Fluorescence-based SSCP Analysis with Automatic Allele Detection Demonstrated for the Factor V Leiden Mutation, Jürgen Geisel,* Tanja Walz, Marion Bodis, Sabine Quast, and Wolfgang Herrmann (Klinisch-Chemisches Zentrallabor, Universitätskliniken des Saarlandes, Oskar-Orth-Strasse, 66421 Homburg/Saar, Germany; * author for correspondence: fax 6841 163109, e-mail kchjgei@medrz.uni-sb.de)

Resistance of coagulation factor V to activated protein C is caused by a point mutation in which a $G\rightarrow A$ substitution at nucleotide 1691 of the factor V gene leads to the replacement of Arg506 by Gln (1). Because of the high prevalence of the factor V Leiden mutation, it would be reasonable to perform a rapid and simple DNA test. In recent publications, several strategies of DNA testing were described. These tests included restriction enzyme digestion, the introduction of new cutting sites, allele-specific PCR, oligonucleotide ligation assay, and single-strand conformation polymorphism (SSCP) analysis (1–5).

The high sensitivity in mutation detection and the simple application have led to wide use of the SSCP method. Currently in most laboratories, SSCP analysis is performed in polyacrylamide gels in combination with silver staining. In this report, we adapted SSCP analysis to capillary electrophoresis. This was the first step toward automation because preparation of a gel was no longer necessary. The denatured DNA samples were placed in a sample tray, and after each electrophoretic run, new gel was injected from the system into the capillary. The SSCP patterns were displayed on the monitor, and the characterization of the mutation was done visually. In a second step, we wanted to automate the identification because the location and height of the peaks were defined, and the software should give suggestions as to the kind of mutation.

The primers used in PCR were described previously (2). For capillary electrophoresis, in PCR the forward and reverse primers were 5'-fluorescently labelled with 6-carboxy-fluorescein (FAM) and 4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein (HEX), respectively. To 1 $\mu$L of the diluted PCR product (diluted 1:30 with $H_2O$), 10.5 $\mu$L of formamide, 0.5 $\mu$L of a size marker (GeneScan-500 Tamra, PE Applied Biosystems), and 0.5 $\mu$L of 0.3 mol/L NaOH were added, and the sample was denatured at 90 °C for 2 min. After the samples were chilled on ice, they were placed in the tray. Capillary electrophoresis was performed with the ABI Prism 310 Genetic Analyzer (PE Applied Biosystems) in a 50 g/L GeneScanTM polymer (PE Applied Biosystems) with 100 mL/L glycerol and 10 Tris-borate-EDTA buffer (0.1 mol/L Tris, 0.077 mol/L boric acid, and 0.0025 mol/L EDTA, pH 8.5). A temperature of 30 °C and a running time of 30 min were chosen. In the case of the wild-type allele, two different peaks

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Fig. 1. Automatic evaluation of the SSCP peak pattern.

(Top) The evaluation of the SSCP peak pattern of an individual heterozygous for the mutation. The characteristic peak patterns of the single strands labeled with either FAM or HEX are displayed. The peaks were named as they were defined before in the Genotyper analysis software. (Bottom) Summary of the evaluation. In the lane for the sense and antisense single strands, both wild-type and mutant peaks were identified. This indicates that the analyzed individual is a heterozygous carrier of the mutation. The analysis of homozygous individuals for the wild-type or mutant alleles was comparable but did not demonstrate the complexity of the heterozygous case.
were found for both sense and antisense single strand. It is likely that more than one stable conformation was formed for each single strand. In the case of the mutation, additional peaks were detected (Fig. 1).

To perform an automatic evaluation of the mutation, an important point is the reproducibility of the complex peak pattern. The analysis of different electrophoretic runs over 15 days confirmed the constant appearance of the peak pattern. Characteristic peaks for the wild-type and mutant alleles were defined by their retention time and height. For the sense strand (FAM), the peaks were defined by the following data points: wild-type 1, 5756; wild-type 2, 5809; mutant 1, 5736; mutant 2, 5788, and for the antisense strand (HEX): wild-type, 5867; mutant, 5738. In the antisense strand, one wild-type and one mutant peak were not considered for automatic evaluation because the two peaks partially overlapped. The variation of the peak retention time was less than ±8 data points for the sense strand and less than ±20 data points for the antisense strand.

The retention time of the peaks and their variations were listed in the Genotyper™ analysis software (Version 2.0; PE Applied Biosystems), and a macro was created for the analysis of the data (clear table; clear labels; select blue lanes; and select green lanes; label category peaks with the category’s name; set up table with one category and one lane per row; append rows to table; show the table window; set row 1 column 1 to sample name; show the plot window). In the diagram, the peaks were named wild-type or mutant, and in the resulting table the summary of analysis was presented (Fig. 1). In the table, the peaks were named wild-type for the sense and antisense single strands in the case of the homozygous presence of the wild-type allele. In the case of homozygosity of the mutation, only peaks defined as mutant were recognized; in the heterozygous state, a combination of both was considered in clinical laboratories in the last 2 years. The main point is the reproducibility of the complex peak pattern. Additional peaks were detected (Fig. 1).

In cell culture studies, MTX inhibits Hcy remethylation, effect of MTX, as demonstrated by an increased plasma tHcy concentrations. Therefore, the present study was undertaken to examine tHcy elimination in subjects with plasma tHcy within reference values and to investigate whether the antifolate effect of MTX had any influence on plasma clearance. This was carried out by monitoring elimination of [14C]Hcy injected intravenously in a dose that did not affect basal tHcy concentrations. By this procedure, elimination kinetics of tHcy at low, fasting tHcy concentrations are obtained. The study group comprised six male cancer patients (mean age ± SD, 40 ± 19 years; Table 1) recruited from the Department of Oncology, Haukeland University Hospital, Bergen, Norway. The protocol was approved by the Institutional Review Board of the University of Bergen.

**References**


**Kinetics of Plasma Total Homocysteine in Patients Receiving High-Dose Methotrexate Therapy,** Anne Berit Guttormsen, Per Magne Ueland, Per Eystein Lønning, Olav Mella, and Helga Refsum (1) Department of Pharmacology, University of Bergen, Armauer Hansens hus, N-5021 Bergen, Norway; 2 Department of Oncology, Haukeland University Hospital, N-5021 Bergen, Norway; *author for correspondence: fax 47 55 974605, e-mail Anne.Guttormsen@farm.uib.no

Homocysteine (Hcy) is a sulfur amino acid formed from methionine during transmethylation. Once formed, it is either remethylated to methionine or irreversibly catabolized to cystathionine. The remethylation is catalyzed by methionine synthase (EC 2.1.1.13), which requires cobalamin as cofactor and 5-methyltetrahydrofolate as substrate (1). This explains why the fasting total homocysteine (tHcy) concentration is related to overall folate or cobalamin status and that increased tHcy has been used to diagnose deficiencies of these vitamins (2–4).

Methotrexate (MTX) is an antifolate drug that inhibits dihydrofolate reductase, thereby depleting the cells of reduced folates, including 5-methyltetrahydrofolate (5). In cell culture studies, MTX inhibits Hcy remethylation, leading to increased Hcy export to the medium (6–8).

Plasma tHcy is a sensitive indicator of the antifolate effect of MTX, as demonstrated by an increased plasma tHcy concentration. The increase is maximal after ~2 days in psoriasis patients given only 25 mg of MTX (9). In cancer patients receiving intermediate or high doses of MTX, there is a rapid increase in plasma tHcy within hours. However, the increased tHcy induced by MTX is normalized after rescue therapy with folinic acid (7, 10).

We have previously shown that tHcy clearance in folate-deficient subjects with hyperhomocysteinemia is close to that observed in healthy individuals (11). However, it has been suggested that folate status predominately affects concentrations of fasting, i.e., low tHcy concentrations. Therefore, the present study was undertaken to examine tHcy elimination in subjects with plasma tHcy within reference values and to investigate whether the antifolate effect of MTX had any influence on plasma clearance. This was carried out by monitoring elimination of [14C]Hcy injected intravenously in a dose that did not affect basal tHcy concentrations. By this procedure, elimination kinetics of tHcy at low, fasting tHcy concentrations are obtained.