Application of real-time PCR methods in population genetics

Ph.D. Thesis

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Introduction

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 since, besides the clinical picture, the genetic background of the host may contribute to the aetiology and pathogenesis of different diseases.

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.

Many currently used techniques rely on the polymerase chain reaction (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.

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, 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.

Ischaemic stroke is considered to be a multifactorial disease. Several genetic mutations and polymorphisms can be associated with stroke. The roles of factor V mutations such as the Leiden G1691A, R306T Cambridge and R306G Hong Kong 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. 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.

The effects of angiotensin-converting enzyme (ACE) I/D polymorphism, methylenetetrahydrofolate reductase (MTHFR) C677T mutations and the apolipoprotein E (ApoE) genotypes in the pathogenesis of stroke has not yet been fully elucidated. Although all these mutations can be associated with arteriosclerosis, thrombophilia or vasoregulation disturbances on a pathophysiological basis, none of them in themselves have been proved to be a major genetic risk factor for ischaemic stroke.

Aims of the study

I. 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 a 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.

II. 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 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.

Patients, materials and methods

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 trans-oesophageal echocardiography where appropriate, and magnetic resonance imaging examinations within 2 days after the first observation of the symptoms.

Genetic examinations

The genetic examinations were carried out without a 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.

Results

The genotyping procedure

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 LightCycler differs in the optimum PCR parameters from the block PCR. Consequently, we had to optimize the PCR reaction. The optimum of the $MgCl_2$, DMSO, primer and probe concentrations were 2 mM, 5%, 0.2 μM/L and 0.1 μM/L, respectively. We found the optimum annealing temperature to be 61 °C.

Patients' description

406 Hungarian patients diagnosed with acutely developing ischaemic stroke were examined.

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.

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%.

Discussion

There were no essential problems in the application of the described real-time methods in the LightCycler.

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 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%.

In order to reduce the time requirements, we suggest a rapid PCR with fluorescently labelled oligonucleotide hybridisation probes on the LightCycler™ instrument (Roche Diagnostics) and subsequent fluorescent probe melting point analysis.

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.

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.

II. In the published clinical studies, the allele frequency of the Leiden mutation in different European areas has been estimated to be 4-7%, and the allele frequency of the prothrombin G20210A mutation as 1-2%. In the case of the MTHFR C677T mutation, the allele frequency is 36-38% in the Caucasian population. The frequency of the ACE D allele was found to be 44% in studies on large numbers of patients (n>1000).

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%. 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.

Conclusions

I. 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.

II. 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.

The R306G Hong Kong and R306T Cambridge mutations could not be detected in any of our examined subjects. 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.

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

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.

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.

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.

Abstracts and other publications

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Evaluation of the roles of common genetic mutations in leukoaraiosis.
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Genotype predisposition to leukoaraiosis.
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Fourth Symposium on Molecular Diagnostics in Laboratory Medicine. Graz, 1-4 May, 2002.
Journal of Laboratory Medicine 2002;26(3/4):224.

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