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Easy and Rapid Detection of Point Mutations in the Human β-hemoglobin Gene with DNA-chips

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Abstract: Study was conducted on hemoglobinopathies the world's most common class of single gene disorders. We selected four point mutations of the human β-hemoglobin gene, giving rise to frequently occurring unstable β-hemoglobin variants or β-thalassemias. Our chip system is based on allele-specific oligonucleotide hybridization to detect single nucleotide polymorphisms (SNPs) and mutations. The probes attached to the chip surface consist of oligonucleotides containing the point mutations and the three corresponding control sequences. The labeled target cDNAs used for hybridization are derived from genomic or subcloned genomic DNA via asymmetric PCR, which is specially adapted for the synthesis of labeled single stranded target DNA. In addition to the useage of a chip reader, evaluation of the hybridized chip is adapted for using enhanced chemiluminescence. With this chip all four hemoglobin point mutations are easily detected and furthermore, homozygous alleles are distinguished from heterozygous alleles. Our SNP chip system is ready for rapid detection of all point mutations and SNPs occurring in the hemoglobin and other genes.

Key words: β-hemoglobin, hemoglobinopathies, enhanced chemiluminescence, oligonucleotide chips, single nucleotide polymorphism

Introduction

Many mutations in the human β-globin gene give rise to abnormal β-hemoglobin or reduced β-hemoglobin levels Clarke and Higgins (1999) and Weatherall (2001). The thalassemias show diverse phenoptypes, but are characterized by a reduction in the amount of the normal globin chain produced. They originate from deletions or base substitutions that result in a lowered transcription rate or mRNA stability, or failures in mRNA splicing. The structural hemoglobin variants or unstable hemoglobins of the hemoglobinopathies are mainly due to amino acid substitutions. Currently 854 Hb variants of which 650 involve the β-chain have been described (Hardison ***et al.***, 2002).

Point mutations or single nucleotide polymorphisms (SNPs) are detectable by direct sequencing or nucleic acid hybridization. Recently nucleic acid probes attached to solid surfaces became available Ramsgy (1998) and Yershov ***et al.*** (1996). We have established a novel chip based method to detect single base pair substitutions and demonstrate the detection of major occurring mutations in the β-hemoglobin gene. In addition to scanning by fluorescence, enhanced chemiluminescence (ECL) has been adapted for SNP chip evaluation.

Materials and Methods

Chip preparation and standardization

All oligonucleotides and XNA on Gold™ DNA chips were synthesized by ThermoHybaid, Interactiva Division, Ulm, Germany. The sensor probes were biotinylated at the amino group of the 5´end. Target DNA was either labeled with Cy5 or FITC at the 5´end. Each chip contained a duplicate.

For immobilization of the oligonucleotides of the chip surface 1 μl of the probe (10 μM in 1 × TBST (TBS (150 mM NaCl, 10 mM Tris-HCl pH 8.0) and 0,1% Tween 20) was loaded onto each spot of an XNA on Gold™ 96 format with a Tecan robot (Cavro Scientific Instruments Inc.). The chips were incubated at 50°C for 1 hour in a humidified box followed by 4°C overnight. The chips were then washed briefly in 0,5% Tween 20 with gentle agitation and dried with nitrogen. Immobilized chips were stored at 4°C.

The chips were scanned with a FUJIFILM FLA-2000 scanner and quantified with the AIDA analysis software (Raytest, Straubenhardt, Germany). The fluorescence intensity obtained from the spots treated with only loading dilution buffer (1×TBST) was used as background.

The sequences for optimization of the probe length (Fig. 1A) were: HMb10, 5´-CGGTGCCGTA; HMb12, 5´-CGGTGCCGTATG; HMb14, 5´-CGGTGCCGTATGGA; HMb16, 5´-CGGTGCCGTATGGAAG and HMb18, 5´-CGGTGCCGTATGGAAGAG. The probes were linked to the chip surface via a 10 mer thymidine spacer at the 5´end. One μl of a 10 μM probe solution in TBST was immobilized onto the chips in duplicate. The hybridization target sequence 5´-ATCTGCCGTACGCAGTCATCAATCTGATCCAATGAAGA-TGAGTAATCTCTCTCTCCTGCCTTTAAATGACTCTTCCATACGGCACCGTT was labeled with Cy5 at the 5´ end. 50 μl of hybridization solution (5 × SSPE, 0.1% Tween 20) containing 1 μM of the target was loaded onto the chips, which were then covered with a glass slide and hybridized in a humidity chamber at 30ºC for 3 h. Three washes were performed in 5 × SSPE, 0.1% Tween 20 at room temperature for 2 min followed by two washes in 5 × SSPE, 0.1% Tween 20 at 40°C for 30 min in order to decrease unspecific binding.

Effects of the probe spacer length was tested with different lengths of polyT spacers inserted beween biotin and probe specific sequences (Fig. 1B). T spacers with 0, 5, 10, 15, 20 and 25 T residues were attached to the specific probe sequence 5´-T_nGGCACTGCCATCTTA at the 5´ end. The target sequence 5´-GTTAAGATGGCAGTGCCCGGTAATCGCATAAAACTTAAAACTTT-ACAGTCAGAGGTTCAATTCCTCTTCTTAACACCA was labeled with Cy5 at the 5´ end. Hybridization and washing conditions were as described in (Fig. 1A).

The effect of the oligonucleotide surface density on hybridization efficiency was tested with varying concentrations of the HMb18 probe (Fig. 1C). Here, immobilization was at 50ºC for 1 h. To optimize the temperature for probe immobilization HMb18 (10 μM) was spotted onto the chip surface and incubated for 1 hour at the indicated temperatures (Fig. 1D). Target sequence and preparation, hybridization and washing conditions were described in (Fig. 1A). The effect of hybridization temperatures was tested at the indicated temperatures for 3 h (Fig. 1E) with 5´-T_{10}GGCACTGCCATCTTA as probe and the corresponding target under conditions as described in. Using 30ºC as hybridization temperature the intra-chip reproducibility for fluorescence intensity (AU/mm^2) of the XNA on Gold™ SNP chip system was 17.83±2.64 and the inter-chip reproducibility using six chips was 17.29 ± 3.10, respectively.

To detect the β-hemoglobin mutations the following 5´ biotinylated T_{15}mer oligonucleotides were synthesized: IVS1-110, 5´-T_{15}GAGA*AATAGGCAGAG; Codon 39 (C->T), 5´-T_{15}AACCTCTA*GGTCCAA; HbE, 5´-T_{15}CCTA*ACCACCAACTT; HbS, 5´-T_{15}CTTCTCCA*CAGGAGT. The asterix after a nucleotide marks the nucleotide where the mutation occurs and which is substituted by all possible bases.

DNA isolation, subcloning and DNA sequencing

Blood samples were collected from patients attending the Children's Hospital, University of Ulm, Ulm, Germany. Variants of β-hemoglobin were analysed Hug ***et al.*** (2002). Genomic human DNA was isolated from white blood cells by using the QIAamp DNA Blood Midi kit (Qiagen) according to the protocol of the manufacturer. For subcloning the genomic β-globin fragments containing the mutations were amplified by the polymerase chain reaction with primer Hb1 5´- GGAGCAGGGAGGGCAGG as forward and Hb3 5´-GGGGAAAGAAAACATCAAGGG as reverse primer. Buffer conditions were according to the PCR kit (Perkin Elmer). The amplification was performed at 94°C for 30, 65°C for 30 and 72°C for 2 min for a total of 5 cycles, followed by 94°C for 30 s, 55°C for 30 and 72°C for 2 min for a total of 30 cycles. The fragments were then ligated with topoisomerase I (Invitrogen) into the vector pCR-XL-Topo (Invitrogen) according to the protocol of the manufacturer. The DNA sequence of the subcloned 592 bp fragments were verified by a using the Big DyeTM Terminator Cycle Sequencing kit (Applied Biosystems) and a 373A automated sequencer (Applied Biosystems).

Asymmetric PCR

The primer 5`-CAACCTCAAACAGACACC labeled with fluorescein at the 5´-end was used as forward and Hb3 as reverse primer. All four mutations were covered by the 497 bp PCR fragment. The conditions used for asymmetric PCR (1999) were adapted and modified. The final concentrations were: genomic DNA, 8 ng μl^{-1}; dNTPs, 0,2 mM each; $MgCl_2$, 1 mM, forward primer (fluorescein labeled), 0.5 μM; reverse primer, 20 nM; Taq polymerase: 0,05 U μl^{-1}. The amplification was performed at 95°C for 30, 58°C for 30 and 72°C for 2 min, for a total of 60 cycles, with an initial denaturation step at 95°C for 2 min and a final elongation step at 72°C for 5 min.

Chip hybridization with labeled β-globin target DNA

The DNA from a total volume of 200 μl of PCR reactions was precipitated with ethanol. The dried pellet was resuspended in 30 μl of 4xSSC and 0.1% SDS. This solution was directly used for chip hybridization. The chips were covered with a glass slide and incubated at 30°C for 3 h. In case of hybridization with different targets in a same chip, 1 μl of sample was loaded separately onto each spot. After hybridization, the chips were washed with 0.2 × SSC and 0.1% SDS for 1 min, followed by the same solution for 30 min at 40°C without shaking. Then the chips were washed 3 times for 2 min in TBST.

For signal amplification chips were incubated with 50 μl Alexa Fluor 488 rabbit anti-fluorescein/Oregon Green antibody (Molecular Probes), diluted to 0.04 mg/μl in 1 × TBST (1:50 from the supplied antibody solution), covered again with a glass slide and incubated in the dark at room temperature for 10 min. Then the chips were washed again 3 times in 1 × TBST for 2 min, and then incubated with Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes). The same dilutions and procedures described above were applied. After the final washing step, the chip was covered with a glass slide, externally dried with a cleaning paper and then scanned with a FujiFilm FLA 2000 scanner.

For evaluation we subtracted the mean of the background values of the negative controls and used the following criterions: If the wildtype/mutation ratio of the signal was = 2 the sample was wildtype. If it was = 0.3 the sample was homozygous, and if it was close to 1 the sample was heterozygous (Sitbon, 1997).

Detection by ECL

Washing steps, buffers and the first antibody were the same as described before. For ECL detection, a second goat anti rabbit IgG-HRP was used (Santa Cruz Biotechnology) at a dilution 1:100. After the final washing step the detection reagent mixture for ECL (Amersham Pharmacia Biotech) was added onto the chip surface according to the protocol of the manufacturer. Then it was covered with a thin glass-slide and finally the light emission was detected with a light sensitive film (Hyperfilm ECL, Amersham Pharmacia Biotech).

Results

Principle of the method

The thermal stability of DNA hybrids formed between the target nucleic acids and the short oligonucleotide probes attached to the chip surface depend on many different parameters, such as nucleic acid length, base composition, amount of probe and target, spacer length, hybridization temperature and time, labeling dyes and ionic strength (Conner ***et al.***, 1983) which have to be optimized for individual chip systems. The XNA on Gold™ microarray platform is based on a streptavidine monolayer to which biotinylated probes are attached (Mecklendurg, 1999). We established this system to reproducibly detect SNPs. The effect of probe lengths on hybridization signals was tested with different chain lengths. The longer the probe length, the stronger the hybridization signal on the chips (Fig. 1A). Fluorescence intensity of the probes with 12 mers reached a plateau. Therefore, 15mers of probe oligonucleotides were chosen in this study.

DNA rigidity may interfere with the hybridization reaction. In order to decrease steric hindrance, a polythymidine spacer is inserted between the specific probe sequence and the support matrix (Ahrendt ***et al.***, 1999; Hacia ***et al.***, 1996; Lopez-Crapez ***et al.***, 2001; Stimpson ***et al.***, 1995). We tested spacer lengths between 0 and 25 T residues (Fig. 1B). Increase of the spacer length increased the hybridization efficiency significantly. In the following examples we used a 15 T long spacer between the biotin attachment site and the specific hybridization sequences.

The concentration of oligonucleotide in a single spot (surface density) influences hybridization. Usually, a lower surface density gives rise a lower hybridization signal. On the other hand, a high surface density can result in steric interferences between the target and the probe and decrease hybridization efficiency. Therefore, varying amounts of probe were immobilized onto the chip surface (Fig. 1C). Concentrations above 10 μM did not result in a significant increase of fluorescence. Therefore, 10 μM of probe concentration were used in all further experiments. The surface density of each spot of a 96 format of XNA on Gold™ chips was $1{,}64\times10^{9}/mm^{2}$ and the amount of detectable nucleotide acid was 135 pg.

To detect the optimal immobilization temperature for binding biotinylated probes to the streptavidine coated chip surface different temperatures were evaluated (Fig. 1D). Fluorescence intensity increased slightly with increasing temperature. The K_{eq} for binding of biotin to streptavidine is approximately 1×10^{15} M^{-1}. Background fluorescence decreased with increasing temperatures. Temperatures above 50°C were not tested in this study, because higher temperatures would probably destroy the intactness of streptavidine and biotin on the chip surface. Therefore, an immobilization temperature of 50°C was chosen.

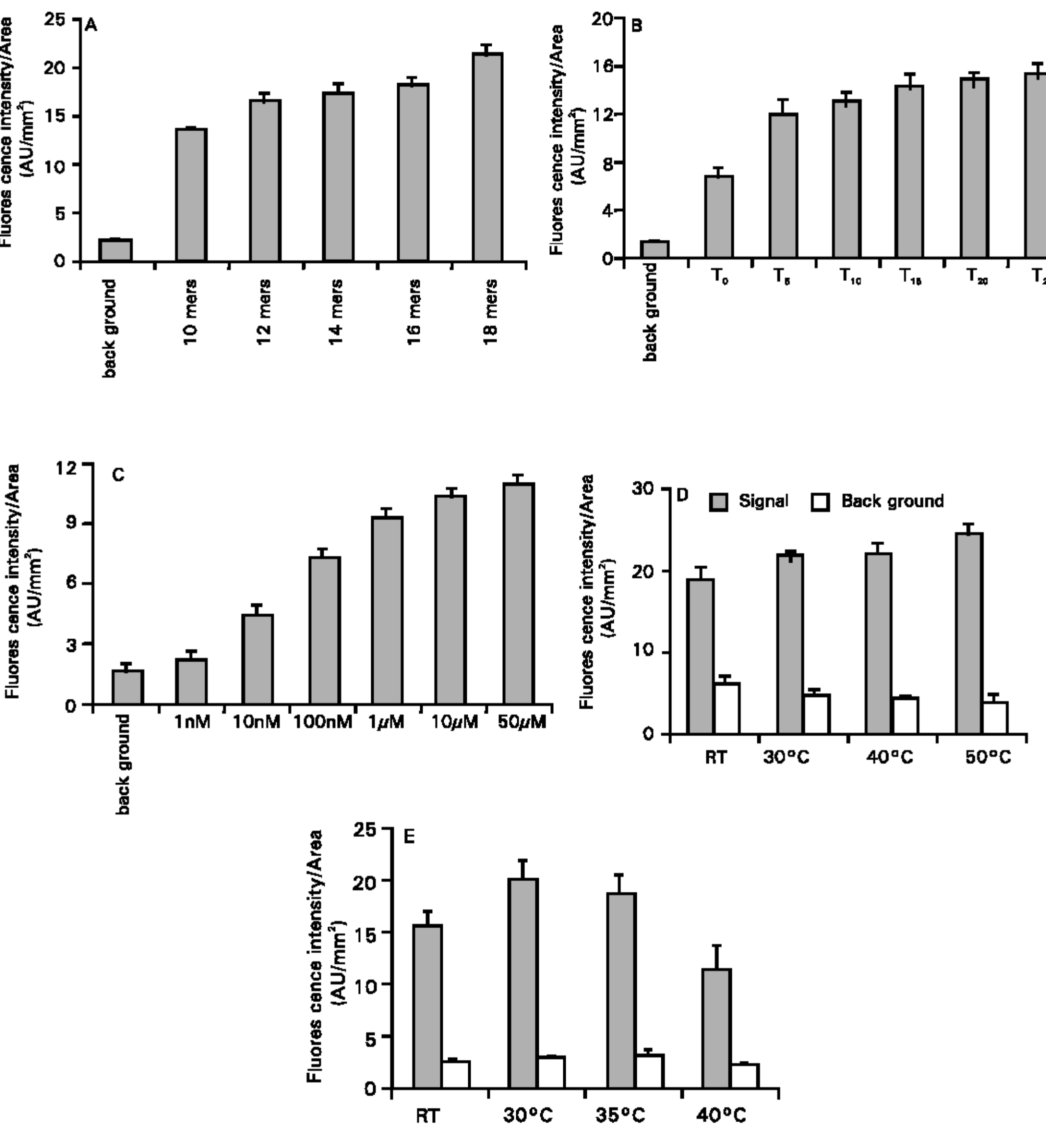

Fig. 1: Prerequisites for XNA on Gold for SNP detection. All probes are immobilized onto XNA on Gold™ chips in duplicate. AU, absorption unit. (A) Effect of the length of the probe on fluorescence intensity. (B) Effect of the spacer length (in bases) on fluorescence intensity. (C) Effect of probe concentrations on fluorescence intensity. (D) Effect of immobilizing temperatures on fluorescence intensity. Background values correspond to dilution buffer. (E) Effect of hybridization temperatures on fluorescence intensity. The mean and standard deviation of at least 48 spots is shown, respectively.

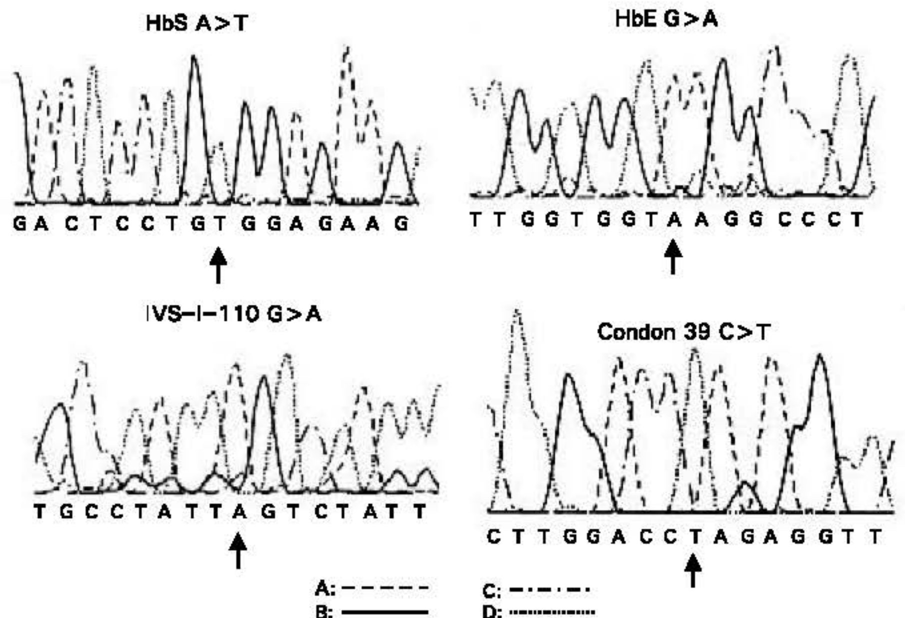

Fig. 2: DNA sequence of the subcloned β-globin mutations. From top to bottom: Codon 39 (C->T), IVS1-101, HbS, HbE. The arrow points to the mutation.

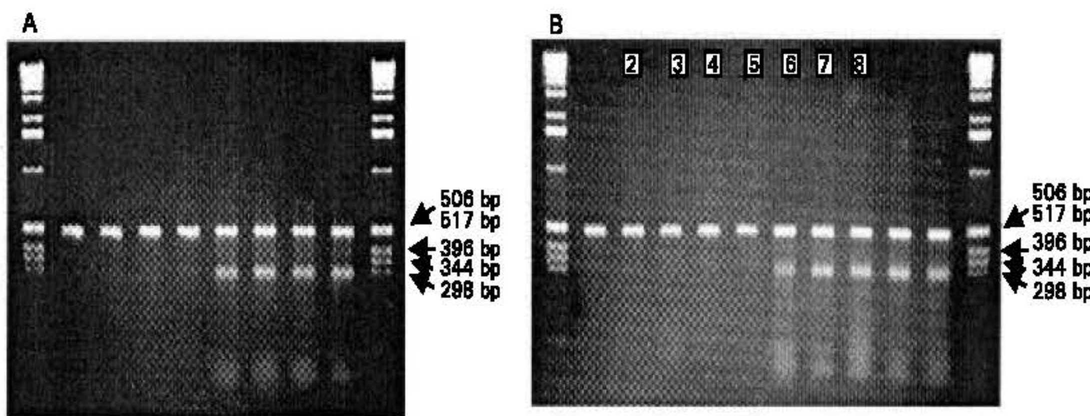

Fig. 3: Agarose gel electrophoresis for comparison of standard and asymmetric PCR products. (A) Lanes 1 to 4; standard PCR of subcloned β-hemoglobin DNA from mutations Cd 39, IVS-I-110, HbS and HbE respectively. Lanes 5 to 8; asymmetric PCR for the same samples in the same order. (B) lanes 1 to 5; standard PCR of genomic DNA homozygous for Codon 39 (C->T), IVS-I-110, HbS, HbE and wildtype, respectively. Lanes 6 to 10; asymmetric PCR of the same samples in the same order. 0,5 μl and 2,0 μl of PCR product was loaded onto 1.5% agarose gels to analyze standard and asymmetric PCR products, respectively. DNA size markers are shown on the right margin.

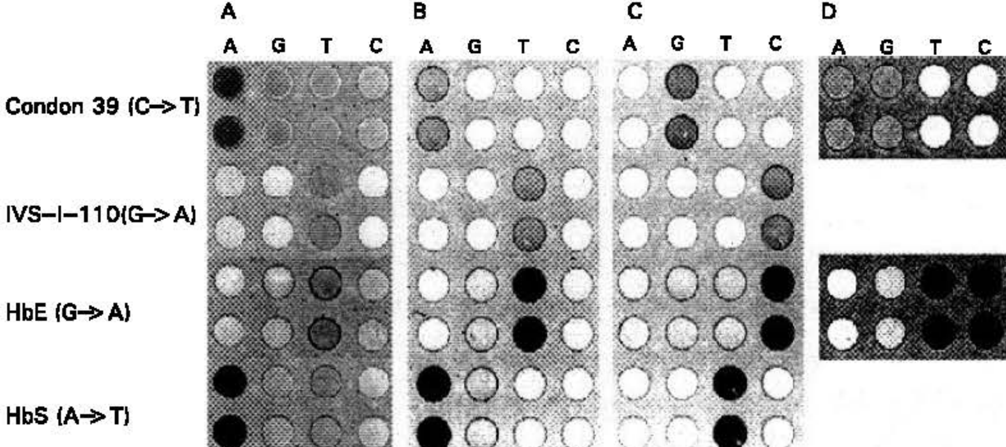

Fig. 4: SNP-Chip analysis of the four selected β-globin mutations as probes and labeled target DNA obtained by asymmetric PCR. Allele-specific oligonucleotides for all four possible replacements (A, G, T, C) were tested in duplicate. (A) Chip hybridization with subcloned β-hemoglobin asymmetric PCR amplified products. (B) An analogous experiment using homozygous mutant genomic DNA as template. (C) Control chip hybridization using wildtype genomic β-hemoglobin DNA. (D) Chip hybridization using genomic DNA from patients heterozygous for two beta-globin gene mutations (Codon 39 (C->T) and HbE).

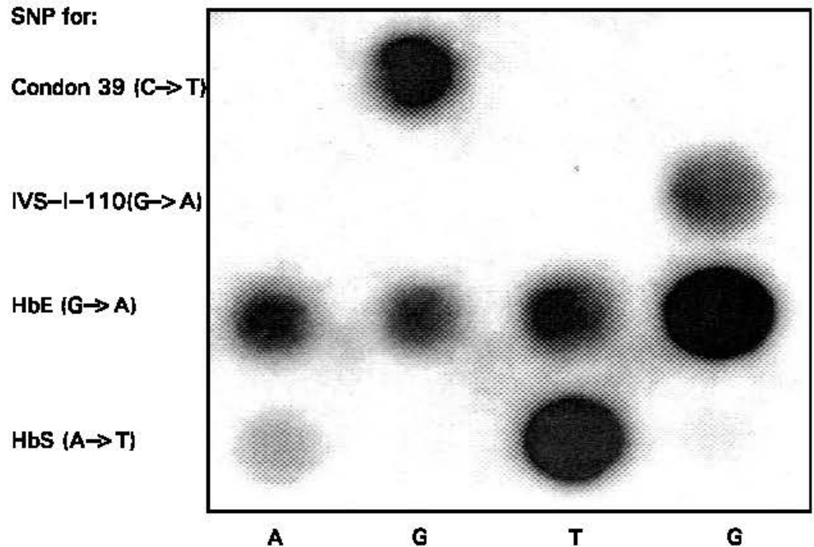

Fig. 5: ECL detection of hybridized wildtype DNA to an SNP-chip containing the four selected β-globin mutations as probes. The oligonucleotide probes were the same as in Fig. 4. The hemoglobin wildtype target DNA was amplified and labeled by asymmetric PCR as shown in Fig. 3.

The optimal buffer conditions for hybridization were 5xSSC, 0.1% SDS (data not shown). The hybridization temperature with the highest signal to noise ratio was at 30°C (Fig. 1E) and this temperature was used for the following experiments. The hybridization between single stranded probe and single stranded target in our system did not follow classical hybridization kinetics (Fig. 1E). It can be performed at lower temperature than T_m.

Analysis of β-hemoglobin point mutations

We selected four frequently occurring point mutations of the human β-hemoglobin gene. Two give rise to β-thalassemias: IVS1-110, which is a G to A substitution at position 110 of the first β-globin intron, and Codon 39 (C->T), which is a C to T substitution at the first position of Codon 39 in the second β-globin exon. Two mutations that result in structural defects of human β-hemoglobin were selected: HbE, which is a G to A substitution at the first position of codon 26 in the first β-globin exon, and HbS, which is an A to T substitution at the second position of codon 6 in the first β-globin exon. PCR amplified genomic DNA fragments containing the mutations were subcloned into plasmids and sequenced to confirm the mutations (Fig. 2).

The 15 mer oligonucleotides attached to the surface of the SNP-DNA-chip consisted of the anti-sense DNA strand of the human β-hemoglobin gene. Sets of four oligonucleotides, respectively, containing all four base substitutions (A, G, C, T) at the position where the point mutation occurs, were used in order to include the wildtype sequence and two additional negative controls as described in the methods section. The hybridization targets were synthesized by asymmetric PCR (Millican and Bird, 1998) adapted for our purpose (Fig. 3). In comparison to standard PCR resulting in a signal of double stranded DNA at 500 bp, a faster migrating band corresponding to the expected single stranded DNA was observed under asymmetric PCR conditions by using β-hemoglobin fragments (Fig. 3A) as well as for the mutations homozygous genomic DNA (Fig. 3B) as template. No significant difference in the DNA amount was obtained by using wildtype or mutant DNA as template.

The FITC labeled target DNA obtained by asymmetric PCR resulted in a stronger hybridization signal with all β-hemoglobin probes attached to a chip surface compared to target DNA obtained by standard PCR (data not shown). Therefore, in all further chip hybridizations targets were synthesized by asymmetric PCR. In the first experiments, targets obtained from subcloned β-hemoglobin DNA were used (Fig. 4A). All targets showed the expected hybridization pattern with the complementary allele specific oligonucleotides on the chip surface. For example, the Codon 39 (C->T) mutation is a replacement of C by T at amino acid position 39 of β-hemoglobin and a signal was only obtained with the oligonucleotide probes containing an A at the corresponding complementary position.

A qualitatively equal hybridization pattern was obtained when asymmetric PCR products from homozygous genomic DNA was used as target (Fig. 4B). But the signal intensity compared to target derived from subcloned DNA varied for unknown reasons even under the same hybridization conditions. As control wildtype DNA was used as target (Fig. 4C). All targets hybridized only to the oligonucleotide probes that contained the complementary wildtype base at the corresponding position. Heterozygous genomic DNAs for two mutations, Codon 39 (C->T) and HbE, were available. Both targets hybridized equally well to the complementary mutant and wildtype positions (Fig. 4D). The lowest signal to noise ratio was 2 (Codon 39 (C->T) in Fig. 4D) and the highest was 7 (HbS in Fig. 4B). According to the criteria given in the methods

section, we were able to distinguish wildtype samples, homozygous and heterozygous mutations. For the wildtype samples, the wildtype/mutation ratio was at least 4.5. For the homozogous mutations this value had a maximum of 0.2 and for the heterozygous it was between 1.0 and 1.2.

Evaluation with ECL

Since not all laboratories are equipped with a chip scanner, we adapted the ECL system for fast and cheap evaluation. On a chip containing the SNPs of the four selected mutations hemoglobin wildtype targets were clearly identified by ECL (Fig. 5.). The signal to noise ratio had a minimum of 2 (HbE (G->A), Fig. 5).

Discussion

We have described a method for the detection of point mutations in the human β-hemoglobin gene with arrayed allele-specific oligonucleotides. Labeled target DNA is prepared via asymmetric PCR. There is no need for a scanner since the signals can be detected easily by ECL.

So far, we are able to diagnose two point mutations of the human β-hemoglobin gene leading to β-thalassemias (IVS1-110, Codon 39) and two point mutations resulting in structural β-hemoglobin variants (HbE, HbS). The long-term goal of this project is to contribute to genetic counseling, decreasing the frequency of mutations in the β-hemoglobin gene leading to disease and to molecular epidemiology.

Our approach can be further extended for the general detection of SNPs and point mutations. It is a reproducible, fast and easy system that could be routinely used in molecular diagnosis. A major advantage is the rapid synthesis of labeled single stranded target DNA by asymmetric PCR and the cheap and fast detection by ECL. The only purification step of the PCR products is ethanol precipitation.

Concerning the time of the analysis a faster chip system is offered by NanoChip™ (Nanogen, San Diego, USA). Here, the target DNA moves in an electric field and thereby becomes concentrated (Gilles ***et al.***, 1999; Hekker ***et al.***, 2000; Sosnowski ***et al.***, 1997 and Westin ***et al.***, 2000). However, the advantage of our system is the applicability in laboratories that cannot afford such sophisticated systems. Alternative systems which could be developed further and would have the advantage of using only one spot per mutation on the chip surface are based on arrayed primer extension (Erdogan ***et al.***, 2001 and Kurg ***et al.***, 2000). Recently, an arrayed primer extension system has been established for the detection of β-thalassemia and glucose 6-phosphate dehydrogenase mutations (Gemignani ***et al.***, 2002).

To detect base substitutions over a larger range (eg. the whole human β-globin gene) we currently establish multiplex asymmetric PCR. A similar approach has been taken for the amplification of genomic DNA directly on a microarray in a single reaction (Huber ***et al.***, 2002).

The easiness of handling of our system should make it useful for the diagnostic screening of inherited diseases with underlying point mutations in laboratories with the equipment for standard molecular biological techniques, or for the mapping of SNPs in populations. In addition to point mutations in the α- and β-hemoglobin genes, all known diseases which are partially or completely due to point mutations, like eg. cystic fibrosis, hemophilias or p53 mutations leading to tumorigenesis, could prove to be faster and cheaper detectable with our SNP chip system.

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