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Original scientific paper

MOLECULAR GENETIC STRATEGY FOR DIAGNOSIS OF CONGENITAL ADRENAL HYPERPLASIA IN SERBIA

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Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency is one of the most common endocrine diseases, yet genetic diagnosis is among the most complicated of all monogenic disorders. It has an overall incidence of 1:10000-1:20000, it is inherited in autosomal recessive pattern and caused by mutations affecting *CYP21A2* gene. Based on the phenotypic expression, this disease is categorized into severe, classical form revealed at birth and mild, non-classical form. Although diagnosis could be established based on biochemical tests and distinctive clinical features, molecular genetic testing is crucial for diagnosis confirmation, detection of carriers and asymptomatic patients, disease prognosis, as well as for providing proper genetic counselling and prenatal diagnosis. Based on *CYP21A2* mutational spectrum and frequencies in Serbia, in this paper we propose an optimal molecular genetic diagnostic algorithm for CAH and discuss genetic mechanisms underlying the disease. The complete diagnostic procedure combines multiplex minisequencing technique (SNaPshot PCR) as a method for rapid detection of common point mutations, direct sequencing of whole *CYP21A2* gene and PCR with sequence specific primers (PCR-SSP) for large gene rearrangements detection (*CYP21A1P/CYP21A2* chimeras). While SNaPshot PCR assay analyses ten common mutations (c.290-13A/C>G, p.P30L, p.R356W, p.G110fs, p.V281L, p.Q318X, p.L307fs, p.I172N, Cluster p.[I236N;V237E;M239K] and p.P453S) which account for over 80% of all *CYP21A2* mutations in Serbian population, direct sequencing of *CYP21A2* gene is needed to identify potential rare or novel mutations present in Serbian population with frequency of 1.8%. Additionally, large gene rearrangements which are present with frequency of 16.7% make PCR-SSP analysis an unavoidable part of molecular characterization of CAH in Serbia. Described molecular genetic strategy is intended to

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facilitate correct diagnosis assessment in CAH affected individuals and their families in Serbia but it will also contribute to molecular genetic testing of CAH patients across Europe.

Keywords: Congenital adrenal hyperplasia, *CYP21A2*, *CYP21A1P/CYP21A2* chimeras, molecular genetic diagnostic algorithm, SNaPshot PCR

INTRODUCTION

Congenital adrenal hyperplasia (CAH) is one of the most common endocrine diseases with a wide range of clinical manifestations. It comprises a group of autosomal recessive disorders characterized by impaired adrenal steroidogenesis (DOLZAN *et al*., 2003). More than 90% of all CAH cases are caused by deficiency of steroid 21-hydroxylase (21OHD) (DOLZAN *et al*., 2003, WAJNRAJCH *et al*., 2010), an enzyme essential for biosynthesis of gluco- and mineralocorticoids in adrenal gland cortex. As a result various degrees of cortisol and aldosterone deficiency, as well as androgen excess occur. Based on severity of the underlying genetic defect, this disorder is categorized into two main phenotypes: classical form, including salt wasting (SW) and simple virilizing (SV) form, and non-classical form (NC). Classical form occurs with a frequency of 1:16000-1:20000 in most populations (WHITE, 2009) and has genital virilization as a main symptom. Simple virilizing form of CAH is characterized by ambiguous external genitalia in female newborns and by hypocortisolism and precocious pseudopuberty due to androgen excess in both sexes. The most severe SW form in addition to cortisol deficiency results in aldosterone deficiency as well and adrenal crisis due to hyponatremia and hyperkalemia, which may prove to be fatal if left untreated, especially in boys (CONCOLINO *et al*., 2010). The milder NC form is more prevalent (1:1000-1:2000) (TRAPP *et al*., 2012) and manifests later in life predominantly in female patients with precocious pseudopuberty and/or hirsutism and decreased fertility. Although CAH mainly occurs due to mutations in *CYP21A2* gene encoding 21-hydroxylase, other genes coding for enzymes involved in adrenal steroidogenesis, could also be the cause of the disease. Deficiencies of CYP11B1, CYP17A1, HSD3B2 and POR are also inherited in recessive pattern and each of them is characterized by a specific biochemical profile and clinical manifestation (KRONE and ARLT, 2009).

The genes and mutations causing CAH are well characterized and their analyses are widely available. Although biochemical tests are sufficient for clinical diagnosis, they are not always straightforward and a degree of overlap between various CAH forms does exist (HUYNH *et al*., 2009). Molecular testing is useful as a complementary tool for diagnosis confirmation, genotype delineation and carrier detection. Knowledge of the underlying molecular genetic defect is also the desirable basis for proper genetic counselling of the affected families as well as prenatal diagnosis.

Over the past few decades this genetically heterogeneous disorder has been extensively studied worldwide. However, only recently a first report regarding genetic basis of CAH in Serbia has been published (MILACIC *et al*., 2015). Based on recent investigation, in this paper we discuss genetic approach used in Serbia and propose an optimal molecular diagnostic algorithm for patients affected with CAH in our country.

Genetic basis of 21-hydroxylase deficiency

The extent of 21-hydroxylase impairment, as well as resulting clinical phenotype, is determined by the severity of the underlying genetic defect in *CYP21A2* gene. The functional gene is 3.4kb long, consists of ten exons and is expressed primarily in the adrenal cortex (WHITE *et al*.,

1984; WHITE *et al*., 1986). It is mapped to the short arm of chromosome 6 (6p21.3) (CARROL *et al*., 1985; HIGASHI *et al*., 1986) in the class III region of the major histocompatibility complex (MHC). In the vicinity, 30 kb upstream, resides highly homologous *CYP21A1P* pseudogene which shares approximately 98% of exon and 96% of intron sequence identity with *CYP21A2* (WHITE *et al*., 1986). Several mutations, accumulated over time, render it inactive. These two genes together with the genes encoding complement proteins (C4A, C4B), serine/threonine nuclear proteins (RP1 and RP2) and tenascin (TNXA and B) are tandemly arranged most frequently as an RCCX bimodule (70-80%), a configuration attributed to an ancient gene duplication (GITELMAN *et al*., 1992; NEW *et al*., 2014b).

The *CYP21A2* gene is highly variable human gene with more than 200 reported mutations (HGMD, http://www.hgmd.cf.ac.uk/ac/index.php), including point mutations, small insertions, small deletions, splice site mutations, as well as large gene rearrangements. Two major molecular mechanisms leading to inactivation of *CYP21A2*, gene conversion and unequal crossing over, are result of high sequence homology between the tandemly repeated RCCX modules (CONCOLINO *et al*., 2010). Due to misalignment of sister chromatids small part of one sister chromatid can be copied to the other thus copping *CYP21A1P* sequence, potentially with a pathogenic mutation, to the *CYP21A2* gene, and vice versa. These gene conversions could be small range gene conversions copping only 50-200bp, but also multi-exon conversions copping much longer sequences. Depending on the severity of transferred pseudogene derived mutation partial or complete inactivation of enzymatic activity can occur (TUSIE-LUNA *et al*., 1990; SPEISER *et al*., 1992). The other mechanism involves large scale gene deletions (30kb) due to misalignment of homologous chromosomes in meiosis and resulting unequal crossing over. Break points can occur at *C4*, *CYP21* or *TNX* genes thus influencing the composition of the resultant hybrid. Whereas unequal crossing over at *C4* genes does not influence CYP21A2 activity, crossover at *CYP21* and *TNX* genes results in chromosomes with nonfunctional *CYP21P/CYP121A1* chimeras or completely deleted *CYP21A2* gene, respectively (NEW *et al*., 2014b). Progeny inheriting those chromosomes are at risk of 21OH deficiency. In approximately 5% of patients with CAH due to 21OH deficiency sporadic, non pseudogene derived mutation exists (CONCOLINO *et al*., 2010; LEE, 2001). Furthermore, in 1% of cases *de novo* mutations arise (CONCOLINO *et al*., 2010) via gametogenesis error or uniparental isodisomy.

Population specific differences regarding mutations and their frequencies exist among European populations (DOLZAN *et al*., 2005; WILSON *et al*., 2007). Therefore, molecular characterization of *CYP21A2* gene and documentation of the mutation spectrum and frequency found in a particular population is the first step in the development of an optimal molecular diagnostic algorithm.

Diagnosis

Worldwide implemented neonatal screening program represents a first step in assessing CAH diagnosis. Measuring 17-hydroxyprogesterone (17-OHP) concentration, the precursor of defective steroid 21-hydroxylase, in dried blood spots enables diagnosis of patients with classical CAH who are at risk of salt wasting crisis in neonatal period (THERRELL *et al*., 1998; NIMKARN *et al*., 2011). This is especially important for boys who don't show ambiguous genitalia and therefore cannot be diagnosed without neonatal screening (FALHAMMAR *et al*., 2015). It also prevents male sex assignment in affected females and reduces long-term morbidities such as short stature, gender confusion, and psychosexual disturbances (WHITE, 2009). Physicians are also urged to recognize

physical characteristics of CAH in newborns and to refer them for a full endocrinological evaluation (NEW *et al*., 2014b). Hormonal diagnosis is determined by performing a corticotropin stimulation test, measuring levels of 17-OHP and androstenedion at baseline and at 60 minutes after intravenous adrenocorticotropic hormone (ACTH) administration, but also measuring other metabolites such as dehydroepiandrosterone (DHEA), cortisol, testosterone, aldosterone and renin. Detailed hormonal profile is essential for determination of different clinical variants. However, due to considerably high false positive rate of newborn screening programs due to prematurity, sickness and stress (WHITE, 2009) and the fact that biochemical evaluation is inadequate for detection of carriers and asymptomatic patients, molecular genetic analysis of *CYP21A2* has become a useful adjunct to hormonal measurements.

The proper treatment of patients affected with CAH based on glucocorticoide replacement therapy has a goal of correcting the deficiency in cortisol secretion and supressing ACTH overproduction (NEW *et al*., 2014b). Treatment also reduces androgen pathway, thus preventing further virilization and allowing normal growth and development. When genital virilization already happened, surgical treatment is possible.

Although prenatal treatment of affected female fetuses to prevent genital virilization is possible, due to numerous contraindications it is controversial and not considered as standard of care, but an experimental procedure (NEW *et al*., 2014a). Dexamethazone is administered in the first trimester, before genital organogenesis begins (at approximately 9 weeks of gestation). Chorionic villus sampling is performed to obtain tissue for karyotyping and molecular genetic analysis. The treatment is then discontinued in male and unaffected female fetuses, and continued to the term in affected female fetuses. Recent investigation (NEW *et al*., 2014a) reported a strategy for noninvasive prenatal CAH diagnosis of fetuses at risk by analysing cell-free fetal DNA from maternal serum before the ninth week of gestation. This way only affected female fetuses would be treated.

Genotype-phenotype correlation studies have suggested that the phenotype of CAH affected individuals can be predicted with reasonable certainty by determining their genotype (NEW *et al*., 2013). Although this predictability of CAH phenotype is not straightforward, identifying predominant phenotype for a given genotype could assist physicians in prenatal diagnosis and genetic counselling of parents who are at risk of having a child with CAH.

Given the wide spectrum of clinical manifestations observed in CAH patients there is a high need for molecular genetic testing for diagnosis confirmation, as well as for carrier detection, prognosis prediction, appropriate genetic counselling and prenatal diagnosis.

Although applied in most European countries (LOEBER *et al*., 2012; BURGARD *et al*., 2012), neonatal screening for CAH has not yet been set up in Serbia and precise incidence of the disease in our population is not known. Therefore, based on a recent study on *CYP21A2* mutational spectrum and frequencies in Serbia, in this paper we propose an optimal molecular genetic diagnostic algorithm for CAH.

MATERIALS AND METHODS

This study is based on molecular characterization of the whole *CYP21A2* gene of 61 Serbian CAH patients (MILACIC *et al*., 2015). Apart from direct sequencing of the PCR product after specific amplification of the *CYP21A2* gene and detection of *CYP21A1P*/*CYP21A2* chimeras using three PCRs with sequence-specific primers (PCR-SSP) (MILACIC *et al*., 2015), we introduced

SNaPshot PCR for detection of the most common mutations in *CYP21A2* gene (KRONE *et al.*, 2002).

We optimized SNaPshot, a rapid screening procedure based on multiplex minisequencing, for detection of the ten most frequent mutations found in Serbian population: c.290-13A/C>G, p.P30L, p.R356W, p.G110fs, p.V281L, p.Q318X, p.L307fs, p.I172N, Cluster p.[I236N;V237E;M239K] and p.P453S. Each mutation detected by SNaPshot was verified by direct DNA sequencing analysis.

RESULTS AND DISCUSSION

Recent studies on molecular characterization of the *CYP21A2* gene in Serbian CAH patients identified the mutational spectrum and frequency and enabled genotype–phenotype correlation analysis (MILACIC *et al.*, 2015). These population specific data of molecular defects in *CYP21A2* gene is of both theoretical and practical interest as it could be used to guide physicians toward precise diagnosis of 21OHD (WILSON *et al.*, 2007).

Each mutation detected by SNaPshot PCR was validated by direct DNA sequencing thus confirming that SNaPshot methodology is precise for the diagnostic purposes (data not shown). Proposed molecular genetic algorithm for CAH diagnosis (Fig 1.) depicts a protocol for patients in Serbia currently being developed and performed at Institute of Molecular Genetics and Genetic Engineering. This diagnostic procedure combines multiplex minisequencing technique (SNaPshot PCR) as a method for rapid detection of common point mutations, direct sequencing of whole *CYP21A2* gene and PCR with sequence specific primers (PCR-SSP) for large gene rearrangements detection (*CYP21A1P/CYP21A2* chimeras detection).

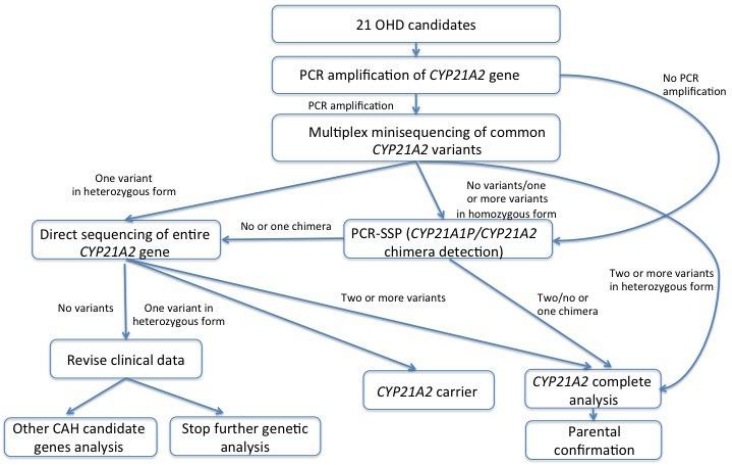

Figure 1. A proposed diagnostic algorithm for molecular genetic diagnosis of congenital adrenal hyperplasia in Serbia. SNaPshot was used for fast detection of common *CYP21A2* mutations (c.290-13A/C>G, p.P30L, p.R356W, p.G110fs, p.V281L, p.Q318X, p.L307fs, p.I172N, Cluster p.[I236N;V237E;M239K] and p.P453S) according to Krone et al., 2002. PCR-SSP was used for detection of large genomic rearrangements (*CYP21A1P/CYP21A2* chimeras) according to Dolzan et al., 2003, and direct sequencing was used for detection of rare variants.

The first step in genetic testing of 21OHD candidates is a whole *CYP21A2* gene amplification using primers created for exclusive amplification of the active gene, thus preventing amplification of highly homologue pseudogene (KRONE *et al*., 2002). Lack of amplification indicates that large gene rearrangement occurred at both chromosomes and that subsequent characterization of *CYP21A1P/CYP21A2* chimeras should be performed combining primers specific for 5' region of *CYP21A1P* gene and 3' region of *CYP21A2* gene (DOLZAN *et al*., 2003). Large gene rearrangements are present with frequency of 16,7% in Serbian population (MILACIC *et al*., 2015), making this analysis an unavoidable part of molecular characterization of CAH in Serbia.

After successful *CYP21A2* gene amplification, ten common mutations reported to account for over 80% of all *CYP21A2* mutations in Serbian population (MILACIC *et al*., 2015) were analysed by SnaPshot PCR. By using this screening procedure it is possible to rapidly genotype the majority of CAH causing mutations in Serbia. Due to frequent coexistence of multiple mutations on the same allele in 21OHD (DOLZAN *et al*., 2005), with high prevalence in Serbian cohort (6.5%) (MILACIC *et al*., 2015), analysis of parental samples for final molecular diagnosis is strongly suggested. This confirmation and segregation analysis could also be rapidly done by SNaPshot PCR. If no mutations are observed after SNaPshot assay, or they are detected in homozygous form, allele dropout, a common diagnostic pitfall characteristic for 21OHD (DAY *et al*., 1996), should be assumed and large genomic rearrangements analysis are carried out. The absence of large gene rearrangement implies that both alleles are amplified and, in the case of homozygous mutations, patient inherited the same mutation from both parents. Absence of heterozygosity in SNaPshot or sequencing analysis, especially in intron 2 abundant with polymorphisms, should always prone attention to potential allele dropout and involvement of large genomic rearrangement.

Detection of only one mutation in heterozygous form after SNaPshot assay, implying presence of both alleles and, hence, no allele dropout, steer further analysis toward direct sequencing of *CYP21A2* gene in order to identify potential rare or novel mutations present in our population with frequency of 1.8% (MILACIC *et al*., 2015). Sanger sequencing is the gold standard for detecting point mutations and small sequence variations (indel) (CHOI *et al*., 2016). It is always advisable to analyse all ten exons and their flanking intron regions due to possibility of multi-exon conversion and presence of multiple mutations. Cases of genotype-phenotype discordance should also be subject to direct sequencing for detection of uncommon mutations that could explain this discrepancy (NIMKARN *et al*., 1999).

Finally, after conducting a thorough *CYP21A2* gene investigation in 21OHD candidates, one of the three possible outcomes are expected: two or more variants detected and genotyping completed, only one variant detected, no variants detected. According to spectrum of Serbian CAH patients with two detected mutations, the utilization of SNaPshot assay, PCR-SSP and direct sequencing methods was assessed and Serbian molecular genetic diagnosis strategy was developed (Fig 2.)

If two or more variants have been detected, analysis of parental samples for final molecular diagnosis is strongly suggested given the inability to differentiate between mutations in *cis* and *trans* when only patient's sample is analysed (KRONE *et al*., 2002). Parental analysis is also crucial for determination of *de novo* mutations present in Serbian cohort with frequency of 4,6% (MILACIC *et al*., 2015).

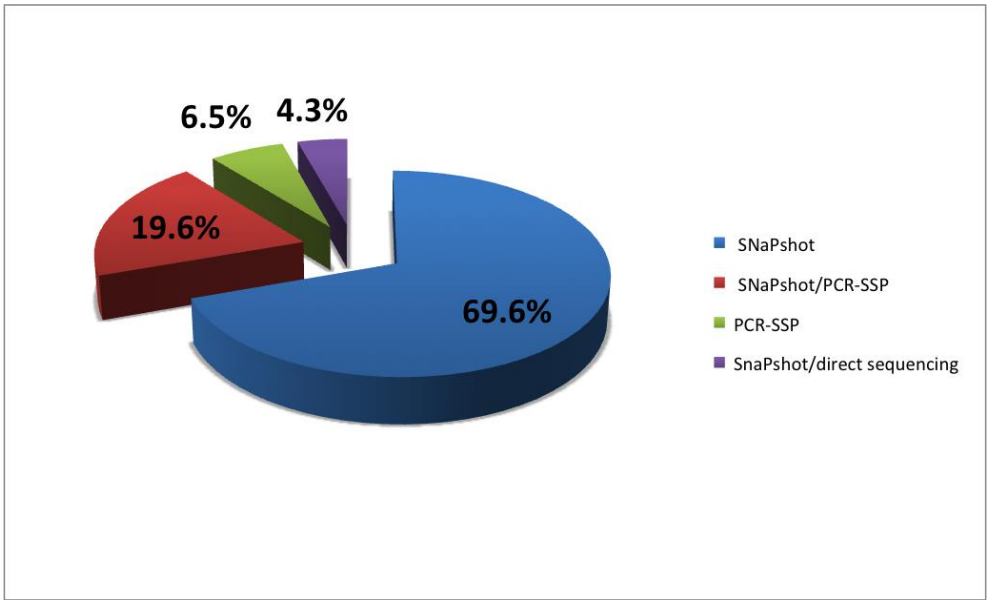

Figure 2. Utilization of SNaPshot assay, PCR-SSP and direct sequencing methods according to spectrum of Serbian CAH patients with at least two detected mutations.

Result of genetic analysis could also conclude that a person with slightly elevated 17-OHP level is only a *CYP21A2* carrier who does not require further medical treatment but should ask for a genetic advice when planning a family. Detecting *CYP21A2* heterozygote carriers among 21OHD candidates is a consequence of existing hormonal overlap with unaffected individuals, which makes genotyping superior to hormonal detection (NEW *et al*., 1983).

In 21OHD candidates in whom normal *CYP21A2* sequence have been found, revising clinical data is advised. When clinical data are strongly suggestive of CAH, mutations in other candidate genes which account for approximately 10% of CAH (*CYP11B1*, *CYP17A1*, *HSD3B2* and *POR*) should be suspected. Furthermore, ours protocol inability of detecting chimeric *TNXA/TNXB* hybrids resulting from unequal crossing over with break points at *TNX* genes should be considered. This chromosome rearrangement is always associated with complete *CYP21A2* gene deletion and a recessive Ehlers-Danlos disorder in addition to 21OHD (LEE, 2005). On the other hand, if no mutations were found and clinical data suggests mild NC-CAH form, it is arguable that patient presents with clinical symptoms that are non-specific and that differential diagnosis in these patients could be broader. For example, only small percentage of individuals presenting with androgen excess are actually affected with NC 21OHD (WHITE and SPEISER, 2000).

Results of genetic analysis must always be interpreted in light of the individual's clinical status and segregation analysis in the family (genotypes of parents and siblings).

CONCLUSION

CAH due to 21-hydroxylase deficiency is one of the most common endocrine diseases, yet genetic diagnosis is among the most complicated of all monogenic disorders. High variability of 17-OHP levels in patients demands molecular genetic testing for disease confirmation, especially in borderline cases. It is also essential for detection of carriers and asymptomatic patients, as well as for providing proper genetic counselling and prenatal diagnosis. Furthermore, genotype is correlated to an extent with clinical severity of 21OHD thus enabling prediction of the clinical course of the disease and prevention of severe complications (JIN-HO *et al*., 2016). Suggested molecular genetic approach is time- and cost-effective, allowing accurate identification of *CYP21A2* mutations. Delineation of molecular genetic strategy based on population specific data is intended to improve assessment of correct diagnosis in CAH affected individuals and their families in Serbia and to facilitate molecular genetic testing of CAH patients across Europe.

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MOLEKULARNO GENETIČKA STRATEGIJA U DIJAGNOSTICI KONGENITALNE ADRENALNE HIPERPLAZIJE U SRBIJI

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Izvod

Kongenitalna adrenalna hiperplazija (KAH) je među najučestalijim naslednim endokrinim poremećajima i odlikuje se narušenom sintezom jednog od enzima neophodnih u sintezi steroidnih hormona nadbubrežne žlezde. Najučestalija forma KAH-a uzrokovana je deficijencijom enzima steroid 21-hidroksilaze (21-OH) i javlja se sa učestalošću od 1:10000-1:20000. Obuhvata grupu autozomno recesivnih oboljenja i posledica je mutacija u *CYP21A2* genu. Na osnovu fenotipske ekspresije bolest se može podeliti na tešku (klasičnu) i blagu (neklasičnu) formu. Mada se dijagnoza bolesti može postaviti na osnovu kliničkih simptoma i hormonalnog statusa pacijenata, molekularno-genetičko testiranje je neophodno za potvrdu dijagnoze, detekciju nosilaca i asimptomatskih pacijenata sa neklasičnom formom, prognozu bolesti, kao i prilikom genetičkog savetovanja pacijenata obolelih od KAH-a ili prenatalne dijagnostike. Na osnovu podataka o spektru i učestalostima *CYP21A2* mutacija u Srbiji, u ovom radu predložen je optimalni molekularno-genetički algoritam. Kompletna dijagnostička procedura kombinuje tehniku multipleks mini-sekvenciranja (SNaPshot PCR) za brzu detekciju čestih tačkastih mutacija, zatim direktno sekvenciranje čitavog *CYP21A2* gena i PCR sa selektivnim prajmerima (PCR-SSP) za detekciju velikih genskih rearanžmana (*CYP21A1P/CYP21A2* himere). SNaPshot PCR-om se istovremeno analiziraju česte mutacije (c.290-13A/C>G, p.P30L, p.R356W, p.G110fs, p.V281L, p.Q318X, p.L307fs, p.I172N, klaster p.[I236N;V237E;M239K] i p.P453S) koje predstavljaju preko 80% svih *CYP21A2* mutacija u srpskoj populaciji, dok direktno sekvenciranje omogućava identifikaciju retkih i novih mutacija (oko 1.8%). Dodatno, veliki genski rearanžmani (16.7%) čine PCR-SSP analizu neizbežnim delom molekularne karakterizacije KAH-a u Srbiji. Opisana molekularno-genetička strategija osmišljena je da omogući preciznu dijagnostiku KAH-a kod pacijenata i njihovih porodica u Srbiji, ali će takođe doprineti i molekularno-genetičkom testiranju KAH pacijenata širom Evrope.

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