Application of molecular biology methods for studying hormonal regulatory mechanisms

Ph. D. theses

dr. Péter Gergics, M.D.
Semmelweis University
Clinical Medicine Doctoral School

Tutor: Prof. Károly Rácz, M.D., D.Sc.

Opponents: Dr. Éva Erhardt, M.D. Ph.D.
Dr. Anna Sebestyén, M.D. Ph.D.

Head of Examination Committee:
Prof. Ida Gerendai, M.D., D.Sc.

Members of Examination Committee:
Dr. Miklós Garami, M.D., Ph.D.
Dr. László Gábor Kovács, M.D., Ph.D.

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I. INTRODUCTION

The appropriate selection of methods from numerous molecular biologic techniques for studying the function and for understanding alterations underlying disorders of the hormonal regulatory system seems mandatory for accurate and rapid diagnosis of clinically relevant genetic abnormalities.

Glucocorticoid receptor (*GR*) gene variants (polymorphisms) may exert an important role on the sensitivity to cortisol, which is responsible for the metabolic and other effects of the hypothalamic-pituitary-adrenal axis. Decreased or increased glucocorticoid sensitivity related to these polymorphisms may increase the risk for different disorders and metabolic abnormalities and, therefore, studies on possible associations between *GR* gene polymorphisms and various disorders or clinical parameters may have clinical relevance. Several methods have been developed for the detection of the BclI polymorphism of the *GR* gene, which has a high prevalence in Caucasian population. However, these methods are labour-intensive, have a high cost and need expensive laboratory equipments and, therefore, they are hardly applicable for population-based studies.

The efficacy of genetic screening for monogenic hereditary endocrine disorders is highly dependent on the available methods. The most commonly used technique, direct DNA sequencing is able to identify disease-causing mutations and small deletions of the gene, but heterozygous copy number changes including large gene deletions cannot be detected using this method. As an example, genetic alterations of the *VHL* gene causing the hereditary von Hippel-Lindau disease include both mutations and partial or complete gene deletions. In addition, *VHL* gene abnormalities may play a role in the pathogenesis of apparently sporadic pheochromocytomas (ASP), although different data have been published on the frequency of disease-causing *VHL* gene defects in geographically different ASP populations.

II. AIMS

In my work I wanted to explore the following issues:

1. The classical methods used for the detection of the Bcl I polymorphism of the *GR* gene, which has a known influence on sensitivity to cortisol that mediates the metabolic and other effects of the hypothalamic-pituitary-adrenal axis, need high-cost equipments (real-time PCR) or are time-

consuming and expensive (Southern-blot and restriction enzyme digestion). Additionally, Southern-blot requires radiation protection equipment. Therefore, my aim was to develop a novel allele-specific PCR method for the detection of the Bcl I polymorphism of the *GR* gene which is time-, labour- and cost-saving and does not need expensive laboratory equipments. My work also included validation experiments by comparing the results obtained from the novel method to those found with the two classical methods (DNA sequencing and restriction enzyme digestion) in a large number of DNA samples in order to prove its applicability in everyday practice. Finally, I wanted to determine the frequency of the polymorphic Bcl I allele in a large cohort of healthy Hungarian subjects.

2. My aim was to identify disease-causing germline *VHL* gene abnormalities in patients with VHL disease and in their relatives, as well as in patients with ASP who were evaluated at the 2nd Department of Medicine, Faculty of Medicine, Semmelweis University between 1998 and 2008. Because VHL disease may be caused not only by mutations but also large deletions of the *VHL* gene, I performed direct DNA sequencing of the *VHL* gene and, in case of a negative result, I

applied two other methods (quantitative real-time PCR and multiple ligation probe amplification analysis, MLPA) for the detection of large deletions of the *VHL* gene. With the combined use of these techniques I wanted to develop a new screening program for the identification of disease-causing *VHL* gene defects which can be applied more efficiently than previous methods. In addition, my aim was to compare clinical and genetic findings obtained from Hungarian VHL patients to those published in recent international literature. During this analysis I also investigated the possibility of a genetic founder effect in order to clarify whether this mutation phenomenon could exist in Hungarian patients as reported in other European cohorts of VHL patients.

III. PATIENTS AND METHODS

Detection of the Bcl I polymorphism of the *GR* gene

For the development of the novel allele-specific PCR method I used the DNA samples of 247 healthy Hungarian adults (60 men and 187 women, mean age 54.5±30.5 years and 55±33 years, respectively). Genomic DNA was isolated from peripheral blood lymphocytes using commercially available standard kits. I designed allele-specific “internal” and non-

specific ”outer” oligonucleotide primers using Primer Premiere software. I separated the fragments of PCR product with agarose gel electrophoresis and visualised with 2% of ethidium-bromide staining using an ultraviolet transilluminator. For validation of the novel method I determined the Bcl I polymorphism in 50 randomly selected DNA samples with the classical restriction enzyme digestion (New England Biolabs, Bcl I enzyme) and with the gold standard direct DNA sequencing (LI-COR 4000L automated infrared DNA sequencer).

To explore whether genotype distribution and allele frequency among the 247 individuals meets the requirements of Hardy-Weinberg-equilibrium I used chi-square test with SPSS for Windows 12.0.1 software.

Patients with VHL disease or ASP

I analysed clinical data and DNA samples of 7 unrelated VHL families with 35 members and 37 unrelated histologically proven unilateral ASP patients who were evaluated at the 2nd Department of Medicine, Semmelweis University between 1998 and 2008. Initial screening included medical history, physical examination, abdominal ultrasonography, computed tomography (CT) or magnetic resonance imaging (MRI), brain

and spinal cord MRI, 131-meta-iodinebenzylguanidine (MIBG)-isotope scan, ophthalmologic examination, and laboratory tests which included routine biochemical testing and 24h urinary catecholamine metabolite determination. All patients underwent genetic counselling, and clinical as well as genetic screening was performed in all relatives who agreed the screening tests.

Patients with VHL disease

The index patients and their mutation carrier relatives were categorised into VHL subtypes based on their clinical findings and according to the international guidelines. During the follow-up no newly detected VHL-associated tumors were found in these families and, therefore, no reclassification was needed.

Patients with ASP

There were 17 men (age, mean±SD, 40.6±15.4; range, 12–67 years) and 20 women (age, mean±SD, 37.7±14.4; range, 19–63 years). Patients with bilateral and familial pheochromocytomas and those with personal or family history of syndromic disease (VHL, multiple endocrine neoplasia type 2, neurofibromatosis type 1, and familial paraganglioma syndromes) were excluded.

Clinical screening for other VHL-associated tumours was performed but no other manifestations were found.

Genetic screening for *VHL* gene mutations

Genomic DNA was isolated from peripheral blood lymphocytes using commercially available standard kits. Using an optimised PCR with newly designed oligonucleotide primers (Primer 3 software) I amplified the corresponding *VHL* gene exons with their splice regions. After purification of the PCR product direct DNA sequencing was performed (Applied Biosystems 310 Genetic Analyzer). When direct DNA sequencing showed no mutation I applied two additional methods for the detection of *VHL* gene large deletions; a SYBRGreen chemistry based novel, real-time PCR method (Power SYBRGreen PCR Master Mix; Applied Biosystems 7500 Fast Real Time PCR) and multiple ligation probe amplification (MLPA) (SALSA MLPA kit; Applied Biosystems 310 Genetic Analyzer) as a reference method. For the evaluation of the MLPA results I applied Peak Scanner and MS-Excel software. With the use of the latter two methods large gene deletion was diagnosed when the test results were under 60% and 55%, respectively, depending on the manufacturer's instructions.

IV. RESULTS

The novel allele-specific PCR method included generation of an internal control fragment and specific wild-type or mutant fragments, or both corresponding to wild-type or heterozygous or homozygous polymorphic BclI alleles, respectively. The novel PCR, restriction enzyme digestion and direct DNA sequencing gave identical result in each of the 50 randomly selected DNA samples. There were 100 homozygous wild, 124 heterozygous and 23 homozygous polymorphic genotypes among the 247 individuals examined and the frequency of the polymorphic allele was 0,344 (q=0.344). Based on the Hardy-Weinberg-equilibrium the measured and calculated genotype distribution was compared with chi-square test and the results indicated no significant differences between the measured and calculated values (0.8122. <5.9915, df=2, alpha=0.05). The measured genotype distribution was concordant with the expected population distribution and the frequency of the polymorphic allele was similar to the reported in other Caucasian populations (mean 36.2 %, between 28.9 – 55 %).

2. There has been no comprehensive study on VHL gene mutations in patients in VHL disease and ASP in

Hungary. In my work I identified 7 different disease-causing *VHL* gene defects in the 7 VHL families and 3 further disease-causing *VHL* gene mutations among the 37 unrelated ASP patients (8.1 %) evaluated at the 2nd Department of Medicine, Semmelweis University. Based on clinical evaluation 5 families were categorised into VHL type 1 while 2 families into VHL type 2. Of the 10 identified disease-causing gene defects DNA sequencing indicated one nonsense (R161X), 6 missense mutations (L158V, R167Q, S80I, L63P, Y156C and R167G) and 1 small deletion (354_355delCT) while the new real-time PCR and MLPA methods showed the presence of 2 large gene deletions (exon 2 and exon 3). Additionally, I detected a benign gene variant of the *VHL* gene (P25L) in one family. Real-time PCR melting temperature analysis showed a single high peak indicating the high specificity of the method. The *VHL* gene mutations detected in our patients were spread throughout the whole gene, and none of them were present in more than one family. With the analysis of genotype-phenotype correlations the nonsense mutation, the 2 basepair small deletion and the two large gene deletions which lead to truncated VHL protein were only found in VHL type 1 while missense mutations were mainly, but not exclusively detected in VHL type 2B and 2C. This mutational spectrum in

Hungarian VHL patients does not differ significantly from those observed in Western, Japanese, or Chinese VHL kindreds. In one of the largest studies 6 mutation hot spots were identified and two of them (R161X and R167Q) were found in our patient cohort.

In my work including 37 patients with unilateral ASP I have found 3 germline *VHL* gene mutations (8.1%). Each of the three patients had a different mutation (L63P, Y156C and R167G) and based on this finding the possibility of founder effect could be excluded. These mutations were described as disease-causing mutations in patients with VHL disease.

V. CONCLUSIONS

I developed a novel, simple allele-specific PCR method for the detection of the Bcl I polymorphism of the *GR* gene which has known influence on the sensitivity to cortisol that mediates the metabolic and other effects of the hypothalamic-pituitary-adrenal axis. I confirmed the results obtained from the novel allele-specific PCR in a large number of DNA samples with two classical method (restriction enzyme digestion and direct DNA sequencing). While preserving the high sensitivity of

earlier techniques, this novel method is time- and labour-saving with a relatively low cost and it does not need robust laboratory or radiation protection equipments. I found that the frequency of the polymorphic allele was 34.4 % in healthy Hungarian individuals, which is similar to data published in other Caucasian populations.

I performed the first comprehensive study for the detection of disease-causing *VHL* gene defects in 35 members from 7 families with VHL disease and 37 unrelated, unilateral ASP patients who were evaluated at the 2nd Department of Medicine, Faculty of Medicine, Semmelweis University between 1998-2008. Apart from the point mutations and small gene deletions which were identified with direct DNA sequencing, I detected large deletions of the *VHL* gene using quantitative real-time PCR and MLPA methods. The combined use of these methods proved to be a useful screening procedure for a more efficient detection of disease-causing genetic defects in the index patients and their relatives. I found disease-causing VHL gene defects in each of the 7 VHL families and I identified 3 disease-causing *VHL* mutations among the 37 unrelated unilateral ASP patients. The 10 disease-causing gene defects included one nonsense (R161X), 6 missense mutations (L158V, R167Q, S80I, L63P, Y156C and R167G), one small

deletion (354_355delCT) and 2 large gene deletions. These mutations were spread throughout the whole gene, and none of them were present in more than one family. With the analysis of genotype-phenotype correlations the nonsense mutation, the 2 basepair small deletion and the two large gene deletions which lead to truncated VHL protein were only found in VHL type 1 while missense mutations were mainly, but not exclusively detected in VHL type 2B and 2C. This mutational spectrum in Hungarian VHL patients does not differ significantly from those observed in Western, Japanese, or Chinese VHL kindreds. Except a small deletion (354_355delCT) each of the 10 disease-causing *VHL* gene defects were previously described in the international literature. Based on my results, *VHL* gene testing is recommended not only in patients /family members with VHL-disease but also in patients with ASP.

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