Noninvasive Prenatal Exclusion of Congenital Adrenal Hyperplasia by Maternal Plasma Analysis: A Feasibility Study, *Rossa W.K. Chiu,[1] Tze K. Lau,[2] Pik T. Cheung,[3] Zhi Q. Gong,[3] Tse N. Leung,[2] and Y.M. Dennis Lo[1*]* (Departments of [1] Chemical Pathology and [2] Obstetrics and Gynaecology, The Chinese University of Hong Kong, Hong Kong SAR; [3] Department of Paediatrics, The University of Hong Kong, Hong Kong SAR; * address correspondence to this author at: Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Room 38023, 1/F Clinical Sciences Building, 30-32 Ngan Shing St., Shatin, New Territories, Hong Kong SAR; fax 852-2194-6171, e-mail loym@cuhk.edu.hk)

The presence of fetal DNA in maternal plasma has allowed the development of strategies for noninvasive prenatal diagnosis *(1)*. However, because fetal DNA in maternal plasma circulates among a background of maternal DNA, strategies for noninvasive prenatal diagnosis with applications of fetal DNA in maternal plasma have been confined to the detection of autosomal dominant, paternally inherited genetic traits, such as fetal gender (for sex-linked disorders) *(1)*, rhesus D *(2, 3)*, myotonic dystrophy *(4)*, and achondroplasia *(5)*. Using congenital adrenal hyperplasia (CAH) as a model system, we present a strategy for the noninvasive prenatal exclusion of an autosomal recessive condition through the detection of fetal DNA in maternal plasma. The approach described in this study may potentially be applicable to other autosomal recessive conditions.

More than 90% of cases of CAH are a result of deficiency of 21-hydroxylase, an enzyme of the adrenal gland involved in the synthesis of glucocorticoids and mineralocorticoids. 21-Hydroxylase is encoded by *CYP21*, a MHC class III gene located on chromosome 6p21.3. Most mutations causing 21-hydroxylase deficiency are caused by either gene deletions or gene conversions, whereby deleterious mutations are transferred from the nearby pseudogene, *CYP21P*, which shares 98% homology with *CYP21 (6)*. Consequent to profound deficiency or the complete absence of activity of 21-hydroxylase, severe forms of CAH manifest as salt-wasting attributable to impaired synthesis of mineralocorticoids and glucocorticoids. In addition, the excess buildup of metabolic precursors causes excessive androgen production, leading to virilization of female fetuses *(6)*. Hence, dexamethasone therapy is customarily prescribed prenatally to prevent in utero virilization of an affected female fetus through suppression of the excessive production of androgens *(7)*. This type of treatment is not indicated for a male fetus. In the antenatal management of CAH, fetal DNA in maternal plasma has been reported to be useful for the stratification of antenatal dexamethasone therapy through noninvasive fetal gender determination *(8)* because such therapy would be unnecessary for male fetuses. However, it would be ideal to further limit the dexamethasone therapy only to female fetuses known to be affected by CAH because the use of antenatal dexamethasone is not without complications *(6)*. Consequently, this study attempted to develop a noninvasive approach that allows the effective in utero exclusion of CAH. The strategy proposed is derived from the reasoning that the presence of a wild-type paternally inherited *CYP21* allele in maternal plasma would infer that the fetus had inherited the nonmutated paternal allele and thus would not manifest CAH.

A set of parents whose first child was diagnosed with the salt-wasting form of CAH attributable to 21-hydroxylase deficiency presented for antenatal care during their second pregnancy. The proband was known to manifest CAH as a result of the inheritance of an exon 8 codon 316 (arginine–stop) mutation of *CYP21 (9)* from the father and a maternally derived de novo mutation that led to the deletion of *CYP21* (data not shown) *(10–12)*. The parents consented to this study, the aim of which was to develop assays to determine, through analysis of maternal plasma, whether the unborn fetus had inherited the wild-type or mutant *CYP21* paternal allele. We propose the use of polymorphic markers within and linked to *CYP21* to differentiate the wild-type from the mutant paternal allele.

Blood samples from the couple and the proband were collected into EDTA-containing tubes. Maternal plasma samples were collected at 11 and 17 weeks of gestation. Buffy coat was collected from the blood samples after centrifugation at 1600*g* for 10 min (Megafuge 1.0R; Heraeus Instruments), whereas maternal plasma was collected after an additional microcentrifugation step at 16 000*g* for 10 min (Eppendorf Centrifuge 5415D) *(13)*. Buffy-coat DNA was extracted with the Nucleon reagent set (Amersham Life Science), whereas DNA was extracted from maternal plasma according to the Blood and Body Fluid protocol of a QIAamp reagent set (Qiagen).

Analysis of the antenatal maternal-plasma samples did not reveal the presence of *SRY* sequences *(14)*, suggesting that the mother was carrying a female fetus. Genomic DNA from the buffy-coat samples from the parents and the proband was first used to select informative markers useful for further analysis. It is common experience that molecular analysis of *CYP21* is often difficult and misleading because of the presence of *CYP21P*, the complex rearrangements and conversion between these two genes, and the reported phenomenon of allele-dropout *(15)*. Therefore, to maximize the reliability of the analyses, both extragenic and intragenic polymorphic markers were selected. The family was informative for three sets of markers, including single-nucleotide polymorphisms in intron 2 of *CYP21 (16)*, linked extragenic microsatellite markers *D6S273* and *D6S299*, and the HLA class II *DRB1* locus. Identification of the intragenic polymorphic markers was established by PCR amplification with primers specific for *CYP21 (16)*, followed by direct sequencing of intron 2 of *CYP21*. The microsatellite markers were analyzed by fluorescence PCR and fragment-size analysis by an ABI Prism 377 automated sequencer. The HLA haplotype of each family member was characterized by serologic methods, and the *DRB1* locus was selected for further analysis. To facilitate detection of the paternally

inherited fetal *DRB1* allele in maternal plasma, amplification refractory mutation system primers *(17)* were designed to amplify the two paternal alleles (*DRB1*03* and *DRB1*12*). The amplification refractory mutation system primers were designed to amplify exon 2 of the *DRB1* alleles, and the amplicons were visualized by agarose gel electrophoresis and ethidium bromide staining. Sequences of the *DRB1* alleles were obtained from the HLA sequence database *(18)*. The primer sequences and pattern of allelic segregation within the family are shown in Table 1. The allele of the respective markers not detectable from the proband was inferred to be associated with the nonmutated paternal allele.

After characterization of a panel of polymorphic markers associated with the wild-type paternal allele, attempts were made to detect the paternally inherited fetal allele in maternal plasma with the same analytical methods as described above. Throughout the process of maternal plasma analysis, the two researchers studying the plasma were blinded from the mutational analysis of the family. On analysis of the maternal plasma, four of the five paternal markers not inherited by the proband were detectable in maternal plasma. Results of the maternal plasma analysis are shown in Table 1. The results inferred the inheritance of the nonmutated paternal allele; thus the autosomal recessive disease in question could be excluded.

The volunteers subsequently gave birth to a healthy girl. Umbilical cord blood was subjected to the same polymorphic marker analyses, and the marker haplotype for the paternally inherited allele was concordant with the maternal plasma analyses. To further confirm the validity of the analyses of fetal DNA in maternal plasma, the mother consented to blood sampling at 24 h postdelivery, and the maternal plasma was subjected to analysis for the paternal polymorphic markers. Postdelivery clearance of fetal DNA from maternal plasma is known to be a rapid process *(19)*, and indeed, we failed to detect the previously detectable paternal polymorphisms in the postdelivery maternal plasma sample.

Rijnders et al. *(8)* reported the use of fetal DNA in maternal plasma for fetal-sex determination to help stratify the need for antenatal dexamethasone therapy. Fetal-sex determination helps to eliminate unnecessary dexamethasone therapy for male fetuses, and the prenatal exclusion of CAH would further reduce unnecessary therapy for nonaffected female fetuses. Using CAH as a model system, we report here that it is feasible to detect polymorphisms associated with the wild-type paternally inherited allele in maternal plasma and, thus, have demonstrated a potential strategy for the noninvasive prenatal exclusion of an autosomal recessive disease. This approach may potentially be applicable to other autosomal recessive conditions, including cystic fibrosis, β-thalassemia, and certain HLA-linked conditions such as hemochromatosis.

Table 1. Detection of polymorphic loci and respective alleles.

A. Polymorphic loci and respective alleles detected from blood samples[a]

Polymorphism	Father	Mother	First child	Primer sequences
HLA *DRB1**	03/**12**	14/0901	03/0901	03F: 5′-ACGTTTCTTGGAGTACTCTACGTC-3′ 03R: 5′-AGTAGTTGTCCACCCGGC-3′ 12F: 5′-CACGTTTCTTGGAGTACTCTACGGG-3′ 12R: 5′-GCGGCGCGCCTGTCT-3′
D6S299	222/**214**	222/232	222/232	F: 5′-(FAM)-AGGTCATTGTGCCAGG-3′[b] R: 5′-TGTCTATGTATACTCCTGAATGTCT-3′
D6S273	129/**127**	135/133	129/133	F: 5′-(HEX)-GCAACTTTTCTGTCAATCCA-3′ R: 5′-ACCAAACTTCAAATTTTCGG-3′
IVS2 564	G/Δ**G**[c]	G/G	G/−	Previously described primers 335F and b73R *(16)*
IVS2 588	G/**A**	G/G	G/−	Previously described primers 335F and b73R *(16)*

B. Detectability of wild-type paternal polymorphic alleles in maternal plasma

Wild-type paternal allele	Positive detection in maternal plasma
HLA *DRB1*12*	Yes
D6S299 214	No amplification
D6S273 127	Yes
IVS2 564 ΔG[c]	Yes
IVS2 588 A	Yes

[a] Blood samples were from each family member. The haplotype of the nonmutated paternal allele is highlighted in bold and is inferred from its absence in the proband's genotype. The HLA *DRB1* alleles are named according to the WHO Nomenclature Committee for Factors of the HLA System *(20)*. The alleles of the microsatellite markers, *D6S299* and *D6S273*, are named by the fragment size measured in basepairs. The polymorphic sequences in intron 2 (IVS2) of *CYP21* are described by the nucleotide found at the particular position.

[b] FAM, 6-carboxy-fluorescein; HEX, hexachloro-6-carboxy-fluorescein.

[c] Δ denotes a single-base deletion.

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Unusual Late Onset of X-linked Chronic Granulomatous Disease in an Adult Woman after Unsuspicious Childhood, *Andreas Lun,[1*] Joachim Roesler,[2] and Harald Renz[3]* ([1] Institut für Laboratoriumsmedizin und Pathobiochemie, Charité, Campus Virchow-Klinikum, Augustenburger Platz 1, D-13353 Berlin, Germany; [2] Klinik für Kinderheilkunde, Universitätsklinikum Carl Gustav Carus, TU, D-01307 Dresden, Germany; [3] Abteilung Klinische Chemie und Molekulare Diagnostik, Philipps-Universität, D-35033 Marburg, Germany; * author for correspondence: fax 004930-450569598, e-mail andreas.lun@charite.de)

The multisubunit enzyme NADPH oxidase [phagocyte oxidase (phox)] of phagocytic cells plays a central role in cellular host defense *(1)*. Phagocytes release large amounts of superoxide in the respiratory extraburst after stimulation with microorganisms. The production and subsequent conversion of superoxide to microbicidal reactive oxygen metabolites [(ROMs); e.g., H_2O_2] are critical for the elimination of pathogens such as *Staphylococcus aureus*, *Serratia marcescens*, and *Aspergillus (2)*.

After activation of phagocytes, p47-phox and p67-phox, together with other proteins such as Rac, translocate from cytosol to the membrane, where they associate with two membrane-bound subunits of cytochrome b_{558}, p22-phox and gp91-phox *(3)*. This initiates an electron flux from NADPH via the activated phox to molecular oxygen leading to the generation of superoxide *(4)*.

The essential role of this oxidase in cellular host defense is clearly demonstrated in patients suffering from a rare inherited disorder known as chronic granulomatous disease (CGD) *(5)*. Mutations affecting any of the four subunits gp91-phox, p22-phox, p47-phox, or p67-phox either render phagocytes from CGD patients incapable of superoxide generation after stimulation or strongly decrease its production *(6)*. Typically, these patients suffer from recurrent and life-threatening bacterial and fungal infections *(3)*.

Here we present the case of a 43-year-old female patient with recurrent serious conditions that are typical of CGD, including *Aspergillus fumigatus* infection and formation of intestinal granulomas. The history of the patient is remarkable because for the first 17 years of her life, no typical CGD infections and no typical symptoms of CGD occurred. In the following years, the patient experienced cutaneous abscesses in the anogenital and back region and recurrent bacterial pneumonia (ages 29, 36, 41, 42, and 43 years). At age 29 years, the patient suffered from a severe therapy-resistant salmonella sepsis with ensuing severe damage of the liver and hepatic coma.

The symptoms and history led to the differential diagnosis of CGD. Stimuli-induced ROM formation of her neutrophils was tested with dihydrorhodamine 123[Phago-Burst; Orpegen; see Ref. *(7)*]. Most of the patient's neutrophils (98%) were unable to produce ROMs aftermaximal stimulation with phorbol-myristate-acetate. Only 2% at most of her neutrophils produced H_2O_2 at concentrations within the reference interval (Fig. 1). To