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Correlation of phenotype with the CYP21 gene mutation analysis of classic type congenital adrenal hyperplasia due to 21-hydroxylase deficiency

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ABSTRACT

Ambiguous genitalia is seen as the most common phenotypic reflection of sexual development disorders. Congenital adrenal hyperplasia (CAH) is the most common cause of ambiguous genitalia, while the most common cause of CAH is a 21-hydroxylase deficiency with a rate of 90-95%. The disease is caused by mutations in the CYP21A2 gene located at 6p21.3. It is inherited in an autosomal recessive manner. Seven previously identified point mutations, an 8-bp deletion and large deletions, have significant role in the etiology of the disease. In this study, we aimed to report CYP21 molecular genetic evaluation by RFLP and MLPA methods in classic CAH patients with 21-hydroxylase deficiency. In this study, 26 patients with pre-diagnosis of Classic Type Congenital Adrenal Hyperplasia due to 21-Hydroxylase deficiency were reported. Seven previously identified point mutations, an 8-bp deletion, and large deletions were analyzed by PCR-RFLP methods in the patient group. For the MLPA study, SALSA MLPA KIT P050-B2 CAH (Lot0408) kit which was produced by MRC Holland was used. In 21 (80.7%) of 26 patients analyzed, causative mutations were found. The most frequent mutation was the large deletions (6 patients, 12 allels), accounting 23% of the patients. In 21 (80.7%) of 26 patients, the causative mutations were found by using PCR (8-bp del. and large deletions) and RFLP (7 known point mutations) methods. MLPA analysis confirmed all of the deletions detected by PCR-RFLP, and the 83% of the detectable point mutations with MLPA. A complete genotype-phenotype relationship could be established in all patients in whom mutation could be detected in the study group.

1. Introduction

Ambiguous genitalia is seen as the most common phenotypic reflection of sexual development disorders. Congenital adrenal hyperplasia (CAH) is the most common cause of ambiguous genitalia, while the most common cause of CAH is a 21-hydroxylase deficiency with a rate of 90-95% (White and Speiser, 2000). 21-Hydroxylase enzyme allows the production of 11-Deoxycortisol from progesterone and 11-Deoxycorticosterone from 17α-OH-Progesterone. The shift of the resulting precursors to androgenic pathways results in an androgenic burden that begins in the prenatal period for a patient with 21-hydroxylase deficiency (21-OHD) (Ohlsson et al., 1999). While this androgen excess does not have a serious effect on the sexual development of male fetuses, it causes virilization in the female fetus. Fetal adrenal glands start the enzyme synthesis in 6-8th weeks; in this

period urogenital sinus is in the vaginal and urethral canalization stage and because of this androgen burden, urogenital sinus may not able to separate, clittoral tissue may enlarge and the labioscrotal folds may fuse. Since the vagina in the 12th week is separated from the urogenital sinus, androgen exposure after this period can cause only the clitoromegaly. The affected female fetus is typically born with Ambiguous genitalia or with external genitalia that is similar to a male with hypospadias and undescended testes (Rimoin, et al., 2007).

Because of the defect in the synthesis pathway leading from the progesterone to Aldosterone, 3 out of 4 patients with **"Classic CAH"** do not produce sufficient levels of aldosterone. Patients with both androgen excess and mineralocorticoid deficiency are associated with the **"Salt-wasting Type CAH"** phenotype. The concentration of potassium in the extracellular fluid is significantly increased, the concentrations of sodium and chlorine are reduced. Total excretion of fluid and blood volume is significantly reduced. On the Clinical side the manifestations could be so severe that shock status may develop. In 21-OHD the newborns with salt wasting, are in the risk of rapid progression to mortal stage because of aldosterone and cortisol deficiency. In untreated cases, hyponatremia-hyperkalemia and hypovolemic shock may cause the newborn's death with Adrenal Crisis within 1-4 weeks after delivery (Rimoin et al., 2007).

The phenotype is defined as **"Simple Virilizing Type CAH"** in cases where the production of mineralocorticoids is not affected very much and androgen excess are at the forefront. In men, accelerated linear growth, pubic hair growth, increased penis size, and testicular enlargement may occur. In females, clitoral enlargement, occurring of facial acne, ovarian dysfunction and hirsutism can be seen.

The patients who are not born with ambiguous genital but are affected by postnatal androgen excess constitute **"Non-classical CAH"** patients. Females are mostly affected by irregular menstruation, hirsutism and occurring of facial acne (Kelestimur et al., 2009). Males are usually asymptomatic, if they are affected, acne and infertility can be seen. The frequency of the disease is estimated at 0.1% in the general population (Torresani and Biason-Lauber, 2007; Concolino et al., 2009).

The disease is caused by mutations in the CYP21A2 gene located at 6p21.3. It is inherited in an autosomal recessive manner. The frequency of the disease is 1 in 15.000 live births in the classical form and 1 in 1000 live births in milder forms Steroid 21-Hydroxylase (P450c21) is encoded by the CYP21 (CYP21B or CYP21A2) gene. There is a pseudogene (CYP21P, CYP21A1P or CYP21A) in the close proximity of the CYP21 gene. Studies in families where CYP21P has been completely lost have proven that this gene is completely dysfunctional. As CYP21 and CYP21P have approximately 98% sequence homology and settle at a distance of approximately 30 kb, the "Unequal Crossing-over" events may occur between these genes. As a result, deletions and duplications may occur in the functional gene. Previously, "Gene Conversion" mechanism of transmission of mutations from the CYP21P gene to the CYP21 gene was suggested. Seven previously identified point mutations, an 8-bp deletion and large deletions, which are mediated by these mechanisms, have a significant role in the etiology of the disease (Torresani and Biason-Lauber, 2007).

The clinical effects of these mutations are associated with the loss of enzyme activity they produce. The mutations are classified according to the degree to which the enzyme activity is affected (Wilson et al., 1995):

- **Type-A:** Genotypes with complete loss of enzyme activity: (Large deletions, wide conversions, 8-base pair deletion, E6 cluster mutations, Q318X and R356W)
- **Type-B:** Genotypes leading an enzyme activity around 1% (IVS2 mutations and I172N)
- **Type-C:** Genotypes leading an enzyme activity around 20-60 % (V281L and P30L)

The CAH phenotypes are associated with these genotypes are defined as follows:

- **Salt-wasting Type CAH:** Type-A/ Type-A genotype
- **Simple virilizing type CAH:** Type-A/ Type-B or Type-B/ Type-B genotypes
- **Non-classical type:** Type-A / Type-C, Type-B / Type-C and Type-C/ Type-C genotype

Previous studies about the relevant genotype-phenotype association, has shown that it brings about 80% accuracy of prediction (White and Speiser, 2000).

In this study, we aimed to report the result of the molecular genetic evaluation of CYP21 gene by two different methods, RFLP and MLPA, in classic CAH patients with 21-OHD and to determine the frequency of the mutations and deletions, correlate the results with the phenotypes and to compare the effectiveness of the two molecular analysis methods used.

2. Material and methods

Patient group

This study involved 26 patients with pre-diagnosis of Classic Type Congenital Adrenal Hyperplasia due to 21-OHD. The patients were followed by the Departments of Pediatric Endocrinology and Medical Genetics of Ondokuz Mayis University, between 2005-2010. Informed consent forms have been signed by the patients or their parents. This study was approved by Ondokuz Mayıs University research ethics committee.

Chromosome analysis

Chromosomes were obtained by peripheral venous blood lymphocyte culture using modified synchronization method (Rooney and Czepulkowski, 2001). GTG banding were used to determine karyotypes.

Isolation of DNA from peripheral blood

Peripheral blood samples were taken from patients and DNA extraction was performed using the High Pure PCR Template Preparation Kit (Roche, Germany).

Cyp21 RFLP analysis

Seven previously identified point mutations, an 8-bp deletion, and large deletions were analyzed by PCR-RFLP methods in the patient group. For the PCR amplifications, 10 different primers were used (Sadeghi et al., 2008). Four different PCR amplifications (Fragments 1, 2, 3, 4) were performed for 7 point mutations. For the PCR amplifications of the CYP21 gene, specific primers were used that did not amplify pseudogene. The four DNA fragments specific to alleles of the 7 point mutations were amplified by PCR. Two additional PCR amplifications (Fragment 5 and 6) were performed to detect 8-bp deletion or large deletions. The resulting PCR products were digested with the appropriate restriction enzymes. The restriction products were visualized on 3% agarose gel prepared with Ethidium Bromide. The DNA fragments analyzed after the enzyme cut-up was normal to the normal allele, mutant allele, or both, and allowed the wild-type, homozygous mutant or heterozygous expression to be assessed, respectively. All PCR reactions and Restrictions were carried out according to the methods that were previously reported (Sadeghi et al., 2008).

In the case of the presence of mutations E6 Cluster (I236N / V237E / M239K), V281L, Q318X, and R356W in the amplified fragment 1, the recognition site of the MboI, Alw21I, PstI, and AciI enzymes were lost respectively. In this case, a single fragment consisting of their total lengths was detected instead of the two fragments in the enzyme-cut products. Similarly, an enzyme recognition site of AciI enzyme was lost in Fragman 4 in the presence of the mutation.

In Fragment 2 and Fragment 3, the corresponding mutations create a novel enzyme recognition site for the AluI and Bse1I enzymes, respectively, where a fragment at that point was divided into two smaller fragments.

The amplified PCR products (Fragments), analyzed mutations and the expected fragment lengths after the restriction reactions were summarised in Table 1.

MLPA

For the MLPA study, SALSA MLPA KIT P050-B2 CAH (Lot0408) kit which was produced by MRC Holland was used. The kit included probes for CYP21A2, CYP21A1P, TNXB, C4A, C4B, and CREBL1 genes. The reactions were carried out according to the manufacturer's protocols.

The PCR products obtained as a result of MLPA reaction were run on the Beckman Coulter CEQ8800 capillary electrophoresis device. As a result of the process, peak images and peak areas of the probes of each sample were obtained in the CEQ program. Excel based Coffalyser 9.4 program was used for the analysis.

3. Results

Patients

Of the 26 patients, 15 were consulted for ambiguous genitalia, 3 were consulted for problems such as genital hyperpigmentation and hirsutism. 8 of the patients presented with complaints such as feeding problems, vomiting, weakness, and prolonged jaundice.

In 8 of 26 patients, consanguinity was defined between parents.

The preliminary phenotype was identified as virilized female genitalia in 16 of 26 patients and all 16 of them were karyotyped as 46, XX.

Table 1. Mutations, fragments, the expected fragment lengths and detected allele frequencies.

Fragment	Mutation	Protein Change/ Other name	Rest. Enz.	Wild Type Frag. (bp)	Mutation Frag. (bp)	Allele Frequencies
F1	c.[710T>A;713T>A;719T>A]	p.[Ile237Asn;Val238Glu;Met 240Lys] **(E6 Cluster)**	MboI	349.336	685	3.5%
F1	c.844G>T	p.Val282Leu **(V281L)**	Alw21I	99.17	1161	1.7%
F1	c.955C>T	p.Gln319Ter **(Q318X)**	PstI	298.154	452	16%
F1	c.1069C>T	p.Arg357Trp **(R356W)**	AciI	189.3	219	7.1%
F2	c.293-13A>G(659A>G)	**(IVS2)**	AluI	51	34,17	14.2%
F3	c.518T>A	p.Ile173Asn **(I172N)**	Bse1I	231	217,14	14.2%
F4	c.92C>T	p.Pro31Leu **(P30L)**	AciI	153.43	196	3.5%
F5	8-bp deletion	-	-	64.56	56	-
F6	Large deletions	-	-	789	-	23%

Additional laboratory findings
Serum levels of 17-hydroxyprogesterone were found to be high in all 26 patients.

In 11 of 26 patients (1,3,9,11,14,15,18,21,24,25,26), hyponatremia and hyperkalemia were detected at admission.

Phenotype-karyotype
In our patient group, the initial determination of sex revealed that the dominant phenotype was male in 11 patients and the dominant phenotype was female in 4 patients. The remaining 11 patients were defined as ambiguous genitalia. Chromosome analysis of 16 of the 26 patients was determined as 46, XX, 10 of the 26 patients was determined as 46, XY. All the patients with XY karyotype had male genitalia.

CYP21 RFLP results
In 21 **(80.7%)** of 26 patients analyzed, causative mutations were found. In 4 more patients the mutation was detected (25 patients; coverage, 96.2%) in the heterozygous state. In one patient, no mutations were detected.

Of the 56 alleles evaluated, 46 (82%) had mutations. The most frequent mutation was the large deletions (6 patients, 12 allels), accounting 23% of the patients. The most frequent point mutations were **c.955C>T** (Q318X) (9 allels, 16%), **c.293-13A>G** (IVS2) (8 allels, 14.2%), **c.518T**>A (I172N) (8 Allels, 14.2%), **c.1069C>T** (R356W) (4 Allels, 7.1%), **c.92C>T** (P30L) (2 Allels, 3.5%) and **E6 cluster** (2 Allels, 3.5%) and **c.844G>T** (V281L) (1 Allel, 1.7%).

The 8-bp deletion was not detected.

MLPA results
All the deletions detected by RFLP (6 patients) were confirmed by MLPA method.

In 10 of our 12 patients who had at least one of the I172N, E6 cluster, and Q318X mutations, MLPA analysis showed RPR values lower than 0.7, supporting the presence of mutations.

All the clinical findings, karyotype, RFLP and MLPA results were summarised in Table 2.

4. Discussion
The cause of more than 90% of cases in virilizing CAH is a 21-OHD In classical disease, females may be born with virilized external genitalia due to prenatal excessive androgen exposure. Since three-quarters of the cases cannot synthesize enough aldosterone, they experience lethal sodium and potassium imbalances if not treated. The disease is the result of mutations in CYP21, a steroid 21-hydroxylase gene. More than 90% of mutations result from intergenic recombinations between CYP21 and pseudogene CYP21P (Wilson et al., 1995; White and Speiser, 2000; Dolž et al., 2005; Torresani and Biason-Lauber, 2007).

Several studies have been conducted in different populations to determine the frequency of mutations in classical CAH due to 21-OHD In 2005, Dolzan et al. performed CYP21 genotyping with PCR-SSP, PCR-SSO, Southern Blotting and sequence analysis in patients with CAH (Classic and Non-Classical) from middle European region (Austria, Czech Republic, Hungary, Slovakia, Slovenia). As a result, 98% of genotyped patients had mutations. The most frequent detected alleles were **IVS2 (31%), deletions (28%), I172N (14.5%).** It was suggested that these 7 known mutations and deletions cover 85.9% of the mutant alleles and that the remaining alleles were formed by rare mutations and minor conversions (Dolž et al., 2005). In 1999, a study was performed by Ohlsson et al. in Denmark. CYP21 genotyping was performed using the sequence analysis method in 68 patients with CAH (Classical and Non-Classical). As a result of the study, deletions (36%) were found to be the most frequent mutations. **The IVS2 mutation (33.8%), I172N mutation (10.3%)**, Q318X mutation (8.8%), V281L mutation (4.4%) were listed as other frequent mutations (Ohlsson et al., 1999). In 2004, Kharrat et al. Performed CYP21 genotyping with PCR, RFLP and sequence analysis in 25 Tunisian patients with classic CAH. **35.3% of the patients had Q318X, 19.6% had deletions, 17.6% had IVS2** and 10.8% had I172N. They found that 5.9% had no mutations.

Similar to the ones in other countries, studies have also been conducted to determine the frequency of mutations in Turkey. In 2008 Sadeghi et al. released the mutation analysis results of 100 patients with classic CAH. In this study, they have reported that the most frequent mutation were IVS2 (28.5%), deletions (17%), Q318X (11.5%), I172N (4%), V281L (3.5%), R356W (3.5%) and 8-bp deletion (3%)(Sadeghi, et al., 2008). In 2009, Baş et al., analysed the eight most common point mutations in 56 patients. They have introduced that the most common mutation were: IVS2 (22.0%), large conversion (14.3%), I172N (9.9%) R356W (8.8%), and large deletion (6.6%) (Baş et al., 2009). In 2013, Toraman et al., published the CYP21A2 mutation analysis results of 48 CAH patients (Toraman et al., 2013). They have reported that among identified mutations, previously described IVS2, large rearrangements and Q318X mutations were the most common mutations (Toraman et al., 2013). In 2014, Kirac et al. characterized the mutations in 124 Turkish CAH patients. They have reported that IVS2, 8-bp deletion, and large rearrangements were the most frequent homozygous mutations in the salt wasting form (Kirac et al., 2014).

In our study, the most frequent mutation was a **large deletion (23%)**. The most frequent point mutations were **c.955C>T (Q318X) (9 alleles, 16%),**

Table 2. Ages, admission cause, phenotype, karyotype results, molecular genetic results and clinical diagnosis.

Patient #	Age	Admission Cause	Genital phenotype	Salt Wasting	Karyotype	CYP21 RFLP	CYP21 MLPA	Diagnosis
#1	11 day-old	**Jaundice**	Virilised female gentalia	(+)	46, XX	Homozygous c.293-13A>G (IVS2)	Normal	Salt Wasting Type Classical CAH
#2	7 day-old	**Ambiguous Genitalia**	Virilised female gentalia	(-)	46, XX	-	Normal	Simple Virilizing Classical CAH ?
#3	26 day-old	**Ambiguous Genitalia**	Virilised female gentalia	(+)	46, XX	Homozygous Large Deletion	CYP21 Exon 2, 3 Deletion	Salt Wasting Type Classical CAH
#4	9 year-old	**Ambiguous Genitalia**	Virilised female gentalia	(-)	46, XX	Homozygous c.518T>A (I172N)	Homozygous c.518T>A	Simple Virilizing Classical CAH
#5	2 year-old	**Ambiguous Genitalia**	Virilised female gentalia	(-)	46, XX	Comp. Het. c.955C>T/ c.518T>A	Heterozygous c.518T>A (c.955C>T:ND)	Simple Virilizing Classical CAH
#6	1 day-old	**Ambiguous Genitalia**	Virilised female gentalia	(+)	46, XX	Homozygous c.955C>T (Q318X)	Homozygous c.955C>T	Salt Wasting Type Classical CAH
#7	2 year-old	**Ambiguous Genitalia**	Virilised female gentalia	(-)	46, XX	Heterozygous c.518T>A (I172N)	Heterozygous c.518T>A	Simple Virilizing Classical CAH
#8	3 day-old	Ambiguous Genitalia	Virilised female gentalia	(+)	46, XX	Homozygous Large Deletion	CYP21 Exon 3, 4 Deletion	Salt Wasting Type Classical CAH
#9	16 month-old	Ambiguous Genitalia	Virilised female gentalia	(-)	46, XX	Homozygous c.518T>A (I172N)	Homozygous c.518T>A	Simple Virilizing Classical CAH
#10	5 day-old	Ambiguous Genitalia	Virilised female gentalia	(+)	46, XX	Homozygous c.955C>T (Q318X)	Homozygous c.955C>T	Salt Wasting Type Classical CAH
#11	5 month-old	Vomiting	Male with hyper pigment.	(+)	46, XY	Homozygous c.1069C>T (R356W)	Normal	Salt Wasting Type Classical CAH
#12	1 month-old	Genital Hyperpigment.	Male with hyper pigment.	(-)	46, XY	Comp. Het. c.844G>T/c.92C>T	Normal	Simple Virilizing / Non Classical?
#13	1 month-old	Vomiting	Male with hyper pigment.	(+)	46, XY	Heterozygous c.955C>T (Q318X)	Heterozygous c.955C>T	Salt Wasting Type Classical CAH
#14	5 month-old	Vomiting	Male	(+)	46, XY	Homozygous c.293-13A>G (IVS2)	Normal	Salt Wasting Type Classical CAH
#15	2 year-old	Hypervirilisation	Male with hyper pigment.	(-)	46, XY	Heterozygous c.518T>A (I172N)	Heterozygous c.518T>A	Simple Virilizing Classical CAH
#16	1 day-old	Vomiting	Male	(-)	46, XY	Comp. Het. c.955C>T/c.92C>T	Normal (c.955C>T: ND)	Simple Virilizing Classical CAH
#17	2 month-old	Vomiting	Male	(+)	46, XY	Homozygous c.1069C>T (R356W)	Normal	Salt Wasting Type Classical CAH
#18	2 day-old	Ambiguous Genitalia	Virilised female gentalia	(+)	46, XX	Homozygous Large Deletion	CYP21 Exon 1-3 Deletion	Salt Wasting Type Classical CAH
#19	1 month-old	Vomiting	Male with hyper pigment.	(+)	46, XY	Homozygous E6 Cluster	Homozygous E6 Cluster	Salt Wasting Type Classical CAH
#20	17 year-old	Ambiguous Genitalia	Virilised female gentalia	(-)	46, XX	Heterozygous c.518T>A (I172N)	Heterozygous c.518T>A	Simple Virilizing Classical CAH
#21	1 day-old	Ambiguous Genitalia	Virilised female gentalia	(+)	46, XX	Homozygous c.955C>T (Q318X)	Homozygous c.955C>T	Salt Wasting Type Classical CAH
#22	18 day-old	Ambiguous Genitalia	Virilised female gentalia	(+)	46, XX	Homozygous Large Deletion	CYP21 Exon 1-3 Deletion	Salt Wasting Type Classical CAH
#23	1 day-old	Ambiguous Genitalia	Virilised female gentalia	(+)	46, XX	Homozygous Large Deletion	CYP21 Exon 1-8 Deletion	Salt Wasting Type Classical CAH
#24	1 month-old	Jaundice	Male	(+)	46, XY	Homozygous Large Deletion	CYP21 Exon 1-8 Deletion	Salt Wasting Type Classical CAH
#25	10 year-old	Hirsutism	Male	(-)	46, XY	Homozygous c.293-13A>G (IVS2)	Normal	Simple Virilizing Classical CAH
#26	2 day-old	Ambiguous Genitalia	Virilised female gentalia	(-)	46, XX	Homozygous c.293-13A>G (IVS2)	Normal	Simple Virilizing Classical CAH

c.293-13A>G (IVS2) (8 alleles, 14.2%), c.518T>A (I172N) (8 Alleles, 14.2%), c.1069C>T (R356W) (4 Alleles, 7.1%), c.92C>T (P30L) (2 Alleles, 3.5%) and E6 cluster (2 Alleles, 3.5%) and c.844G>T (V281L) (1 Allele, 1.7%). The 8-bp deletion was not detected. These results were consistent with the previously reported studies.

The genotypes of **15 patients with salt wasting phenotype** were associated with Type-A mutations. Of these 15 patients, 6 had Homozygous large deletions, 3 had Homozygous Q318X mutations, 2 had Homozygous R356W mutation, 2 had homozygous IVS2 mutation, 1 had Homozygous E6 Cluster mutation and 1 had heterozygous Q318X mutation.

The 6 patients with homozygous large deletions (#3,#8,#18,#22,#23,#24) were showing Salt wasting severe phenotype as expected. **The IVS2 mutation** disrupts the splice site of intron 2 and causes a shift in the translational reading frame. Because of this, almost all of the enzyme was abnormally spliced, whereas in the cultured cells there can be little normal enzyme activity. For this reason, the IVS2 mutation is associated with both a salt-wasting and simple virilizing types of the phenotype. In our study, 2 patients (#1,#14) with homozygous IVS2 mutation were found to have a salt wasting phenotype, while the other 2 (#25,#26) showed the simple virilizing phenotype. This situation was appropriate for the nature of the mutation (Forsham and Greenspan, 1983; Goossens et al., 2009).

All of the **11 patients with a simple virilizing phenotype** had Type-A / Type-B or Type-B / Type-B genotypes. Of these 11 patients, 2 had Homozygous I172N mutation, 3 had Heterozygous I172N mutation, 2 had homozygous IVS2 mutation, 1 had compound heterozygous Q318X/I172N mutations,1had compound heterozygous V281L/P30L mutations, 1 had compound heterozygous Q318X/P30L mutations. One patient had no mutations.

The Q318X mutation is classified as a Type-A mutation and this mutation leads to complete loss of enzyme activity in the homozygous state. However, Kharrat et al. suggested that Q318X mutation with I172N mutation resulted in a simple virilizing phenotype in the compound heterozygous state (Kharrat et al., 2004). Our patient #5 with Q318X / I172N Compound Heterozygote genotype had also had a simple virilizing phenotype. **The I172N mutation** leads to up to 1% of normal enzyme activity and is the most common cause of simple virilizing phenotype. I172N allele was detected in 6 patients. 2 of them (Patient #4 and #9) were in homozygous state and they were showing a simple virilizing phenotype. In patient #12, compound heterozygous V281L / P30L mutations were found. These two mutations were defined as Type-C mutations which are leading an enzyme activity around 20-60%. Our patient was removed from the follow-up after receiving medical treatment for a while. This mild phenotype (Non-Classical?) was consistent with the genotype. In patient #12, compound heterozygous Q318X / P30L mutations were detected. The Q318X mutation was classified as Type-A (null group) and P30L was classified as Type-C. Our patient (#12) was showing genital hyperpigmentation and followed-up as simple virilizing/Non-Classical CAH Phenotype. This mild phenotype was consistent with the genotype (Delague et al., 2000; Gonçalves et al., 2007).

In conclusion, all patients with a salt wasting phenotype participating in our study had Type-A/ Type-A genotype and all patients with a simple virilizing phenotype had Type-A / Type-B or Type-B / Type-B genotype. All these results show that in CAH a good genotype-phenotype correlation can be established.

RFLP and MLPA methods were used to determine the CYP21 genotypes of 26 patients. In many studies, 7 known point mutation, 8-bp deletion, and large deletions were detected in more than 90% of cases. In the literature, the deletions in most studies have been investigated by genomic blot hybridization but nowadays, they are replaced by PCR based methods that require less labor and provide more information. This data obtained by PCR is limited in determining the extent of the deletion and not being able to detect duplications frequently. The MLPA method has been identified as a sensitive method for detecting deletions and duplications. Concolino et al. (2009) reported in their study that, of the 7 known subjects with CYP21A2 deletions and 2 with gene duplications previously characterized by Southern Blot, all were successfully identified by the MLPA). Researchers have argued that the MLPA method may be a highly informative method in the molecular diagnosis of CAH but, due to the complex nature of the CYP21A2 gene, which is one of the most known polymorphic genes, these studies require profound experience in the genetics of CYP21A2. In our study, MLPA confirmed the deletions in all 6 cases with deletions detected by PCR. Additionally, the four probes had been designed for the detection of 4 common mutations (8-bp deletion, I172N, E6 cluster, and Q318X). In our study, in 10 of 12 patients who had at least one of the I172N, E6 cluster, and Q318X mutations, MLPA analysis showed decreased peaks, supporting the presence of mutations.

Conclusion

Major results of the study;

1. The study was conducted on 7 known point mutations and 2 deletions. Although small in number, these 9 mutations did cover more than 96% of the mutant alleles.
2. The most frequent mutation was the large deletion (23%). The most frequent point mutations were c.955C>T (Q318X) (9 alleles, 16%), c.293-13A>G

(IVS2) (8 allels, 14.2%), c.518T>A (I172N) (8 Allels, 14.2%), c.1069C>T (R356W) (4 Allels, 7.1%), c.92C>T (P30L) (2 Allels, 3.5%) and E6 cluster (2 Allels, 3.5%) and c.844G>T (V281L) (1 Allel, 1.7%). The 8-bp deletion was not detected.

3. Order of mutation frequency did not differ in between our cohort which included 9 mutations and 26 families and between the cohort of New and Rosenwaks (2019), which included 113 known mutations and 1507 families. Briefly, in both deletions and Q318Xwere the most frequent mutations followed by I172N and IVS2 (New and Rosenwaks, 2019).
4. In 21 (80.7%) of 26 patients, the causative mutations were found by using PCR (8-bp del. and large deletions) and RFLP (7 known point mutations) methods.
5. MLPA analysis confirmed all of the deletions detected by PCR-RFLP, 83% of the detectable point mutations with MLPA.
6. A complete genotype-phenotype relationship could be established in all patients in whom mutation could be detected in the study group.

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