ORIGINAL ARTICLE

# DETECTION OF MUTATIONS IN THE *CYP21A2* GENE: GENOTYPE-PHENOTYPE CORRELATION IN SLOVENIAN COUPLES WITH CONCEIVING PROBLEMS

Stangler Herodež Š[1,*], Fijavž L[2], Zagradišnik B[1], Kokalj Vokač N[1,2]

**\*Corresponding Author:** Dr. Špela Stangler Herodež, Laboratory of Medical Genetics, University Clinical Centre Maribor, Ljubljanska ulica 5, 2000 Maribor, Slovenia. Tel: +386-2-321-27-37. Fax: +386-2-321-27-55. E-mail: spela.sh@ukc-mb.si

## ABSTRACT

The objective of this study was to compare the CYP 21A2 genetic profiles of couples with unexplained fertility problems (UFP) with genetic profiles of healthy controls (HCs). Furthermore, we analyzed associations between mutations in the *CYP21A2* gene and various clinical and laboratory parameters. Allele-specific polymerase chain reaction (PCR) was used in 638 probands with UFP and 200 HCs. Statistic analysis with $\chi^2$ was used to study the association of mutations with infertility. The effect of mutations on particular clinical and laboratory parameters was assessed with the analysis of variance (ANOVA) test. With regard to the *CYP21A2* gene, 0.6% of probands with UFP and 0.5% of HCs were positive for the c.290-13A/C>G mutation; 0.6% of probands with UFP and 1.5% of HCs were positive for the p.I172N mutation; there were no probands with UFP positive for the p.P30L mutation, whereas 0.5% of HCs were; and 0.2% of probands with UFP and 0.5% of HCs were found to have the p.V281L mutation. We found a significant association between c.290-13A/C>G mutation and the frequency of significant hormone deviations ($\chi^2 = 6.997$, $p = 0.008$). Similar association was also observed between the c.290-13A/C>G mutation and the frequency of polycystic ovary syndrome (PCOS) ($\chi^2 = 16.775$, $p = 0.000$). Our findings indicate that no significant difference in the prevalence of CYP 21A2 mutations can be found in probands with UFP when compared with HCs without infertility history. The results also imply the significant association of the c.290-13A/ C>G mutation in the *CYP21A2* gene, not only with the frequency of PCOS, but also with the frequency of significant hormone deviations.

**Keywords:** *CYP21A2* gene; genetics; infertility; mutations; unexplained infertility problems (UFP); healthy controls (HCs).

## INTRODUCTION

The World Health Organization (WHO) estimates that 10.0-15.0% of couples in the Western World develop issues regarding fertility. In addition, we can estimate that nearly 50.0% of couples with infertility have an underlying genetic abnormality [1].

Mutation in the *CYP21A2* gene results in a deficient 21-hydroxilase (21-OH) enzyme, which in turn leads to diminished aldosterone and cortysole production. Contrarily, the production of testosterone and dihydrotestosterone increases [2,3]. Congenital adrenal hyperplasia is a hereditary autosomal recessive disease. A defective 21-OH enzyme is responsible for 90.0% of disrupted steroid hormone production. The 21-OH catalyzes the conversion of progesterone and 17-hydroxyprogesterone (17-OHP) into 11-deoxycorticosterone (11-DOC) and 11-deoxycortisol. The enzyme is coded by the *CYP21A2* gene at position 6p21.3 [1,4]. Genes involved in steroidogenesis usually comprise 7 to 9 exons but the *CYP21A2* gene has 10

[1] Laboratory of Medical Genetics, University Clinical Centre Maribor, Maribor, Slovenia

[2] Medical Faculty, University of Maribor, Maribor, Slovenia

exons. In humans, there are two genes associated with coding 21-OH, both located on 6p21.3, on a region 35 kb in length in the major histocompatibility complex. The first of two is a disfunctional pseudogene (*CYP21A1* or *CYP21P*), the second is a functional gene (*CYP21A2* or *CYP21*). The pseudogene encodes an enzyme, nonfunctional due to the frameshift and occurence of a premature stop codon [5].

Although we cannot establish the phenotype only from the genetic profile, there are certain associations between the two. Patients with a detected mutation in the *CYP21A2* gene are divided into groups by the severity of the mutation on 21-OH [6]. Clinical findings associated with the phenotype are not always proportional to the degree of disfunction of 21-OH. We assume there are other genes involved in the manifestation of symptoms. There is marked concordance between genotype and phenotype in patients with mild or severe manifestation of congenital adrenal hyperplasia (CAH) [7,8]. Nevertheless, the most common manifestations are virilizing effects, clinically evident as hirsutism or androgenic alopecia. Oligomenorrhea or amenorrhea and ultrasound-evident polycystic ovaries may also be present [2,9,10].

Reduced fertility in female patients with CAH is often associated with excessive androgen levels. Adrenal androgens directly inhibit aromatase activity in granulosa cells of the ovary, thus indirectly hindering foliculogenesis. The high androgen levels result in gonadotropin-releasing hormone (GnRH), inhibition, leading to anovulation [10,11].

Thankfully, with glucocorticoid replacement therapy of patients with classical CAH, females mature into adults, so the focus has shifted to infertility treatment [2]. These patients suffer not only from salt wasting, they also have concomitant excessive androgens, excessive progesterone, post-reconstructive surgery states, PCOS, ovarian adrenal rest tumors and various psychosexual factors [1,5,11-14].

In our study, we presumed that the mutations c.290-13A/C>G, p.I172N, p.P30L and p.V281L in the *CYP21A2* gene are associated with infertility and influence clinical and laboratory parameters of women with UFP. For this purpose, we compared genetic profiles of couples with UFP with genetic profiles of healthy controls (HCs). Furthermore, we have also analyzed associations between mutations in the *CYP21A2* gene and various clinical and laboratory parameters.

## MATERIALS AND METHODS

**Probands.** Our study was a retrospective study and included 838 probands representing two different groups: couples with UFP and HCs. Couples with UFP (*n* = 638, 319 females and 319 males), were referred to our laboratory from the Department of Reproductive Medicine and Gynecologic Endocrinology, University Clinical Centre Maribor, Maribor, Slovenia, for routine karyotyping. At the time of enrollment in the study, all probands were childless as a couple and had not undergone CAH diagnosis. Clinical data of interest was extracted from a questionnaire designed for the needs of the study and existing medical records collected between 2005 and 2012. The HCs (*n* = 200, 100 females and 100 males) were blood donors with an unremarkable reproductive history, who were recruited through the Department of Transfusiology, University Clinical Centre Maribor, Maribor, Slovenia.

All samples were from individuals of Caucasian origin who were residents of different geographical areas of Slovenia. Informed consent was obtained from all participating individuals and ethical approval was granted prior to conducting the study.

**Clinical and Hormonal Investigations.** For female probands, the following clinical and biochemical parameters were determined: the concentration of prolactin in serum (nmol/L), the concentration of dihydroepiandrosterone in serum (nmol/L), the concentration of free testosterone in serum (nmol/L), the concentration of testosterone in serum (nmol/L), concentration of Anti-Muller hormone levels in serum (nmol/L), body mass index (BMI), the presence of a thyroid disease, the presence of an autoimmune disease, the presence of PCOS.

The follicular phase of the menstrual cycle was determined: the concentration of follicle-stimulating hormone in serum (nmol/L), the concentration of luteinizing hormone in serum (nmol/L), the concentration of 1,2-estradiol in the serum (nmol/L). In the luteal phase of the menstrual cycle, the concentration of progesterone in the serum (nmol/L) was determined. Basic laboratory parameters of female probands are given in Table 1.

**DNA Extraction and Analysis.** Peripheral venous blood was collected in standard collection vacutainers containing EDTA as anticoagulant. Genomic DNA was extracted from blood leukocytes

with a simple salting-out method [15]. All DNA samples were screened for the four most common mutations (p.P30L in exon 1, c.290-13A/ C>G in intron 2, p.I172N in exon 4 and p.V281L in exon 7) in the *CYP21A2* gene using allele-specific polymerase chain reaction (PCR). Four PCR reactions were performed per sample with the Multiplex PCR Kit (Cat. #206143; Qiagen GmbH, Hilden, Germany). The mix included 1X master mix, 1 μM of each primer and 50 ng of genomic DNA. In reaction 1, the p.P30L mutation in exon 1 was detected with CAH-30LT-P-F (5'-AAG CTC CGG AGC CTC CAC CTA CT-3') and CAH-I1-A-R1 (5'-AGC ATA GCA AGA ACC CAT CTG TT-3'). In reaction 2, the c.290-13A/C>G mutation in intron 2 was detected with CAH-I2-A2-F (5'-CTA ACT ACA TAT CTG GTG GGG AGA AAG C-3') and CAH-In2-M-G-R (5'-CAG CTT GTC TGC AGG AGG CGC-3'). In reaction 3, the p.I172N mutation in exon 4 was detected with CAH-172NA-F (5'-TCT CTC CTC ACC TGC AGC ATG AA-3') and CAH-I172N-A-RL (5'-TTC ATG TCG TCC TGC CAG AAA AGC AG-3'). In reaction 4, the p.V281L mutation in exon 7 was detected with CAH-I6-A-F (5'-CAG CAC AAG GTG GGG ACT GGA C-3'). The annealing temperature was 63 °C in 30 amplification cycles. Successful PCR amplification was confirmed by electrophoresis on 3.0% agarose gel, stained with SYBR Green I, and photographed for documentation and mutation scoring.

**Statistical Analyses.** For statistical analysis, the Statistical Package for the Social Science (SPSS), version 21 software program (IBM Corporation, Armonk, NY, USA), was used. Comparison between the means was done by non parametric tests. The results were expressed as an arithmetic mean ± standard deviation (SD). The relations between variables were analyzed by the analysis of variance (ANOVA) test. The distribution of the mutations was compared using the $\chi^2$ test; $p \leq 0.05$ was considered statistically significant.

## RESULTS

We analyzed 319 couples, a total of 638 individuals (average age 32.9 years, median age 33 years), 319 females (average and median age 32 years, range 21-44 years) and 319 males (average age 33.8 years, median age 34 years, range 23-44 years). The control

**Table 1.** Ages, mean ages and age ranges of the male and female probands.

|  | Total | Males | Females |
|---|---|---|---|
| *n* (probands) | 638 | 319 | 319 |
| Mean age | 32.9 | 33.8 | 32.0 |
| Age range (years) | 21-44 | 23-44 | 21-44 |

**Table 2.** Number and percentage of female probands with the deviation of laboratory parameters from the reference value and the presence of the clinical indicators.

| Parameters/Clinical Indicator | *n* | % |
|---|---|---|
| A thyroid disease | 19 | 6.1 |
|   Hor | 40 | 12.5 |
|   Est | 22 | 6.9 |
|   Prog | 3 | 0.9 |
| An autoimmune disease | 6 | 1.9 |
|   PCOS | 18 | 5.6 |
|   BMI | 44 | 13.8 |

Hor: deviation of estrogens and/or 17-OHP and/or testosterone in serum; Est: deviation of estrogens in serum; Prog: deviation of 17-OHP in serum. Autoimmune disease: celiac disease; Chron's disease; psoriasis; rheumatoid arthritis; lupus and ulcerative colitis. PCOS: polycystic ovary syndrome; BMI: body mass index.

group included 200 healthy blood donors (average and median age 43 years), 100 females (average and median age 42 years, range 27-66 years) and 100 males (average and median age 44 years, range 21-71 years) (Table 1).

For female probands, significant clinical parameters, biochemical parameters and the simultaneous presence of: a thyroid disease, increased BMI, an autoimmune disease, PCOS, conditions related to the concentration of sex hormones and conditions related to the concentration of progesterone, were determined. Number and proportions of female probands with the deviation of laboratory parameters from the reference value and the presence of the clinical indicators are presented in Table 2.

From Table 2, we can see that in the study group the proportion of females with the irregularities within the concentration of hormones (12.5%) stand out. At the same time, the smallest proportion belongs to the females with the deviation of 17-OHP in serum (0.9%). The c.290-13A/ C>G, p.I172N, p.P30L and p.V281L mutation frequencies were compared between probands and controls. There were no significant differences in mutation frequencies for all

four mutations ($\chi^2$ = 2.167, $p$ = 0.141). Results are presented in Table 3.

Additionally, the c.290-13A/C>G, p.I172N, p.P30L and p.V281L mutation frequencies were compared between male and female probands. There were no significant differences in mutation frequencies for all four mutations ($\chi^2$ = 2.254, $p$ = 0.324) (Table 4).

The possible impact of most common mutations in the *CYP21A2* gene on clinical and laboratory parameters were determined by the ANOVA test for the whole group and separately, only for female individuals. The ANOVA test showed no effect of mutations on laboratory parameters for the female group of individuals. For the analysis of the impact of mutations on the presence of clinical parameters, association analysis for the statistical processing of using data was used (Table 5).

We found a statistically significant association between clinical indicator for irregularities within the concentration of hormones and the p.V281L mutation ($\chi^2$ = 6.997, $p$ = 0.008) (see Table 5). We have also found a statistically significant association between the PCOS and the p.V281L mutation ($\chi^2$ = 16.775, $p$ = 0.000) (see Table 5). In the case of other clinical indicators, we did not find a statistically significant correlation with the studied mutations.

**Table 3.** The c.290-13A/C>G, p.I172N, p.P30L and p.V281L mutation frequencies in couples with unexplained fertility problems and controls.

| Mutations | Probands ($n$=638) (%) | Controls ($n$=200) (%) | $\chi^2$ | $p$ Value |
|---|---|---|---|---|
| c.290-13A/C>G | 4 (0.6) | 1 (0.5) | 0.043 | 0.836 |
| p.I172N | 4 (0.6) | 3 (1.5) | 1.389 | 0.239 |
| p.P30L | 0 (0.0) | 1 (0.5) | 3.183 | 0.074 |
| p.V281L | 1 (0.2) | 1 (0.5) | 0.749 | 0.387 |
| Total | 9 (1.4) | 6 (3.0) | 2.167 | 0.141 |

**Table 4.** Number and percentage of mutations discovered in the male and females probands.

| | **Males** | | **Females** | | $\chi^2$ | $p$ Value |
|---|---|---|---|---|---|---|
| Mutations | $n$ | % | $n$ | % | | |
| c.290-13A/C>G | 2 | 0.6 | 2 | 0.6 | 0.043 | 0.979 |
| p.I172N | 2 | 0.6 | 2 | 0.6 | 1.389 | 0.499 |
| p.P30L | 0 | 0.0 | 0 | 0.0 | **0.000** | 0.000 |
| p.V281L | 1 | 0.3 | 0 | 0.0 | 1.405 | 0.495 |
| Total | 5 | 1.6 | 4 | 1.3 | 2.254 | 0.324 |

**Table 5.** Comparison of clinical parameters of the entire group of female probands with the c.290-13A/C>G, p.I172N, p.P30L and p.V281L mutations and female probands without the c.290-13A/C>G, p.I172N, p.P30L and p.V281L mutations.

| Clinical Parameters | c.290-13A/C>G ($\chi^2$; $p$ value) | p.I172N ($\chi^2$; $p$ value) | p.P30L ($\chi^2$; $p$ value) | p.V281L ($\chi^2$; $p$ value) |
|---|---|---|---|---|
| A thyroid disease | 0.257; 0.613 | 0.257; 0.613 | –; – | 0.064; 0.801 |
| Hor | 0.581; 0.446 | 0.581; 0.446 | –; – | **6.997; 0.008** |
| Est | 0.300; 0.584 | 0.300; 0.584 | –; – | 0.074; 0.785 |
| Prog | 0.038; 0.845 | 0.038; 0.845 | –; – | 0.010; 0.922 |
| An autoimmune disease | 0.078; 0.781 | 0.078; 0.781 | –; – | 0.019; 0.890 |
| PCOS | 0.242; 0.623 | 0.242; 0.623 | –; – | **16.775; 0.000** |
| BMI | 0.648; 0.421 | 0.648; 0.421 | –; – | 0.161; 0.689 |

Hor: deviation of estrogens and/or 17-OHP and/or testosterone in serum; Est: deviation of estrogens in serum; Prog: deviation of 17-OHP progesterone in serum. Autoimmune diseases: celiac disease; Chron's disease; psoriasis; rheumatoid arthritis; lupus and ulcerative colitis. PCOS: polycystic ovary syndrome; BMI: body mass index.

## DISCUSSION

In our research, we determined the frequency of the c.290-13A/C>G, p.I172N, p.P30L and p.V281 mutations in couples with conceiving problems comparing them with a healthy control group. We did not observe any statistical differences between frequency of heterozygous carriers in infertile couples and HCs. Although we did not find any statistically important association, we cannot exclude the importance of the aforementioned mutations in infertility. We attribute it to an insufficient number of test subjects and controls. Also, we cannot draw an indefinite conclusion, as the presence of mutations in the *CYP21A2* gene may be associated with infertility [16]. It is also important to point out that in samples without the c.290-13A/C>G, p.I172N, p.P30L and p.V281 mutations, we cannot exclude the presence of other mutation variants in the *CYP21A2* gene [17].

In female test subjects, we examined the hormonal profiles during stages of the menstrual cycle and determined concomitant diagnosed illnesses from available medical documentation. We did not find any statistically significant association between hormonal deviations, concomitant illnesses and mutation frequency. However, we can conclude that the presence of a single mutation in the *CYP21A2* gene does not distinctively affect the hormonal status [9,18].

In general, fertility in people with CAH ranges from normal to severely impaired, as Reichman *et al.* [19] found that most CAH patients encountered fertility issues. In addition, these patients had less successful natural conceptions with more patients reporting spontaneous abortions. Similarly, Reichman *et al.* [19] found the frequency of successful conceptions is inversely proportional to the severity of mutations. To explain, females with an evidently more significant structural anomaly encountered more difficulties in conception [7,8,19]. Moreover, structurally milder mutations did not significantly affect fertility.

Çakir *et al.* [20] found that males with CAH have an evident diminished reproductive capability due to the presence of hipogonadotropic hipogonadism and consequently testicular adrenal rest tumors (TART). The latter, being more common, have adrenocorticotropic hormone (ACTH) and angiotensin II receptors and they synthesize similar steroid hormones as the adrenal glands. Permanent localized high steroid concentration may in turn damage the testes [16,20].

Peritubular testicular fibrosis results in impaired spermatogenesis [21]. In addition, the size of TART is inversely proportional to testosterone production and sperm ejaculation count [22].

We also studied the association between mutations in CAH and most frequently found concomitant illnesses. We did not find any association between female test subjects and the frequency of associated illnesses. Deckx *et al.* [23] found estrogens and progesterins to have immunomodulatory properties. In addition, Gleicher *et al.* [24] found that infertile females not only possess a diminished ovarian reserve but also serological profiles associated with autoimmune diseases. Moreover, he implicates an existence of an androgen derived cofactor, responsible for immunologic disorders [24]. In our study, we did not find any statistically significant association between the presence of a mutation and the level of testosterone in female subjects.

The *CYP21A2*, *RP*, *C4* and *TNX* genes are structurally adjacent, thus forming a genetic module RCCX complex. Olsson and Holmdahl [25] determined that CAH is closely associated with autoimmunity. Chen *et al.* [26] found that a mutation in the *CYP21A2* gene results not only in 21-OH deficiency but also in a variable copy count of the *C4* gene. To clarify, the *C4* gene encodes a homonymous protein, involved in classic complement activation [26]. In our study, we did not find a statistically significant association between autoimmunity and our studied mutations.

Thyroid disorders are closely associated with infertility. They not only affect female fertility but also hinder most of the human organs [27,28]. Sinha *et al.* [29] found that in thyrotoxicosis there is an increase of all sex hormones and their plasma binding proteins. As mentioned, excess estrogens hamper conception and pregnancy [2,10, 29]. Mansourian [30] and Mnif *et al.* [11] found that pathologic hormonal profiles of women with hyper- and hypothyroidism often result in abortions, low birth weight and premature labor. In our study, we did not find any association between mutations in the *CYP21A2* gene and the presence of thyroid disease.

On the other hand, we found an association between the p.V281L mutation in the *CYP21A2* gene and a marker indicating any hormonal deviance ($p = 0.008$). Next, we also found an association between the p.V281L mutation in the *CYP21A2* gene and the

frequency of PCOS ($p = 0.000$). Although we found this association, we cannot categorically confirm an actual association due to the small number of enrolled test subjects.

Surprisingly, we did not find a firm association between mutations and deviance in hormonal profiles. Of course, our results do not disprove that the c.290-13A/ C>G, p.I172N, p.P30L and p.V281L mutations in the *CYP21A2* gene are involved in infertility. Further research is necessary in order to clarify whether CAH is involved in infertility. According to Montgomery *et al.* [31], genome-wide association studies (GWAS) represent an evolution in polymorphism, mutation and gene research. Broad population GWAS, using multiple gene markers and extensive clinical information will be important in helping clinicians in not only disease treatment but also in predicting the risk of concomitant disease occurrence [29,31].

Whether it is necessary to enroll all couples diagnosed with infertility in genetic counseling, is still debatable. Many studies confirm the necessity of such a protocol, as there is a confirmed higher incidence of gene mutations among these couples [32]. Conversely, Papanikolaou *et al.* [33] disagrees with mandatory genetic testing of couples with infertility. He proposed that genetic testing should be executed only upon a valid indication.

In conclusion, does an early diagnosis of CAH essentially reduce the consequence of CAH? Moreover, should we be actively screening the population for mutations in the *CYP21A2* gene? To date, in the USA and 40 other countries, screening of CAH is done at birth [34]. The multitude of disease manifestations develop with age, therefore, in order to minimize disease outcomes, many stress the necessity of early detection of CAH [35,36].

## ACKNOWLEDGMENTS

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