Detection of the Hemoglobin E Mutation Using the Color Complementation Assay: Application to Complex Genotyping

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The color complementation assay (CCA) is a method of allele-specific DNA amplification by which competitive priming and extension of fluorescently labeled oligonucleotide primers determine the color of DNA amplification product. This diagnostic method precludes the need for radioisotopes, electrophoresis, and multiple high-stringency reaction conditions. The multiplicity of mutant globin genes present in Southeast Asians complicates clinical diagnosis and underscores the importance of DNA-based diagnostic methods. We have applied CCA to distinguish $\beta^s$ and $\beta^t$ alleles. Competing 15mer primers were a fluorescein-labeled complement to $\beta^t$ and a rhodamine-labeled complement to $\beta^s$, identical except for their central nucleotides. A common unlabeled primer was used to amplify DNA product, the color of which was determined by the perfectly complementary primer. Color photography and spectrofluorometry, as well as a method of black-white photography that we developed to distinguish fluorescein- and rhodamine-labeled DNA, were used to record results. We applied CCA to define the complex genotype of a Thai woman having thalassemia intermedia, 96% Hb E, and 4% HbF whose possible genotypes included several permutations of $\alpha$-thalassemia, $\beta$-thalassemia, and $\beta^s$ genes. $\gamma$-Globin gene mapping of DNA doubly digested with BglII and Asp 718 showed the $-\alpha^3.7/-\alpha^4.2\beta^a$ genotype, and CCA confirmed homozygous $\beta^s/\beta^s$. The CCA is useful for diagnosing the compound hemoglobin genotypes of Southeast Asians and could be applied also to prenatal diagnosis in this population.

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MATERIALS AND METHODS
CCA of $\beta^s$ Versus $\beta^t$ Alleles

Testing of DNA for $\beta^s$ versus $\beta^t$ sequences by CCA used as template either genomic DNA or DNA preamplified using unlabeled primers. Preamplification primers were a sense primer complementary to bases $128$ to $130$ of the $\beta$ cap site (5' GTAGACCACCCAGCAAGC3') and an antisense primer (5' GTAGACACCCAGCAAG3') complementary to bases $2$ through $16$ of exon $2$ of the $\beta$-globin gene. PCR was performed using the GeneAmp DNA Amplification Reagent Kit (Perkin Elmer Cetus, Norwalk, CT) and $20$ pmol of each primer in a $100$-$\mu$L reaction with an initial $5$-minute ($30$ seconds in subsequent cycles) denaturation at $95^\circ$C, a $30$-second annealing at $55^\circ$C, and a $30$-second ($5$ minutes in the final cycle) extension at $72^\circ$C for $35$ cycles using a DNA Thermal Cycler (Perkin Elmer Cetus).

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Figure 1 demonstrates the strategy for distinguishing $\beta^A$ from $\beta^B$ by CCA of preamplified DNA. For the amplification of a 270-nucleotide sequence, competing primers were a pair of antisense 15mers complementary to $\beta^A$ (5'-AGGGCGTACCACCACTA-3') labeled with 5'-carboxylfluorescein, and to $\beta^B$ (5'-AGGCCTACCACCACAAT-3') labeled with 6-carboxy-X-rhodamine (both dyes from Applied Biosystems, Foster City, CA), identical except for the central bases underlined. Fluorescent labeling of the primers was via a 5'- amino linker (Aminolink-2, Applied Biosystems), as described. The common primer was the same unlabeled sense 26mer used for preamplification. CCA template was 1 µL of the preamplification reaction, and reaction conditions were as described for PCR. For CCA without preamplification, 0.5 µg genomic DNA was used as template.

Electrophoresis of DNA product was on 8% polyacrylamide gels. When detection of CCA product in solution was used, product was separated from primers by repeated ultrafiltration through Centricon C 100 microconcentrators (Amicon, Danvers, MA).

Methods of Assessing CCA Product

Color photography of electrophoretic gels. Gels were ultraviolet (300 nm)-transilluminated (Fotodyne Incorporated, New Berlin, WI) for double-exposure photography using Polaroid type 668 film (Polaroid Corp, Cambridge, MA) to discern green, red, and yellow fluorescing CCA product. Both exposures were at f4.5 for 1 to 2 minutes, using written gelatin filters 23A and 2B for the first and 21 and 2B for the second. Confirmation of the bands as DNA fragments was by black-white photography of the same gels after ethidium bromide staining.

Differential black-white photography. To provide a more convenient way of photographing and publishing CCA results, we developed a method of black-white photography in which differential filtration was used for a pair of photographs using Polaroid type 667 film with exposures at f11 for 6 to 8 seconds. For the first photograph we combined one 2B and two 23A filters to filter 520 nm emission from fluorescein-labeled $\beta^B$ DNA. For the second photograph we combined one 2B and one 60 filter to filter 605 nm emission from rhodamine-labeled $\beta^B$ DNA. With fluorescein filtration only rhodamine-labeled product (homozygous or heterozygous $\beta^A$) appeared on black-white photos, with rhodamine filtration only fluorescein-labeled product (homzygous or heterozygous $\beta^B$) appeared.

Color photography of CCA product in solution. After separation of fluorescently labeled primer from CCA product, a double-exposure color photography method similar to that described for electrophoretic gels was used to photograph microcentrifuge tubes containing solutions of fluorescently labeled DNA. Both exposures were at f4.5, the first for 2 minutes with one 23A and one 2B filter, and the second for 30 seconds with one 40 and one 2B filter.

Spectrofluorometry. Ultralifted CCA product was assayed using a Perkin Elmer Model 650-40 Fluorescence Spectrophotometer. Optimal detection of fluorescein was at excitation/emission wavelengths of 485/520 nm and of rhodamine at 585/605 nm. Relative emissions of CCA product from known $\beta^A/\beta^A$, $\beta^B/\beta^B$, and $\beta^A/\beta^B$ DNA were recorded for each excitation wavelength. Ratios of 520 nm/605 nm ≈ 1 were diagnostic of the $\beta^A$ allele, and 605 nm/520 nm ratios ≥ 1 of the $\beta^B$ allele.

Control DNA Samples

DNA was prepared using standard sodium dodecyl sulfate/phenol/ chloroform extraction from peripheral blood leukocytes of individuals having $\beta^A/\beta^A$, $\beta^B/\beta^B$, $\beta^A/\beta^B$ genotypes and from the subjects described below.

Subject With Thalassemia Intermedia and a Complex $\beta$ Genotype

A 34-year-old Thai woman in her second trimester of pregnancy had Hb concentration = 9.1 gm/dL, Hct = 28.0%, and MCV = 54 FL by electronic cell counting (Coulter plus, Coulter Electronics, Miami, FL), hypochromia, microcytosis, target cells, anisocytosis, and polikilocytosis on peripheral smear, and 96% Hbe (A2) and 4% Hbf detected using standard techniques. A 4-year-old daughter by a previous white husband had Hb = 12.7 gm/dL, Hct = 37.4%, MCV = 79 FL, 72% Hb A, 27% Hb E, and 0.7% Hb F. A 4-year-old daughter by her current white husband had Hb = 12.5 gm/dL, Hct = 36.1%, MCV = 76 FL, 73% Hb A, 25% Hb E, and 1.7% Hb F.

Globin Biosynthetic Ratio

Globin chains from low-density, reticulocyte-rich erythrocyte fractions of a StraClant II (St Regis Paper Co, NY) gradient were radioiodinated. With fluorescein filtration only $\beta^A$ DNA. With rhodamine filtration only $\beta^B$ DNA. Assay of CCA product in solution was used to further define the $\beta$-globin genotype. With this method the $\beta$-globin gene yields a 4-kilobase (kb) fragment with each of the above haplotypes, and the $\beta^A$ gene yields a fragment of 12 kb from the $\alpha^A$, $\alpha^B$, and $\alpha^S$ haplotypes, and was used to further define the $\alpha$-globin genotype. With this method the $\beta$-globin gene yields a 4-kilobase (kb) fragment with each of the above haplotypes, and the $\beta^A$ gene yields a fragment of 12 kb from the $\alpha^A$, $\alpha^B$, and $\alpha^S$ haplotypes.

RESULTS

The electrophoretic gel in Fig 2A demonstrates that the fluorescence of products from control DNA of genotype $\alpha^A/\beta^B$ was green, $\beta^A/\beta^B$ was red, and $\beta^A/\beta^A$ was yellow. Product from the propositus fluoresced red, indicating the presence of the $\beta^A/\beta^A$ genotype. The results shown used preamplified DNA templates, but identical results were obtained using genomic templates (data not shown). Ethidium bromide staining of this same gel confirms the presence of discrete DNA bands where each of the colored bands had been observed (Fig 2B).

To further simplify recording and publishing CCA results,
Fig 2. Photographs of the same gel showing the 270 NT CCA products from template DNA of genotype (1) $\beta^A/\beta^A$, (2) $\beta^A/\beta^B$, (3) $\beta^B/\beta^E$, and (4) unknown (propositus). (A) A selectively filtered double-exposure color photograph. The green DNA in lane 1 demonstrates the presence of the $\beta^A/\beta^E$ genotype, the red DNA in lanes 2 and 4 of the $\beta^A/\beta^B$ genotype, and the yellow DNA in lane 3 of the heterozygous $\beta^A/\beta^E$ genotype. The purified DNA products in solution shown below the gel correspond to the above lanes. (B) A black-white photograph of an ethidium bromide permeated gel. Discrete 270 NT bands are seen in each lane. (C) A black-white photograph from which red fluorescence (605 nm) has been filtered. Only the fluorescein-labeled DNA in lanes 1 (BA/BA) and 3 (BA/BE) are seen. (D) A black-white photograph from which green fluorescence (520 nm) has been filtered. Only the rhodamine-labeled DNA in lanes 2 (BE/BE), 3 (BE/BE), and 4 (propositus) are seen.

We devised a differential black-white photography method, the results of which are shown in Figs 2C and 2D. These photographs are of the same gel seen in Figs 2A and 2B. In Fig 2C red 605 nm emission was selectively filtered so that only the green emission from the products of the wild type $\beta^A$ alleles in lanes 1 and 3 was detected. In Fig 2D green 520-nm emission was selectively filtered so that only the red emission from the products of the mutant $\beta^E$ alleles in lanes 2, 3, and 4 was detected. The data from this pair of black-white photographs, taken together, demonstrate that only green emission comes from $\beta^A/\beta^A$, only red emission comes from $\beta^A/\beta^E$, and both green and red emissions come from $\beta^A/\beta^B$. Detection of propositus DNA with green but not red filtration was diagnostic of the homozygous $\beta^B/\beta^E$ genotype.

Separation of dye-labeled primers from amplified product using Centricon C 100 ultrafiltration allowed the different colored products to be recorded by color photography of the microcentrifuge tubes containing DNA in solution (Fig 2A). Spectrofluorometry also distinguished $\beta^A$ and $\beta^E$ alleles. The relative 520 nm/605 nm fluorescence ratios ($\cong 1$ diagnostic of the $\beta^A$ allele) were 1.5 for $\beta^A/\beta^A$, 1.0 for $\beta^A/\beta^B$, 0.1 for $\beta^B/\beta^B$, and 0.1 for the propositus. The relative 605 nm/520 nm fluorescence ratios ($\cong 1$ diagnostic of the $\beta^E$ allele) were 8.2 for $\beta^E/\beta^E$, 1.0 for $\beta^A/\beta^E$, 0.7 for $\beta^A/\beta^B$, and 7.6 for the
propositus. These data confirm $\beta^B/\beta^E$ as the propositus genotype.

Among the possible causes of thalassemia intermedia were $96\%$ HbE and $4\%$ HbF in the pregnant Thai woman were $\beta^B/\beta^B$ thalassemia associated with $\alpha^b_2$ or $\alpha^b_2/\alpha^a_2$; $\beta^B/\beta^B$ associated with pregnancy; and $\beta^E/\beta^E$ associated with $\alpha^A_2/\alpha^A_2$ or, theoretically, with supernormal numbers of $\alpha$ loci. Her $\alpha^b_2$ synthetic ratio was 1.28. Blot hybridization of her DNA with an $\alpha$-specific probe showed only 10-kb $BamH\text{I}$ and 15.8-kb $Bgl\text{II}$ fragments, indicative of the $-\alpha^A_2/-\alpha^A_2$ or the $-/-\alpha^A_2$ genotype. DNA from both of her daughters showed 7, 12.5, and 15.8 kb $\alpha$-specific $Bgl\text{II}$ fragments, diagnostic of the $-\alpha^A_2/-\alpha^A_2$ genotype. As shown in Fig 3, $\alpha$-specific hybridization of propositus DNA doubly digested with $Bgl\text{II}$ and $Asp\text{I}$ revealed 4, 10, and 15.3 kb fragments, diagnostic of the $-\alpha^A_2/-\alpha^A_2$ genotype. The limited stringency used for competitive oligonucleotide hybridization in allele-specific amplification assays greatly favors hybridization of the perfectly matched primer over one mismatched by only a single base. Thus, provided both alleles are present, the fluorescence of CCA product accurately reflects the allelic nature of the template DNA. No deletion or sequence variation known at the time of this writing to cause $\beta^B$ thalassemia in Asian populations would invalidate our CCA parameters. While the Thai $\beta^E$ thalassemia mutation, $\beta^E\text{C}^\text{T}A\text{A}$, could have confounded our use of a preamplification primer overlapping codon 35, our verification of the $\beta^E/\beta^E$ genotype without preamplification precluded this artifact. Thus, the data derived from our propositus is interpreted to be diagnostic of homozygous $\beta^E/\beta^E$ rather than HbE-$\beta^B$ thalassemia.

Recording the results of CCA can be by color photography of DNA product in electrophoretic gels or in solution after purification by ultrafiltration. Alternatives to color photography include spectrofluorometry and the paired black-white photographic method that we describe. In this differential filtration method, green DNA appears in one photograph, red DNA in the other, and heterozygous yellow DNA in both.

Diagnosis of inherited hemoglobin disorders in Southeast Asians is a particularly cogent application for CCA, as the myriad of high frequency globin-gene mutations interacting in this group commonly results in complex, difficult to diagnose genotypes. The ability to detect the Constant Spring mutation using allele-specific hybridization of amplified $\alpha_2$ DNA provided an important diagnostic advantage for this population. Now the $\beta^E$ mutation can be differentiated from normal $\beta^E$ and $\beta^O$ thalassemia using CCA. DNA-based genotypic diagnosis was particularly informative in the propositus, because the absence of Hb Bart's normally seen with her genotype may have confused a clinical diagnosis. We speculate that the absence of Hb Bart's in this case may have been the result of diminished, adult level $\gamma$-globin gene expression, as was demonstrated by the temporal decline of Hb F and Bart's levels in an Hb AS individual having the $-/-\alpha$ genotype.

In this case, genetic counseling was straightforward, as her husband had no clinically detectable Hb mutation. However, in areas of the world where Hb mutations have the high frequency found in Southeast Asia, diagnosis and counseling are more complex. The importance of accurate DNA-
based diagnostic methods to the American medical community is underscored by the recent large-scale influx of Southeast Asian emigrants and the birth of children having serious Hb disorders of the variety found in Southeast Asia. CCA complements other DNA-based methods for diagnosing complex Hb genotypes. Only through precise genotypic analysis can these populations be afforded accurate genetic counseling and prenatal diagnosis.

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