individuals without the deletion. Numbers at the bottom of the figure represent distance from the chromosome 16p telomere.

possibility that this large deletion is responsible for more than the hematologic changes observed.

Deletions Causing α-Thalassemia

To determine the ability to characterize *HBA* deletions by array CGH, DNA samples that had been characterized previously by gap-PCR were studied using the globin array. Figure 4 shows results from patients with *cis* (two-gene, α-thalassemia-1) deletions. A sample heterozygous for the Filipino deletion (αα/–FIL) yielded a 50% decrease in signal spanning nucleotides chr16:150,821-182,671, including the *HBZ*, the two *HBA*, and the *HBQ1* genes (Figure 4A), consistent with accurate mapping of the mutation. A Southeast Asian deletion (αα/–SEA) sample similarly resulted in the expected 50% decrease in signal from nucleotides chr16:165,401-184,701, sparing the *HBZ* gene, and removing the *HBM*, the two *HBA*, and the *HBQ* genes (Figure 4B). Finally, a case of a homozygous –SEA deletion, associated with Hb Bart hydrops fetalis, reveals a complete loss of signal in the deleted region (Figure 4C). Of interest, the –SEA specimens (heterozygous and homozygous) had a shorter α-globin inter-ζ hypervariable region (HVR)[13] when compared with the reference DNA, telomeric to the large deletion. This finding appears to be in linkage disequilibrium with the –SEA deletion because it is present in all –SEA specimens examined for this study. Similarly, the –FIL specimen appears to have a shorter α-globin 3′ HVR[14] than the reference DNA, seen as a decrease in signal just centromeric to the deleted region (Figure 4A) and the –SEA homozygous specimen apparently has a longer 3′ HVR as evidenced by increased signal (Figure 4C).

We also investigated the utility of this array to detect the common single *HBA* gene deletions. Interpretation of the plots is difficult due to the sequence homology between the two *HBA* genes because DNA from the remaining (nondeleted) *HBA* gene in *cis* hybridizes to the probes spanning the deleted *HBA* gene, as well as its own probes. Although it is not possible to determine the extent of these deletions from array CGH a priori, the patterns of signal loss for each deletion are reproducible. Examples are shown in Supplemental Figure S2.

Discussion

Molecular diagnosis of thalassemia is a useful adjunct to the more common techniques of Hb electrophoresis and HPLC when genetic counseling or prenatal diagnosis is a concern. It is especially important when standard methods cannot be used for a definitive diagnosis, for example, in the setting of β-thalassemia or HbE traits, where α-thalassemia can be masked, or in the setting of deletional β-thalassemia, where the characteristic increase in HbA$_2$ is often not present. In this study, we found that array CGH can be accurately used to diagnose patients as having deletions of the *HBA* and *HBB* loci.

We designed a custom oligonucleotide array representing 400- to 450-kbp genomic segments surrounding the *HBA* and *HBB* loci with a median probe spacing of 60 to 64 bp, enabling high-resolution mapping of deletions involving these loci. As a demonstration of the ability of this technique to map known deletions, we accurately map the 5′ and 3′ breakpoints of the previously characterized HPFH-1, HPFH-2, and Hb Kenya deletions (Figure 1). We then reveal that deletions that were mapped previously using lower-resolution DNA blots can be mapped at high resolution using the CGH array, allowing a definitive molecular characterization after the design of a single set of PCR primers and a single Sanger sequencing run. This technique allows the rapid identification of novel mutations and facilitates their rapid sequencing. Finally, we reveal that CGH produces reproducible patterns for deletional α-thalassemia. Two other groups have reported using similar technology to identify *HBA* and *HBB* deletions,[15,16] but sequencing of breakpoints was not reliable. In contrast, the higher resolution of our array has facilitated one-step PCR and sequencing when breakpoints occur in the high-density region of the array.

HBB Deletions

Diagnosis of deletional β-thalassemia can be challenging. Most β-thalassemias are due to point mutations of the *HBB* structural or regulatory sequences and are routinely characterized by DNA sequencing.[17] In populations where specific mutations are very common, methods are available to detect these mutations using allele-specific reagents.[18–21] Similarly, gap-PCR methods, in which primers are used that flank specific deletion breakpoints, may be used to confirm specific mutations when suspected from less specific studies or family history.[22] To detect unknown deletions, DNA blot analysis has been the standard approach but is not commonly used in clinical laboratories because it is labor intensive, low resolution, and slow. Quantitative PCR using primer sets spaced throughout the *HBB* locus followed by capillary electrophoresis has been reported for characterizing *HBB* deletions.[23] More recently, multiplex ligation-dependent probe amplification has been used to characterize deletions of both the *HBA* and *HBB* loci.[24–27] These latter techniques, although useful to reveal the presence of deletions, do not have the resolution available with the CGH method presented in this study.

Our CGH approach was able to map previously uncharacterized *HBB* locus deletions with high enough resolution to design PCR primers that could amplify the DNA sequences flanking the deletion breakpoints and obtain definitive sequence analysis in a single run. This finding resulted in the identification and characterization of novel HPFH and (γ) (δ)β⁰-thalassemia deletions as well as definitive sequencing of prior mutations that were only imprecisely mapped. Subject 4, an adult Cambodian woman, was a compound heterozygote for HbE trait and

β-thalassemia with 44.2% HbF. CGH array analysis revealed chromosome 11 breakpoints that correspond to previously described Vietnamese/Laotian/Thai HPFH deletions.[8,28,29] In a previously reported case,[28] the HbF level was 21.3%. In our case, the HbF level is roughly twice that in previous reports. This can be accounted for by the fact that expression of β^E is approximately half that of β^A.

Both subjects 6 and 10, of Sri Lankan ethnicity, had elevated HbF levels but with quite different phenotypes. Subject 6, a 36-year-old man, has a very high HbF level of 40.8% with low-normal MCV. CGH revealed the 49,772-bp HPFH-3 deletion.[10] Subject 10, a 4.5-year-old girl, was microcytic with a moderately elevated HbF of 16.8% and a novel 38,225-bp deletion we define as Sri Lankan $(\delta\beta)^0$-thalassemia.[10] The telomeric breakpoints were almost identical for these two patients (chr11:5,194,453 for subject 6 versus chr11:5,194,461 for subject 10), whereas the centromeric breakpoints were 11,539 bp apart (chr11:5,244,225 for subject 6 versus chr11:5,232,686 for subject 10). The region between chr11:5,244,225 and chr11:5,232,686, which is immediately centromeric to the *HBD* gene, contains sequences that repress HBG expression based on the large difference in HbF levels between these two subjects, and chromatin immunoprecipitation has reveasled a binding site for the key HbF silencing factor BCL11A in this interval,[30] along with binding sites for corepressors and repressive histone and chromatin marks.[10] This site has also been associated with regulation of HBG expression in genome-wide association studies.[31,32] Subject 7, a 30-year-old Kurdish woman, has β-thalassemia with typical microcytosis and a significantly elevated HbA_2 level but no significant increase in HbF. CGH analysis revealed two noncontiguous deletions removing the *HBB* and *HBBP1* genes but sparing the *HBD* gene. The retained sequences include this BCL11A binding site.[10] Other mechanisms must exist for elevated HBG expression, however, because the deletions in subjects 4 and 5 do not remove the BCL11A binding site centromeric to the *HBD* gene, yet these subjects have elevated HbF levels of 44.2% and 20.6%, respectively. Perhaps the increased HbF in these subjects is due to a telomeric enhancer brought into proximity of the *HBG* genes.

One other interesting feature of subject 10's Sri Lankan $(\delta\beta)^0$ thalassemia deletion is that the *HBD* gene is intact, yet there is no increase in HbA_2. The breakpoint occurs approximately 200 bp 3' (telomeric) to the *HBD* structural sequences and 3' untranslated region. This result suggests the presence of an *HBD*-positive regulatory element located somewhere telomeric to this breakpoint.

HBA Deletions

We also report the utility of array CGH for detecting *HBA* gene deletions. For subjects heterozygous for large deletions of the *HBA* locus, such as the $-^{SEA}$ and $-^{FIL}$ deletions, the expected 50% decrease in signal intensity is observed throughout the deleted region. CGH is also able to characterize some of the polymorphic repeat regions in the *HBA*

locus, including the inter-ζ HVR, a polymorphic region on chromosome 16 that lies between the *HBZ* and *HBZP1* genes[13] and the 3' HVR centromeric to the *HBQ* gene.[14] It appears that the inter-ζ HVR is smaller and the 3' HVR is larger for the $-^{SEA}$ allele than for the corresponding regions of the reference DNA used for the CGH study (Figure 4, A and B). The $-^{FIL}$ allele appears to have a shorter 3' HVR (Figure 4A). Identification of alleles lacking one of two *HBA* genes, such as the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions, is more difficult because the two *HBA* genes are highly homologous, and when only one is deleted, labeled DNA fragments from the nondeleted *HBA* genes (and their homologous pseudogenes) cross-hybridize to probes covering the deleted regions, resulting in a signal ratio of subject to reference DNA of >0.5 (Supplemental Figure S2). Standard gap-PCR is adequate for detecting the common *HBA* deletions leading to α-thalassemia,[1] but the array CGH method still has potential utility to identify unusual deletions not tested for by gap-PCR and novel deletions of the *HBA* locus. Although we did not have samples to test, array CGH should also be able to identify and characterize triplicated *HBA* genes.

Conclusions

We found that CGH using a high-density custom oligonucleotide array can be used to rapidly and efficiently define both previously described and unique deletions of the *HBA* and *HBB* loci. This method will be a useful adjunct to current diagnostic methods for patients suspected of having thalassemia where standard diagnostic techniques are inconclusive. The typical patient would be microcytic with normal iron studies and have a normal level of HbA_2. If gap-PCR to detect the common *HBA* deletions is negative, an *HBB* or uncommon *HBA* deletion is likely. The lack of HbA_2 elevation makes an *HBB* point mutation unlikely, so Sanger sequencing of *HBB* would not likely be diagnostic, and thus proceeding directly to array CGH would be the most efficient diagnostic pathway. The other clinical scenario where *HBB* deletion analysis would be useful is HPFH, where there is a large variety of deletions that are not amenable to gap-PCR, particularly in an ethnically diverse population. Next-generation sequencing should eventually replace CGH and most other genetic technologies for clinical testing, although currently CGH represents a less labor- and data-intensive way to characterize deletions, such as those in the globin loci, and thus could remain more accessible to laboratories with limited resources.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.jmoldx.2015.07.011*.

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