Hereditary Intrinsic Factor Deficiency in Chaldeans

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Abstract Juvenile vitamin B₁₂ or cobalamin (Cbl) deficiency is notoriously difficult to explain due to numerous acquired and inherited causes. The consequences of insufficient Cbl are megaloblastic anemia, nutrient malabsorption, and neurological problems. The treatment is straightforward with parenteral Cbl supplementation that resolves most health issues without an urgent need to clarify their cause. Aside from being clinically unsatisfying, failing to elucidate the basis of Cbl deficiency means important information regarding recurrence risk is not available to the individual if the cause is contagious or inherited. Acquired causes have largely disappeared in the Modern World because they were mostly due to parasites or

malnutrition. Today, perhaps the most common causes of juvenile Cbl deficiency are Imerslund-Gräsbeck syndrome and inherited intrinsic factor deficiency (IFD). Three genes are involved and genetic testing is complicated and not widely available. We used self-identified ancestry to accelerate and confirm the genetic diagnosis of IFD in three families of Chaldean origin. A founder mutation limited to Chaldeans from Iraq in the intrinsic factor gene *GIF* was identified as the cause. World events reshape the genetic structure of populations and inherited diseases in many ways. In this case, all the patients were diagnosed in the USA among recent immigrants from a single region. While IFD itself is not restricted to one kind of people, certain mutations are limited in their range but migrations relocate them along with their host population. As a result, self-identified ancestry as a stratifying characteristic should perhaps be considered in diagnostic strategies for rare genetic disorders.

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Introduction

Juvenile cobalamin deficiency (JCD) can be attributed to dietary, infectious, or hereditary causes (Gräsbeck 2006). In modern societies, dietary reasons and infectious agents are largely a thing of the past. As a result, inborn causes of JCD such as cobalamin (Cbl) malabsorption, serum transport defects (transcobalamin deficiency), or metabolic mutations have become increasingly important in pediatric care (Watkins and Rosenblatt 2011). Cbl deficiency leads to various hematological problems that range from mild weakness to life-threatening acute megaloblastic anemia. Neurological symptoms are often overlooked since they can vary from mild learning difficulties to overt antisocial outbursts and may be recognized only if carefully sought

Fig. 1 Pedigrees of four patients affected by inherited intrinsic factor deficiency (IFD, filled symbols) due to the intronic mutation c.1073+5G>A in the intrinsic factor gene, *GIF*. The parents are all of Chaldean ancestry

for (Gräsbeck 2006; Luder et al. 2008). Due to the myriad of potential causes and varying degree of symptoms, explaining the etiology of JCD can be daunting and diagnostic testing can take months (Carmel et al. 2003; Watkins and Rosenblatt 2011). Even if malabsorption of Cbl is established as the cause of JCD by measurement of transcobalamin-bound Cbl (Obeid and Herrmann 2007) or with the Schilling test (Schilling 1953), clarification remains challenging and clinical testing for gastric causes can be invasive. Genetically, pathogenic mutations in either *CUBN* (Aminoff et al. 1999) or *AMN* (Tanner et al. 2003) cause Imerslund-Gräsbeck syndrome (IGS; OMIM261100; Imerslund 1960; Gräsbeck et al. 1960) and in intrinsic factor deficiency (IFD; OMIM261000; Katz et al. 1972), mutations in the *GIF* gene are causative (Yassin et al. 2004; Tanner et al. 2005). Proteinuria is sometimes indicative of IGS but lack thereof is not necessarily diagnostic for IFD (Gräsbeck and Tanner 2011), while mono-symptomatic proteinuria due to *CUBN* mutations is not necessarily associated with Cbl deficiency (Ovunc et al. 2011). Although all three genes can be sequenced (Tanner et al. 2004, 2005; Storm et al. 2011), the screening task is complex given the genetic heterogeneity.

Recent human migrations have an impact on the clinical and epidemiological picture of diseases that are encountered in the Western World. While infectious diseases come to mind first, hereditary conditions similarly move about with migrants. Resulting population bottlenecks in resettled communities might lead to founder effects that change the incidence and the mutational spectrum of disorders in the new home country. The current focus on personalized medicine has to adapt to that challenge. Self-identified ethnicity might offer rapid shortcuts for diagnostics in certain cases if the ancestry of a patient is carefully evaluated. We present the results of genetic testing for JCD in three families of Chaldean origin. While the first family with two patients had to wait several years for the analysis to be completed because the significance of the mutation was not obvious, genetic confirmation in a second family permitted the last patient to be diagnosed within weeks. Thus, patients with rare disorders might be readily and reliably diagnosed if their ethnicity is taken into account.

Patients and Methods

Patients and Samples

Family 24 came to our attention because of two boys (ages 14 and 8) with macrocytic anemia (mean cell volume 111.6 fL, normal range 86–98 fL) and Cbl deficiency (exact values were not reported, Fig. 1 and Table 1). No neurological problems were noted and nutritional deficiency was excluded. The macrocytosis and anemia were responsive to parenteral Cbl replacement therapy. A Schilling test in the older sibling (24-1) was positive for malabsorption but the younger boy (24-6) was not tested. The older brother did not respond to the addition of intrinsic factor (IF) during the second stage of the Schilling test (Schilling 1953). Proteinuria was absent in both brothers. The family reported Chaldean ancestry and lives in the USA. Family history was significant for a paternal cousin reportedly with a similar problem, although he also had neurologic symptoms that were not further specified.

Family 63: A 4-year-old boy, one of four siblings, was noted to have Cbl deficiency (Cbl 74 pg/mL) (Fig. 1). Intramuscular injections with Cbl resulted in a significant improvement from his anemia and macrocytosis. He was then lost to follow-up until 3 years later when he presented after an episode of syncope. Hemoglobin at that time was 11.1 gm/dL, mean cell volume (MCV) was elevated at 103 fL, and the Cbl level was severely decreased at <70 pg/mL. A screen for Anti-IF antibodies was negative. Proteinuria was absent and nutritional deficiency was excluded. Family history was significant for multiple cousins who also received regular Cbl injections. He was restarted on monthly parenteral Cbl with hydroxocobalamin 1,000 mcg. On this regimen, his hemoglobin has remained normal, and his MCV and Cbl level have normalized. Both parents of family 63 were born in the same region of Northern Iraq and reported no consanguinity. The family resides in the USA and they identify themselves as Chaldeans.

Family 92: A 4-year-old Iraqi Chaldean girl was seen with a diagnosis of acute lymphoblastic leukemia (ALL) in Michigan, USA (manuscript in preparation). She was originally diagnosed in Turkey and had received chemotherapy there until recently. She had a history of Cbl

Table 1 Genetic analysis of *GIF* in three Chaldean families with inherited intrinsic factor deficiency

Sample ID	Family information	<i>GIF</i> intron 7 ^a	<i>GIF</i> M1	<i>GIF</i> M3b	<i>GIF</i> M4	<i>GIF</i> M5	<i>GIF</i> M6	<i>GIF</i> M7
24-1	Male patient	hom c.1073+5G>A	176-176	235-235	160-160	256-256	204-204	238-238
24-2	Father	het c.1073+5G>A	176-176	235-239	160-160	nd	nd	nd
24-3	Mother	het c.1073+5G>A	176-176	235-235	160-160	nd	nd	nd
24-4	Brother	het c.1073+5G>A	176-176	235-235	160-160	nd	nd	nd
24-5	Brother	c.1073+5G	176-176	235-239	160-160	nd	nd	nd
24-6	Brother, patient	hom c.1073+5G>A	176-176	235-235	160-160	256-256	204-204	238-238
63-1	Male patient	hom c.1073+5G>A	176-176	235-235	160-160	256-256	204-204	238-238
63-2	Father	het c.1073+5G>A	176-176	223-235	160-160	nd	nd	nd
63-3	Mother	het c.1073+5G>A	176-176	235-243	160-160	nd	nd	nd
63-4	Sister	het c.1073+5G>A	176-176	235-243	160-160	nd	nd	nd
63-5	Brother	c.1073+5G	176-176	223-243	160-160	nd	nd	nd
63-6	Brother	het c.1073+5G>A	176-176	235-243	160-160	nd	nd	nd
92-1	Female patient	hom c.1073+5G>A	176-176	235-235	160-160	256-256	204-204	238-238
92-2	Father	het c.1073+5G>A	nd	nd	nd	nd	nd	nd
92-3	Mother	het c.1073+5G>A	nd	nd	nd	nd	nd	nd
92-4	Brother	c.1073+5G	nd	nd	nd	nd	nd	nd

hom homozygous, het heterozygous, nd not done

^a Numbering relative to adenine in the ATG start codon of *GIF* (GenBank accession AP002347)

deficiency, which was diagnosed in Iraq when she was 18 months of age and was treated there for 4–5 months by injections. Until the diagnosis of ALL in February 2011, she had not received any further treatment for Cbl deficiency, though she was given an oral Cbl supplement monthly during chemotherapy before relocating to the USA according to the parents. Repeated Cbl measurements were low at 161 and 92 pg/mL (normal low = 211) and she had elevated serum and urine methylmalonic acid levels (0.74 mmol/L (normal <0.4) and 9.6 mmol/mol creatinine (normal <3.6), respectively). She had no proteinuria as the urine microalbumin/creatinine ratio was within normal limits. Her diet was appropriate.

Methods

Peripheral blood samples were collected after informed consent was obtained under Ohio State University review board protocol 2005H0201 according to the Declaration of Helsinki. Genomic DNA samples were isolated from peripheral blood by standard phenol-chloroform-ethanol-precipitation. For mutational analysis, families 24 and 63 were studied for suspected IGS (*AMN* and *CUBN*) and IFD (*GIF*) as previously described (Tanner et al. 2003, 2004, 2005). For family 92, only *GIF* exon 7 was amplified from genomic DNA by PCR. For haplotype characterization, genotyping was performed with the six microsatellite markers, *GIF* M1 (28 kb proximal of *GIF*), *GIF* M3b (intron 7), *GIF* M4 (2 kb distal), *GIF* M5 (12 kb distal), *GIF* M6 (56 kb distal),

and *GIF* M7 (83 kb distal), and analyzed using fluorescent-labeled primers as described (Ament et al. 2009).

Results and Discussion

The two patients in family 24 were initially thought to suffer from IGS. A two-stage Schilling test was performed, where in stage 1 the patient was given radiolabeled Cbl orally, followed by parenteral Cbl to saturate tissue absorption so that any absorbed radiolabeled Cbl was released into the urine for measurement (Schilling 1953). The patient's urine over the next 24 h was negative for radiolabeled Cbl, indicative of intestinal malabsorption. For stage 2, the test was repeated by adding oral IF but this did not correct the malabsorption as would be expected for IFD. Consequently, the defect was thought to be in the intestinal cubam receptor, thereby causing IGS (Fyfe et al. 2004). As a result, the molecular genetics analysis initially focused on screening *CUBN* and *AMN* in the two patients of family 24. However, we quickly realized that the genotypes of several polymorphisms in both genes differed in the two brothers, thus excluding *CUBN* and *AMN* as the cause for this recessive disease. Given our previous experience with other cases of IGS that turned out to suffer from *GIF* mutations (Tanner et al. 2005), we proceeded with sequencing of *GIF*. Unfortunately, no obviously pathogenic mutation was detected. The only noteworthy change that cosegregated with the phenotype in the family was intronic, c.1073+5G>A in intron 7. Although

Wildtype Sequence Amplicon <i>GIF</i> exon 7:							
Length: 285 nucleotides.							
28.8% A, 26.7% C, 18.9% G, 25.6% T, 0.0% X, 45.6% G+C							
Donor splice sites, direct strand							

	pos 5' ->3'	phase	strand	confidence	5'	exon intron	3'
	178	0	+	0.71	GACCATCAAT^GTTAGTGTGA		
	195	2	+	0.00	TGAAAAGTGG^GTCAGTGTTA		
Native donor site	249	2	+	0.00	CTATGTTCAA^ <u>GTGAG</u> TATTA		
Mutated Sequence Amplicon <i>GIF</i> exon 7:							
Length: 285 nucleotides.							
29.1% A, 26.7% C, 18.6% G, 25.6% T, 0.0% X, 45.3% G+C							
Donor splice sites, direct strand							

	pos 5' ->3'	phase	strand	confidence	5'	exon intron	3'
	178	0	+	0.71	GACCATCAAT^GTTAGTGTGA		
	195	2	+	0.00	TGAAAAGTGG^GTCAGTGTTA		

Fig. 2 Splice site prediction with NetGene2 of the 195bp amplicon covering exon 7 of *GIF* and the flanking splice site sequences. The upper panel shows the prediction using the wild-type sequence with

the native donor splice site underlined and c.1073+5G marked in bold. The lower panel shows that the native donor splice site is predicted to be lost when c.1073+5G>A is present

it was homozygous in both patients and heterozygous in the parents and one sibling (Table 1), the molecular consequence of this change was not evident and could not be studied because of lack of RNA samples. The canonical donor splice site position +5 was G in 81.4% followed by A in 7.1% of over 22,000 intron sequences analyzed (Burset et al. 2001), thus the change was not obviously deleterious. This inconclusive result lingered while we studied additional candidate genes potentially involved in the Cbl malabsorption pathway (Shah et al. 2011).

Family 63 was referred to us for molecular analysis of suspected JCD and because of lack of proteinuria we first sequenced *GIF*. The same change, c.1073+5G>A in intron 7, was detected which cosegregated with the phenotype in a recessive pattern. No additional changes were identified by sequencing the complete gene. This result strongly supported that this was the pathogenic change in the patients. Since both families were of Chaldean origin, we genotyped the three flanking markers *GIF* M1, *GIF* M3b, and *GIF* M4 in the two families. The disease haplotype was identified as 176-mutation-235-160 in both sibships but markers *GIF* M1 and *GIF* M4 were not informative in the parents. However, the tetra-nucleotide marker *GIF* M3b (GGAA/GAAA with 19 different alleles observed among 93 European- and 93 African-American controls with >75% heterozygosity; Ament et al. 2009) is only 1.5 kb downstream of the mutation in intron 7. The marker was informative in three of the four parents in family 24 and 63. We concluded that *GIF* c.1073+5G>A is the likely culprit causing IFD in these two families because of identical haplotypes. Reverse-transcriptase PCR-analysis of *GIF* using RNA isolated from peripheral blood cells from family 63 failed because *GIF* was not expressed in these cells and stomach RNA from the patient was not obtained for ethical reasons.

Further empirical proof that c.1073+5G>A is an IFD mutation was provided by analyzing family 92. The clinical history with lack of proteinuria in the female patient supported a diagnosis of IFD. Since the family was of Iraqi Chaldean origin, we sequenced *GIF* exon 7 and again found c.1073+5G>A homozygous in the patient and heterozygous in the parents (Table 1). Genotyping of the patients in the three families with six microsatellite markers established a common disease haplotype. This ancestral haplotype was found unaltered in all four patients (Table 1). We concluded that the c.1073+5G>A mutation represents a founder event for IFD patients of Chaldean origin and was never seen in over 150 additional cases with Cbl malabsorption or in any of the 176 normal controls sequenced (Tanner et al. 2005). In addition, a splice site sequence prediction with NetGene2 (Brunak et al. 1991) showed that the native donor splice site is lost when c.1073+5G>A is present (Fig. 2), which strengthens the evidence that c.1073+5G>A is pathogenic. This particular change is also not listed in the dbSNP variation database. Unfortunately, Chaldean controls were not available. Iraqi Chaldeans are ethnic Assyrians. They are indigenous to, and have traditionally lived all over, Iraq, North East Syria, North West Iran, and Southeastern Turkey (Parpola 2004).

Although it could be argued that the mutation illustrated is a rare polymorphism exclusive to the Chaldean people, the phenotype of Cbl malabsorption, lack of proteinuria, and response to treatment support a defect in *GIF*. No other causal variant was found by sequencing most of the intronic sequences. Despite the bioinformatics results that indicate a loss of the native donor splice site (Fig. 2), the splicing defect might not remove 100% of the IF and thus some residual IF might be present. However, the range of clinical and laboratory manifestations in IFD varies considerably among patients and we do not see clear phenotype-genotype correlations. The negative Schilling test result

when adding IF during stage 2 in individual 24-1 was likely due to residual general nutrient malabsorption. Lack of Cbl leads to enterocyte malfunction that in turn exacerbates Cbl uptake, leading to a vicious cycle that needs to be corrected with parenteral Cbl before any malabsorption testing is informative (Gräsbeck and Tanner 2011).

In certain cases, self-identified ethnicity to group patients for targeted genetic testing and interpretation might be advantageous to accelerate and/or confirm a diagnosis. Using this approach, we identified a novel *GIF* mutation that is responsible for Chaldean cases who suffer from inherited Cbl deficiency. Ethnic stratification also revealed the significance of a genetic variant that otherwise might have remained unexplained. The focus on personalized medicine in this example benefited a rare heritable disorder.

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Declaration

The authors confirm independence from the sponsor; the content of the article has not been influenced by the sponsors. The authors have no competing financial interests.

Synopsis

Ethnicity as a shortcut to diagnosis, the case of hereditary intrinsic factor deficiency in Chaldeans.

Authors' Contributions

Amy C. Sturm and Elizabeth C. Baack coordinated DNA sample collection, genetic counseling, and helped to draft the manuscript. Michael B. Armstrong, Deborah Schiff, Ayesha Zia, and Sureyya Savasan performed clinical evaluations and sample collection. Albert de la Chapelle commented on the manuscript draft. Stephan M. Tanner carried out the molecular genetic studies, coordinated the research, and wrote the final manuscript. All authors read and approved the manuscript.

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