Application of real-time PCR methods in population genetics

Ph.D. Thesis

Ferenc Somogyvári

Department of Medical Microbiology

Faculty of Medicine
University of Szeged
Szeged, Hungary

2003

Contents

List of publications related to the Ph.D. thesis

I.

Szolnoki Z, **Somogyvári F**, Szolics M, Szabó M, Fodor L:

Common genetic mutations as possible aetiological factors in stroke.

European Neurology 2001;45:119-20.

Impact factor: 1.179

II.

Szolnoki Z, **Somogyvári F**, Szabó M, Fodor L:

A clustering of unfavourable common genetic mutations in stroke cases.

Acta Neurologica Scandinavica 2000;102:1-5.

Impact factor: 1.304

III.

Somogyvári F, Szolnoki Z, Szabó M, Fodor L:

Real-time PCR assay with fluorescent hybridisation probes for exact and rapid genotyping of the angiotensin-converting enzyme gene insertion/deletion polymorphism.

Clinical Chemistry 2001;47(9):1728-9.

Impact factor: 4.371

1. Introduction

DNA-based analysis is routinely used in a wide range of genetic settings, including evolutionary relationship studies in biology (Kondrashov et al.), identification of susceptibility to common diseases in clinical diagnostics (Foy et al.) and monitoring of infectious diseases in microbiology (Tang et al.). Advances in DNA analysis with a view to the development of methods that are increasingly specific, sensitive, fast, simple, automatable and cost-effective are considered paramount. These demands are driving the evolution of a diverse range of newer technologies. Clearly, some of these developing technologies will offer benefits to the services provided by clinical laboratories (Reiss).

The most promising technique is **DNA microarray genotyping** (McGlennen). This is a hybridization-based method that allows the simultaneous analysis of many polymorphisms. The DNA sample of interest is polymerase chain reaction (PCR) amplified to incorporate fluorescently labelled nucleotides and then hybridized to the array. Perfectly matched sequences hybridize more efficiently to their corresponding oligomers on the array and therefore give stronger fluorescent signals over mismatched probe-target combinations. The hybridization signals are quantified by high-resolution fluorescence scanning and analysed with computer software (Shi, Chee et al., Wang et al.). The application of these chip-based genotyping technologies is now under development. A number of arrays have been generated to detect variants in the different microbial genomes and in human polymorphism (Gershon). The current limitation is that homozygous variants are readily detected, whereas heterozygous ones may be missed and deletions and insertions will not be recognized specifically.

Another recently described method is **real-time pyrophosphate DNA sequencing** (Alderborn et al., Aldeborn). Pyrosequencing detects the *de novo* incorporation of nucleotides based on the specific template. The incorporation process releases a pyrophosphate that is converted in the presence of 5'-phosphosulfate to adenosine triphosphate (ATP), which in turn stimulates luciferase. The light production in the luciferase-catalysed reaction is detected by a charge-coupled device camera. ATP and unincorporated deoxynucleotide triphosphates (dNTP) are continuously degraded by apyrase. The light is switched off and the next dNTP is added. As the process continues, the complementary DNA strand is built up and the

nucleotide sequence is determined from the signal peak in the program (Pyrosequencing AB, Uppsala, Sweden *http://www.pyrosequencing.com*). The major disadvantage of the method is the limited length of the investigated DNA (Garcin et al., O'Meara et al., Gharizadeh et al.).

Platforms available for **non-PCR-based techniques** include the BDProbeTecET™ system (BD Biosciences, *http://www.bd.com/biosciences*), based on strand displacement amplification (Little et al.), and the Abbott LCx™ system (Abbott Laboratories, *http://www.abbottdiagnostics.com*), based on the ligase chain reaction (Austina et al.).

Many currently used techniques rely on the **PCR amplification** of a target molecule, followed by a variety of post amplification analyses, such as restriction enzyme digestion, agarose gel electrophoresis or melting-point analysis, to detect the specific analyte amplified (Wolk et al.). One of the recent advances is the development of technologies with the ability to amplify, detect and quantify DNA targets in a single closed-tube reaction as the PCR proceeds. These real-time homogeneous systems offer many advantages over traditional methods, including speed, a reduced risk of contamination and the ability to quantify the amount of starting material present more accurately. These technologies have been used for the on-line quantification of nucleic acid targets, mutation detection and sequence confirmation.

Several platforms are available for the performance of **real-time PCR analyses**. These range from ultra rapid, heated-air thermocyclers, where the PCR is performed in glass capillaries (Roche LightCycler, Roche Diagnostic Systems, *http://www.roche.com/diagnostics*), to tube and microtitre plate-based systems (ABI Prism 7700 Sequence Detection System, Abbott Laboratories, *http://www.abbottdiagnostics.com*). As the use of real-time PCR assays gains popularity, a range of alternative real-time instruments are becoming available, including the iCycler (Bio-Rad), the Sentinel (Stratagene), the DNA Engine Opticon (MJ Research) the MCA System (HYBAID) and the SmartCycler (Cepheid).

The **ABI Prism 7700 Sequence Detection System** utilizes an array of optical fibres to distribute laser light across all 96 samples contained in either thin-walled tubes or microtitre plates. The fluorescence emitted returns via the fibres to a spectrograph with a charged coupled device camera. Fluorescence between 500 and 660 nm can be detected. The built-in thermal cycler usually needs 2 hours to complete the amplification of the reactions.

Basically two kinds of detection methods are used in real-time PCR assays. In the case of **non-specific detection methods,** dyes such as SYBR Green produce enhanced fluorescence signals upon binding to dsDNA duplexes. Increases in signals are monitored in real time as the dye binds to the newly synthesized DNA molecules as the PCR proceeds. The signals are measured at the end of either the annealing or elongation step of each PCR cycle. Although the application of dsDNA-binding dyes is a simple, fast and inexpensive way of monitoring amplicon production, the major disadvantage of the method is that the dye binds non-specifically to all dsDNA and thus primer-dimer species and non-specific amplification products cannot be distinguished from the specific amplicon. Careful assay optimization is critical to minimize the production of non-specific species. Most platforms offer the opportunity to "melt" the amplicons after amplification is completed. If the temperature is increased slowly, the point at which the strands dissociate can be identified by the subsequent drop in signal as the dye falls from the denatured duplex (Ririe et al.).

The **specific, fluorescence probe detection methods** require fluorophore-conjugated probes for investigation. Many probe-based systems rely on the principle of fluorescence resonance energy transfer (FRET) for signal generation. FRET involves the non-radiative energy transfer from a donor molecule to an acceptor molecule. When two fluorophores whose excitation and emission spectra overlap are in close physical proximity, excitation of one fluorophore will cause it to emit light at wavelengths that are absorbed by and stimulate the second fluorophore. The excited acceptor molecule releases its captured energy and returns to a lower energy state. The released energy can be dissipated into the environment as light or heat. Most of the probe-based systems, such as TaqMan™ probes (Livak et al., Chen et al.), molecular beacons (Giesendorf et al., Smit et al.) and Scorpion™ primers (Whitcombe et al.), use non-fluorescent acceptors that will accept energy from a donor without any resulting fluorescence emission. These acceptors as a group are known as "dark quenchers", and include Dabcyl, QSYTM-7 and BlackHoleTM dyes. The donor dyes are generally FAM, fluorescein, TET HEX or TAMRA. Upon hybridization to the target, the fluorophore and quencher become separated, either through conformational changes that occur upon hybridization (molecular beacons and Scorpion primers) or through enzymatic cleavage of the fluorophore from the quencher upon hybridization as a result of the 5'-nuclease activity of *Taq* polymerase (TaqMan probes). The physical separation of the fluorophore and quencher moieties produces an increase in signal.

TaqMan probes consist of a probe sequence labelled at one end with a fluorophore and at the other end with a quencher moiety. In the unhybridized state, fluorescence is quenched because of the close proximity of the fluorophore and quencher. Upon hybridization of the probe to the target sequence during PCR, the 5'-3'-exonuclease activity of *Taq* polymerase cleaves the probe; the subsequent separation of the fluorophore from the quencher produces a concomitant increase in fluorescence.

Molecular beacon probes are designed such that a target-specific hybridization domain is positioned centrally between short sequences (unrelated to the target). The fluorophore and quencher moieties are situated on the 5' and 3' ends of the probe in the native state; the molecular beacon forms a hairpin structure, with the reporter and quencher groups directly adjacent.Upon hybridization to the target sequence, the loop opens out; the resulting separation of the fluorophore from the quencher produces an increase in the signal. Molecular beacons have been reported to have a higher specificity than that of TaqMan probes, because of the presence of a stem structure (Tyagi et al.). Hybrids between the beacons and mismatch targets dissociate at lower temperatures than do hybrids between linear probes and mismatch targets. Thus, a wider temperature range is observed between the melting points of perfect and mismatch hybrids.

Scorpion primers consist of a probe sequence linked to the 5' end of a primer via a non-amplifiable stopper moiety. The probe is held in a hairpin loop structure by complementary sequences on the 5' and 3' sides of the probe. A fluorophore at the 5' end of the probe is quenched by a moiety at the 3' end of the loop in the unhybridized format (similar to molecular beacons). As the primer extends during PCR, the probe sequence is capable of hybridizing to a target on the newly formed strand. Upon hybridization, the loop is opened, producing a physical separation of the fluorophore and quencher, so that the signal increases.

Probe-based systems can be used for both **quantitative and qualitative applications** (Ruitz-Ponte et al., Rasmussen). For qualitative applications, the probes are designed to hybridize to the region containing the specific sequence or mutation under investigation. Amplification is performed by using an annealing temperature intermediate to the melting temperatures (T_m) of the probe on its matched and mismatched targets so that a signal will be generated only when the probe perfectly matches the target. In this way, the amplification and the analysis are finished at the same time.

The LightCycler utilizes a blue-light-emitting diode with fluorescence measured by three photodetection diodes with different wavelength filters. The capillaries are placed in a carousel rotated over the diodes. The ultrarapid heating and cooling of the capillaries with an airstream allows amplifications to be completed in typically <20 min (Wittwer et al.).The donor dye in the LightCycler probe-based system is either fluorescein or FAM. The fluorescent acceptors that accept energy from a donor to result in fluorescence emission with a wavelength of 640 or 705 nm are LCRed640 (or Cy5) and LCRed705 (or Cy5.5) (Wittwer). This probe method uses two fluorescently-labelled oligonucleotide probes. The donor and acceptor fluorophores are conjugated to the 3' and 5' ends of different oligonucleotides. Thus, they are originally separated. Additionally, any probe used in PCR must be blocked at the 3' end in order to prevent extension by polymerase. FRET occurs as the two probes hybridize in a head-to-tail arrangement to adjacent sequences on the target DNA. One of the probes is a tightly binding 'anchor probe', whereas the adjacent 'sensor probe' spans the region of sequence variation. As the reaction mixture is heated, the probe-target duplex is denatured, the fluorophores are separated and the fluorescence of the acceptor dye drops to the background. When the probe hybridizes over a sequence variant, a mismatch is formed and the duplex is destabilized. This is reflected as a shift in T_m, the temperature at which 50% of the probe is denatured. The final analysis is more easily discerned by plotting a derivative melting curve, where the centre of the melting peak corresponds to the point of inflection of the fluorescence-temperature curves. Although the temperature dependence of the detection probe can be observed during rapid thermal cycling (Lay et al.), a slower temperature transition performed as an addendum to PCR is optimum for single base discrimination. Hybridization probes have been used for mutation detection by both slow cooling (annealing curves) and slow heating (melting curves) (Gundry et al.); melting curve analysis is more commonly used. With the aid of two different acceptor dyes, multiple mutation detection is possible.

Disease-causing mutations have been identified by means of molecular genetic techniques in an increasing number of genes in recent years. The incidence of 'disease genes' is accelerating rapidly as a result of the progress of the Human Genome Project. Since the majority of mutations in genetic disorders are due to single base pair changes or insertions or deletions, it is highly desirable to develop DNA diagnostic tests that are sensitive, specific, cost-effective and easily carried out in any clinical laboratory.

The detection of mutations in individuals who carry such gene mutations can be of significance as concerns early diagnosis, genetic counselling and disease prevention (Kohli-Seth et al.).Moreover, it may be important to carry out large prospective studies in which the roles of the potential genetic risk factors can be examined under standardized circumstances. (Szolnoki et al.)

Ischaemic stroke is considered to be a multifactorial disease (Hassan et al.). Different environmental and genetic factors together might lead to its development. Several genetic mutations and polymorphisms can be associated with stroke.

Such presumptive mutations could have been the mutations of the **thromboembolic factors**. The most important mutations associated with the thromboembolic events are the factor V mutations and the factor II prothrombin G20210A mutation (Bowen et al., Zent et al.).

Hyperhomocysteinaemia has also been shown to be a risk factor for venous thromboembolic diseases (Den Heier et al.). A genetic risk factor that is thought to cause a folic acid deficiency level that gives rise to hyperhomocysteinaemia is the methylenetetrahydrofolate reductase (MTHFR) C677T mutation (Frosst et al., Arruda et al.). A second common mutation in the MTHFR gene (A1298C) has been described which, in combination with a heterozygous MTHFR C677T mutation, can have a similar pathobiochemical effect to that of a homozygous MTHFR C677T mutation (Put et al., Cunha et al.). In this study, we investigated only the MTHFR C677T mutation, but, with regard to future surveys, we developed a new multiplex real-time PCR method capable of investigating both MTHFR mutations together (Somogyvári et al.).

In **the renin-angiotensin system,** angiotensin-converting enzyme (ACE) is a peptidyl dipeptide hydrolase that plays a role in the conversion of angiotensin I into the active octapeptide angiotensin II and also inactivates the vasodilator bradykinin (Hooper NM). The blood and cellular levels of ACE are determined genetically. The variability of the plasma ACE levels is a result of an I/D polymorphism of the ACE gene (Tiret et al.). The presence of the D allele is associated with higher circulating ACE levels and several human disorders (Lin et al.).

Apolipoproteins (Apo) play a central role in the **cholesterol transport** by their association to lipoproteins and their function as ligands for receptors. Genetic variation of the ApoE locus in humans is an important determining factor of plasma lipid concentrations. The E4 isoform is associated with increased cholesterol, thereby enhancing the risk of circulatory diseases (Davignon et al.). In addition, E4/E4 individuals are at high risk of the development of Alzheimer disease (Corder et al.). There is growing evidence of interactions between the renin-angiotensin system and hypercholesterolaemia (Batalla et al., Nakai et al.), and between the renin-angiotensin system and hyperhomocysteinaemia (Fernandez-Arcas et al.) as concerns enhancement of the risk of different circulatory diseases. This is the pathobiochemical background upon which these mutations were chosen as the objects of the present study.

The **roles of factor V mutations** such as the Leiden G1691A (Catto et al., De Lucia et al., Eskandari et al., Gaustadnes et al.), R306T Cambridge (Williamson et al.) and R306G Hong Kong (Chan et al.) in the pathogenesis of stroke are still debated. It has been reported that the prothrombin G20210A mutation can be detected more frequently in young patients who have suffered a stroke than in healthy persons (de Stefano et al, Giardano et al., Ridker et al., Huberfeld et al.). The Leiden and prothrombin G20210A mutations might give rise to stroke if they are associated with hypertension, diabetes mellitus or some other clinical risk factors (Huisman et al., Zenz et al.).

The **effects of ACE I/D polymorphism** (Agerholm et al., Doi et al., Takami et al., Zee et al.), MTHFR C677T mutations (Harmon et al., Nakata et al., Press et al., Salooja et al.) and the ApoE genotypes (Chowdhury et al., MacLeod et al., Perdro-Botet et al., Topic et al.) in the pathogenesis of stroke has not yet been fully elucidated. Although all these mutations can be associated with arteriosclerosis or thrombophilia or vasoregulation disturbances (Bortolotto et al., Bostom et al., Clarke et al., Okamura et al., Perry) on a pathophysiological basis, none of them in themselves have been proved to be a major genetic risk factor for ischaemic stroke (Elbaz et al., Kontula et al.).

2. Aims of the study

A correct diagnosis is essential for adequate therapy. For this purpose, the usage of new diagnostic methods is advisable. Molecular biology tests are currently undergoing continuous improvement, refinement and differentiation. The diagnostic resolution may be enhanced by direct analysis of genes because, besides the clinical picture, the genetic characteristics of the host may contribute to the aetiology and pathogenesis of different diseases.

2.1. One of our aims was to increase the efficiency and decrease the time consumption and handling requirements of human genetic examinations. Efficiency is important in population studies, where large number of examinations should be performed. The time factor could be crucial at the patient level because the rapid elucidation of certain hereditary diseases might influence the efficacy of the therapy and the outcome of the disease. Finally, handling and cost-effectiveness are key issues for clinical laboratory practice.

The genotyping procedure can generally be divided into two steps: sample preparation and allele detection. To accomplish this goal, we first changed the DNA extraction from a home-brew method to use of the rapid manufactured kit. All of the previously used traditional PCR-RFLP (restriction fragment length polymorphism) assays were then replaced with real-time PCR tests. In the case of ACE polymorphism, we have developed a new real-time PCR method. This was necessary because real-time PCR methods were originally established for the detection of point mutations. We could not find any publication in the literature that reported a method for the reliable detection of long insertions/deletions by using real-time PCR technology.

In detail, the aims were as follows:

To find a generally applicable method for the detection of different alleles with long insertions/deletions, using fluorescence-labelled detection probes.

To design detection probe-pair with which to examine ACE insertion/deletion polymorphism with the Roche LightCycler instrument.

To optimize the conditions of real-time PCR reactions on use of the Roche LightCycler instrument:

Optimization of the $MgCl_2$ concentration

Optimization of the dimethyl sulfoxide (DMSO) concentration

Optimization of the annealing temperature

Optimization of the concentrations of the primers

Optimization of the concentrations of the detection probes

2.2. Our second aim was to apply these methods in clinical practice. For this purpose, we chose to examine the genetic background of a multifactorial disease, and the possible value of the examinations in the therapeutic strategy.

Our final goal was to examine the role of genetic factors in stroke cases when these factors were not associated with other well-known clinical risk factors.

In detail:

The screening of different mutations in a satisfactorily large population of stroke patients was performed in order to ascertain whether these genetic alterations could themselves be risk factors for stroke.

The examined mutations were:

factor V Leiden G1691A mutation

factor V Cambridge R306T mutation

factor V Hong Kong R306G mutation

prothrombin G20210A mutation

MTHFR C677T polymorphism

ACE I/D polymorphism

ApoE polymorphism

3. Patients, materials and methods

3.1. Stroke patients

406 stroke patients were identified by screening the medical records of the Neurological and Neurophysiological Department at Pándy Kálmán County Hospital in Gyula. All subjects underwent a detailed clinical scrutiny, including an exploration of the medical history and the family history, an evaluation of vascular risk factors, general physical and neurological examinations, urine analysis, extended laboratory examinations to detect coagulopathies such as deficiencies of antithrombin III, protein C and S, vasculitis, underlying infections or immunodeficiencies, electrocardiography, extracranial and transcranial Doppler sonography of the brain-supplying arteries, transthoracic and/or transoesoephageal echocardiography where appropriate, and magnetic resonance imaging examinations within 2 days after the first observation of the symptoms.

3.2. Genetic examinations

The genetic examinations were carried out without knowledge of the results of the clinical work-up.

By means of the PCR technique, the coagulation factor V (R506Q Leiden, R306G Hong Kong, and R306T Cambridge), prothrombin G20210A and MTHFR C677T mutations, and the ACE and ApoE polymorphisms were examined.

The R306G Hong Kong and R306T Cambridge mutation examinations were carried out in the Department of Human Genetics and Child Development at the University Medical School, Pécs, Hungary.

A Perkin Elmer GeneAmp PCR System 2400 block thermocycler was used between 1 January 1998 and 1 April 1999. The amplified DNA was genotyped by using restriction enzyme digestion. The restriction products were resolved on agarose gel. The restriction

patterns were enrolled on polaroid film after the visualization of bands with ethidium bromide.

DNA isolation (Miller et al.) and the PCR technique for the Leiden V (Greengard et al.), MTHFR 677C-T (Clark et al.) and prothrombin 20210G-A mutations (Danneberg et al.) and ApoE polymorphism (Crook et al.) were carried out in accordance with the original descriptions. ACE polymorphism was examined by a modification of the original (Rigat et al.) description for the increased detection of heterozygotes (Chiang et al.).

The following reagents were used: PCR mastermix, 25 mM $MgCl_2$ and ΣdNTP (Perkin Elmer, Norwalk, USA), proteinase K, dimethyl sulfoxide, agarose, ethidium bromide and REDTaq DNA polymerase (Sigma Aldrich, Munich, Germany), and HindIII, HinfI and Hin6I (HhaI) restriction enzymes (Fermentas AB, Vilnius, Lithuania).

The primers were synthetized in the Biological Research Centre of the Hungarian Academy of Sciences, Szeged.

In order to reduce the time requirements, real-time PCR (a LightCycler™ instrument, Roche Diagnostics) was used after 1 April, 1999.

At the same time, we changed the DNA extraction from a home-brew method to the rapid manufactured kit. Genomic DNA was extracted from 200 µl of peripheral blood anticoagulated with EDTA with the High Pure PCR Template Purification Kit from Roche Diagnostics and the QIAmp DNA Blood Mini Kit from Qiagen Incorporated, according to the manufacturer's instructions.

The PCR technique for the Leiden V and prothrombin 20210G-A mutations (Berg et al.), the MTHFR 677C-T mutation (Ahsen et al.) and the ApoE polymorphism (Bernard et al. and Aslanidis et al.) was carried out in accordance with the original descriptions.

The following reagents were used: PCR mastermix, and 25 mM $MgCl_2$ (Roche, Mannheim, Germany).
The primers and probes were synthetized by Tib MolBiol, Berlin, Germany.

The ACE polymorphism was examined by a PCR method developed with a view to decreasing the examination time and to providing a better detection of heterozygotes. This method consisted in fluorescent probe melting point analysis performed with fluorescently labelled oligonucleotide hybridization probes on the LightCycler™ instrument (Roche Diagnostics).

3.3. Primer design

The amplification primers were reported by Rigat et al.
The insertion-specific confirmatory primer pair were demonstrated by Lindpaintner et al.
The Oligo Analyzer 2.5 program was used to check the annealing temperatures of the primers. Use of the program is available free on the home page of Integrated DNA Technologies (*http://www.idtdna.com/*).
The primers were manufactured by Life Technologies Ltd. (3 Fountain Drive, Inchinnan Business Park, Paisley, 9A4 9RF, Scotland). The Hungarian dealer is Csertex Kft., H-1035 Budapest, Derű u. 8.

3.4. Probe design

The National Center for Biotechnology Information (NCBI) home page was utilized to collect the sequence information (*http://www.ncbi.nlm.nih.gov/*).
The ACE gene sequence (GenBank accession number AF118569) was applied to design the FRET probe because the antisense primers are over the 3' end of the sequence demonstrated by Rigat et al. (GenBank accession number X62855).
The Qiagen Operon Oligo Toolkit (*http://www.operon.com/oligos/toolkit.php*) was used to calculate the melting temperatures of the probes.
The probes were manufactured by Tib MolBiol Syntheselabor (Tempelhofer Weg 11-12, D-10829 Berlin, Germany). The Hungarian dealer is Roche Hungary Kft., H-1123 Budapest, Győri u. 20.

4. Results

4. 1. The genotyping procedure

The purity of the prepared DNA differed if we used the HighPure, the Qiagene kits or the salting-out procedure. The OD 260/280 was 1.5-1.6, 1.7-1.8 and 1.8-1.9 respectively.

To perform an analysis of the ACE gene sequence (GenBank accession number AF118569), we designed a detection probe specific for the 3' end of the insertion (underlined four bases in the 5' end of the probe): 5'-CGT GAT ACA GTC ACT TTT ATG-3'. The probe was labelled at the 3' end with fluorescein. The designed anchor probe (5'- GGT TTC GCC AAT TTT ATT CCA GCT CTG -3') was labelled with LightCycler Red 640 at the 5' end and was modified at the 3' end by phosphorylation to block extension. There was a 1 bp gap between the detection and the anchor probe.

For the amplification, we used the original primer pair described by Rigat et al.
The relative placements of the primers and the hybridization probes detecting the ACE gene insertion/deletion polymorphism are shown in Figure 1.

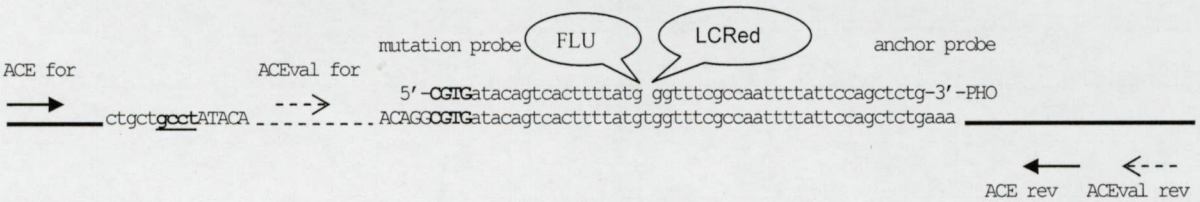

Figure 1.
(The insertion is indicated by capital letters and by the chopped line; ACE for/rev: ACE primers, amplicon length 490 bp with, and 190 bp without insertion; ACEval for/rev: ACE insertion-specific primer pair, amplicon length 335 bp only in the presence of an insertion; the site of the mismatch in the case of the D allele is underlined.)

The LightCycler differs in the optimum PCR parameters from the block PCR. Consequently, we had to optimize the PCR reaction.

The $MgCl_2$ concentration was examined in 0.5 mM steps in the range 1-3 mM. The optimum was found to be 2 mM.

The DMSO concentration was tested in 2.5 % steps in the range 0-12.5%. The optimum was 5%.

The calculated annealing temperature of the forward primer used was 61.8 °C.
The calculated annealing temperature of the reverse primer used was 61.3 °C.
The annealing temperature tests ranged between 56 and 65 °C, in 1 °C steps. Empirically, we found the optimum annealing temperature to be 61 °C.
The optimization studies were repeated with the iCycler. (Special thanks are expressed to József Paulik, BioRad Hungary Kft.) This is a conventional block PCR with temperature gradient capabilities. The gradient used ran from 55 to 65 °C. The optimum temperature here too proved to be 61 °C.

The primer concentration was optimized in 3 steps between 0.1 and 0.3 μM/L. The optimum was 0.2 μM/L, in accordance with Rigat et al.

The probe concentration was optimized in 5 steps between 0.05 and 0.25 μM/L. The optimum was 0.1 μM/L. Here, the criteria for optimum performance were the peak heights of the derivative melting curves and the ratio between the peaks characteristics of the two alleles.

4. 2. Patients' description

406 Hungarian patients diagnosed with acutely developing ischaemic stroke were examined.
The typical risk factors included any of the following conditions: diabetes mellitus, hypertension, ischaemic heart disease, cardiac valvular disease, cardiac arrhythmia, cardiac failure, stenosis of the internal carotid artery or vertebral artery, arterial dissection, atherosclerotic plaques in the internal carotid artery and vertebral artery, aneurysm diseases, age above 60 years, hypertriglyceridaemia, hypercholesterinaemia, presence of anticardiolipin antibody or lupus anticoagulant, heavy smoking or drinking.

After a detailed clinical analysis, 5 patients were found to have no classical clinical risk factors. No clinical explanation for the aetiology of stroke could be established in these

subjects. In these 5 cases, the results of clinical examinations were as follows: carotid, vertebral duplex and cardiac transoesophageal ultrasonography provided negative results. A right to left cardiac shunt could be excluded by means of an injection of agitated saline during the transoesophageal echocardiogram. Ultrasound examinations screening the lower extremities for deep vein thrombosis were negative. 24-hour arterial blood pressure monitoring did not register abnormal values. Magnetic resonance angiography did not reveal any vascular anomalies. The transcranial Doppler did not indicate any embolic events. ECG examinations demonstrated no rhythmic alterations. Myeloproliferative disorders, malignancy and complications of chemotherapy were not present. Both abdominal ultrasonographic and chest X-ray findings were negative. Routine blood tests and urine analyses presented normal values. Routine cerebrospinal fluid analyses showed no alteration. Oligoclonal bands were negative. Routine fungal and mycobacterium cultures of cerebrospinal fluid were negative. The serum immunoglobulin A, G and M levels were normal. Anticardiolipin, antileukocyte, antimitochondrial, anti-DNA antibody and lupus anticoagulant were not detected. Protein C, protein S and antithrombin deficiencies were not found. The antithrombin III levels were in the normal range.

An activated protein C resistance was discovered in all 5 patients.

Heterozygous Leiden V mutations were detected in all 5 patients. The R306T Cambridge, R306G Hong Kong and prothrombin G20210A mutations were not observed in any of the 5 subjects. Heterozygous MTHFR C677T mutations were detected in patients 1-4, and the homozygous MTHFR C677T mutation in patient 5. I/D genotypes were detected for ACE polymorphism in patients 1-4, and D/D genotype in patient 5. ApoE 3/3 genotypes were found in all 5 subjects.

Among the 406 stroke patients, R306T Cambridge, R306G Hong Kong, homozygous Leiden V and homozygous prothrombin G20210A mutations were not detected. The frequencies of heterozygous Leiden V mutations was 5%, that of heterozygous prothrombin G20210A mutations was 1%, those of homozygous and heterozygous MTHFR C667T mutations were 11% and 31%, respectively; and the distribution of the DD, II and ID genotypes was 32%, 23% and 45%, respectively. The frequency of the ApoE 2 allele was 7%, and that of the ApoE 4 allele was 25%.

5. Discussion

5.1. The genotyping procedure

We compared two rapid DNA extraction methods to replace the tedious and time-consuming salting-out procedure. The kits used were the High Pure PCR Template Purification Kit from Roche Diagnostics and the QIAmp DNA Blood Mini Kit from Qiagen Incorporated. The purity of the prepared DNA was better with the Qiagen kit, and we therefore used this.

There were no essential problems in the application of the described real-time methods in the LightCycler. However, detection of the Leiden V and the prothrombin mutations did not succeed in the multiplexed mode as described by Berg et al., and we changed the $MgCl_2$ concentration in the case of the MTHFR C677T mutation, and we had to combine the primers and the probes from two papers (Bernard et al. and Aslanidis et al.) in the case of ApoE polymorphism because information obtainable from the publications separately was not enough to emulate the described techniques successfully.

In the case of the ACE polymorphism, we developed a new detection mode.
The PCR method reported by Rigat et al. was considered; this presents several problems. It has been modified several times (Shangmugam et al., Weissensteiner et al., Chiang et al.) because of the preferential amplification of the D allele. The I/D heterozygotes were earlier mistyped as D/D homozygotes. The extent of this misclassification has been estimated at ~5% (Lindpaintner et al., Singer et al., Ueda et al.). One good solution to solve this problem is to use a second, independent PCR amplification (Lindpaintner et al.) with a primer pair recognizing an insertion-specific sequence. In this way, the I/D genotypes that were mistyped as D/D in the first PCR reaction can be detected. Unfortunately, this method is time-consuming and laborious.

We attempted using real-time PCR with the non-specific SybrGreen dye as this was published later (Lin et al., Sakai et al.), but the detectability of the I allele of the heterozygotes was not superior to the agarose or acrylamide gel assay.

For the detection of a long insertion, a possible general solution could be the design of a probe to overlap 1-5 bases on the insertion. The sensitivity of the detection is known to be better when specific probes are used, and we therefore designed and constructed one for this purpose.The fluorescence hybridization probe was constructed to fit in the insertion-specific sequence, which can be amplified by the confirmatory primer pair described by Lindpaintner et al. This proved to be a useful way to adapt an independent second PCR method to the LightCycler.

With our specific probe, we were readily able to detect I/D heterozygosity and differentiate between D/D and I/D genotypes. During optimization of the new assay system, however, another complication arose, which forced us to modify the original method described by Rigat et al. further. Using the "nearest neighbour" approach (Ahsen and Schütz, Ahsen et al. 2000, Ahsen et al. 2001) for determination of the annealing temperature, we calculated it to be 61 °C instead of 58 °C, as indicated in the source article. When the experimental trial was performed on the optimization of the annealing temperature, it proved that the later estimation was correct.

We determined the reliability of the new assay system by comparing it with the Lindpaintner method modified for the LightCycler. In 103 independent tests, we found no differences between the parallel results obtained with the two techniques.

5.2. Patients' description

In the published clinical studies, the allele frequency of the Leiden mutation in different European areas has been estimated to be 4-7% (Bowen et al, Hoppe et al., Rees et al., Schroder et al.), and the allele frequency of the prothrombin G20210A mutation as 1-2% (Cumming et al., Swibertus et al., Szén et al.). In the case of the MTHFR C677T mutation, the allele frequency is 36-38% in the Caucasian population (Bowen et al., Lachmeijer et al., Schindler et al., Somogyvári et al.), and likewise in the Japanese (Arai et al.) and French-Canadian (Frosst et al.) populations. The frequency of the ACE D allele was found to be 44% (Fatini et al., Lindpaintner et al., Malik et al.) in studies on large numbers of patients (n>1000), and 36% (Barnas et al.) when a low number of cases were tested (n=40). In our studies on the frequencies of these mutations we obtained that were very similar, to those reported for different allele frequencies in the cited studies.

Very heterogeneous data have been given for different ApoE allele frequencies: in different papers ApoE2 allele frequencies of 2-20%, and ApoE4 allele frequencies of 6-16% (Batalla et al, Nakai et al, Szalai et al., Traykov et al., Tsuboi et al.). We found an even higher allele frequency (25%) for the E4 isoform in stroke patients.

There are no data on the incidences of R306G Hong Kong and R306T Cambridge mutations in the Caucasian population. We did not observe these mutations in any of our stroke patients. This fact, in combination with the above observations, points to the conclusion that these mutations probably do not play any significant role in the pathogenesis of stroke.

In our study, 406 patients were examined because of ischaemic stroke. After a detailed clinical scrutiny, 5 were found who did not exhibit any classical clinical risk factors. The aim of the compilation was to create a clinically homogeneous subgroup in which the role of the previously mentioned genetic factors in the aetiology of stroke could be analysed. Heterozygous Leiden V mutations, MTHFR 677T and ACE D allele were found in all 5 stroke subjects without conventional risk factors. This type of clustering of the mutations was not observed in the remaining 401 stroke patients. According to the allele frequencies discussed above, the probability of the simultaneous presence of these three mutations in one person is calculated to be only 0.36-1.4%. In our 5 young stroke patients who did not carry any classical clinical risk factors, the frequency of the Leiden V mutations was 100%, whereas the occurrence of the Leiden V mutations in our overall examined stroke population was 5%.

The PCR technique and the mutation pattern analysis could provide more exact data related to the pathogenesis of stroke. On the basis of our results, the possibility of a Leiden mutation in patients with ischaemic stroke should be considered when no other aetiology is evident. The contributions of the MTHFR C677T mutation and ACE I/D polymorphism to a thrombotic event (not as major risk factors, but as risk-augmenting ones) should also be kept in mind under such these circumstances. The presented cases suggest that it might be important to carry out large prospective studies in which the roles of all potential genetic risk factors should be examined together under standardized circumstances in special subgroups of patients of different ages. For such a purpose, the application of the real-time PCR technique appears to be convenient and excellently suitable with the methodology elaborated in our laboratory and presented here.

6. Conclusions

6.1. We have replaced the time-consuming and laborious PCR-RFLP mutation analysis methods with real-time PCR tests in the cases of consequential mutations such as the factor V Leiden G1691A and the prothrombin G20210A mutations, and the MTHFR C677T and ApoE polymorphisms.

In the case of ACE I/D polymorphism, we have constructed a new method based on fluorescence-labelled oligonucleotide hybridization probes and subsequent fluorescent probe melting point analysis. This method is more sensitive and exact than the previous ones for the detection of I/D heterozygotes. This procedure appears generally suitable for the detection of long insertions with the help of fluorescence-labelled detection probes, although the original recommendation is to use them for the detection of point mutations.

6.2. The aetiological role of common genetic mutations was analysed in a subgroup of stroke patients. The results suggest that the Leiden mutation might possibly be a major aetiological factor for stroke in a rare subgroup of patients where none of the classical risk factors could be found. The pathogenetic roles of ACE D polymorphism and the MTHFR C677T mutation should also be taken into consideration in this particular subgroup of stroke patients. These unfavourable genetic features might be considered to be aetiological factors if they are clustered together in a stroke patient who does not presenting any classical clinical risk factors.

Among the 406 stroke patients, the allele frequencies of the factor V Leiden and prothrombin G20210A mutations and the MTHFR C677T, ACE I/D and ApoE polymorphisms were similar to those described in previous studies. The R306G Hong Kong and R306T Cambridge mutations could not be detected in any of our examined subjects.

These data demonstrate the value of these molecular genetic examinations in facilitating an understanding of the aetiology and pathogenesis of stroke. In the future, it might become possible to predict the relative risk of a stroke event in subgroups of patients by using the genetic allele pattern results

References

1. Agerholm-Larsen B, Tybjaerg-Hansen A, Frikke-Schmidt R, Gronholdt ML, Jensen G, Nordestaard BG: ACE gene polymorphism as a risk factor for ischemic cerebrovascular disease. Annals of Internal Medicine 1997;127:346-55.
2. Ahsen N, Oellerich M, Armstrong VW, Schüttz E: Application of a thermodynamic nearest-neighbor model to estimate nucleic acid stability and optimize probe design: prediction of melting points of multiple mutations of apolipoprotein B-3500 and Factor V with a hybridization probe genotyping assay on the LightCycler. Clinical Chemistry 2000;45(12):2094-101.
3. Ahsen N, Schütz E: Using the nearest neighbor model for the estimation of matched and mismatched hybridization probe melting points and selection of optimal probes on the LightCycler. In: Meuer S, Wittwer C, Nakagawara K: Rapid cycle real-time PCR. Springer Verlag Berlin Heidelberg New York 2001;43-56.
4. Ahsen N, Wittwer CT, Schütz E: Oligonucleotide melting temperatures under PCR conditions: nearest-neighbor corrections for Mg^{2+}, deoxynucleotide triphosphate, and dimethyl sulfoxide concentrations with comparison to alternative empirical formulas. Clinical Chemistry 2001:47(11);1956-61.
5. Ahsen N, Schütz E, Armstrong VW, Oellerich M: Rapid detection of prothrombotic mutations of Prothrombin (G20210A) Factor V (G1691A), and Methylenetetrahydrofolate reductase (C677T) by real-time fluorescence PCR with the LightCycler. Clinical Chemistry 1999;45(5):94-6.
6. Alderborn A, Kristofferson A, Hammerling U: Determination of single-nucleotide polymorphisms by real-time pyrophosphate DNA sequencing. Genome Research 2000;10(8):1249-58.
7. Arai K, Yamasaki Y, Kajimoto Y, Watada H, Umayahara Y, Kodama M, Sakamoto K, Hori M: Association of methylenetetrahydrofolate reductase gene polymorphism with carotid arterial wall thickening and myocardial infarction risk in NIDDM. Diabetes 1997;46(12):2102-4.
8. Arruda VR, von Zuben PM, Chiaparini LC, Annichino-Bizzacchi JM, Costa FF: The mutation Ala677 to Val in the methylene tetrahydrofolate reductase gene: a risk factor for arterial disease and venous thrombosis. Thrombosis Haemostasis 1997;77:818-21.

9. Aslanidis C, Schmitz G: High-speed Apolipoprotein E genotyping and Apolipoprotein B3500 mutation detection using real-time fluorescence PCR and melting curves. Clinical Chemistry 1999;45(7):1094-7.
10. Austina V, Gamboa F, Gazapo E, Manterola J, Lonca J, Matas L. Evaluation of the semiautomated Abbott LCx *Mycobacterium tuberculosis* assay for direct detection of *Mycobacterium tuberculosis* in respiratory specimens. Journal of Clinical Microbiology 1997;35:1996-2002.
11. Barnas U, Schmidt A, Illievich A, Kiener HP, Rabeinsteiner D, Kaider A, Prager R, Abrahamian H, Irsigler K, Mayer G: Evaluation of risk factors for the development of nephropathy in patients with IDDM: insertion/deletion angiotensin converting enzyme gene polymorphism, hypertension and metabolic control. Diabetologia 1977;40:327-31.
12. Batalla A, Alvarez R, Reguero JR, Hevia S, Iglesias-Cubero G, Alvarez V, Cortina A, González P, Celada MM, Medina A, Coto E: Synergistic effect between Apolipoprotein E and angiotensinogen gene polymorphism in the risk for early myocardial infarction. Clinical Chemistry 2000;46:1910-5.
13. Bergh FAJTM, Oeveren-Dybicz AM, Bon MAM: Rapid single-tube genotyping of the factor V Leiden and prothrombin mutations by real-time PCR using dual-color detection. Clinical Chemistry 2000;46(8):1191-5.
14. Bernard PS, Pritham GH, Wittwer CT: Color multiplex hybridization probes using the Apolipoprotein E locus as a model system for genotyping. Analytical Biochemistry 1999;273:221-228.
15. Bonnet G, Tyagi S, Libchaber A, Kramer FR: Thermodynamic basis of the enhanced specificity of structured DNA probes. Proceedings of the National Academy of Sciences of the United States of America 1999;96:6171-6.
16. Bortolotto LA, Safar ME, Billaud E, Lacroix C, Asmar R, London GM, Blacher J: Plasma homocysteine, aortic stiffness, and renal function in hypertensive patients. Hypertension 1999;34:837-42.
17. Bostom AG, Rosenberg IH, Silbershatz H, Jacques PH, Selhub J, D'Agostino RB, Wilson PB, Wolf PA: Nonfasting plasma total homocysteine levels and stroke incidence in elderly persons: the Framingham Study. Annals of Internal Medicine 1999;131:352-5.
18. Bowen DJ, Bowley S, John M, Collins PW: Factor V Leiden (G1691A), the prothrombin 3'-untranslated region variant (G20210A) and thermolabile methylenetetrahydrofolate reductase (C677T): a single genetic test genotypes all three loci - determination of

frequencies in the S. Wales population of the UK. Thrombosis Haemostasis 1998;79:949-54.

19. Catto A, Carter A, Ireland H, Bayston TA, Philippou H, Barrett J, Lane DA, Grant PJ: Factor V Leiden gene mutation and thrombin generation in relation to the development of acute stroke. Arteriosclerosis, Thrombosis, and Vascular Biology 1995;15:783-5.
20. Chan WP, Lee CK, Kwong YL, Lam CK, Liang R: A novel mutation of Arg 306 of factor V gene in Hong Kong Chinese. Blood 1998;91:1135-9.
21. Chee M, Yang R, Hubbell E, Bemo A, Huang XC, Stem D, Winkler J, Lockhart DJ, Morris MS, Fodor SP: Accessing genetic information with high-density DNA arrays. Science 1996;274(5287):610-4.
22. Chen X, Zehnbauer B, Gnirke A, Kwok P: Fluorescence energy transfer detection as a homogenous DNA diagnostic method. Proceedings of the National Academy of Sciences of the United States of America 1997;94:10756-61.
23. Chiang FT, Hsu K, Chen W, Tseng C, Tseng Y: Determination of angiotensin-converting enzyme gene polymorphisms: stepdown PCR increases detection of heterozygotes. Clinical Chemistry 1998;44(6):1353-6.
24. Chowdhury AH, Yokoyama T, Kokubo Y, Zaman MM, Haque A, Tanaka H: Apolipoprotein E genetic polymorphism and stroke subtypes in a Bangladesh hospital-based study. Journal of Epidemiology 2001;11:131-8.
25. Clark ZE, Bowen DJ, Whatley SD, Bellamy MF, Collins PW and McDowell IFW: Genotyping method for methylenetetrahydrofolate reductase (C677T) thermolabile variant using heteroduplex technology. Clinical Chemistry 1998;44(11):2360-2362.
26. Clarke R, Joachim C, Esiri M, Morris J, Bungay H, Molyneux A, Budge M, Frost C, King E, Barnetson L, Smith AD: Leukoaraiosis at presentation and disease progression during follow-up in histologically confirmed cases of dementia. Annals of the New York Academy of Sciences 2000;903:497-500.
27. Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW Roses AD, Haines JL, Pericah-Vance MA: Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science 1993;261:828-9.
28. Crook R, Hardy J, Duff K: Single-day Apolipoprotein E genotyping. Journal of Neuroscience Methods 1994;53:125-7.
29. Cumming AM, Keeney S, Salden A, Bhavnani M, Shwe KH, Hay CRM: The prothrombin gene G20210A variant: prevalence in a U.K. anticoagulant clinic population. British Journal of Haematology 1997;98:353-5.

30. Cunha ALA, Hirata MH, Kim CA, Guerra-Shinohara EM, Nonoyama K, Hirata RDC: Metabolic effects of C677T and A1298C mutations at the MTHFR gene in Brazilian children with neural tube defects. Clinica Chimica Acta 2002;318:139-43.
31. Danneberg J, Abbes AP, Bruggeman BJM, EngelH, Gerrits J and Martens A: Reliable genotyping of the G-20210-A mutation of coagulation factor II (prothrombin). Clinical Chemistry 1998;44(2):349-350.
32. Davignon J, Gregg RE, Sing CF: Apolipoprotein E polymorphism and atherosclerosis. Arteriosclerosis1988;8:1-21.
33. De Lucia D, Papa ML, Ammendola F, Pezzela S, Del Giudice V, Marotta R, Renis V, Di Mauro C, Maisto G, Masi S, Nina P, Franco A, Schisano G: Association of elevated level of prothrombin fragment 1+2 and Arg506 to Gln mutation in patients with a history of ischemic stroke. Journal of Neurosurgury Sciences 1999;43:45-50.
34. Den Heier M, Koster T, Blom HJ, Bos GMJ, Briet E, Reitsma PH, Vandenbroucke JP, Rosendaal FR: Hyperhomocysteinemia as a risk factor for deep-vein thrombosis. New England Journal of Medicine 1996;334:759-62.
35. De Stefano V, Chiusolo P, Paciaroni K, Casorelli I, Rossi E, Molinari M, Servidei S, Tonali PA, Leone G: Prothrombin G20210A mutant genotype is a risk factor for cerebrovascular ischemic disease in young patients. Blood 1998;91:3562-5.
36. Doi Y, Yoshinari M, Yoshizumi H, Ibayashi S, Wakisaka M, Fujishima M: Polymorphism of the angiotensin-converting enzyme (ACE) gene in patients with thrombotic brain infarction. Atherosclerosis 1997;132:145-50.
37. Elbaz A, Amarenco P: Genetic susceptibility and ischemic stroke. Current Opinion of Neurology 1999;12:47-55.
38. Eskandari MK, Bontempo FA, Hassett AC, Furaki H, Makaroun MS: Arterial thromboembolic events in patients with the factor V Leiden mutation. American Journal of Surgery 1998;176:122-5.
39. Fatini C, Gensini F, Sticchi E, Battaglini B, Angotti C, Conforti ML, Generini S, Pignone A, Abbate R, Matucci-Cerinic M: High prevalence of polymorphisms of angiotensin-converting enzyme (I/D) and endothelial nitric oxide synthase (Glu298Asp) in patients with systemic sclerosis. American Journal of Medicine 2002;112(7):540-4.
40. Fernandez-Arcas N, Dieguez-Lucena JL, Munoz-Moran E, Ruiz-Galdon M, Espinosa-Caliani S, Aranda-Lara P, Martinez-Espigares S, Banderas-Donaire MJ, Teresa-Galvan ED, Reyes-Engel A: The genotype interactions of methylenetetrahydrofolate reductase

and renin-angiotensin system genes are associated with myocardial infarction. Atherosclerosis 1999;145:293-300.

41. Foy CA, Parkes HC: Emerging homogeneous DNA-based technologies in the clinical laboratory. Clinical Chemistry 2001;47(6):990-1000.
42. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthewes RG, Boers GJH, den Heijer M, Kluijtmans LAJ, van den Heuvel LP, Rozan R: A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nature Genetics 1995;10:111-3.
43. Garcin CA, Ahmadian A, Gharizadeh B, Lundeberg J, Ronaghi M, Nyren P: Mutation detection by pyrosequencing: sequencing of exon 5-8 of the p53 tumor suppressor gene. Gene 2000;253(2):249-57.
44. Gaustadnes M, Rudiger N, Moller J, Rasmussen K, Bjerregaard LarsenT, Ingerslev J: Thrombophilic predisposition in stroke and venous thromboembolism in Danish patients. Blood Coagulation and Fibrinolysis 1999;10:251-9.
45. Gharizadeh B, Kalantari M, Garcia CA, Johansson B, Nyren P: Typing of human papillomavirus by pyrosequencing. Laboratory Investigations 2001;81(5):673-9.
46. Giardano P, De Lucia D, Coppola B, Iolascon A: Homozygous prothrombin gene mutation and ischemic cerebrovascular disease. A case report. Acta Haematologica 1999;102:101-3.
47. Giesendorf BAJ, Vet JAM, Tyagi S, Mensink EJMG, Trijbels FJM, Blom HJ: Molecular beacons: a new approach for semiautomated mutation analysis. Clinical Chemistry 1998;44(3):482-6.
48. Gershon D: Microrray technology. An array of opportunities. Nature 2002;416:885-93.
49. Gershon D: DIY or off the shelf? Nature 2002;416:885-6.
50. Greengard J S, Xu X, Gandrile S and Griffin J H: Alternative PCR method for diagnosis of mutation causing activated protein C resistant Gln506-Factor V. Thrombosis Research 1995;80:441-443.
51. Gundry CN, Bernard PS, Herrmann MG, Reed GH,Wittwer CT: Rapid F508del and F508C assay using fluorescent hybridization probes. Genetic Testing 1999;3:365-70.
52. Harmon DL, Doyle RM, Meleady R, Doyle M, Shields DC, Barry R, Coakley D, Graham IM, Whitehead AS: Genetic analysis of the thermolabile variant of 5,10-methylenetetrafolate reductase as a risk factor for ischaemic stroke. Arteriosclerosis, Thrombosis, and Vascular Biology 1999;19:208-11.
53. Hassan A, Markus HS: Genetics and ischaemic stroke. Brain 2000;123:1784-812.

54. Hooper NM: Angiotensin converting enzyme: implications from molecular biology for its physiological functions. International Journal of Biochemistry 1991;23.641-7.

55. Hoppe C, Matsunaga A: Pediatric thrombosis. Pediatric Clinics of North America 2002;49(6):1257-67.

56. Huberfeld G, Kubis N, Lot G, Ripoll L, Chaine P, Drouet L, Woimant F: G20210A prothrombin gene mutation in two siblings with cerebral venous thrombosis. Neurology 1998;51:316-7.

57. Huisman MV, Rosendaal F: Thrombophilia. Current Opinion of Hematology 1999;6(5):291-7.

58. Kohli-Seth R, Oropello JM: The future of bedside monitoring. Critical Care Clinics 2000;16(4):557-78.

59. Kondrashov FA, Rogozin IB, Wolf YI, Koonin EV: Selection in the evolution of gene duplications. Genome Biology 2002;3(2):1-9.

60. Kontula K, Ylikorkala A, Miettinen H, Vuorio A, Kauppinen-Makelin R, Hamalainen L, Palomaki H, Kaste M: Arg506Gln factor V mutation (factor V Leiden) in patients with ischaemic cerebrovascular disease and survivors of myocardial infarction. Thrombosis Haemostasis 1995;73:558-60.

61. Lachmeijer AMA, Arngrimsson R, Bastiaans EJ, Pals G, ten Kate LP, de Vries JIP, Kostense PJ, Aarnoudse JG, Dekker GA: Mutations in the gene for methylenetetrahydrofolate reductase, homocysteine levels, and vitamin status in women with a history of preeclampsia. American Journal of Obstetrics and Gynecology 2001;184(3):394-402.

62. Lay MJ, Wittwer CT: Real-time fluorescence genotyping of factor V Leiden during rapid-cycle PCR. Clinical Chemistry 1997;43(12):2262-7.

63. Lin MH, Tseng CH, Tseng CC, Huang CH, Chong CK, Tseng CP: Real-time PCR for rapid genotyping of angiotensin-converting enzyme insertion/deletion polymorphism. Clinical Biochemistry 2001;34:661-6.

64. Lindpaintner K, Pfeffer MA, Kreutz R, Stampfer MJ, Grodstein F, LaMotte F, Buring J, Hennekens CH: A prospective evaluation of an angiotensin-converting-enzyme gene polymorphism and the risk of ischemic heart disease. New England Journal of Medicine 1995;332:706-11.

65. Little M, Andrews J, Moore R, Bustos S, Jones L, Embres C, Strand displacement amplification and homogeneous real-time detection incorporated in a second-generation DNA probe system, BDProbeTecET. Clinical Chemistry 1999;45:777-784

66. Livak K, Flood S, Marmaro J, Giusti W, Deetz K: Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Application 1995;4:357-362.

67. MacLeod MJ, De Lange RP, Breen G, Meiklejohn D, Lemmon H, St Clair D: Lack of association between apolipoprotein E genotype and ischaemic stroke in a Scottish population. European Journal of Clinical Investigation 2001;31:570-3.

68. Malik FS, Lavie CJ, Mehra MR, Milani RV, Re RN: Renin-angiotensin system: genes to bedside. American Heart Journal 1997;134(3):514-26.

69. McGlennen RC: Miniaturization technologies for molecular diagnostics. Clinical Chemistry 2001;47(3):393-401.

70. Miller SA, Dykes DD, Polesky HF: A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research 1998;16:1215-8.

71. Nakai K, Fusazaki T, Zhang T, Shiroto T, Osawa M, Kamata J, Itoh M, Nakai K, Habano W, Kiuchi T, Yamamori S, Hiramori K: Polymorphism of the apolipoprotein E and angiotensin I converting enzyme genes in Japanese patients with myocardial infarction. Coronary Artery Disease 1998;9(6):329-34.

72. Nakata Y, Katsuya T, Takami S, Sato N, Fu Y, Isikawa K, Takiuchi S, Rakugi H, Miki T, Higaki J, Ogihara T: Methylenetetrahydrofolate reductase gene polymorphism: relation to blood pressure and cerebrovascular disease. American Journal of Hypertension 1998;11:1019-23.

73. Okamura T, Kitamura A, Moriyama Y, Imano H, Sato S, Terao A, Naito Y, Nakagawa Y, Kiyama M, Tamura Y, Iida M, Suzuki H, Komachi Y: Plasma level of homocysteine is correlated to extracranial carotid-artery atherosclerosis in non-hypertensive Japanese. Journal of Cardiovascular Risk 1999;6:371-7.

74. O'Meara D, Wilbe K, Leitner T, Hejdeman B, Albert J, Lundeberg J: Monitoring resistance to human immunodeficiency virus type 1 protease inhibitors by pyrosequencing. Journal of Clinical Microbiology 2001;39(2):464-73.

75. Perdro-Botet J, Senti M, Rubies-Prat J: Apolipoprotein E polymorphism and ischaemic cerebrovascular disease. Stroke 1994;25:521.

76. . Perry IJ: Homocysteine, hypertension and stroke. Journal of Human Hypertension 1999;13:289-93.

77. Press RD, Beamer N, Evans A, Deloughery TG, Coull BM: Role of a common mutation in the homocysteine regulatory enzyme methylenetetrahydrofolate reductase in ischaemic stroke. Diagnostic Molecular Pathology 1999;8:54-8.

78. Put NMJ, Gabreels F, Stevens EMB, Smeitink JAM, Trijbels FJM, Eskes TKAB, Heuvel LPVD, Blom HJ: A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects. American Journal of Human Genetics 1998;62:1044-51.

79. Rasmussen R: Quantification on the LightCycler. In: Meuer S, Wittwer C, Nakagawara K: Rapid cycle real-time PCR. Springer Verlag Berlin Heidelberg New York 2001;21-34.

80. Rees DC, Cox M, Clegg JB: World distribution of factor V Leiden. Lancet 1995;346(8983):1133-4.

81. Reiss J: The polymerase chain reaction and its potential role in clinical diagnostics and research. Journal of Internal Medicine 1991;230:391-5.

82. Ridker PM, Hennekens CH, Miletich JP: G20210A mutation in prothrombin gene and risk of myocardial infarction, stroke, and venous thrombosis in large cohort of US men. Circulation 1999;99:999-1004.

83. Rigat B, Hubert C, Corvol P, Soubrier F. PCR detection of the insertion/deletion polymorphism of the human angiotensin converting enzyme gene (DCP1) (dipeptidyl carboxypeptidase 1). Nucleic Acids Research 1992;20:1433.

84. Ririe K, Rasmussen R, Wittwer C. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Analitical Biochemistry 1997;245:154-160.

85. Ruitz-Ponte c, Loidi L, Vega A, Carracedo A, Barros F: Rapid real-time fluorescent PCR gene dosage test for the diagnosis of DNA duplications and deletions. Clinical Chemistry 2000;46(10):1574-82.

86. Sakai E, Tajima M, Mori M, Inage R, Fukumoto M, Nakagawara K: Genotyping of angotensin-converting enzyme and angiotensinogen polymorphisms with the LightCycler System. In: Meuer S, Wittwer C, Nakagawara K: Rapid cycle real-time PCR. Springer Verlag Berlin Heidelberg New York 2001;135-42.

87. Salooja N, Catto A, Carter A, Tudenham EG, Grant PJ: Methylenetetrahydrofolate reductase C677T genotype and stroke. Clinical Laboratory of Haematology 1998;20:357-61.

88. Schindler K, Zauner C, Buchmayer H, Fodinger M, Wolfl G, Bieglmayer C, Heinz G, Wilfing A, Horl WH, Sunder-Plassmann G: High prevalence of hyperhomocysteinemia in critically ill patients. Critical Care Medicine 2000;28(4):991-5.

89. Schroder W, Koesling M, Wulff K, Wehnert M, Herrmann FH: Large-scale screening for factor V Leiden mutation in a north-eastern German population. Haemostasis 1996;26(5):233-6.

90. Shangmugam V, Sell KW, Saha BK: Mistyping ACE heterozygotes. PCR Methods Application 1993;3:120-1.

91. Shi MM: Enabling large-scale pharmacogenetic studies by high-throughput mutation detection and genotyping technologies. Clinical Chemistry 2001;47(2):164-72.

92. Singer DRJ, Missouris CG, Jeffery S: Angiotensin-converting enzyme gene polymorphism. What to do about the confusion? Circulation 1996;94:236-9.

93. Smit ML, Giesendorf BAJ, Vet JAM, Trijbels FJM, Blom HJ: Semiautomated DNA mutation analysis using a robotic workstation and molecular beacons. Clinical Chemistry 2001;47(4):739-44.

94. Somogyvári F, Szolnoki Z, Márki-Zay J, Fodor L, Szabó M: Rapid Single-Tube Genotyping of the Methylenetetrahydrofolate Reductase Gene C677T and A1298C Polymorphisms by Real-Time PCR Using Dual-Colour Detection. Journal of Laboratory Medicine 2002;26(3/4):224. (Abstract)

95. Szalai C, Császár A, Czinner A, Szabó T: Apolipoprotein E és A-IV polimorfizmusok gyakorisága és összefüggése a szérum lipidszintekkel IDDM-es gyermekekben. (Frequencies of apolipoprotein E and A-IV polymorphism and correlation with serum lipid levels in children with IDDM.) Klinikai Kísérletes Laboratóriumi Medicina 2000;26(1):21-4.

96. Szén L, Kremmer T, Horányi M, Sas G: A protrombinképzés örökletes variánsa (F II 20210 A): a trombózishajlam lehetséges oka. (Inherited variant of prothrombin formation (F II 20210 A): possible cause of thrombosis tendency) Lege Artis Medicinae 1997;7(7-8):500-3.

97. Szolnoki Z, Somogyvári F, Kondacs A, Szabó M, Fodor L: Evaluation of the roles of the Leiden V mutation and ACE I/D polymorphism in subtypes of ischaemic stroke. Journal of Neurology 2001;248(9):756-61.

98. Swibertus R, Poort R, Frits R, Rosendaal R, Pieter H, Retsima H, Bertina RM: A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood 1996;88(10):3698-703.

99. Takami S, Imai Y, Katsuya T, Ohkubo T, Tsuji I, Nagai K, Satoh H, Hisamichi S, Higaki J, Ogihara T: Gene polymorphism of the renin-angiotensin system associated with risk for lacunar infarction. The Ohasama study. American Journal of Hypertension 2000;13:121-7.

100. Tang YW, Procop GW, Persing DH: Molecular diagnostics of infectious diseases. Clinical Chemistry 1997;43(11):2021-38.

101. Tiret L, Rigat B, Visvikis S et al.: Evidence from combined segregation and linkage analysis that a variant of the angiotensin I converting enzyme (ACE) controls plasma ACE levels. American Journal of Human Genetics 1992;51:197-205.

102. Topic E, Timundic AM, Ttefanovic M, Demarin V, Vukovic V, Lovrencic-Huzjan A, Zuntar I: Polymorphism of apoprotein E (APOE), methylenetetrahydrofolate reductase (MTHFR) and paraoxonase (PON1) genes in patients with cerebrovascular disease. Clinical Chemistry and Laboratory Medicine 2001;39:346-50.

103. Traykov L, Rigaud AS, Baudic S, Smagghe A, Boller F, Forette F: Apolipoprotein E ε4 allele frequency in demented and cognitively impaired patients with and without cerebrovascular disease. Journal of the Neurological Sciences 2002;203-204:177-81.

104. Tsuboi Y, Josephs KA, Cookson N, Dickson DW: APOE E4 is a determinant for Alzheimer type pathology in progressive supranuclear palsy. Neurology 2003;60(2):240-5.

105. Tyagi S, Kramer F. Molecular Beacons: probes that fluoresce upon hybridization. Nature Biotechnology 1996;14:303-308.

106. Ueda S, Heeley RP, Lees KR, Elliott HL, Connel JM. Mistyping of the human angiotensin-converting enzyme gene polymorphism: frequency, causes and possible methods to avoid errors in typing. Journal of Molecular Endocrinology 1996;1:27-30.

107. Zee RY, Ridker PM, Stampfer MJ, Hennekens CH, Lindpainter K: Prospective evaluation of the angiotensin-converting enzyme insertion/deletion polymorphism and the risk of stroke. Circulation 1999;99:340-3.

108. Zenz W, Bodo Z, Plotho J, Streif W, Male C, Bernert G, Rauter L, Ebetsberger G, Kaltenbrunner K, Kurnik P, Lischka A, Paky F, Ploier R, Hofler G, Mannhalter C, Muntean W: Factor V Leiden and prothrombin gene G20210A variant in children with ischemic stroke. Thrombosis Haemostasis 1998;80:763-6.

109. Wang DG, Fan JB, Siao CJ, Berno A, Young P, Sapolsky R, Ghandour G, Perkins N, Winchester E, Spencer J, Kruglyak L, Stein L, Hsie L, Topaloglou T, Hubbell E, Robinson E, Mittmann M, Morris MS, Shen N, Kilburu D, Rioux J, Nasbaum C, Rozen S, hudson TJ, Lander ES et al.: Large-scale identification, mapping, and genotyping of single-nucleotide polymorphism in the human genome. Science 1998;280(5366):1077-82.

110. Weissensteiner T, Lanchburg JS: Strategy for controlling preferential amplification and avoiding false negative in PCR typing. Biotechniques 1996;21:1102-8.

111. Williamson D, Brown K, Luddington R, Baglin C and Baglin T: Factor V Cambridge: a new mutation (Arg306 -Thr) associated with resistance to activated protein C. Blood 1998;91:1140-4.

112. Whitcombe D, Theaker J, Guy S, Brown T, Little S. Detection of PCR products using self-probing amplicons and fluorescence. Nature Biotechnolology 1999;17:804-807.

113. Wittwer C: Rapid cycle real-time PCR: methods and applications. In: Meuer S, Wittwer C, Nakagawara K: Rapid cycle real-time PCR. Springer Verlag Berlin Heidelberg New York 2001;11-20.

114. Wittwer C, Ririe K, Andrew R, David D, Gundry R, Ballis U. The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. Biotechniques 1997;22:176-181.

115. Wolk D, Mitchell S, Patel R: Principles of molecular microbiology testing methods. Infectious Disease Clinics of North America 2001;15(4):

Abstracts and other publications

Articles:

Szolnoki Z, **Somogyvári F**, Kondacs A, Szabó M, Fodor L:
Evaluation of the roles of common genetic mutations in leukoaraiosis.
Acta Neurologica Scandinavica 2001;104:281-7.

Szolnoki Z, **Somogyvári F**, Kondacs A, Szabó M, Fodor L:
Evaluation of the roles of the Leiden V mutation and ACE I/D polymorphism in subtypes of ischaemic stroke.
Journal of Neurology 2001;248(9):756-61.

Szolnoki Z, Szabó M, **Somogyvári F**:
Genotype predisposition to leukoaraiosis.
Journal of Neurology, Neurosurgery, and Psychiatry 2002;73(3):352-3.

Szolnoki Z, **Somogyvári F**, Kondacs A, Szabó M, Fodor L:
Evaluation of the interactions of common genetic mutations in stroke subtypes.
Journal of Neurology 2002;249(10):1391-7.

Posters:

Szolnoki Z, **Somogyvári F**, Szólics M, Szabó M:
Common genetic mutations as possible etiological factors in stroke (case report).
31st International Danube Symposium for Neurological Sciences and Continuing Education 25-28 August, 1999 Szeged, Hungary. Abstract book P-147.
Cephalalgia Hungarica 1999;5:60.

Somogyvári F, Szolnoki Z, Márki-Zay J, Fodor L, Szabó M:
The possible role of common genetic mutations in thrombotic processes.
Third Symposium on Molecular Diagnostics in Laboratory Medicine. Graz, 3-6 May, 2000.
Journal of Laboratory Medicine 2000;24(4):218.

Szolnoki Z, **Somogyvári F**, Szabó L, Fodor L:
Clustering of common genetic mutations may possibly cause thrombotic processes in a clinically healthy circulatory system.
10th Meeting of the European Neurological Society. Jerusalem, Israel, 18-22 June, 2000.
Journal of Neurology 2000;247(Suppl 3):192.

Szolnoki Z, **Somogyvari F**, Szabo M:
Evaluation of the roles of the Leiden V mutation and the ACE I/D polymorphism in subtypes of stroke.
XVII World Congress of Neurology, London, 17-22 June, 2001.
Journal of the Neurological Sciences 2001;187(Suppl 1):191.

Somogyvári F, Szolnoki Z, Márki-Zay J, Fodor L, Szabó M:
Rapid Single-Tube Genotyping of the Methylenetetrahydrofolate Reductase Gene C677T and A1298C Polymorphisms by Real-Time PCR Using Dual-Colour Detection
Fourth Symposium on Molecular Diagnostics in Laboratory Medicine. Graz, 1-4 May, 2002.
Journal of Laboratory Medicine 2002;26(3/4):224.

Abbreviations

ACE	angiotensin-converting enzyme
ApoE	apolipoprotein E
ATP	adenosine triphosphate
bp	base pair
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide triphosphates
dsDNA	double-stranded DNA
FRET	fluorescence resonance energy transfer
LCRed640	LightCycler Red 640 fluorescent dye
LCRed705	LightCycler Red 705 fluorescent dye
MTHFR	methylenetetrahydrofolate reductase
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
T_m(°C)	melting temperature

Acknowledgements

My grateful thanks are due to Dr habil. Lajos Fodor, who made this work possible by providing me with all the material basis necessary to carry out experimental work, together with his professional advice and friendly encouragement.

I wish to convey similar thanks to Professor Erzsébet Nagy and Dr Judit Deák, who supported and encouraged my work in the final stages.

I owe special thanks and recognition to Dr Zoltán Szolnoki, without his invaluable help and guidance in the clinical subjects this work would never have been done. I consider him my mentor in topics relating to stroke and related genetic mutations.

I also wish to acknowledge the valuable contributions by Professor Béla Melegh and Dr János Márki-Zay to the experimental work in different parts of this study and to scientific consultations on related matters.

I would like to express my deep appreciation to Professor László Seres, Dr Sándor Tóth and Dr David Durham for their professional advice.

My warmest thanks are due to my supervisor, Professor Yvette Mándi, who guided me throughout the whole process, for her kindness, wisdom and humanity.

Most of all, I wish to dedicate this work to my late wife Dr Ildikó Seres. Without her, I could not have achieved what I have so far, and would not have been able to perform this work. I would like to honour her memory by completing and presenting this study.