


SSCP, the target DNA sequence is amplified by PCR, and then the resulting double-stranded product is rendered single-stranded by heating in a denaturing buffer. Rapid cooling prevents the DNA strands from reannealing, and the single strands fold back on themselves into a conformation determined by the primary DNA sequence. The different conformers are separated on a nondenaturing polyacrylamide gel and detected either radioactively or with silver staining. Although the concept of SSCP is simple, because of the complexity of thermodynamics of single-stranded folding, it currently is impossible to predict which nucleotide change will alter the conformation. Thus, development of these methods remains largely empirical. In a previous work, we described a rapid semiautomated non-radioactive PCR-SSCP method for genotyping the above-described pentanucleotide deletion in A2M (22). Here we report a similar method that is capable of screening large numbers of samples for the A and G coding variants in exon 24 of the A2M gene.

The study was approved by the review board of the National Institute of Mental Health as part of a larger study following people at risk of developing AD. All subjects gave informed consent. Genomic DNA was extracted from EDTA-anticoagulated whole blood with the QIAamp blood kit (Qiagen). For PCR-SSCP, the A2M target DNA sequence was amplified using a forward primer (5'-GAGACATATTAGGCTCTGCC-3') and the reverse primer (5'-GTAACTGAAACCTACTGGAA-3'). The PCR reaction mixture of 100 µL contained 1 µg of human genomic DNA, 20 pmol of each primer, 200 µmol/L dNTPs, 2.5 mmol/L MgCl₂, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 0.1 g/L gelatin, and 3 U of AmpliTaq Gold polymerase (PE Applied Biosystems). In addition, 5 µmol/L dUTP and 10 kU/L uracil glycosylase (GeneAmp Carryover Prevention Kit; PE Applied Biosystem) were added to the PCR mixtures to prevent contamination. PCR conditions were as follows: 37 °C for 10 min; 95 °C for 9 min; 35 cycles of 94 °C for 1 min, 58 °C for 40 s, and 72 °C for 40 s; and then final extension at 72 °C for 5 min. The PCR products were checked for purity by electrophoresis in 1.5% agarose gel (ethidium bromide staining), and the DNA content of the 245-bp band was quantified with reference to a DNA Gel Marker (Research Genetics) (22).

SSCP analysis of the PCR fragments was carried out essentially as described previously (22). Briefly, PCR products were mixed with an equal volume of denaturing/loading solution containing 980 mL/L formamide (Amresco), incubated at 95 °C for 3 min, and then immediately chilled on ice. Precast mini gels with 20% homogenous polyacrylamide and native buffer strips (Pharmacia Biotechnology) were used for electrophoresis. Both gel electrophoresis and band development was carried out with the semiautomated PhastSystem™ (Pharmacia). The gels were prerun at 400 V, 10 mA, 1.0 W for 100 V·h at 4 °C. Sample (1 µL) was applied at 25 V, 10 mA, 1.0 W for 2 V·h at 4 °C, and then electrophoresed at 200 V, 5 mA, 1.0 W for 700 V·h at 4 °C. Gels were silver-stained according to the manufacturer’s protocol.

Representative PCR-SSCP patterns for the three different A2M genotypes that were classified according to the nomenclature of Wavrant-DeVrieze et al. (15) are shown in Fig. 1A. There were two distinct groups of bands: four bands for the A allele, and three bands for the G allele. The presence of multiple bands for both alleles is likely caused by alternative conformations of single-stranded DNA. The non-denatured, double-stranded PCR product migrated much faster than the single-stranded forms and therefore is not seen in Fig. 1. After dilution of PCR products from a heterozygote (Fig. 1D), the two fastest moving bands of both alleles were reproducible over a wide range of DNA loads (0.3–19.0 ng/lane). In turn, the two slower moving bands of the A allele and the single

**Fig. 1.** PCR-SSCP, PCR-RFLP, and DNA sequence patterns of the three different genotypes for a novel sequence polymorphism (Ile1000Val) of the human A2M gene.

Unless otherwise specified, each lane contained 17–20 ng and 200–400 ng of PCR products for SSCP and RFLP analysis, respectively. (A and D), lane M, linear double-stranded DNA marker (Gelmarker™, Research Genetics); (B), lanes M1 and M2, different DNA size markers (Gelmarker and 10-bp DNA ladder (Life Technologies), respectively); (C), DNA sequencing. A, adenosine; C, cytidine; G, guanosine; R, A or G; T, thymidine. (D), serial dilution of a heterozygous specimen showing dependence of band detection with SSCP on the DNA load.
slow moving band of the G allele only were detectable at higher DNA loads (>5 ng/lane). Although these slower moving bands confirm a genotype, they are not necessary for genotyping (Fig. 1A). Nevertheless, under our usual experimental conditions, they were appropriately present in the SSCP patterns of the 57 patients studied.

Results of the PCR-SSCP analysis were confirmed in two ways. The first method involved PCR-restriction fragment length polymorphism (RFLP) analysis by minor modification of the method of Wavrant-DeVrieze et al. (15). Briefly, the A2M gene target sequence was amplified by PCR using 1 μg of extracted genomic DNA as a template. The forward primer was 5’-GAGACATATTAGGCTCTGCC-3’, and the reverse primer was 5’-CAGTGTAGAGATAGCCCAATG-3’. Apart from a smaller number of cycles (30) and no final extension for 5 min at 72 °C, the PCR was carried out as described above, and the DNA content of the resulting 180-bp PCR band was quantified with reference to a DNA Gel Marker (Research Genetics) (22). PCR products (200–400 ng) were subjected to restriction enzyme digestion with 10 U of DpnII (New England BioLabs) for 16 h. After electrophoresis in a 6% polyacrylamide gel, the 140- and 40-bp restriction fragments for the A allele (type 1) and the uncut 180-bp fragment for the G allele (type 2) were visualized with SYBR Green staining (Molecular Probes). Images were captured with a Storm 840 system (Molecular Dynamics). For the 57 human blood specimens that were analyzed, a complete match was found between the PCR-SSCP and PCR-RFLP results that included 22 cases of type 1/1, 28 cases of heterozygous type 1/2, and 7 cases of type 2/2 (Fig. 1B). The distribution of these genotypes is similar to those reported for much larger populations: 39% type 1/1, 49% type 1/2, and 12% type 2/2 in our study subjects vs 42%, 46%, and 12% respectively, in controls (n = 12,000) and 12% type 2/2 in our study subjects vs 42%, 46%, and 12% respectively, in controls (n = 12,000) and 12% type 2/2 in our study subjects vs 42%, 46%, and 12% respectively, in controls (n = 12,000).

For additional confirmation of the PCR-SSCP and PCR-RFLP results, a subset of genomic DNA specimens was sequenced with the primer 5’-GAGACATATTAGGCTCTGCC-3’, using an ABI Prism 310 genetic analyzer (PE Applied Biosystem) and Big Dye Terminator cycle sequencing kit (PE Applied Biosystem). Before sequencing, PCR products were purified with a PCR Product Purification kit (Roche). All 27 different specimens that were analyzed by DNA sequencing (10 cases each of type 1/1 and type 1/2, and 7 cases of type 2/2) revealed the expected alleles (A/A for type 1/1, A/G for type 1/2, and G/G for type 2/2; Fig. 1C).

The semiautomated nonradioactive PCR-SSCP method described here for detection of a novel single nucleotide polymorphism (A-to-G transition, which produces Ile100Val) in the human A2M gene is reliable and is simpler to perform and less labor-intensive than previously reported methods such as DNA sequencing (13, 14) and PCR-RFLP (9, 11, 15, 21). From the time a whole blood specimen is received, our PCR-SSCP would take ~10 h to complete (~2 h of labor), our DNA sequencing would take ~16 h to complete (~3 h of labor), and our PCR-RFLP would take ~24 h to complete (~3 h of labor). PCR-SSCP reduces the likelihood of misinterpretation that may occur because of partial digestion in the PCR-RFLP method, and it is easier to use than DNA sequencing for interpreting heterozygotes. Furthermore, PCR-SSCP requires less expensive instrumentation than sequencing (PhastSystem vs DNA sequencer). These advantages favor the use of PCR-SSCP in routine clinical laboratory testing and for large-scale screening.

References
Role of Heart-Type Fatty Acid-binding Protein in Early Detection of Acute Myocardial Infarction, Farooq Chani,1, Alan H.B. Wu,1 Louis Graff,2 Christoph Petry,3 Glenn Armstrong,4 Florence Prigent,4 and Milton Brown1 (1 Department of Pathology and Laboratory Medicine, Hartford Hospital, Hartford, CT 06102; 2 New Britain General Hospital, New Britain, CT 06050; 3 Bayer Diagnostics, Tarrytown, NY 10591; 4 Winthrop University Hospital, Mineola, Long Island, NY 11501; * author for correspondence: fax 601-545-3733, e-mail fghani@harthosp.org)

Biochemical evidence of acute myocardial infarction (AMI) is delayed by the delay of appearance of serum cardiac markers in the blood after myocardial injury. Heart-type fatty acid-binding protein (H-FABP), a small (15 kDa) cytoplasmic protein (1) involved in lipid homeostasis, is abundant in heart muscle (2). H-FABP is ~10-fold lower in skeletal muscle than in heart muscle, and the amounts in the kidney, liver, and small intestine are even lower (3). After myocardial damage, H-FABP is released into the intercellular space and appears in the bloodstream (4). The magnitude of the increase in plasma H-FABP has also demonstrated a good correlation with the size of the infarction (5). Myoglobin, another small protein (18 kDa), appears in the plasma within 2–3 h after myocardial infarction and is considered a useful marker in the early detection of AMI (6). Myoglobin lacks specificity because myoglobin released from skeletal muscles cannot be distinguished from that released from the heart. Cardiac troponin I (cTnI) and creatine kinase MB isoenzyme (CK-MB) are more specific for myocardial injury but lack early sensitivity because their blood concentrations do not increase appreciably until 6–8 h after the onset of AMI (7).

The aim of this multicenter study was to compare H-FABP with myoglobin, cTnI, and CK-MB in the early detection of AMI in patients presenting with chest pain in the emergency departments of participating centers.

Serial plasma samples were collected from 460 consecutive patients (253 men, 207 women) presenting with chest pain in to the emergency departments of Hartford Hospital, Hartford, CT; New Britain General Hospital, New Britain, CT; and Winthrop University Hospital, Mineola, NY. The mean age was 62.7 years. In 95 patients, a diagnosis of AMI, based on the WHO criteria (8), was made by the emergency department physician or cardiologist, blinded to the results of H-FABP. Patients were classified as having unstable angina if they experienced new-onset angina, angina that came on with less exertion, or angina that increased in severity, duration, or frequency relative to their baselines. Chest pain patients who did not have AMI, unstable angina, or stable angina were classified as having non-cardiac chest pain. Samples were collected at 4-h intervals for the first 16 h and on the basis of clinical need thereafter. Blood samples from 39 healthy individuals were assayed for all cardiac markers. Samples were centrifuged, and plasma was separated and stored at −20°C until assayed. Myoglobin, cTnI, CK-MB, and H-FABP were assayed on the Bayer Immuno t1™ system (Bayer Diagnostics), using sandwich immunoassay. H-FABP was an experimental assay.

Total CK (EC 2.7.3.2) was assayed using the Technicon RA Analyzer (Bayer Diagnostics). The recommended reference interval for total CK is 10–180 U/L. For H-FABP, CK-MB, myoglobin, and cTnI, the AMI cutoff concentrations are 12, 4.0, 84, and 0.9 µg/L, respectively.

Data were analyzed based on the discharge diagnosis for clinical sensitivity, specificity, and diagnostic accuracy. ANOVA with post hoc comparisons (Tukey’s Honestly Significant Difference) was used for analysis of continuous data, and the χ² test was used to analyze nominal data. ROCKIT software, Ver. 0.9 (Dr. Charles Metz, University of Chicago), was used for ROC analysis. The area test, using univariate z-scores, was used to compare areas under the curve.

The mean and central 95% ranges for samples collected from 39 healthy individuals were as follows: H-FABP, 4.27 µg/L (1.57–8.97 µg/L); CK-MB, 1.41 µg/L (0.33–3.7 µg/L); myoglobin, 30.5 µg/L (9.96–67.5 µg/L); and cTnI, 0.0 µg/L. Imprecision (CV) measured on three concentrations of H-FABP control material in 20 consecutive assays over 21 days was 7.0%, 3.4%, and 2.3% at mean values of 8.3, 54, and 324 µg/L, respectively.

H-FABP differed among patients with AMI (59 µg/L), non-cardiac chest pain (14 µg/L), and healthy individuals (4.3 µg/L; P <0.001).

We compared the diagnostic performance of H-FABP with myoglobin, CK-MB, and cTnI. At a specificity of 95%, the sensitivity of H-FABP was 39%, compared with 28% for myoglobin for the first sample at the time of admission. Performance of all the markers worsened after 12 h except for cTnI, whose specificity was still high at 48 h. Positive likelihood ratios (LR+) for H-FABP and myoglobin for the 0–4 h interval were 2.6 and 2, respectively.

There was no statistically significant difference in the myoglobin/H-FABP ratio for AMI [4.92; 95% confidence interval (CI), 4.41–5.43], non-AMI (7.37; 95% CI, 5.32–9.42), and healthy individuals (7.49; 95% CI, 6.56–8.42). The areas under the ROC curves (with 95% CI) at 0–4 h were as follows: H-FABP, 0.80 (0.73–0.85); myoglobin, 0.73 (0.65–0.79); CK-MB, 0.79 (0.72–0.85); and cTnI, 0.91 (0.87–0.94), as shown in Fig. 1A. Fig. 1B shows the improvement in the performance of CK-MB and cTnI at 5–8 h post admission.

Van Nieuwenhoven et al. (9) have suggested that the ratio of plasma myoglobin to H-FABP could be used in the diagnosis of AMI. In our study, there was no statistically significant difference in the myoglobin/H-FABP ratio among the three groups because of considerable overlap in the values. For the diagnosis of AMI, the ratio did not add value to the measurement of H-FABP alone.