Investigation of genetic polymorphisms of ABC transporters by melting curve analysis after asymmetric PCR

Ph.D Thesis

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Introduction

Ischemic heart disease (CHD) is the leading cause of death, and stroke is the third commonest cause of death worldwide. Both are multifactorial disorders, those classic risk factors include hypertension, elevated serum cholesterol and LDL (low density lipoprotein) levels, increased alcohol intake, smoking, etc. In contrast, elevated HDL (high density lipoprotein) level has a protective effect against disease manifestation. The classic risk factors account for approx. 60-75% of these diseases, while in the development of the remaining of cases other (for example genetic) factors may play an important role. Familial enrichment of diseases and the results of twin studies are both evidences for genetic predisposition of these polygenic disorders. In addition, the importance of genetic factors is also proved by the hereditary component of the development of the classical risk factors.

Studies of the genetic background of multifactorial disorders contributes to the understanding of disease pathomechanism and function of proteins involved in disease developing processes. In the future, these results can be applied in personalized treatment or they can point to new medical targets.

The ABC (ATP-binding cassette) transporters make up a large protein superfamily. They transport a wide variety of substrates across extra- and intracellular membranes. To date, 13 diseases are known to be caused by 14 ABC transporters. Several ABC transporters play a role in lipid metabolism. Mutations in the genes of ABCA1 and ABCG5/ABCG8 cause rare autosomal recessive disorders, namely Tangier disease and sitosterolemia. Tangier disease is caused by mutations in ABCA1 gene and is characterized by an almost complete absence of serum HDL. The ABCA1 protein is expressed in several cell types: macrophages, endothel

cells, hepatocytes and adipose cells. ABCA1 functions as a homodimer and mediates the efflux of cholesterol to apolipoprotein-A1, thus has an important role in HDL formation. Sitosterolemia is caused by mutations in ABCG5 or ABCG8 genes and is characterized by elevated serum plant sterol (mainly β-sitosterol) levels and premature development of xanthomas. ABCG5/ABCG8 function as a heterodimer localized in the apical surface of enterocytes and hepatocytes and promotes plant sterol efflux into the bile or to the lumen of intestine. Both diseases can lead to premature atherosclerosis or even to CHD, as a complication.

Mutations in any of ABCA1, ABCG5 and ABCG8 lead to the defect of lipid metabolism, and may lead to lipid metabolism associated disorders. However, considerable numbers of missense polymorphisms are also identified in the above genes. The importance of these polymorphisms in lipid metabolism and in lipid metabolism related disorders (such as stroke or CHD) is still unclear. ABCA1 R219K, V771M, I883M variants and ABCG8 D19H, Y54C, T400K, A632V variants have been investigated in many human clinical studies. Among others, serum lipid level alterations and genotype-CHD association were also studied, but in many of these issues published data are controversial, or results could not be proved by other research groups.

Aims

Our aims were to investigate associations between the polymorphism of two ABC transporters, which are involved in lipid metabolism and two multifactorial disorders (namely ischemic stroke and CHD); and to investigate associations of mentioned polymorphisms and serum lipid levels.

First, our aim was the optimization of our allele-discrimination method. Based on previous results, melting curve analysis seemed to be more reliable and effective after asymmetric PCR than after symmetric PCR conditions. Therefore we aimed to determine the optimal primer ratio, and test whether asymmetric PCR could be adapted to other gene settings.

Second, our aim was to investigate associations between missense polymorphisms of two ABC transporters (ABCA1: R219K, V771M, I883M; ABCG8: D19H, Y54C, T400K, A632V) in groups of cerebro- and cardiovascular patients. To reveal potential polymorphism-disease association we aimed: (1) to determine AFs and genotype frequencies of polymorphisms in stroke, CHD and control groups; (2) to compare AFs and genotype frequencies between groups of patients and controls; (3) to compare AFs and genotype frequencies between subgroups of patients and controls, based on stratification on gender and/or age at disease onset. Finally, to reveal important polymorphism-lipid level association, i.e. test whether genotypes have any affect on lipid levels, we aimed to investigate correlations between genotypes and lipid (cholesterol, triglyceride [TG], LDL, HDL) levels in all investigated groups (stroke, CHD and control) separately.

Methods

Subjects

Our study involved 241 unrelated ischemic stroke patients (164 males and 77 females; mean age of disease onset: 53.4 ± 14.5 years), 148 unrelated CHD patients (107 males and 41 females; mean age of disease onset: 61.4 ± 9.3 years) and 191 healthy blood donors, as controls (92 males and 99 females; mean age: 35. ± 11.8 years)

Methods

DNA isolation was performed from anticoagulated peripheral blood with the standard ‘‘salting-out’’ procedure. Genotype analyses were based on melting curve analysis of hybridization probes on the Light-Cycler. Serum total cholesterol, TG and HDL levels were measured by standard colorimetric techniques. LDL was calculated using the Friedewald formula if total cholesterol, TG, and HDL values were all available.

Statistical analyses

We used Student’s t-test to compare areas under melting curves after symmetric and asymmetric PCR conditions. We used 2 x 2 contingency tables, Fisher’s exact test, two-sided p value and crude odds ratio with its 95% confidence interval (OR [95% CI]) for comparison of allele-frequencies (AFs) of polymorphisms of ABC transporters. Tests of Hardy-Weinberg equilibrium and linkage disequilibrium were also carried out. Normal distributions of lipid levels were tested by the method of Kolmogorov-Smirnov. The Mann-Whitney (non-parametric) test was used to compare lipid values observed in groups of individuals with different genotypes. According to the general consensus, $p<0.05$ was considered significant.

Results

Asymmetric PCR

By the use of asymmetric PCR, we successfully eliminated the inconsistent non-specific peak observed on the melting curve of our factor V (FV) Leiden mutation genotyping system after the use of symmetric PCR conditions in a part of the normal genotype cases. Beside this, use of asymmetric PCR resulted in significant (approx. 8-fold) increases in fluorescence signal intensity. To determine the optimal primer ratio, we evaluated the fluorescent signal intensity increase on melting curve analysis in four different genotyping systems (ABCG2 Q141K, FII g.20210G>A, FV Leiden, HFE H63D) subsequently to change to asymmetric PCR. After the use of excess amounts of amplification primers allowing the preferential synthesis of the strand complementary to the hybridization probes, gradual increases of fluorescent signals were observed as a consequence of using 1:3.3 and 1:6.7 primer ratios, while the use of further increase in primer ratio (1:13 or 1:16) did not show further increase in signal intensity in all the investigated systems. Fluorescent signal increased by a mean of 11.2-fold in case of an amplification primer ratio of 1:6.7 compared to symmetric primer conditions in four different SNP-genotyping systems. The use of excess amounts of the opposite amplification primers allowing the preferential synthesis of the strand, which is in competition with the hybridization probes during melting analysis, fluorescent signal intensity decreased dramatically. After PCR carried out with 3.3:1 primer ratios, it was impossible to distinguish between different genotypes.

Polymorphisms of ABCA1 and ABCG8 transporters

For investigation of ABC transporter polymorphisms in cerebro- and cardiovascular disease we carried out the genotyping of 580 cases. We first determined the AFs of polymorphisms in the Hungarian population. The AF values of the healthy controls were the following. ABCA1: R219K: 31.2%; V771M: 5.0%; I883: 12.1%; and ABCG8: D19H: 4.7%; Y54C: 40.8%; T400K: 18.8%; A632V: 21.2%. AF of V771M was lower in CHD group compared to control (1.4 ± 1.3% vs. 5.0 ± 2.2%; p=0,010). Lower AF in patients' group indicated protecting effect of the polymorphism, which was also confirmed by odds ratio (OR [95% CI]: 0.3 [0.1-0.8]). In case of other polymorphisms, no such associations were detected, neither in whole groups, nor in subgroups based on stratification by gender. Subgroups were defined based on stratification by age of disease onset in groups of stroke and CHD patient. In the group of stroke patients diagnosed under the age of 50 (n=106), frequencies of carriers of minor alleles of R219K or V771M were lower compared to control (R219K: 35.8 ± 4.7% vs. 50.3 ± 3.6%; p=0.022; OR [95% CI]: 0.6 [0.3-0.9]; V771M: 2.8 ± 1.6% vs. 9.9 ± 2.2%; p=0.035; OR [95% CI]: 0.3 [0.1-0.9]). Carriers of minor alleles of R219K or V771M may be protected against stoke under the age of 50. In case of other variants no such associations were detected in subgroups based on stratification by age of disease onset. Subgroups based on stratification by age of disease onset were further stratified by gender. Genotype-disease associations were also investigated in these subgroups. In male subgroup of stroke patients diagnosed under the age of 50 (n=62), lower frequency of ABCG8 54YY genotype was observed as compared to the male subgroup of control (24.2 ± 5.4% vs. 41.3 ± 5.1%; p=0.038; OR [95% CI]: 2.2 [1.1-4.5]). Carriers of the major allele of Y54C

may be protected against stoke under the age of 50. All investigated populations were in Hardy-Weinberg equilibrium with one exception. In the stroke group, R219K variant showed alteration from Hardy-Weinberg equilibrium. We suggested, this alteration may have been caused by a biased selection related to the disease phenotype. By linkage disequilibrium test, genetic associations were detected between the R219K-V771M and R219K-I883M variants of ABCA1; D19H-Y54C, Y54C-T400K, T400K-A632V and D19H-A632V variants of ABCG8. The carriers of 771M allele (n=37) also carried the 219K allele with only one exception (n=36). We could not demonstrate whether these two alleles are independent protecting factors or one of the above allele's protection effect is due to its linkage to the other allele.

Investigation of the association of genotypes and lipid (cholesterol, TG, LDL, HDL) levels revealed significant differences in cholesterol levels between genotypes of Y54C variant in the control group. Cases with 54YY genotype had lower level of cholesterol compared to other genotypes (n=71; median: 4.51 mM; 25-75 percentiles: 4.19-5.43 vs. n=120; median: 4.95 mM; 25-75 percentiles: 4.42-5.88; p=0.009). Similar associations between genotypes and lipid levels in patients' groups or in case of the other lipid levels were not observed.

Finally, during genotyping of the T400K variant, we revealed a new, missense genetic variant: T401S (c.1201A>T), next to the investigated variant in a particular sample.

Conclusions

Amplification primer ratios have a significant effect on genotyping based on melting curve analysis of hybridization probes. Use of excess amount of primer allowing the preferential synthesis of the strand complementary to the hybridization probes resulted in elevated efficiency and reliability of the method, while use of excess amounts of the opposite amplification primers allowing the preferential synthesis of the strand, which is in competition with the hybridization probes during melting analysis lead to inconclusive results. Asymmetric PCR has been successfully applied to resolve different types of problems in melting curve based genotyping by other research groups as well.

Alteration in efficiency of amplification primers may occur due to several reasons, for example, sub-optimal annealing temperature for one of the primers, degradation of primer quality or technical error. We suppose that, in unfavorable conditions mentioned before, unfavorable amplification conditions observed in our model system may occur, which may have a negative effect on allele discrimination. The advantage of asymmetric PCR condition lies in the fact that the above problems -- within certain limits -- could be corrected or prevented using asymmetric PCR. We suggest that, determination of optimal primer ratios should be an integral part of optimization of all genotyping systems based on melting curve analysis of hybridization probes.

Comparism of AFs or genotype frequencies of ABC transporter polymorphisms (ABCA1: R219K, V771M, I883M; ABCG8: D19H, Y54C, T400K, A632V) between patient groups and controls revealed association in case of three polymorphism with stroke, and one polymorphism with CHD. Minor allele of V771M may have a protecting effect against CHD. Under the age of 50, carriers of the minor allele of

R219K or V771M and carriers of major allele of Y54C may have a protecting effect against stroke. This protecting effect of R219K and V771M may be gender-independent, while the effect of Y54C may be male-specific. Several of investigated polymorphisms are in linkage disequilibrium with others. Minor alleles of R219K and V771M are associated to each other. In our study we could not prove, whether protecting effect of above polymorphisms are independent, or it's due to their linkage. The comparison of plasma lipid parameters and genotypic data revealed significant differences between Y54C genotypes and blood cholesterol levels in the control group, where cases with the 54YY genotype had the lowest cholesterol levels. Since reduced cholesterol level is a classical risk factor for stroke, we suggest that, the protective effect of 54Y allele in young males may be due to its cholesterol reducing effect.

In summary, we conclude that the R219K, V771M (ABCA1) and Y54C (ABCG8) polymorphisms of studied transporters directly (by altering transporter function), or indirectly (by being in linkage with a function-altering polymorphism), may play a role in lipid metabolism. Thereby they also may play a role in the manifestation of atherosclerosis, CHD or stroke. The protective effect of these polymorphisms against multifactorial diseases is minor, therefore environmental factors, or gender can significantly influence or modify these effects.

Publications

Publications with relevance to the current work

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