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Review article
Pregledni članak

INTRODUCTION TO MOLECULAR GENETIC DIAGNOSTICS

UVOD U MOLEKULARNU GENETIČKU DIJAGNOSTIKU

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Summary: Molecular genetic testing is part of modern medical practice. DNA tests are an essential part of diagnostics and genetic counseling in single gene diseases, while their application in polygenic disorders is still limited. Pharmacogenetics studies DNA variants associated with variations in drug efficacy and toxicity, and tests in this field are being developed rapidly. The main method for molecular genetic testing is the polymerase chain reaction, with a number of modifications. New methods, such as next generation sequencing and DNA microarray, should allow simultaneous analysis of a number of genes, even whole genome sequencing. Ethical concerns in molecular genetic testing are very important, along with legislation. After molecular genetic testing, interpretation of results and genetic counseling should be done by professionals. With the example of thrombophilia, we discuss questions about genetic testing, its possibilities and promises.

Keywords: molecular genetics, test, methods, genetic counselling

Kratak sadržaj: Molekularno genetički testovi su deo savremene medicinske prakse. Primenjuju se pre svega u dijagnostici i genetičkom savetovanju kod monogenskih bolesti, dok su kod poligenskih poremećaja još uvek u razvoju. Farmakogenetski testovi su vezani za ulogu DNK varijanti u različitom odgovoru na terapiju i već dobijaju punu afirmaciju. Metode izvođenja molekularno genetičkih testova su bazirane na reakciji lančane polimerizacije DNK (PCR) i njenim modifikacijama. Nove metode, kao što su sledeća generacija sekvenciranja i primena genskih čipova, omogućiće simultano dobijanje velikog broja podataka o ispitivanoj DNK, pa čak i analizu čitavog genoma. Molekularno genetičko testiranje je povezano sa striktnim etičkim postulatima, koji su i zakonski regulisani. Tumačenje rezultata testiranja i sledstveni genetički savet treba da da profesionalac. Na primeru trombofilije iznosimo savremene stavove o genetičkom testiranju, razmatramo njegove mogućnosti i nove smernice.

Ključne reči: molekularna genetika, testovi, metode, genetičko savetovanje

Introduction

Molecular genetic tests are defined as any analysis of genetic material that helps to establish diagnosis, choice of treatment, long term follow-up of a patient and family counseling. Nowadays, these tests are an integral part of medicine, available for a large number of human disorders, including single gene diseases, polygenic diseases and chromosomal syndromes (1, 2).

In the case of single gene disorders, molecular tests provide exact detection of genetic changes in symptomatic patients, as well as in asymptomatic or presymptomatic carriers. Genetic testing is an essential part of prenatal, even pre-implantation diagnosis also, usually based on a specific DNA change detected in an index patient. Even molecular testing of *post mortem* material sometimes helps in establishing a diagnosis and genetic counseling for a family. At this moment, more than 3000 single gene diseases are known, and for the majority of them molecular genetic tests are available. The direct approach in testing is predominant, which comprises exact analysis of

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the disease-causing gene with precise detection of the mutation. However, in some cases, direct analysis is not possible, usually due to technical/technological deficiencies. Then, indirect molecular genetic analysis tries to provide the necessary information. Indirect analysis is a family-based study of polymorphic markers closely linked to the disease gene. Such studies have lower sensitivity compared to direct ones, because of the possibility of separation between markers and the disease-causing mutation during DNA recombination; expected percentage of error should be ≤2% (1, 2).

Polygenic or multifactor diseases are the result of interaction between a genetic base (represented by a number of genes) and environmental factors. So, in this field, the focus of molecular testing is on susceptibility genes, i.e. gene polymorphisms that predispose to disease. Despite massive efforts and the large number of conducted associative and candidate-gene studies, progress in this area is not so impressive and strong susceptibility genes have been detected for a small proportion of disorders (3–7). Today, there are only a few genetic tests for polygenic disorders in medical practice, such as thrombophilia testing (8).

A new and promising field of application for molecular genetic tests is pharmacogenetics. Pharmacogenetics studies DNA variants associated with variations in drug efficacy and toxicity, with the final goal of developing personalized therapy, tailored for a particular individual. Pharmacogenetic tests are now available for a number of drugs, and they have been rapidly and widely adopted into clinical practice. Moreover, genotyping to predict drug response has the potential to become more widespread than genotyping to predict risk (9, 10).

Methods of molecular genetic testing

Many different methods of DNA analysis have been described and applied. In every laboratory, a method is chosen based on the required sensitivity, necessary equipment, number of samples and economic capacity (1, 2). Genomic DNA is required for genetic analysis, but quantity and quality of DNA preparation may vary, depending on the requirements of the assay. Majority of laboratories routinely purify DNA for the analysis, using some of the manual methods or commercial kits. The source of DNA could be any tissue with nuclear cells: usually, it is blood or bucal swab; in prenatal diagnosis these are chorionic villi samples, cells from amniotic fluid etc (1, 2, 10).

Most of the methods of molecular genetic analysis are based on amplification of the DNA region of interest by the polymerase chain reaction (PCR), using synthetic oligonucleotide primers and the enzyme Taq polymerase. PCR with a number of its modifications has been the golden standard in molecular genetic testing for more than 20 years (10). An important and innovative modification of standard PCR is Real Time PCR, which provides elegant and effective quantification or genotypisation of the tested material. Detection of the amplification range in real time is enabled by the measurement of increasing fluorescence from dye linked to DNA. In some cases, however, time consuming classic tests of hybridization (Southern blotting, dot blot) are still the methods of choice. Recently, the MLPA (multiplex ligation-dependent probe amplification) method has been developed as rapid and effective for detection of gene deletions/duplications as well as targeted substitutions. Since 2003, several hundred commercial MLPA assays have been available for routine usage.

Direct DNA sequencing, as an extremely sophisticated method of analysis, gives exact data about the primary structure of a particular DNA segment. Classic, famous and well established is Sanger's method of sequencing, but in the last few years several new platforms of »next generation sequencing« (NGS) have been developed, in order to provide faster and cheaper sequencing analyses (11, 12). The available personal whole genome/exome sequencing is one of the NGS goals, likely to be achieved in the next few years. Microarray format of DNA testing is also technically sophisticated, modern and has perspective, allowing more than 100.000 simultaneous microhybridization tests in one assay. Gene chips created for molecular genetic testing of some groups of diseases are based on this method (13).

Ethical concerns and interpretation of results

From an ethical point of view, in molecular genetic testing the issues of confidentiality and privacy, the use of individual-specific information and protection of individual rights are of general interest. It is postulated that the results of genetic tests should be confidential, and could not be used for discrimination in health insurance, or education and job opportunities. Vulnerable groups, such as children, should be particularly protected (14, 15). It is necessary to emphasize that the results obtained by DNA-based methods show very high specificity and sensitivity. However, for exact interpretation of results, good knowledge of the genetic basis of disease is necessary. For example, a negative result could be a consequence of the genetic heterogeneity of disease or method limitations.

Molecular genetic diagnostics: State-of-the-art in Serbia

In Serbia, the molecular genetic service is well-developed, with almost 20 years of experience. However, this kind of tests is not yet covered by health

insurance. According to recommendations for developing countries, Serbian geneticists perform molecular genetic diagnostics of cystic fibrosis, Duchenne/Becker muscular dystrophy, trinucleotide repeat disorders, spinal muscular atrophy, hemoglobinopathies, and male infertility, as well as thrombophilia testing, and a number of neurogenetic and pharmacogenetic tests. Some laboratories are members of different quality control networks, with inter-laboratory and external – foreign checking (www.dgsgenetika.org.rs/sekcije-medicinska-genetika).

The example of thrombophilia

Genetic testing in thrombophilia is a good example of the power, specificity and sensitivity of molecular genetic testing in disease management.

Standard genetic testing in thrombophilia

By definition, thrombophilia is increased tendency to develop thrombosis and its clinical manifestations, which are familial, recurrent or unusual in age and site of occurrence. Prothrombotic phenotype results from the interaction of genetic predisposing factors and »clinical« risk factors such as obesity, immobility, major and minor surgery, hormone therapy, malignancy, etc (16). The most common congenital disorders associated with thrombophilia are: a deficiency of antithrombin, protein C and protein S, variants of factor V Leiden and prothrombin 20210, and mild hyperhomocysteinemia. Individually or in combination, these traits are present in about 40% of patients with venous thromboembolism, and in approximately the same percentage of women with disorders of pregnancy and puerperium, such as fetal loss, fetal growth restriction and preeclampsia (17).

Direct molecular genetic detection of genetic thrombophilia risk factors including factor V Leiden, prothrombin G20210A, and MTHFR (methylenetetrahydrofolate-reductase) C677T mutations is offered by many clinical diagnostic laboratories (16–18). The Laboratory of Molecular Genetics at the Institute of Human Genetics, University of Belgrade School of Medicine, Belgrade, in cooperation with the Clinic of Hematology and Neurology Clinic, Clinical Center of Serbia, have been performing these analyses for one decade.

New data about the genetics of thrombophilia

In order to determine new genetic markers of thrombophilia, beside the analysis of candidate genes, genome-wide association studies (GWAS) have been performed. Recent family-based GWAS showed new association of locus rs973117 with hyperhomocysteinemia, which is considered to be an independent prothrombotic risk factor (19). This association was independent of known confounders, including creatinine clearance and plasma fibrinogen concentration. Polymorphism rs973117 is located on chromosome 9, near the PTPRD gene which encodes for receptor-type tyrosine-protein phosphatase delta, a member of the protein tyrosine phosphatase (PTP) family. PTPs are known to be signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation, but no previous data on their involvement in homocysteine metabolism exist. In the cited multicentric GWAS study, rs973117 A allele was associated with a higher homocysteine level in the cohorts GAIT (Genetic Analysis of Idiopathic Thrombophilia) and PROCARDIS (Precocious Coronary Artery Disease).

In our investigation, we have analyzed the association of polymorphism rs973117 and plasma homocysteine level in a group of Serbian patients with cerebrovascular insult (CVI). Our group consisted of 72 patients (mean age 53.75 y) with no other significant CVI risk factors. Plasma homocysteine level was measured by the HPLC (high pressure liquid chromatography) method. Genotyping of the rs973117 locus was performed by the Real-Time PCR method (ABI 7500 RT PCR System), using the predesigned TaqMan genotyping assay (Life Technologies, USA) (*Figure 1*). Initially, we found no significant association of rs973117 genotypes and plasma homocysteine ($p=0.898$) (*Figure 2*). Mean homocysteine level was 14.44 ± 6.92, 14.67 ± 4.57 and 15.18 ± 5.00 μmol/L in the AA, AC and CC genotype, respectively. However, in the group of patients under 50 y of age, a significantly higher homocysteine level in individuals with the rs973117 CC genotype has been detected ($p=0.007$). Our results confirm the role of the PTPRD gene as a novel determinant of plasma homocysteine and implicate new pathways in homocysteine metabolism, as well as new thrombophilia susceptibility loci.

Pharmacogenetics of thrombophilia

Among anticoagulant drugs, warfarin has been the standard of care for more than 50 years to prevent and treat thromboembolism. One of the major problems with its use in clinical practice is large inter-individual variability in dosage requirement. Pharmacogenetic studies showed that polymorphisms in genes VKORC1, CYP2C9, and CYP4F2 are responsible for this variability, and a genome-wide association study confirmed their role (20–22). VKORC1 (vitamin K epoxide reductase complex 1) is the target enzyme inhibited by warfarin, resulting in interruption of the recycling of vitamin K in the liver, and CYP enzymes are responsible for the metabolic clearance of S-warfarin, the more potent isomer of warfarin. It is established that approximately 30% of the dose variance is

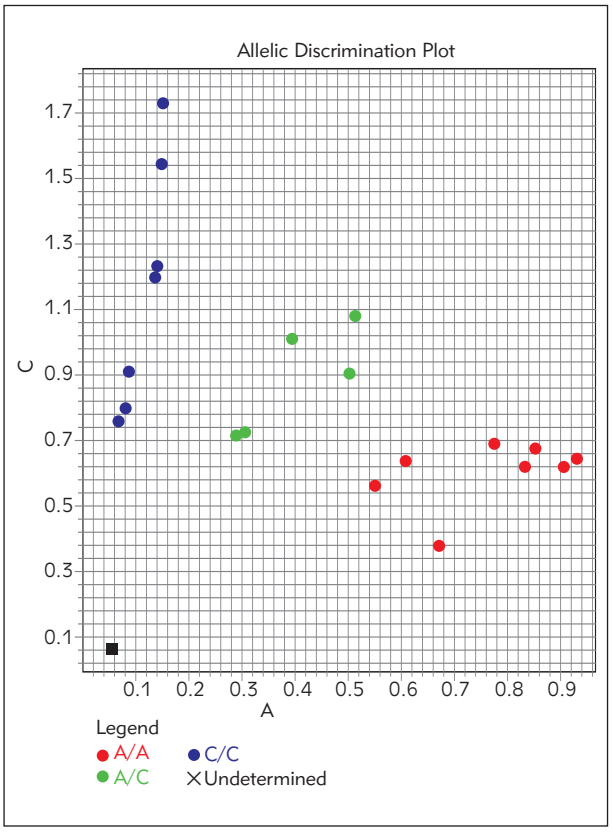

Figure 1 Results of rs973117analysis using TaqMan genotypisation assay.

explained by single nucleotide polymorphisms (SNPs) in the VKORC1 and another approximately 12% by two non-synonymous SNPs (*2, *3) in the CYP2C9 gene. Other important factors include: age, dietary vitamin K intake, the presence of other comorbidities and interaction with other drugs. A number of studies have shown that algorithms for warfarin dosing that incorporate pharmacogenomic information are better than those using clinical data alone. However, some experts think that routine genetic testing before warfarin initiation should not be recommended (23).

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

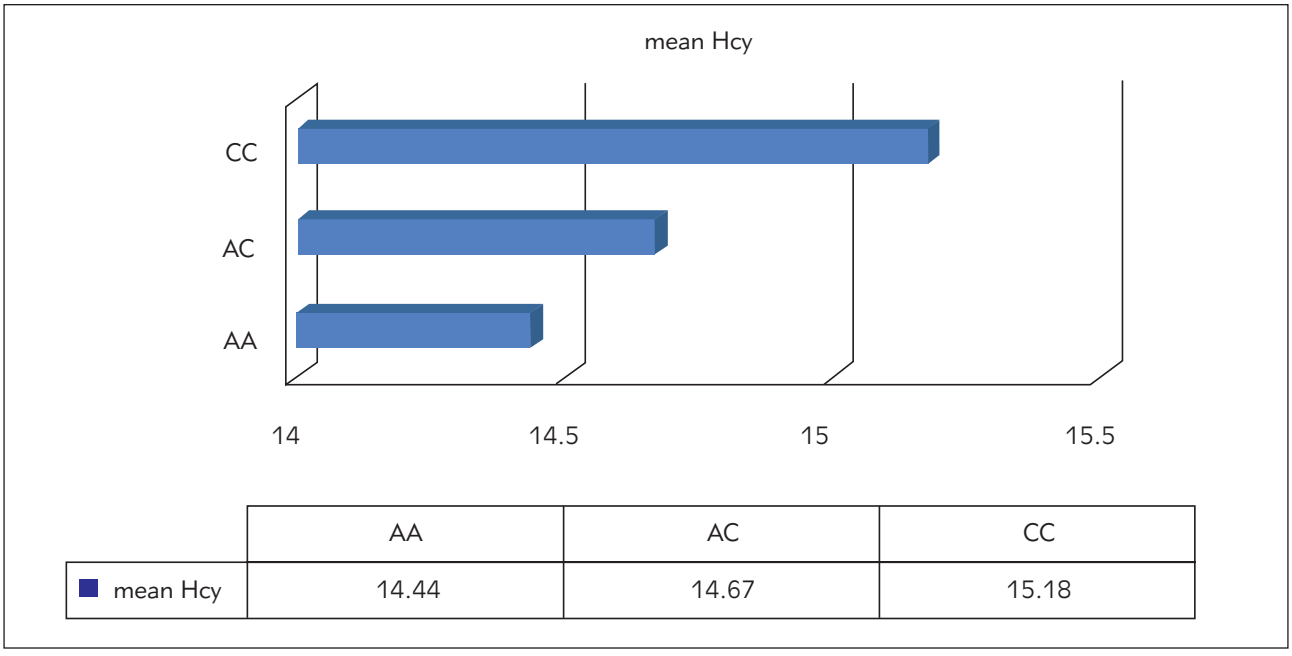

Figure 2 Mean plasma homocysteine levels (in μmol/L) in different rs973117 genotypes.

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