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Complement component 4 variations may influence psychopathology risk in patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency

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Abstract

Background: *CYP21A2* defects result in congenital adrenal hyperplasia (CAH), an autosomal recessive disorder characterized by impaired adrenal steroidogenesis. *CYP21A2* lies within the major histocompatibility complex in an area of the genome highly susceptible to genetic variation. Alterations in the neighboring complement component 4 isotypes *C4A* and *C4B* have been associated with psychiatric and autoimmune disease. The purpose of this study was to evaluate *C4A* and *C4B* in patients with CAH in relation to *CYP21A2* genotype and psychiatric and autoimmune comorbidity.

Methods: We determined the copy numbers of *C4A* and *C4B* in 145 patients with CAH (median age: 15.5 yrs, IQR: 16.8) and 108 carrier relatives (median age: 41.5 yrs, IQR: 12.0) and evaluated serum C4 concentrations. Comorbidity was determined by medical record review.

Results: Only 30% of subjects had the expected two copies each of the two *C4* genes. *C4B* copy number determined total *C4* copy number and serum C4 concentration, negatively correlated with carriership of a 30-kb deletion ($P < 10^{-5}$), and positively correlated with carriership of p.V281L ($P < 10^{-5}$). High *C4A* copy number (≥ 3) was associated with increased risk of having an externalizing psychiatric condition (relative risk: 2.67, 95% CI: 1.03–6.89, $P = 0.04$). No association was found between *C4* copy number and autoimmune disease.

Conclusion: Mutation specific *C4* structural variations commonly occur in patients with CAH and may have important clinical consequences, including increased risk of psychiatric morbidity.

Trial registration: (November 7, 2005)

Keywords

Complement component 4; C4; congenital adrenal hyperplasia; CAH; *CYP21A2*

INTRODUCTION

Congenital Adrenal Hyperplasia (CAH) due to 21-hydroxylase deficiency (21-OHD; *CYP21A2*; OMIM201910) is an autosomal recessive disease of the adrenal cortex resulting in altered steroidogenesis and manifests as a broad spectrum of phenotypes. CAH is classified based on clinical severity: the classic (severe) form includes two subtypes, salt-wasting (SW) and simple virilizing (SV), and affects approximately 1 in 15,000 live births (Speiser et al. 2018); the mild but more common nonclassic (NC) form is estimated to occur in 1 in 200 to 1 in 1,000 Caucasians. (Hannah-Shmouni et al. 2017) Twelve common mutations and large (30-kb) deletions account for the majority of *CYP21A2* defects (Finkelstein et al. 2011).

The *CYP21A2* gene is mapped at a locus of low copy repeats composed of *RP-C4-CYP21-TNX (RCCX)* module(s), in the human histocompatibility complex on chromosome 6 (p21.33) (supplemental Fig. S1) (Blanchong et al. 2000). *RP* signifies *RPI* (synonym *STK19*) encoding a serine/threonine nuclear protein kinase of unknown function and pseudogene *RP2 (STK19P)*; *C4* signifies *C4A* and *C4B* encoding two isotopes of complement component 4; *CYP21* signifies *CYP21A2* and pseudogene *CYP21A1P*; *TNX* signifies *TNXB* encoding tenascin-X and pseudogene *TNXA*. The locus is error prone during meiosis sometimes leading to structural variations in daughter alleles. These variations are mainly caused by unequal crossovers between highly homologous gene pairs, resulting in gene deletion or duplication. Amongst *CYP21A2* pathogenic alleles are 30-kb deletions resulting in *CYP21A1P/CYP21A2* or *CYP21A1P-TNXA/TNXB* chimeras and accounting for over 30% of *CYP21A2* mutations associated with the classic form (Burch et al. 1997; Chen et al. 2012b; Finkelstein et al. 2011; Merke et al. 2013; Morissette et al. 2015). A point mutation in exon 7, p.V281L, accounts for the majority of NC alleles worldwide (Falhammar and Nordenstrom 2015; Hannah-Shmouni et al. 2017).

In the general population, a bimodular *RCCX* with one copy each of *C4A* and *C4B* is the most common haplotype with an allele frequency of approximately 75 percent (Banlaki et al. 2012; Blanchong et al. 2000; Sekar et al. 2016; Wu et al. 2008). *C4* plays an important role in the activation of immune defenses and the clearance of immune complexes. The two isotopes of *C4* have different chemical reactivities. *C4A* is more reactive to amino group containing antigens, while *C4B* primarily binds to antigens with hydroxyl groups (Blanchong et al. 2001; Law et al. 1984). These isotopes have been implicated as playing a role in a variety of diseases. High *C4A* gene dosage has been identified as a risk factor for schizophrenia and high copy number of either *C4A* or *C4B* has been found to be a risk factor for Alzheimer's disease (Sekar et al. 2016; Zorzetto et al. 2017). Low *C4* copy number likely contributes to the development of autoimmune diseases such as systemic lupus erythematosus (Li et al. 2017; Wu et al. 2008). Patients with CAH suffer from multiple comorbidities including mental illness (Engberg et al. 2015; Falhammar et al. 2014). The purpose of this study was to evaluate *C4A* and *C4B* copy number in patients with CAH and to evaluate the clinical implications of *C4* copy number variation, specifically in relation to psychiatric morbidity and autoimmunity.

MATERIALS AND METHODS

We evaluated 145 patients with CAH due to 21-OHD and their 108 carrier relatives. (Table 1). All subjects were enrolled in an ongoing Natural History Study at the NIH Clinical Center in Bethesda, MD, USA (). All subjects (and parents of patients < 18 years old) gave written informed consent and the study was approved by the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development Institutional Review Board. Serum C4 concentrations were determined with a Vista analyzer (NIH Clinical Center Laboratory, Bethesda, MD). Individuals were selected based on the availability of high quality genomic DNA samples at the time of study.

All subjects had previously completed *CYP21A2* genotyping (Finkelstein et al. 2011). *C4A* and *C4B* copy numbers were determined by a droplet digital PCR (ddPCR) method described by Sekar *et al* with minor modifications in primers and probes (supplemental Table S1) (Sekar et al. 2016).

Psychiatric morbidity and autoimmunity were determined by retrospective chart review. Clinical data was not available for carriers. A mental health condition was recorded if the patient was treated with a psychotropic medication for a diagnosed condition. A psychiatrist (ME), who was blinded to the genetic data, reviewed all psychiatric data and categorized the condition as “internalizing” representing disorders such as prominent anxiety, depressive, and somatic symptoms or “externalizing” disorders representing disorders such as prominent impulsive, disruptive conduct, and substance use symptoms according to a widely accepted clinical guidelines (American Psychiatric Association 2013).

Raw *C4* copy number calls (supplemental Fig.S2), were rounded to integers. Statistical analysis was performed using KaleidaGraph version 4.1 (Synergy Software, PA). Associations between total *C4*, *C4A* and *C4B* copy number and *CYP21A2* genotype and psychiatric and autoimmune comorbidity were evaluated. ANOVA was used for comparison among groups and average values were shown as mean $\pm$ standard deviation. Open sourced R program was used for chi-square, Pearson correlation, Spearman rank correlation and relative risk analyses (R Core Team 2017). A *P*value of <0.05 was considered significant.

RESULTS

Copy number variations in the two *C4* genes were commonly observed with a broad range of configurations (Fig. 1). Only 30% of the cohort (43/145 of CAH patients; 33/108 of carriers) had two copies of each *C4* gene in diploid, a criteria for having the bimodular *RCCX* with *C4A-C4B* haplotype in both alleles.

The copy number of *C4B* ($P<10^{-11}$), but not *C4A* ($P=0.20$), was associated with total serum C4 level (Fig 2). In addition, the total number of *C4* genes was more strongly influenced by the *C4B* copy number than by the *C4A* copy number ($r_p=0.81$, $P<10^{-5}$ and $r_p=0.35$, $P=1.9\times 10^{-4}$ respectively). Similar associations were also found in carriers (supplemental Fig.S3).

The *C4B* copy number was negatively associated ($r_p = -0.50$, $P < 10^{-5}$) with carrying a 30-kb deletion (Fig 3A). The majority or 66.7% (40/60) of patients with one 30-kb deletion allele and 90% (18/20) of patients with two 30-kb deletion alleles had a *C4B* copy number 1, compared to 24% (16/65) of patients carrying other mutations ($P < 10^{-5}$). The mean *C4B* copy number varied in relation to the number of 30-kb deletion alleles (0, 1, 2: 2.00 ± 0.88 vs. 1.33 ± 0.73 vs. 0.7 ± 0.66 ; $P < 10^{-4}$). No association was observed between *C4A* copy number and the number of 30-kb deletion alleles.

A positive correlation ($r_p = 0.60$, $P < 10^{-5}$) was observed between copy numbers of *C4B* and p.V281L. Fifty three percent (7/13) of patients with one p.V281L allele and all four patients with two p.V281L alleles had high *C4B* copy number (3), compared to 4% (5/128) of patients with other mutations ($P < 10^{-5}$) (Fig. 3B). The mean *C4B* copy number varied in relation to the number of p.V281L alleles (0, 1 and 2: 1.36 ± 0.75 vs. 2.62 ± 0.65 vs. 4.00 ± 0.00 ; $P < 10^{-4}$). No correlation between *C4A* copy number and p.V281L was observed.

Forty-two of our patients with CAH suffered from mental disorders including depression, anxiety, oppositional/defiant disorder, anorexia nervosa, attention deficit hyperactivity disorder and bipolar mood disorder categorized into internalizing ($n=27$) and externalizing ($n=15$) subgroups. Forty percent (6/15) of patients with externalizing conditions had high *C4A* copy number (3), significantly more frequent than patients with internalizing conditions (7% or 2/27) or without mental disorders (20% or 21/103) ($P=0.04$) (Fig. 3C). The *C4A* copy number was highest in patients with an externalizing disorder (externalizing, internalizing, no mental disorder: 2.47 ± 0.64 vs. 2.00 ± 0.40 vs. 2.13 ± 0.58 ; $P=0.03$). The relative risk of having an externalizing mental condition with *C4A* copy number 3 was 2.67, 95% CI: 1.03–6.89, $P=0.04$. No association was observed between mental conditions and *C4B*, total *C4* copy number, total serum level or the subtypes of CAH. Overall, female CAH patients were more likely to suffer from a mental disorder compared to their male counterparts (26/69 vs. 16/76, $P=0.03$).

Five patients suffered from autoimmune diseases including ulcerative colitis ($n=2$), juvenile rheumatoid arthritis ($n=1$), Grave's disease ($n=1$), and Hashimoto's thyroiditis ($n=1$). The prevalence of autoimmune disease was not associated with *C4* copy number or serum *C4* level.

It is notable that our CAH patient cohort is relatively young (median age of 15.5 yrs with an IQR of 16.8). Many psychiatric or autoimmune diseases develop with time, therefore our data may be underestimating specific risks.

Discussion

In our study, we confirm that individuals affected by CAH, including both patients and carriers, have unusual copy number variations of the *C4* genes, which play an important role in immunity, health and disease. In this population, we demonstrate that the variations in *C4* copy number and serum *C4* level, as well as *CYP21A2* mutation specific associations, are determined by the copy number of *C4B* rather than *C4A*. We also show for the first time an association at the genomic level between *C4* copy number and susceptibility to

psychopathology in patients with CAH. Although CAH is a monogenic disorder, *CYP21A2* defects may influence contiguous genes, expanding the phenotypic spectrum.

We found that *C4B* copy number was the determinant of C4 serum levels in CAH patients. *C4B* copy number varied in those carrying 30-kb deletions and NC patients carrying the p.V281L mutation. Carrying a 30-kb deletion signifies the presence of a *CYP21A2P/CYP21A2* or *CYP21A2P-TNXA/TNXB* chimera caused by unequal crossovers in which a copy of *C4* is lost. Thus, the presence of a 30-kb deletion is expected to be associated with a lower *C4* copy number. These results are in agreement with the human genome assembly in that *C4B* rather than *C4A* is more likely to conjugate with *CYP21A2*; therefore *C4B* and *CYP21A2* are vulnerable to be lost together in the event of a 30-kb deletion. Conversely, the *CYP21A2* p.V281L mutation that causes NC CAH was previously correlated with high total *C4* copy number (Chen et al. 2012a), and we found that this association is due to an increase in *C4B*, not *C4A*. The clinical implications are uncertain; however, distinctions between the C4A and C4B proteins are known. C4A has an important role in immunoclearance; while C4B is important in propagating the complement activation pathways (Yang et al. 2004). Deficiencies of both have been related to infectious and/or autoimmune diseases.

A recent study found high *C4A* expression due to high *C4A* gene dosages (e.g. haplotypes of *C4A-C4A*) as a risk factor for schizophrenia (Sekar et al. 2016), suggesting the importance of the *C4* genes in mental health. Although our patients displayed a range of mental disorders, the one patient in our cohort with early childhood onset of psychosis had high *C4* copy number, predominantly *C4A*. Patients with CAH are at higher risk for mental illness than the general population, most notably substance abuse, adjustment disorders and suicidality, with multiple confounders at play (Engberg et al. 2015; Falhammar et al. 2014). In Sweden, high risk of psychotic disorder was observed in CAH patients with the P30L genotype and SV phenotype (Falhammar et al. 2014). We did not find an association between psychiatric disorders and *CYP21A2* genotype, but did find an association between *C4A* copy number and externalizing psychiatric comorbidity. The stress of having a chronic disease with multiple hormonal imbalances is likely a stronger determinant of mental health status than *C4* gene dosage, as implicated by a recent Swedish study showing CAH carriers have even lower risk of psychiatric morbidity compared to the general population (Nordenstrom et al. 2017). However, we show that genotype as it relates to the neighboring *C4* genes may play a role in the development of psychiatric disorders in CAH patients and even carriers of CAH.

Low total *C4* gene copy number (<4) has been identified as a risk factor for autoimmune disease (Li et al. 2017). We did not find this association in our CAH cohort despite the large number of patients (52/145) having low total *C4* copy number (<4). It is possible that long term glucocorticoid therapy had some protective effects. The young age of our CAH cohort may also have underestimated the lifetime risk of autoimmune disease.

As expected, *RCCX* structural variations were commonly observed. Only 30% of our CAH patients or carriers had two copies each of *C4A* and *C4B* resulting in a low odds of having a haplotype match the genome assembly reference of both alleles. This is in contrast to an estimated 50% chance of matching the reference genome in the general population

(calculated from 70% frequency of *C4A-C4B* haplotype). Thus, the use of next generation sequencing platforms in testing the *RCCX* genes is challenging; highly homologous pseudogene originated reads and great copy number variations remain important obstacles (Mueller et al. 2013).

The strengths of our study are the large cohort size, availability of longitudinal medical records, and the comprehensive *CYP21A2* genotyping that included phase configuration. Nevertheless, due to the lack of allele frequency reference for the *RCCX* locus in the CAH population, it was impossible to conclusively determine the haplotype configuration of the families with 4 *C4* copies. We therefore analyzed and presented our *C4* copy number in diploid without haplotype specifications. However, based on the diploid data, we did observe notable patterns implicating CAH genotype specific *C4* haplotypes. For example, all 17 patients carrying p.V281L likely had at least one allele of *C4A-C4B-C4B*. Limitations of our study include the relatively young age of the cohort and the lack of clinical data for carriers.

The study of *CYP21A2* in relation to the *C4* neighboring genes provides insight into the complex genetics of the human histocompatibility complex, an important immune modulator affecting a spectrum of disorders, and provides evidence that *RCCX* genotype as it relates to *C4* may be an additional risk factor for psychiatric comorbidity in patients with CAH. Large population based studies are needed to better evaluate the impact of *C4* gene dosage on mental health in relation to *CYP21A2* genotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Frequency of various *C4A* and *C4B* copy number configurations in the three clinical subtypes of congenital adrenal hyperplasia. SW, salt-wasting; SV, simple virilizing; NC, nonclassic.

Fig. 2.

Copy number of *C4B*, but not *C4A*, was associated with the serum C4 level in patients with congenital adrenal hyperplasia. (A) Total *C4* copy number, (B) *C4A* copy number, and (C) *C4B* copy number are shown in relation to serum C4 levels. Spearman Rank Correlation test results are shown on the top of each panel.

Fig. 3.

Frequency of *C4B* copy number groups according to *CYP21A2* mutations and psychiatric comorbidity. (A) Copy number of *C4B* was negatively associated with carriership of a 30-kb deletion and (B) positively associated with carriership of p.V281L mutation. (C) Copy number of *C4A* was positively associated with having an externalizing psychiatric disorder. Del, deletion.

Table 1.

Demographics and serum complement levels of patients with congenital adrenal hyperplasia and related subjects.

	Female	Male	subtotal	Age (years)	Serum C4 (mg/L)
Salt-wasting	42(2)	42(1)	84(3)	18.0±12.5	20.2±7.7 ^{**}
Simple virilizing	9	27	36	21.0±15.6	21.2±6.2
Nonclassic	16	6	22	23.9±18.4	27.6±7.7 [*]
CAH subtotal	67(2)	75(1)	142(3)	19.6±14.4	21.6±7.9
CAH carriers	68(3)	36(1)	104(4)	42.0±10.2	22.9±7.3
cohort	135(5)	111(2)	246(7)	29.0±16.9	22.1±7.6

Numbers in parentheses are those with missing serum C4 concentration and thus were excluded in the analyses related to serum C4 level. Age refers to the age when the serum C4 was tested.

^{*}
P<0.05 vs carriers;

^{**}
P<0.05 vs all other groups.