

## Original Article

# *In Vitro* Enzyme Assay of *CYP21A2* Mutation (R483Q) by A Novel Method Using Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS)

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**Abstract.** Congenital adrenal hyperplasia (CAH) is one of the most common autosomal recessive disorders in humans, and 21-hydroxylase deficiency (21-OHD) accounts for 90 to 95% of all cases of CAH. Approximately 95% mutations are a consequence of recombination between the *CYP21A2* and its highly homologous pseudogene *CYP21A1P*. Recently, other rare mutations have been identified, increasing the number of reported mutations to more than eighty. The *in vitro* enzyme assay for the detection of mutated 21-hydroxylase is a well-established method. In this study, we report the characterization of the R483Q mutation using a novel *in vitro* enzyme assay, liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). With this system, we evaluated the activity of the R483Q mutation. The enzyme activities of 21-hydroxylase in the conversion of progesterone to deoxycorticosterone (DOC), and 17-hydroxyprogesterone (17-OHP) to 11-deoxycortisol (11-DOF), were measured as  $2.00 \pm 0.25\%$  and  $1.89 \pm 0.30\%$  of the wild type, respectively. This result was in agreement with that of a previous report, which measured the activities using the <sup>3</sup>H labeled steroid assay. Our results suggest that the R483Q mutation is compatible with the simple virilizing form of 21-OHD and that the LC-ESI-MS/MS assay using picolinoyl derivatives is an alternative to the existing <sup>3</sup>H-labeled steroid assay for the characterization of the *CYP21A2* mutation.

**Key words:** 21-hydroxylase deficiency (21-OHD), *CYP21A2*, enzyme assay, LC-ESI-MS/MS

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### Introduction

Congenital adrenal hyperplasia (CAH) [OMIM #201910] is one of the most common

autosomal recessive disorders in humans; and 21-hydroxylase deficiency (21-OHD) accounts for 90 to 95% of all cases of CAH. It is an inherited hormonal disorder with impaired synthesis of glucocorticoids and mineralocorticoids (1–5). This disorder leads to adrenal insufficiency and androgen excess. Adrenal insufficiency is a critical emergency that is often life threatening. Androgen excess causes virilization of female external genitalia, precocious puberty, and short stature. Adequate and prompt treatment with

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glucocorticoid supplementation is necessary. Consequently, neonatal mass-screening for CAH was initiated in Japan twenty years ago (6, 7).

According to the severity of symptoms, CAH is classified into three types: the salt wasting, simple virilizing, and nonclassical forms. The salt wasting form is the most severe phenotype with androgen excess and adrenal insufficiency. The salt wasting and simple virilizing forms are also known as the classical type. The nonclassical type has a milder phenotype than that of the classical type (1–5).

21-OHD is caused by the mutation of *CYP21A2* encoding 21-hydroxylase. This gene is located in human chromosome 6 and codes 494 amino acids (1–5). Pseudogene *CYP21A1P* is located adjacent to *CYP21A2*. This gene has 98% homology with *CYP21A2* and does not code for any protein. The major mutations of *CYP21A2* are derived from this pseudogene through conversion (1–5). In general, there exists a mutual correlation between the severity of the phenotype and the retained enzyme activity caused by *CYP21A2* mutation (1–5). Prediction of the enzyme activity is important for determining clinical treatment and diagnosis. The *in vitro* assay for detection of mutated 21-hydroxylase enzyme activity is a well-established method (8–10).

Recently, we identified a patient with the R483Q mutation (compound heterozygous genotype with deletion/conversion) in *CYP21A2*, who showed clinical symptoms of the classical form of CAH. This is a rare mutation and has previously been reported in only two patients (11, 12). One was a nonclassical male patient with compound heterozygous deletion/conversion and the other was a simple virilizing female patient with compound heterozygous I172N mutation. From the enzyme activity that has been previously reported, the phenotype of this mutation is predicted to be of the simple virilizing form (12).

In our case, despite normal levels of sodium and potassium, plasma renin activity was elevated, and poor weight gain was observed.

These symptoms indicated that the patient was showing mild salt wasting. To confirm the enzyme activity of the R483Q mutation, we performed an *in vitro* enzyme assay of the mutant protein. However, this conventional method presented some problems, such as complicated procedure, radioisotope handling, and indirect measurement of synthesized steroids.

Liquid chromatography-mass spectrometry (LC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (also known as HPLC) with the mass analysis capabilities of mass spectrometry. LC-MS is used for applications that require high sensitivity and specificity (13, 14). Electrospray ionization (ESI) is one of the methods frequently used to ionize and measure steroids (15, 16). Previously, the choice of ionization techniques used in LC-ESI-MS/MS to obtain adequate sensitivity was dependent on the instrument and the steroid compounds being measured. This complicated the procedure when measuring multiple steroid compounds by LC-MS. However, an improved LC-ESI-MS/MS technique has recently been developed, which makes use of picolinoyl derivatization of steroids (17, 18). In this method, derivatives of steroids are obtained by a simple one-step procedure, allowing the simultaneous measurement of multiple steroids with excellent sensitivity and specificity.

We assayed the enzyme activity of R483Q using this novel technique. The result obtained was in agreement with previously reported enzyme activity (12). Therefore, we concluded that this assay system is a potent method for the evaluation of *in vitro* enzyme assay of 21-hydroxylase.

## Materials and Methods

### Patient

At the age of 10 d, a female patient was referred to our hospital due to high levels of 17-hydroxyprogesterone (17-OHP), 81.9 ng/ml, detected during neonatal mass screening. She

**Table 1** Results of endocrinological profiles of the case study

	Normal range	case
ACTH (pg/ml)	7–56	478
17-OHP (ng/ml)	0.56–1.35	141
DHEA (ng/ml)	0.10–0.48	3.59
DHEAS (ng/ml)	980–4920	1490.0
Androstenedione (ng/ml)	0.16–0.50	2.59
Testosterone (ng/ml)	0.16–0.29	0.4
21-deoxycortisol (21-DOF) (ng/ml)	0.03–0.40	62.2
11-deoxycortisol (ng/ml)	0.11–0.60	3.79
17-OH pregnenolone (ng/ml)	0.1–4.0	34.40
U-Pregnanetriol (mg/g · Cre)	0.013–0.041	12.5
U-Pregnanetriolone (mg/g · Cre)	<0.1	21.4
PRA (ng/ml/h)	3.7–11.1	66.3
Aldosterone (ng/dl)	28.7–75.6	85.1

The elevated values of 17-OHP and 21-DOF indicate a diagnosis of 21-hydroxylase deficiency.

was a full term infant and her body weight at birth was 2,948 g. There was no previous history of 21-OHD in her family; her father's parents were cousins. She was hospitalized immediately and examined more thoroughly.

The patient's body weight on admission was 2,685 g and weight gain was poor. Her skin was pigmented and icteric. Clitoromegaly and minor labial fusion were observed. Urogenital sinus was undetectable by cystography. There were no symptoms that indicated severe adrenal crisis or shock. Serum levels of electrolytes were within the normal range (Na: 136 mEq/l, K: 4.9 mEq/l, Cl: 100 mEq/l), and hypoglycemia was not observed (Glu: 78 mg/dl). However, endocrinological data revealed adrenal insufficiency and androgen excess (Table 1). Considering the remarkably elevated levels of 17-OHP and 21-deoxycortisol (21-DOF), we diagnosed 21-OHD, and administered 22 mg/d of hydrocortisone. With this treatment, the patient's body weight gain became normal and her skin pigmentation disappeared. Based on improvement in the clinical signs and endocrinological data, we decreased the dose of hydrocortisone to 11 mg/d gradually, and at the age of 74 d, the patient was discharged from our hospital.

### DNA analysis

Genomic DNA of the patient was obtained from peripheral blood lymphocytes by standard procedures.

*CYP21A2* amplification, RFLP, and direct sequencing were performed as previously described (19).

Southern blot analysis was performed to detect gene deletions or large conversions. Genomic DNA was digested with the restriction enzyme, *Taq I*. The digests were separated on a 0.8% agarose gel, transferred on to a nylon membrane, and hybridized with the human cDNA probe, pC21/3c (ATCC) (20), which hybridizes with *CYP21A1P* and *CYP21A2*. Probe labeling, hybridization and signal detection were performed using the AlkPhos Direct<sup>®</sup> system (GE Healthcare).

Written informed consent was obtained from the parents of the patient, in accordance with the institutional review board of Tokyo Medical and Dental University.

### Construction of plasmids and site-directed mutagenesis

Expression plasmid pCMV4-CYP21-WT, containing the cDNA of *CYP21A2*, was kindly

provided by Dr. Anna Wedell (Department of Molecular Medicine and Surgery, Karolinska Institutet/Karolinska University Hospital, Stockholm, Sweden) (21).

Mutant plasmid of *CYP21A2* (R483Q), pCMV4-CYP21-R483Q, was generated from pCMV4-CYP21-WT plasmid, using the QuikChange<sup>®</sup> site directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. The identity of the mutated nucleotides was confirmed by DNA sequencing.

### Cell culture and transfection

COS-1 cells that do not express *CYP21A2* were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin (Gibco BRL). Transient transfection of each plasmid was performed using FuGENE<sup>®</sup> (Roche) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were harvested and prepared for examination (12).

### Western blotting and antibodies

COS-1 cells were transiently transfected with pCMV4-CYP21-WT, pCMV4-CYP21-R483Q, or an empty vector using FuGENE<sup>®</sup>. Transfected cells were trypsinized, pelleted, and lysed in lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM PMSF) containing 1/100 $\times$  protease inhibitor cocktail (Sigma) for 30 min on ice. After sonication and centrifugation, the supernatants were fractionated in 10% sodium dodecylsulfate-polyacrylamide gel, transferred to polyvinylidene difluoride membranes (Millipore), and immunoblotted using the rabbit polyclonal anti-CYP21 antibody (Corgen) (1:5000) in 3% non-fat milk. The signals were detected by an ECL system (Amersham).

### Functional analysis

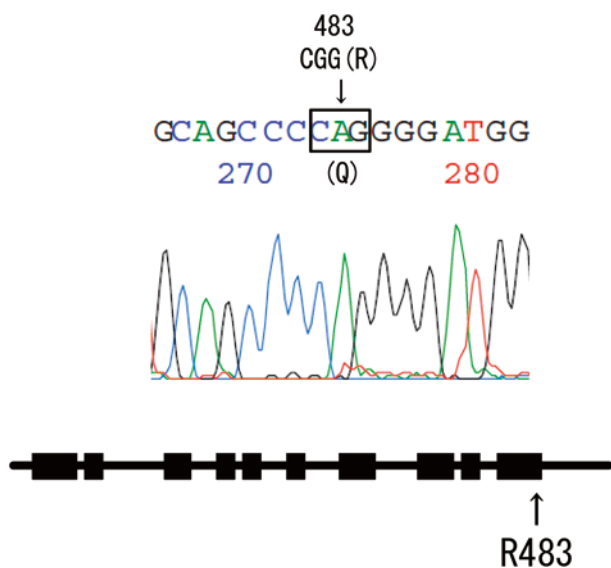
Functional analysis was performed according to a previous report (10,12). COS-1 cells were

seeded in a 24-well plate and 0.2  $\mu$ g of the pCMV4-CYP21-WT, pCMV4-CYP21-R483Q or empty vector were co-transfected with 0.05  $\mu$ g of pSV- $\beta$ -galactosidase control vector (Promega) in each well. Twenty-four hours after transfection, cells were washed twice with phosphate-buffered saline and were incubated for 15 min at 37°C with 2.0  $\mu$ mol/l of substrate, progesterone or 17-OHP, and 4.0 mmol/l of reduced  $\beta$ -nicotinamide adenine dinucleotide phosphate (NADPH) (Sigma). We collected the media and measured the level of steroid products, deoxycorticosterone (DOC), and 11-deoxycortisol (11-DOF), using LC-ESI-MS/MS. Total protein content was determined directly after cell harvest using BCA Protein Assay Kit<sup>®</sup> (Pierce).  $\beta$ -galactosidase activity was measured according to standard protocols (Promega). The efficiency and reproducibility of the transfection was estimated by determining the ratio of  $\beta$ -galactosidase activity to the total protein content. We defined wild type enzyme activity as 100%.

### Liquid chromatography electrospray-ionization tandem mass spectrometry (LC-ESI-MS/MS)

Four wells in each group were measured, and the measurement was repeated twice in independent experiments. Detailed description of the LC-ESI-MS/MS has recently been reported (17). A LC-ESI-MS/MS (API 5000 system, Applied Biosystems, CA, USA) equipped with an ESI ion source was used. Briefly, HPLC was run on a Shimadzu system (SCL 10A system controller, LC-20AD pump, Kyoto, Japan) equipped with a CTO-20A autosampler. The column was a Cadenza CD-C18 (150 mm  $\times$  4.6 mm I.D., 3  $\mu$ m, Imtakt, Kyoto, Japan). The mobile phase consisted of CH<sub>3</sub>CN and 0.25% CH<sub>3</sub>COOH (45:55, v/v) with gradient elution at a flow rate of 0.4 ml/min.

Derivatization was carried out as previously described microscale derivatization and purification method 1 (18). Briefly, a mixed solution of reagents (2-methyl-6-nitrobenzoic



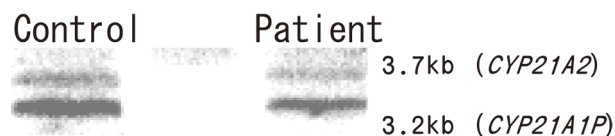
**Fig. 1** Chromatograms of the *CYP21A2* DNA sequence at position 2669 of exon 10 in the patient. Chromatograms obtained by direct sequencing of PCR products reveal a substitution of G for A in exon 10 of the *CYP21A2* in the patient. In this missense mutation, coded amino acid arginine is substituted by glutamine.

anhydride, 4-dimethylaminopyridine and picolinic acid in dry tetrahydrofuran) was added to a dried tube containing steroid extract. Then triethylamine was added to this reaction mixture and purified by the solid-phase column.

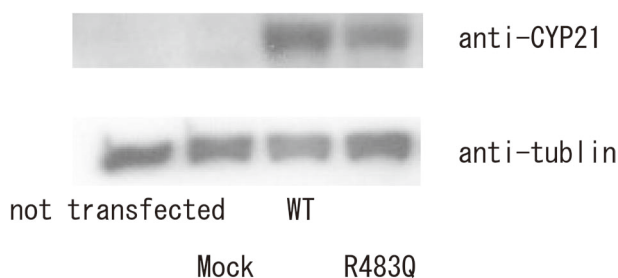
## Results

We screened the genomic DNA of the patient for nine major mutations with the PCR-RFLP method. We were unable to detect these major mutations. We performed direct sequencing and detected the R483Q (2669G>A) mutation in exon 10 (Fig. 1). The result of Southern blot analysis suggested that one allele of *CYP21A2* was lost (Fig. 2). Thus, we concluded that this patient had a compound heterozygous genotype for the R483Q missense mutation and *CYP21A2* gene deletions.

We further evaluated the *in vitro* enzyme



**Fig. 2** Southern blot analysis of *CYP21A2* gene. Compared to the control, the intensity of the 3.7 kb band is reduced in the patient. This indicates that one allele of *CYP21A2* in this patient is lost through gene deletion or translocation.

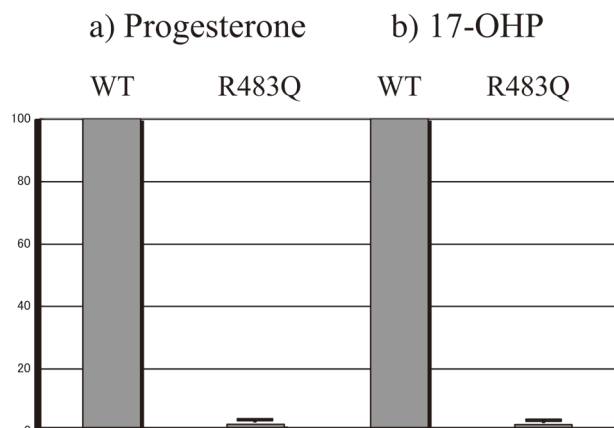


**Fig. 3** The 21-hydroxylase expression detected using western blot analysis in transiently transfected COS-1 cells. pCMV vector carrying 21-hydroxylase wild type and the R483Q mutation was transiently transfected into COS-1 cells. Western blot analysis was performed. Protein expression revealed no differences between the wild type and the R483Q mutants.

activity. The level of the R483Q mutant protein expression was the same as that of the wild type protein, suggesting that this mutation did not affect the level of 21-hydroxylase protein expression (Fig. 3). The activity of the R483Q mutant protein is shown in Fig. 4. The enzyme activities of mutant 21-hydroxylase, which converts progesterone to DOC, and 17-OHP to 11-DOF, were measured as  $2.00 \pm 0.25\%$  and  $1.89 \pm 0.30\%$  of the wild type. These results indicate that the mutant enzyme activity was profoundly impaired.

## Discussion

In this study, we report a case of 21-OHD



**Fig. 4** Measurement of enzyme activity of R483Q mutants using LC-ESI-MS/MS. Enzyme activity of R483Q mutant is expressed in relative values as a percentage of wild type activity, defined as 100%. Rates were determined for the conversion of a) progesterone to DOC and b) 17-OHP to 11-DOF. All values are mean  $\pm$  1SD. Progesterone:  $2.00 \pm 0.25$  (%), 17-OHP:  $1.89 \pm 0.30$  (%).

with a R483Q mutation. The enzyme activity of the R483Q mutant 21-hydroxylase was measured *in vitro* using the LC-ESI-MS/MS method and was extremely impaired (conversion rate  $2.00 \pm 0.25\%$  for progesterone;  $1.89 \pm 0.30\%$  for 17-OHP). These results were in agreement with a previous report (12). Previous studies have shown that *in vitro* activities from approximately 1 to 14% are associated with a simple virilizing phenotype (8, 12, 22). Thus, the R483Q mutation found by us may belong to the group of *CYP21A2* mutations that result in the simple virilizing form of 21-OHD.

Five types of R483 mutations (R483Q, R483P, R483W, 2668delGGinsC, and 2669insC) have been reported (12, 23–26). The R483Q missense mutation is one of these mutations. Thus, amino acid R483 is thought to play an important role in the proper functioning of 21-hydroxylase (12, 27). R483 is a conserved amino acid in other mammals, such as porcine, bovine, and murine species. However, in other

steroidogenic enzymes, such as P450c11, P450scc, and P450c17, this homology is not maintained (12, 27). This suggests R483 plays a role in substrate specificity. A recent report suggested that R483 interacts with D322 located in helix J and plays an important role as the macro dipole stabilizer of  $\alpha$ -helix (28).

So far, two other cases of R483Q mutation have been reported. One case was a male patient diagnosed with the nonclassical form and the other was a female patient with the simple virilizing form (11, 12). The male patient had a compound heterozygous genotype with a deletion/conversion in his other allele. His case was detected during neonatal screening, and he had only slightly elevated levels of serum 17-OHP and androstenedione with no salt loss or elevated plasma renin concentration. Elevated 17-OHP and androstenedione decreased without treatment during the first year of the patient's life (11). However, based on the clinical phenotype, it is difficult to distinguish between the simple virilizing and nonclassical forms in male patients. To describe the exact phenotype, careful long-term observation and re-evaluation of the phenotype may be necessary. On the other hand, the female patient had compound heterozygous genotype with I172N mutation (12). The residual I172N enzyme activity was reported as 3–7% (22), which was slightly more than that of R483Q. The phenotype in this case could be explained by the residual activity of the I172N mutation.

In our case, clitoromegaly and minor labial fusion were observed. There were no symptoms of adrenal crisis or shock with normal serum levels of electrolytes and glucose. The patient had poor weight gain and showed an increased level of plasma renin activity. These findings indicate that our case had a more severe phenotype than the female patient, who was a compound heterozygote of R483Q and I172N (12). Our case had compound heterozygous genotype with deletion/conversion and R483Q mutation. The difference of residual enzyme

activity between I172N and R483Q may cause these alternations in phenotype.

Steroid molecules are characterized by a carbon skeleton with four fused rings, and have different functions according to the molecules attached to these rings and the oxidation state of the rings. Hundreds of different steroids are found in animals, therefore, to evaluate a specific steroid, high performance measurement systems are required. Conventional methods for analyzing the steroidogenic enzyme activity *in vitro* are a combination of <sup>3</sup>H-labeling of steroid substrate followed by thin-layer chromatography. This method has some limitations, such as complicated procedures, radiolabel handling, and indirect measurement of the synthesized substances.

High performance liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) is used for quantitative bioanalysis of small molecules due to its outstanding sensitivity and selectivity. To improve the detection sensitivity of the LC-MS, the derivatization of steroids is essential (17). Previously reported derivatization methods were suitable for limited types of steroid molecules. For example, HMP (2-hydrazino-1-methylpyridinium) derivatization is not appropriate for steroids with two carbonyl groups, e.g. androstenedione and progesterone (29). Picolinoyl derivatization is a newly developed method (17, 18). Using this method, steroids are derivatized easily by following a one-step procedure, and it is possible to simultaneously measure multiple steroid molecules. In our method, synthesized steroids were measured directly and their absolute values were reported. Our results were in agreement with the previous data measured by the conventional method (12). Thus, the LC-ESI-MS/MS system is an improvement over the existing *in vitro* enzyme assay.

In conclusion, we identified the R483Q mutation in *CYP21A2*. Because the residual enzyme activity of this mutant can be determined by a novel LC-ESI-MS/MS using picolinoyl ionization, the LC-ESI-MS/MS method is a viable

new *in vitro* enzyme assay method for evaluating the activity of 21-hydroxylase in CAH patients.

## References

1. Newfield RS, New MI. 21-hydroxylase deficiency. *Ann NY Acad Sci* 1997;816:219–29.
2. White PC, Speiser PW. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Endocr Rev* 2000;21:245–91.
3. White PC, New MI, Dupont B. Congenital adrenal hyperplasia. *N Engl J Med* 1987;316:1519–24.
4. Speiser PW, White PC. Congenital adrenal hyperplasia. *N Engl J Med* 2003;349:776–88.
5. Merke DP, Bornstein SR. Congenital adrenal hyperplasia. *Lancet* 2005;365:2125–36.
6. Shimozawa K, Saisho S, Saito N, Yata J, Igarashi Y, Hikita Y, *et al.* A neonatal mass-screening for congenital adrenal hyperplasia in Japan. *Acta Endocrinol (Copenh)* 1984;107:513–8.
7. Naruse H, Suzuki E, Irie M, Tsuji A, Takasugi N, Fukushi M, *et al.* Neonatal screening for congenital adrenal hyperplasia in Japan. *Ann NY Acad Sci* 1985;458:103–10.
8. Tusie-Luna MT, Traktman P, White PC. Determination of functional effects of mutations in the steroid 21-hydroxylase gene (*CYP21*) using recombinant vaccinia virus. *J Biol Chem* 1990;265:20916–22.
9. Higashi Y, Fujii-Kuriyama Y. Functional analysis of mutant P450(*C21*) genes in COS cell expression system. *Methods Enzymol* 1991;206:166–73.
10. Robins T, Barbaro M, Lajic S, Wedell A. Not all amino acid substitutions of the common cluster E6 mutation in *CYP21* cause congenital adrenal hyperplasia. *J Clin Endocrinol Metab* 2005;90:2148–53.
11. Stikkelbroeck NM, Hoefsloot LH, de Wijs IJ, Otten BJ, Hermus AR, Sistermans EA. *CYP21* gene mutation analysis in 198 patients with 21-hydroxylase deficiency in the Netherlands: six novel mutations and a specific cluster of four mutations. *J Clin Endocrinol Metab* 2003;88:3852–9.
12. Robins T, Bellanne-Chantelot C, Barbaro M, Cabrol S, Wedell A, Lajic S. Characterization of novel missense mutations in *CYP21* causing congenital adrenal hyperplasia. *J Mol Med*

- 2007;85:247–55.
13. Mück W. Quantitative analysis of pharmacokinetic study samples by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). *Pharmazie* 1999;54:639–44.
  14. Ma S, Subramanian R. Detecting and characterizing reactive metabolites by liquid chromatography/tandem mass spectrometry. *J Mass Spectrom* 2006;41:1121–39.
  15. Samtani MN, Jusko WJ. Quantification of dexamethasone and corticosterone in rat biofluids and fetal tissue using highly sensitive analytical methods: assay validation and application to a pharmacokinetic study. *Biomed Chromatogr* 2007;21:585–97.
  16. Spyridaki MH, Kiouisi P, Vonaparti A, Valavani P, Zonaras V, Zahariou M, *et al.* Doping control analysis in human urine by liquid chromatography-electrospray ionization ion trap mass spectrometry for the Olympic Games Athens 2004: determination of corticosteroids and quantification of ephedrine, salbutamol and morphine. *Anal Chim Acta* 2006;573–574:242–9.
  17. Yamashita K, Takahashi M, Tsukamoto S, Numazawa M, Okuyama M, Honma S. Use of novel picolinoyl derivatization for simultaneous quantification of six corticosteroids by liquid chromatography-electrospray ionization tandem mass spectrometry. *J Chromatogr A* 2007;1173:120–8.
  18. Yamashita K, Okuyama M, Watanabe Y, Honma S, Kobayashi S, Numazawa M. Highly sensitive determination of estrone and estradiol in human serum by liquid chromatography-electrospray ionization tandem mass spectrometry. *Steroids* 2007;72:819–27.
  19. Koyama S, Toyoura T, Saisho S, Shimozawa K, Yata J. Genetic analysis of Japanese patients with 21-hydroxylase deficiency: identification of a patient with a new mutation of a homozygous deletion of adenine at codon 246 and patients without demonstrable mutations within the structural gene for CYP21. *J Clin Endocrinol Metab* 2002;87:2668–73.
  20. White PC, Grossberger D, Onufer BJ, Chaplin DD, New MI, Dupont B, *et al.* Two genes encoding steroid 21-hydroxylase are located near the genes encoding the fourth component of complement in man. *Proc Natl Acad Sci USA* 1985;82:1089–93.
  21. Nikoshkov A, Lajic S, Holst M, Wedell A, Luthman H. Synergistic effect of partially inactivating mutations in steroid 21-hydroxylase deficiency. *J Clin Endocrinol Metab* 1997;82:194–9.
  22. Larsen P, Kronenberg H, Memed S, Polonsky K. *Williams textbook of endocrinology* 10<sup>th</sup> edition. Philadelphia: WB Saunders; 2003. p.913–27.
  23. Nikoshkov A, Lajic S, Vlamis-Gardikas A, Tranebjærgi L, Holst M, Wedell A, *et al.* Naturally occurring mutants of human steroid 21-hydroxylase (P450c21) pinpoint residues important for enzyme activity and stability. *J Biol Chem* 1998;273:6163–5.
  24. Barbat B, Bogyo A, Raux-Demay MC, Kuttann F, Boué J, Simon-Bouy B, *et al.* Screening of CYP21 gene mutations in 129 French patients affected by steroid 21-hydroxylase deficiency. *Hum Mutat* 1995;5:126–30.
  25. Kharrat M, Tardy V, M'Rad R, Maazoul F, Jemaa LB, Refai M, Morel Y, *et al.* Molecular genetic analysis of Tunisian patients with a classic form of 21-hydroxylase deficiency: identification of four novel mutations and high prevalence of Q318X mutation. *J Clin Endocrinol Metab* 2004;89:368–74.
  26. Wedell A, Ritzén EM, Haglund-Stengler B, Luthman H. Steroid 21-hydroxylase deficiency: three additional mutated alleles and establishment of phenotype-genotype relationships of common mutations. *Proc Natl Acad Sci USA* 1992;89:7232–6.
  27. Wedell A, Luthman H. Steroid 21-hydroxylase (P450c21): a new allele and spread of mutations through the pseudogene. *Hum Genet* 1993;91:236–40.
  28. Robins T, Carlsson J, Sunnerhagen M, Wedell A, Persson B. Molecular model of human CYP21 based on mammalian CYP2C5: structural features correlate with clinical severity of mutations causing congenital adrenal hyperplasia. *Mol Endocrinol* 2006;20:2946–64.
  29. Higashi T, Yamauchi A, Shimada K. 2-hydrazino-1-methylpyridine: a highly sensitive derivatization reagent for oxosteroids in liquid chromatography-electrospray ionization-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2005;825:214–22.