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A promoter variant in the *OTC* gene associated with late and variable age of onset hyperammonemia

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Abstract

Ornithine transcarbamylase deficiency (OTCD) is an X-linked inborn error caused by loss of function variants in the *OTC* gene typically associated with severe neonatal hyperammonemia. Rare examples of late-onset OTCD have also been described. Here we describe an *OTC* promoter variant, c.−106C>A, in a conserved HNF4a binding site, identified in two male siblings in family 1 whose first and only recognized episodes of severe hyperammonemia occurred at ages 14 and 39 years, respectively. We identified the same *OTC* variant segregating in a large family with late-onset OTCD with variable expressivity (family 2). We show that this *OTC* promoter variant reduces expression >5-fold in a dual luciferase assay that tests promoter function. Addition of an upstream *OTC* enhancer increases expression of both the wild type and the c.−106C>A variant promoter constructs >5 fold with the mutant promoter still about 4-fold lower than the wild type. Thus, in both contexts, the promoter variant results in substantially lower *OTC* expression. Under normal demand on urea cycle function, *OTC* expression in hemizygous males, although reduced, is sufficient to meet the demand for waste nitrogen excretion. However, in response to severe metabolic stress with attendant increased requirements on urea cycle function, the impaired promoter function results in inadequate *OTC* expression with resultant hyperammonemia. In the absence of precipitating events, hemizygotes with this allele are asymptomatic, explaining the late

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age of onset of hyperammonemia in affected individuals and the incomplete penetrance observed in some individuals in family 2.

Keywords

OTC deficiency; promoter variant; regulatory DNA sequence; non-coding DNA; late onset

INTRODUCTION

OTC deficiency (OTCD) [MIM: 311250] is an X-linked disorder of ureagenesis typically presenting with hyperammonemia, vomiting, lethargy, and cerebral edema in male infants. In the absence of treatment, the symptoms usually progress to hyperammonemic coma and death in the first few days after birth[1]. In a minority of cases, symptoms do not occur until later in life (> 28 days of life)[1,2]. This late onset OTCD can occur in female heterozygotes with some residual OTC activity or[3] in hemizygous males where it is often associated with *OTC* variants of milder functional consequence[4].

The *OTC* gene [NM_000531] at Xp11.4 encodes ornithine transcarbamylase (OTC) [MIM: 300461, EC 2.1.3.3], a homotrimeric, intramitochondrial enzyme that catalyzes an early step in the urea cycle: condensation of carbamyl phosphate and ornithine to form citrulline[5]. The urea cycle is a dynamic homeostatic system necessary for removal of waste nitrogen and *de novo* synthesis of arginine. The physiological demands on waste nitrogen removal depend on the intake of dietary protein and the balance of anabolism and catabolism of endogenous protein. Accordingly, expression of OTC varies by as much as ten-fold depending on the daily demands for waste nitrogen disposal [6,7]. In OTCD, hyperammonemia results when the demand on waste nitrogen (in the form of ammonium) disposal exceed the maximal flux catalyzed by available OTC activity.

In adults, acute hyperammonemia with attended cerebral edema can proceed rapidly to death[1]. The rapidity of onset and nonspecific constellation of neurologic symptoms together with failure to carefully review family history, may result in delayed, or even failure, to diagnose late onset OTCD with sometimes lethal consequences[2,8]. The proband in our Family 1 denied any symptoms suggestive of a metabolic disorder before his first recognized episode of hyperammonemia at the age of 39. During the course of our work on Family 1, we became aware of a large multigeneration family segregating late onset OTCD[2] and the same *OTC* allele as in our family. Since the initial report in 1990, this family has expanded significantly to include over 10 affected or at-risk males with variable age of onset of symptoms (7-52 years of age) and examples of incomplete penetrance of OTCD with transmission through males; no female heterozygotes of this variant were found or suspected to present with symptoms of hyperammonemia. We confirmed that affected individuals in Family 2 carried the same c.−106C>A variant on the same haplotype background, demonstrating likely inheritance from a common ancestor. We show that this variant reduces *OTC* promoter function, preventing maximal *OTC* expression and impairing the upregulation of the *OTC* expression in times of increased metabolic demand. In contrast to neonatal OTCD, most cases of which are caused by *OTC* coding variants resulting in

an OTC enzyme that is both qualitatively and quantitatively abnormal, this *OTC* promoter variant results in expression of a qualitatively normal enzyme (normal amino acid sequence) but expressed at reduced levels. We conclude that this regulatory variant explains the modestly compromised urea cycle function in affected individuals in these two families enabling adequate waste nitrogen homeostasis except when affected males experience an episode of severe and sustained high demand on waste nitrogen excretion.

RESULTS

Family 1

Proband 1 was a 39 year old previously healthy Caucasian male who was transferred to Johns Hopkins Hospital with altered mental status of three days duration[9]. Two weeks prior to admission he developed persistent fever and pharyngitis. One week prior to admission he went to a local ER and received IV fluids, steroids, and antibiotics. Three days prior to admission he developed altered mental status and was seen again at the outside hospital where he had a cranial CT, a lumbar puncture, and blood and pharyngeal cultures, all of which were negative. His confusion persisted, and, on the day of admission, he was seen again at his local ER and where his plasma ammonia was 249 μmol/L (nl 9-32 μmol/L). He was transferred emergently to Johns Hopkins Hospital, where he was admitted to the ICU in early coma with a plasma ammonia of 438 μmol/L and respiratory alkalosis (pCO_2 22 mmHg, nl 35-45 mmHg and arterial pH 7.55, nl 7.35-7.45) while a head CT showed diffuse cerebral edema. Despite acute treatment with placement of a subarachnoid bolt, IV fluids and intravenous sodium phenylacetate and benzoate, his hyperammonemia persisted and hemodialysis was instituted nine hours after arrival. Hemodialysis decreased his plasma NH_4 over 36 hours (Figure 1A) with concomitant improvement in his mental status. Following hemodialysis, he was managed with protein restriction and intravenous sodium phenylacetate and benzoate with further improvement of his mental status over the subsequent 24 hours. His pre-dialysis plasma amino acids were abnormal with elevated glutamine and low normal citrulline (Figure 1B). His urinary orotic acid excretion was 199 mmol/mol creatinine (normal range 2.1 ± 0.71), all consistent with a primary urea cycle disorder[10]. Over the next 24 months he gradually recovered neurological function while his metabolic profile consistently demonstrated elevated orotic acid excretion supporting a diagnosis of OTCD, while his plasma amino acids returned to normal (Supplemental Table).

Family history was initially interpreted as “negative” with regards to metabolic disease. The proband’s brother was reported to have died by drowning at age 14, nearly 20 years prior to presentation of the proband. However, the contemporaneous police and autopsy reports revealed several abnormalities suggestive of OTCD. Multiple accounts described the brother as being “confused” in the 24 hours prior to his death. He vomited several times for no apparent reason and the events culminated in him falling off a dock into the lake and drowning. At autopsy, in addition to findings typical of acute drowning, the medical examiner noted cerebral edema, an abnormality not typical for acute drowning but consistent with hyperammonemia. We tested several members of family 1 for impaired *OTC* function by allopurinol challenge[11] and found that in addition to the proband, the mother had abnormal results consistent with OTCD[11] while the proband’s nephew did not (Figure

1C). Genotyping of leukocyte DNA from the proband's maternal aunt revealed that she was also a heterozygote for the variant. She and her two adult sons declined further evaluation.

Family 2

The proband of Family 2, a 7 year old Caucasian male[12], was initially seen in a local emergency department with altered mental status after 24 h of vomiting. He had a two year history of recurrent, unexplained vomiting and abdominal pain associated with fasting, for which he had undergone outpatient GI evaluations. Twenty minutes prior to presentation in the ED he developed altered mental status. Abdominal ultrasound, an abdominal CT, a cranial CT, a lumbar puncture, and blood cultures were obtained and were negative. Progressive worsening of confusion and agitation prompted transfer to a tertiary hospital. On arrival, the patient had tachycardia and was rigid, prompting lorazepam administration, which resulted in some relaxation. A repeat head CT showed no acute intracranial abnormalities. Plasma ammonia was 374 μmol/L (nl 21-50 μmol/L) without respiratory alkalosis (pCO_2 33.4 mmHg, nl 30-43 mmHg and venous pH 7.41, nl 7.33-7.39). Urine orotic acid was extremely elevated at >800 mmol/mol creatinine while plasma amino acids showed elevated glutamine and low normal citrulline (Figure 1B). Due to suspicion of a urea cycle disorder, metabolic labs were obtained and the child was started on intravenous calories (glucose and lipids 20%) with a bolus of sodium benzoate and sodium phenylacetate followed by continuous infusion. Review of the child's newborn screening revealed normal results, specifically with normal glutamine/glutamate and arginine/citrulline ratios. Hemodialysis was performed with reduction of plasma NH_4 to normal. Despite this, he did not regain consciousness and with evidence of poor neurologic outcome, the family elected to discontinue treatment. This clinical course was consistent with OTCD.

In initial consultations, family history was reported to be negative for a metabolic disease with no atypical male deaths. On further review, his maternal grandmother's brother died a few days after receiving steroids for lung cancer and the mother's grandmother had a sister with two sons who died unexpectedly in their 20s of unclear causes. Investigation into the family's genealogy revealed that other patients/families known to the clinic had clinical diagnoses of late-onset OTCD with a common ancestor[13] (Figure 2C). The female ancestor at the head of the extended pedigree came to Utah from England in the 19th century with the Mormon pioneers[2]. Since the initial identification of a late-onset OTCD phenotype in branches of Family 2, several family members underwent metabolic testing. A distant cousin of the proband had hepatic OTC activity measured to be 26% of normal[14]. His daughter had an ammonia loading study which showed above normal ^{15}N incorporation to glutamine, consistent with an OTCD carrier. Additionally, the mother of the proband[2] underwent allopurinol challenge[11] which indicated impaired OTC function. Since identification of this promoter variant, more than 80 members of this extended family have undergone molecular and biochemical testing. Males hemizygous for the variant have ranged from asymptomatic with normal plasma amino acids to unrecognized symptoms with elevated ammonia and glutamine requiring nitrogen scavenging medication and protein restriction. The uncle to Proband 2 was one such individual who reported recurrent severe headaches, which have improved since beginning nitrogen scavenger therapy.

Sequencing and genotyping implicate c.−106C>A as the pathogenic variant.

Clinical sequencing of *OTC* in the proband of Family 1 revealed four variants: a promoter variant, c.−106C>A; a missense variant p.Lys46Arg, and 2 intronic variants, ivs3-(40-39)dupT, and ivs3-(−8T>A). The missense variant has a minor allele frequency (MAF) in gnomADv2.1.1 of 0.19 and is interpreted as benign in ClinVar. Similarly, both intronic variants are common polymorphisms in the general population and are predicted to be benign. Therefore, we focused on the promoter variant, c.−106C>A (rs749748052). Genotyping by Sanger sequencing verified the variant in the proband as well as in DNA from the deceased brother extracted from decidual teeth saved by the parents (Figure 2A). Sequencing of DNA from members of the extended family revealed that the mother and maternal aunt were both heterozygote carriers, demonstrating transmission from a maternal grandparent. The sister of the proband (III-2) did not inherit the disease associated allele, and she and her son (IV-1) show no biochemical signs of OTCD. Individuals III-6 and III-7 declined genetic testing (Figure 2B).

The *OTC* c.−106C>A variant is extremely rare with only one observation across publicly available DNA sequence databases, interestingly in a heterozygous female from Utah. The 1000 Genomes Project dataset contains the lone observation of this variant in 3775 sequenced alleles, but it is otherwise not reported in any other population variant database (ExAC/gnomAD/TOPMed). A recent publication describing individuals from 38 families with biochemical features of OTC deficiency but without OTC coding variants, found three individuals with this variant[15]. Of these, two individuals had late onset OTCD and one was an asymptomatic adult male whose adult brother died from hyperammonemic encephalopathy. The c.−106C>A variant was also found in the proband of Family 2 by whole genome sequencing. Subsequent testing of other members of this large family demonstrate segregation amongst multiple males with late onset[2] OTC deficiency (Anderson, K and Longo N unpublished). The variant has also been identified in an isolated case of late onset OTCD in Australia (personal communication, Bruce Bennetts, New South Wales Ministry of Health, Australia). This promoter variant, rare in the general population, has therefore been observed in several independent families segregating late onset OTCD. We identified five other single nucleotide variants in the 5'-flanking 1 kB sequence of the disease-associated *OTC* allele (Figure 3A). All are common SNPs (allele frequency for all of 0.28) that mark a common *OTC* promoter haplotype block in the general population (Figure 3B). The exact *OTC* haplotype with the c.−106C>A variant also segregated with OTC deficiency in males in Family 2 suggesting that the mutation producing this variant is not recurrent, but rather occurred in a distant, unrecognized ancestor of both families.

Dual luciferase assay demonstrates reduced function and significantly impaired enhancer modulated activity.

The promoter variant alters a nucleotide position conserved across mammals within a HNF4a binding site known to regulate *OTC* expression[16]. This HNF4a binding site maps to c.−105_−110[17] (Figure S2) and disruption of this binding site prevents HNF4a binding and impairs promoter activity[17]. The 800bp segment of DNA sequence upstream of the *OTC* transcription start site is highly conserved across the mammalian lineage and contains the promoter variant. We cloned this DNA segment in a luciferase reporter vector and

demonstrated that it functions as a promoter driving expression of a luciferase reporter gene when transfected into an immortalized hepatocyte (HEPG2) cell line. Under these same conditions, the promoter construct containing the c.−106C>A variant had significantly decreased activity (10% normal activity, $p = 2.2x10^{-16}$) compared to the wild-type (WT) allele (Figure 3C). To test the effect of the *OTC* enhancer located 9kb upstream on this activity[17], we added a 500 bp sequence encompassing the enhancer sequence to our luciferase constructs and tested its effect on the WT (WT + enh) and variant (OTCD + enh) promoters in HEPG2 cells. The presence of the enhancer increased the activity of both the WT and the variant promoter above basal activity, but the variant promotor only achieved 25% of the WT promoter activity with the enhancer (1.6 fold vs 5.93 fold relative to WT promoter alone; $p = 9.9x10^{-11}$). In the presence of the enhancer the expression driven by the c.−106C>A variant promoter achieves a level slightly higher than that of basal activity of the WT allele but significantly lower than the WT allele with the enhancer.

The high frequency of the haplotype carrying the promoter variant (each SNP has MAF ~0.3) and the fact that none of the SNPs on this haplotype affect known transcription factor binding sites, suggest that this haplotype is neutral with respect to *OTC* function. However, we still considered if it could account for the reduced activity in our luciferase assays. To address this, we utilized site directed mutagenesis to measure the promoter activity of the haplotype with and without the c.−106 variant. The activity of the c.−106 C>A variant, when measured in the context of the reference haplotype, was reduced to levels indistinguishable from those of its native background haplotype. Moreover, correction of the c.−106 C>A variant back to reference on the proband's less common haplotype restored function to WT levels (Figure 3D). These reconstructed alleles also showed significant increases in activity in the presence of the enhancer, as observed with the native promoter alleles (Figure S1). We conclude that the c.−106 C>A promoter variant explains the reduced promoter function and that this effect is independent of haplotype background.

DISCUSSION

Late onset OTCD is a potentially deadly disorder and one that may be difficult to recognize due to rapid onset of hyperammonemia[1]. In this study, we confirmed extensive segregation analysis of a variant in the *OTC* promoter in two separate families with OTCD. We tested the functional consequence of this variant using a dual luciferase system and found that the variant impaired, but did not abolish, basal activity of the promoter as well as its activity in the presence of its native enhancer. These data suggest that the variant allows moderate expression of *OTC* but is unable to achieve maximal expression levels which may be necessary under high metabolic load. This explanation is consistent with negative newborn screens for affected individuals as well as appearing healthy until the time of metabolic stress.

This promoter variant affects the quantity but not the quality of OTC enzyme produced, allowing males who carry this allele to remain asymptomatic until a severe metabolic challenge requires increased enzymatic activity beyond what they are capable of producing, with resultant disruption of metabolic homeostasis. The sudden onset of hyperammonemia can occur at any age as demonstrated by the wide range in symptom onset in these patients.

Onset in Proband 1 appears to have been caused by the increased nitrogen load caused by more than 10 days of excessive catabolism associated with severe pharyngitis and possibly the accompanying treatment with steroids. Administration of steroids has previously been described to aggravate OTCD[18] and has been associated with hyperammonemic crises on several occasions in members of Family 2. In the case of the brother of Proband 1 who also carried the variant, the specific trigger of his fatal episode is not known. The *OTC* promoter variant shared by all affected individuals in Families 1 and 2 reduced *OTC* expression to levels which are adequate for the demand on waste nitrogen removal typical of non-stressed conditions, but inadequate when the demands on waste nitrogen excretion is excessive such as during extensive catabolism or the administration of steroids. Of particular interest is the observation of incomplete penetrance via apparent transmission of this OTCD allele through males in Family 2; and while this did not occur for the proband in Family 1, he was well into his reproductive years at time of his clinical presentation. So, in the case of this allele, transmission through apparently unaffected males to their daughters would not rule out OTCD as it typically would for most X-linked disorders. Rapid onset of symptoms beyond the neonatal period combined with transmission through males and negative newborn screen, when available, makes this a particularly challenging diagnosis and one very likely to be an underreported.

Functional testing in HepG2 cells demonstrated impaired activity of the promoter carrying c.−106 C>A. However, this variant does not completely abolish activity and expression was elevated by the presence of the native enhancer sequence. This distinction is consistent with the clinical presentation of all affected individuals by suggesting that the OTCD allele is capable of modest levels of expression sufficient for processing daily metabolic needs but unable to reach maximal expression levels which may be needed during times of metabolic stress or crisis; these precipitating events may include treatment with steroids, increased dietary protein, inadequate nutrition with attendant weight loss, chemotherapy, or certain hormone therapies[8]. This is also consistent with previous studies which noted that onset of symptoms in individuals carrying partial function OTC alleles was more dependent upon genetic and environmental modifiers than those who carried severe loss of function alleles[4]. The physiological result of this would be an individual possessing an underlying risk for metabolic crisis at a threshold higher than classic OTCD but lower than most people in the general population.

Although few regulatory variants are currently known to cause OTCD[19], noncoding variants should be considered in cases of suspected OTCD. Interpretation and classification of regulatory variants is poised to significantly improve in the near future, due in large part to increased availability of whole genome sequence and collaborative data sharing initiatives implemented in recent years [20-22]. Implementation of whole genome sequencing will allow for identification of genetic variants in regulatory elements and it is likely that many of them will associate with cases of mild, late onset, or incompletely penetrant monogenic disease. As with other recessive metabolic disorders, OTCD is prevented or ameliorated by a relatively low levels of residual enzyme function[4], thus it is reasonable that a regulatory variant that retains some level of basal expression will not manifest any disease symptoms unless significantly stressed. This likely explains the observed incomplete penetrance of OTCD in these families[23]. Incomplete penetrance may also be due to genetic

modifiers, epistatic genes, or other environmental effects[24], which collectively modulate the level of enzyme activity needed to maintain metabolic homeostasis. In aggregate, these considerations also explain, in part, the challenge of interpreting the consequences of potential regulatory variants particularly in conditions for which there are not clear biochemical markers[23,25]. It is likely that other pathogenic regulatory variants of *OTC* would cause similar disease, raising the possibility that they will be identified in instances of late or adult onset OTCD rather than neonatal onset. Thus, it is likely that consideration of OTCD in adults with unexplained hyperammonemia will lead to the discovery of additional conditionally pathogenic regulatory variants.

METHODS

Study Approval

Subjects were consented under the "Clinical Studies of Inborn Errors of Metabolism" study approved by the Johns Hopkins Office of Human Subjects Research (NA_00036487)

Allopurinol Challenge

Ethyl acetate extraction of acidified urine using 1,3-[N15]orotic acid as an isotopic internal standard and BSTFA + 1% TMCS as derivatizing agent. Gas chromatography performed on a 0.2mm x 25 m methylsilicone (0.33μm phase) capillary column programmed from 70 to 290°C at 4°C per min. Quantification by selected-ion mass spectrometry at m/u = 254 and 256 in the electron impact mode at 70 eV and a source temperature of 200°C.

DNA extraction/sequencing of proband and family

Whole blood was collected from proband and family members and genomic DNA was extracted using the Blood and Tissue DNeasy Kit (69504, Qiagen, Germantown MD). Five sets of PCR primers were used to generate overlapping PCR fragments spanning exon 1 and 1kb upstream of the start codon. PCR products were visualized by agarose gel electrophoresis, purified by Exo/SAP digestion (78205 Thermo Fisher Scientific, Waltham, MA), and sequenced by Sanger sequencing. Sequences were aligned to the human reference genome (GRCh37) and evaluated for sequence variants.

DNA extraction and sequencing of deceased brother

Deciduous teeth from the deceased brother of the proband, which had been saved by his mother, were cleaned by washing using HPLC grade water and 20-minute UV irradiation. Teeth were soaked in solution of 0.5 M EDTA + 25mg/ml proteinase K overnight at room temperature with gentle rocking. DNA was purified from extraction solution by running through DNA purification kit (28104 Qiagen, Germantown MD).

Promoter and enhancer cloning

800bp of genomic DNA upstream of the translation start site of OTC were cloned from the proband and his unaffected relative using PCR primers that added KpnI 5’ overhang and HindIII 3’ overhang restriction enzyme recognition sequences. PCR products were run on agarose gel and fragments were purified using a QIAquick Gel Extraction Kit (28706X4

Qiagen, Germantown MD) and incubated for 1 hour at 37C with KpnI (R0142S New England Biolabs, Beverly MA) and HindIII (R0104S New England Biolabs, Beverly MA) to generate sticky ends. pGL4.10[luc2] (E6651 Promega, Milwaukee WI) reporter plasmid containing the luciferase gene was linearized using the same restriction enzymes, and cloned promoter fragments were ligated into the multiple cloning region of the plasmid using the In-Fusion HD Cloning Kit (638910 Clontech, Mountain View CA). The ligated product was used to transform DH5a competent cells (18265017 Invitrogen, Carlsbad CA) and then plated on LB agar plates containing 100μg/mL ampicillin. Colonies were picked and cultured for 8 hours in LB with 1% ampicillin and plasmid was extracted using the Plasmid Miniprep Kit (27104 Qiagen, Germantown MD). Plasmids were digested using KpnI and HindIII to verify that a fragment of appropriate size had inserted into the plasmid. Plasmids containing appropriately sized inserts were sequenced using a priming site in the plasmid backbone to confirm the presence of the promoter fragment. Enhancer sequences were cloned from the proband using PCR primers containing overhangs that introduced a SalI restriction recognition sequence on both ends. PCR products were gel extracted and digested with SalI (R0138S New England Biolabs, Beverly MA) enzyme and ligated into vectors containing promoter sequences. Plasmids were digested to confirm insertion of enhancer fragments and sequenced using a backbone priming site to determine the orientation of enhancer insertion.

Dual Luciferase Assay

HepG2 cells were grown in MEM with 10% FBS and 1% Penicillin/streptomycin. Cells were co-transfected with reporter constructs containing the cloned promoter fragments and enhancer sequences, and Renilla plasmids (E2231 Promega, Milwaukee WI) in triplicate using Lipofectamine 3000 (L3000015 Invitrogen, Carlsbad CA). Lysates were harvested after 48 hours using passive lysis buffer (Promega, Milwaukee WI), and luminescence was measured using the Dual Luciferase Reporter Assay (Promega, Milwaukee WI).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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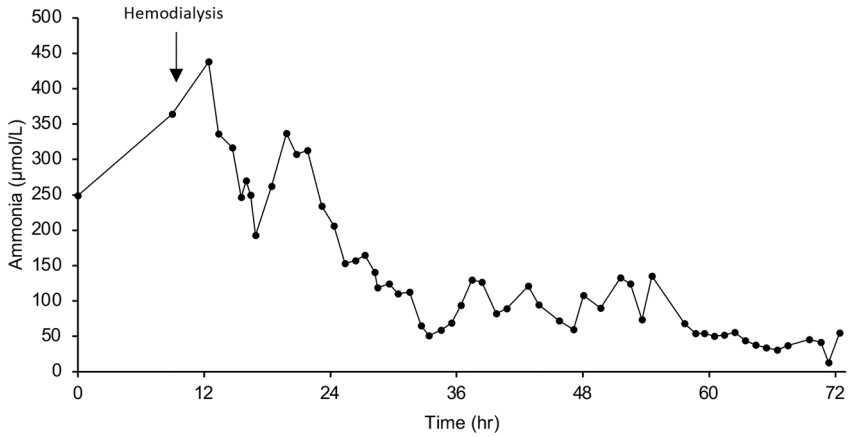
Synopsis:

Late onset OTC deficiency caused by genetic variants in regulatory regions can cause sudden onset of hyperammonemia under times of metabolic stress without suspicion of underlying disease.

A



B

	Control Range JHH	Proband 1	Control Range Utah	Proband 2
Glutamine (mmol/L)	337 – 673	988	410 - 700	1,413
Citrulline (mmol/L)	10 – 39	19	10 - 60	14
Urinary orotic acid (mmol/mol creatinine)	1.4 – 2.21	199	0.2 - 1.5	>800
Peak plasma ammonium (μmol/L)	9 – 32	438	21-50	374

C

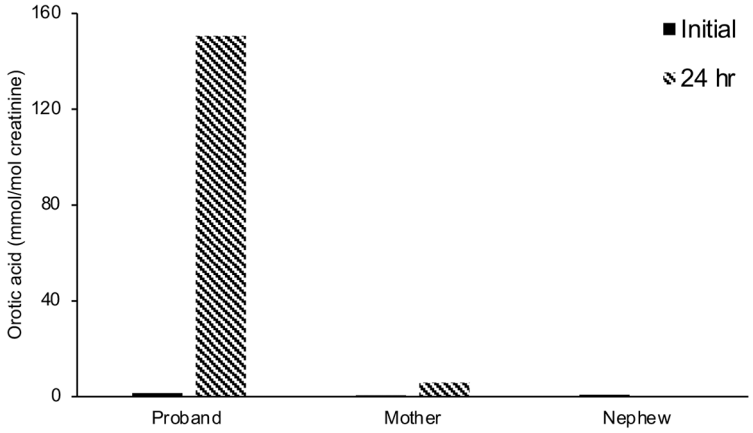


Figure 1. Results of biochemical testing

(**A**) Plasma ammonia levels for proband measured over time, hemodialysis was begun 14.5 hours after admission at constant 500 μmol/L. Peak ammonia in proband measured to be 438 μmol/L and dropped to normal range (1-32) after 72 hours. (**B**) Plasma amino acids, urinary orotic acid, and peak plasma ammonia of probands taken at admission are consistent with OTCD. (**C**) Orotic acid levels (mmol/mol creatinine) of proband, mother, and nephew (normal range 2.1 +/− 0.71) at baseline or following allopurinol challenge. Results demonstrate normal baselines for all three individuals, however allopurinol challenge demonstrated results consistent with OTCD and OTCD carrier for proband and mother, respectively, but not for the nephew.

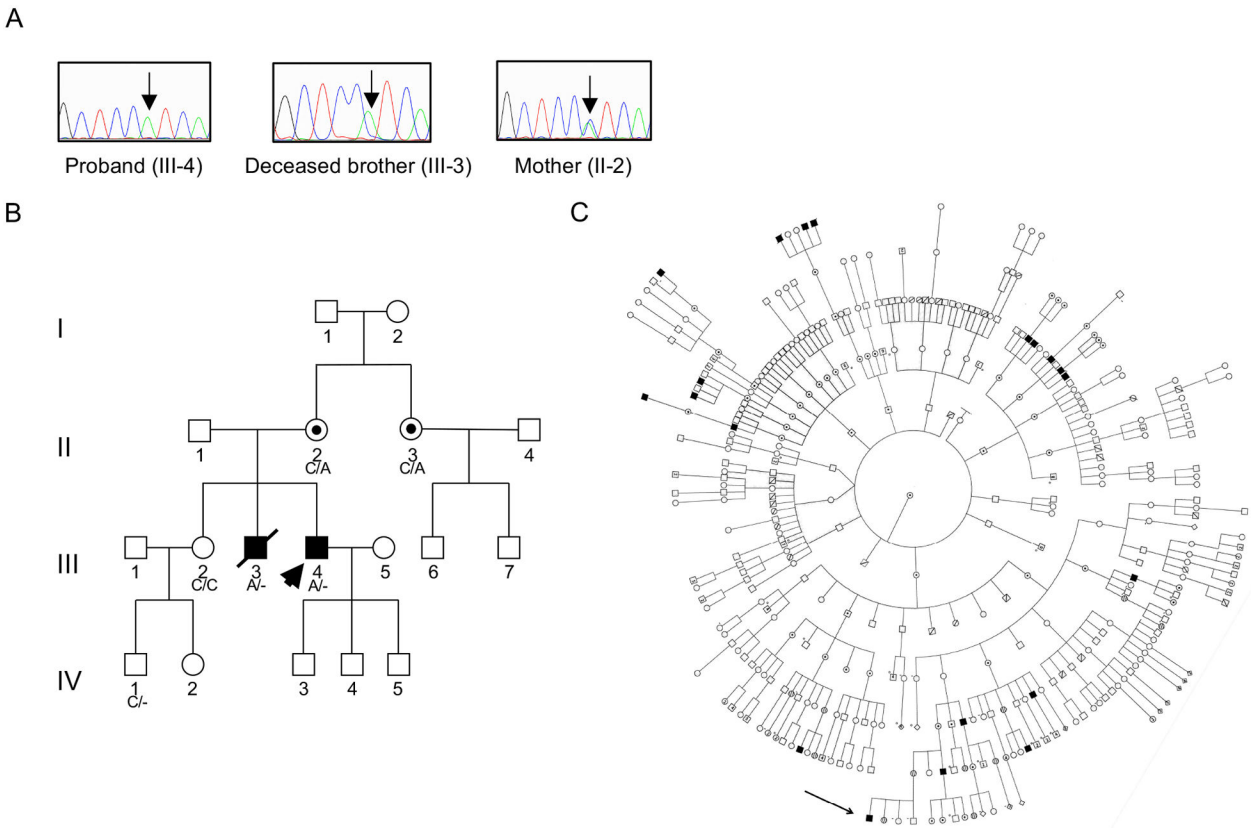


Figure 2. Co-segregation of c.−106 C>A with late onset OTCD phenotype

(**A**) PCR amplification and Sanger sequencing of *OTC* upstream sequence confirmed transmission of the c.−106 C>A variant from the mother to the proband and deceased brother. (**B**) Pedigree of Family 1. The proband (III-4) and his brother (III-3) carry the pathogenic allele. Individual IV-1 carries the alternate X inherited from II-2 and showed no signs of metabolic disease by age 8. (**C**) Pedigree of Family 2 living in Utah demonstrates transmission of OTCD allele from common ancestors diagnosed with late onset OTCD.

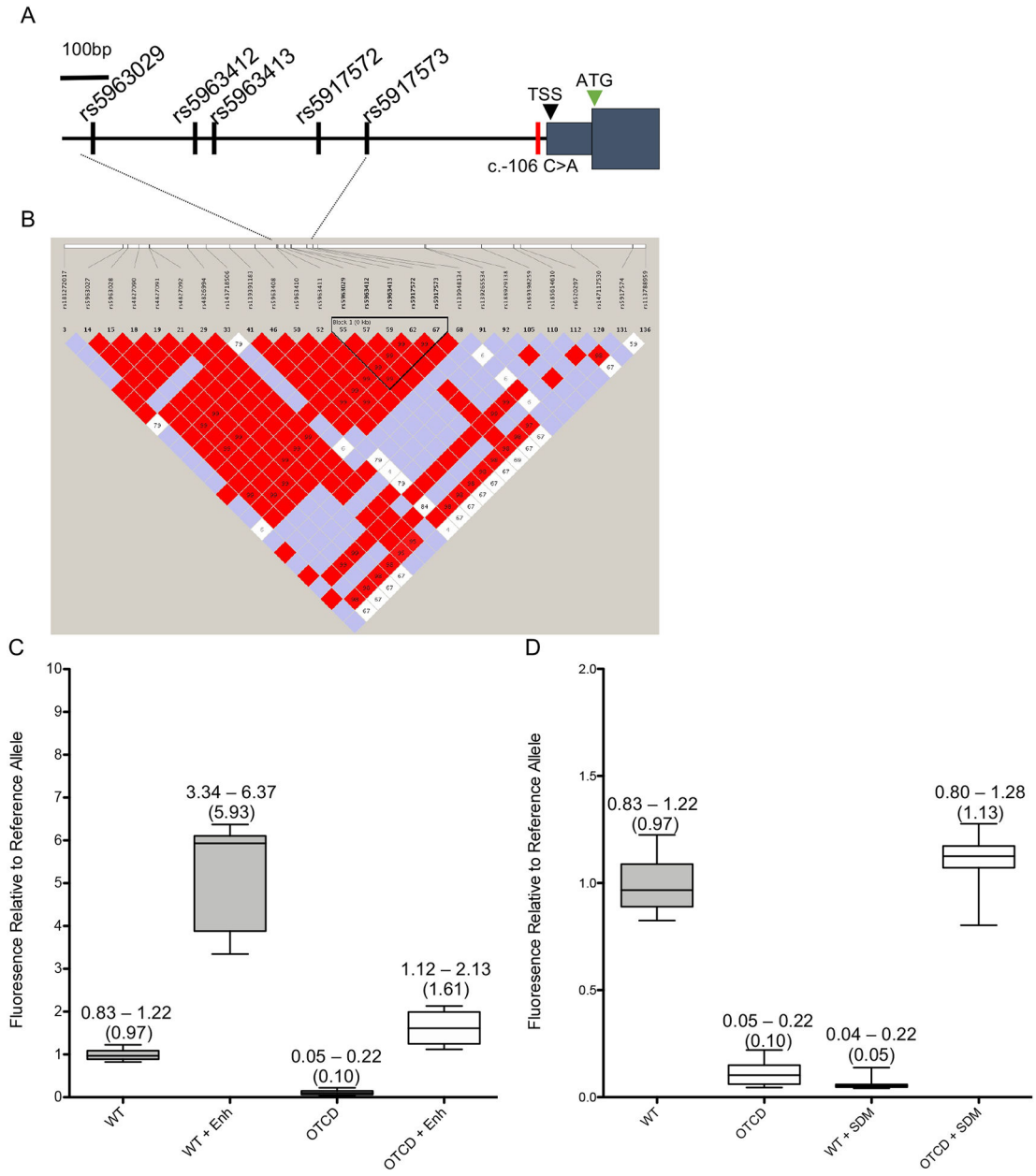


Figure 3. The c.−106C>A variant is sufficient to impair promoter activity independent of haplotype background

(**A**) Sanger sequencing confirmed the presence of the c.−106 C>A variant upstream of the *OTC* transcription start site (TSS) and start methionine (ATG). Five additional variants were identified within 1kb upstream of *OTC.* All five variants are known polymorphisms and each has a population frequency of 0.28 reported in dbSNP. (**B**) The five SNPs identified on the pathogenic haplotype are found in high LD with one another, demonstrating that they are often inherited as a single unit. The pathogenic variant is a rare variant on this haplotype. (**C**) Dual luciferase assay demonstrated that the promoter allele cloned from the proband was significantly less active than the WT allele. Inclusion of the 500 bp enhancer (Enh) element increased function of both the WT and OTCD alleles, however the OTCD allele

was not able to achieve activity significantly higher than the basal activity of the WT allele. (**D**) The c.−106C>A variant was introduced onto the WT haplotype (WT + SDM) while the c.−106C>A variant was reverted back to reference on the OTCD haplotype by site directed mutagenesis (OTCD + SDM). Introduction of the c.−106C>A variant lowered the function of the WT allele to levels comparable to the OTCD allele. Correction of the c.−106C>A variant restored function of the OTCD allele to WT levels. All values displayed as min – max and (median).