Luminal expression of cubilin is impaired in Imerslund-Gräsbeck syndrome with compound *AMN* mutations in intron 3 and exon 7

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ABSTRACT

Juvenile megaloblastic anaemia 1 (OMIM # 261100) is a rare autosomic disorder characterized by selective cobalamin malabsorption and inconstant proteinuria produced by mutations in either *CUBN* or *AMN* genes. Amnionless, the gene product of *AMN*, is a transmembrane protein that binds tightly to the N-terminal end of cubilin, the gene product of CUBN. Cubilin binds to intrinsic factor-cobalamin complex and is expressed in the distal intestine and the proximal renal tubule. We report a compound *AMN* heterozygosity with c.742C>T, p.Gln248X and c.208-2A>G mutations in 2 siblings that led to premature termination codon in exon 7 and exon 6, respectively. It produced a dramatic decrease in receptor activity in urine, despite absence of *CUBN* mutation and normal affinity of the receptor for intrinsic factor binding. Heterozygous carriers for c.742T and c.208-2G had no pathological signs. These results indicate that amnionless is essential for the correct luminal expression of cubilin in humans.

Key words: Imerslund-Gräsbeck syndrome, megaloblastic anemia, vitamin B12, malabsorption, amnionless.

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Introduction

Intrinsic factor (IF) is required for intestinal uptake of cobalamin (Cbl, also named vitamin B12). Imerslund Gräsbeck Syndrome or juvenile megaloblastic anemia 1 (IGS or MGA1, OMIM # 261100) is a rare autosomic recessive disorder characterized by selective Cbl malabsorption in the distal intestine. It is sometimes accompanied by proteinuria, which results from a defective reabsorption of low molecular weight proteins in renal proximal tubules. Initially, the disease was almost simultaneously described in Norway and Finland.[1,2] Usually, IGS is diagnosed in children aged between three and ten years old who present signs of Cbl deficiency and proteinuria without any other cause of cbl malabsorption. IGS remains a rare inherited disorder. There are notable clusters in Finland and Norway although an increasing number of cases are being reported in eastern Mediterranean countries.[3]

IGS is caused by abnormal expression of the IF-Cbl receptor. Presentation is hetereogeneous and includes absence of expression, increased degradation or decreased affinity of IF-Cbl receptor.[4,5,6] IGS has also been explained as mutations in the cubilin gene (*CUBN* OMIM # 602997) located on chromosome 10 or in the amnionless gene (*AMN* OMIM # 605799) located on chromosome 14.[7-10] These two proteins are parts of the IF-Cbl receptor complex, also called cubam, which is expressed in the ileal mucosa and in the renal proximal tubules.[7,9] Cubilin is a 460 kDa multiple ligand binding protein, which cannot internalize the IF-Cbl complex by itself.[7] Amnionless (AMN), originally identified as a visceral endoderm specific protein essential for embryonic development, is a 50 kDa transmembrane protein expressed in polarized epithelia that binds tightly to the cubilin N-terminal end.[8,9] Mutations in *CUBN* and *AMN* account for 54.8% (23/42) and 23.8% (10/42) of IGS, respectively, whereas mutations in other still unidentified genes account for 21% (9/42).[10] More recently, IGS was caused by a compound heterozygous mutation in *CUBN* comprising a missense mutation in the paternal allele (c.1010 C>T) and a partial gene deletion in the maternal allele.[11]

AMN is involved in apical targeting as well as internalization of cubilin in a spontaneous Imerslund–Gräsbeck canine model with a mutated *AMN* homolog.[9] But its role in the mechanisms of the Cbl malabsorption of IGS has not yet been documented in humans. In the present study, we describe a compound heterozygous mutation in *AMN* and its consequence on the activity of intrinsic factor receptor that illustrates the role of amnionless in the luminal expression of cubilin in humans.

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Design and Methods

Genetic analyses consisted of sequencing *GIF, CUBN* and *AMN* genes in all family members. Informed consent was obtained from all members and the Ethics Committee of the University Hospital of Salzburg has approved the study. Genomic DNA was extracted from peripheral blood samples using Nucleon™ BACC Genomic DNA extraction kit (GE Healthcare Life Sciences). Mutation nomenclature follows the guidelines of the Human Genome Variation Society (*http://www.hgvs.org/mutnomen/*). *CUBN* exons and intron-exon junctions were sequenced as described.[12] Exons and intron-exon junctions of the *AMN* and the *GIF* genes were analyzed by sequencing PCR amplicons, with experimental conditions and primer pairs available upon request. PCR products were purified with a QIAquick PCR purification kit (Qiagen) and directly sequenced with the BigDye terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) by using an automatic sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems).

Homocysteine and methylmalonic acid were determined from 50 μL of plasma by UPLC-MS/MS, as described.[13] IF-Cbl receptor activity was assayed in urine by radioisotope binding assay, as previously described.[4] Briefly, urine samples were concentrated 50-fold through a YM-5 membrane and urine IF-Cbl receptor activity was determined by phenyl-Sepharose radioisotope assay using 50 μL aliquots. For this a two-step procedure that involved the washing of receptor adsorbed to the gel with EDTA was used to remove the endogenous ligands. The radio-labeled IF-Cbl ligand was then added. The receptor activity was determined by Scatchard plot. Results were expressed in femtomoles of IF-Cbl binding activity per mg of creatinine. Renal tubular cells from fresh urine samples of control subjects were isolated and cultured as described.[14] Expression of cubiline and amnionless was assessed by RT-PCR and by immunofluorescence examination in cultured permeabilized cells as described.[15,16] IF-Cbl receptor activity was assessed in cell culture medium by gel filtration chromatography, as previously described.[4]

Results and Discussion

The probands are a brother (YM) and a sister (YS) born to 2 healthy parents of Turkish origin (father: FaY; mother: MoY) living in Austria. The parents have a second daughter (YH) with no pathological symptoms. YM and YS were suspected of Cbl absorption defect on the basis of the association of anemia with low serum Cbl and normal folate levels, in the absence of any known gastric and intestinal disease. YM was two years old when he presented with anemia, macrocytosis, low Cbl and normal folate in the blood (Table 1). Parietal cell antibodies and anti intrinsic factor antibodies were undetectable. The Schilling test could not be performed as it was no longer available in Europe. The repetitive intramuscular injections of Cbl were effective in treating the anemia. The patient was ten years old at the time of genetic investigations. At this time, biological investigations reported a proteinuria and a Cbl deficit with low serum Cbl, low holo-transcobalamin, increased plasma homocysteine and methylmalonic acid despite the absence of anemia (Table 1). The assay for IF-Cbl receptor showed a very low activity at 0.56 fmol/ mg creatinine and a normal affinity for intrinsic factor binding (Figure 1).

YS was the symptomatic sister of YM. She was born in 1990 in Turkey where a Cbl deficiency with anemia and proteinuria was diagnosed between 1991 and 1999. After she came to Austria in 2000, she had several episodes of respiratory tract infection and of anemia with proteinuria which were treated with intramuscular Cbl. Brain MRI identified a focal cortical atrophy in the left insular region. She was 20 years old at the time of genetic diagnosis and received Cbl i.m. one month before the last biological analyses presented in Table 1. The biological investigation showed a slightly elevated homocysteine plasma level, but no sign of macrocytic anemia and normal Cbl concentration in serum. The parents and the sister of the 2 probands had normal laboratory data except for homocysteine, which was slightly elevated in the father (Table 1).

No mutations were detected in the *CUBN* or *GIF* genes in the study subjects or in family members. Two mutations were identified in the *AMN* gene. One affected a splicing site in intron 3 (c.208-2A>G) and was previously described.[10] The c.208-2A>G mutation causes complete skipping of exon 4 (88bp) with a subsequent frame shift and a premature termination codon in exon 6. The other was a yet unknown mutation in exon 7 replacing glutamine by a stop codon on codon 248, c.742C>T, p.Gln248X (Figure 2). The family genetic profile was established as follows: the 2 affected siblings, YM and YS, were com-

Table 1. Clinical and laboratory data (in blood and urine) in the 2 siblings with Imerslund-Gräsbeck disease (YM and YS), their sister (YH), and their father (FaY) and mother (MoY).

Patient	Sex	Age (years)	Hb (12-16 g/dL)	MCV M=77-95 fL F=80-100 fL	Cbl (208-964 pg/mL)	Hcy (<15 μM)	MMA (<0.50 μM)	HoloTC (37-170 pmol/L)	Proteinuria (<0.2 g/L)
FaY	M	40	15	81.6	201.9	18.5	0.21	ND	0.11
MoY	F	37	ND	ND	ND	13.4	0.19	ND	0.03
YM	M	2	9.4	106	44	ND	ND	ND	ND
YM	M	10	13.7	80.4	<60.0	16.6	1.6	9.08	0.60
YS	F	12-15	10.1	105.3	83	ND	ND	ND	0.75
YS Under Cbl*	F	20	13,8	89.4	325.3	21.9	0.14	ND	0.20
YH	F	16	11.4	72.4	284.2	10.4	0.39	ND	0.13

**YS was supplemented with Cobalamin one month before the blood workup. Hb: hemoglobin; MCV: mean corpuscular volume; Cbl: cobalamin; Hcy: homocysteine; MMA: methylmalonic acid.*

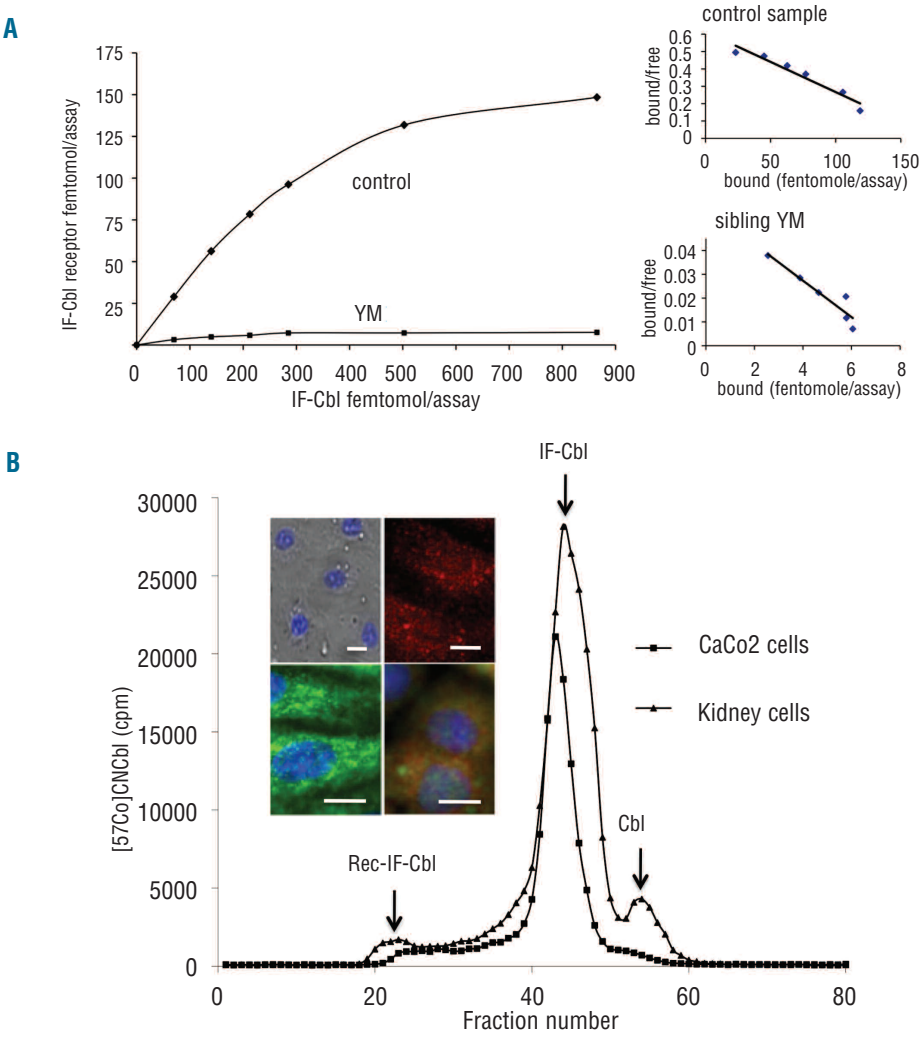

Figure 1. (A) Intrinsic factor-cobalamin receptor (Rec-IF-Cbl) assay in urine concentrate from YM, the proband with proteinuria, and a control sample. IF-Cbl receptor activity is very low in YM. YM has B_{max}= 0.56 fmol/mg creatinine and K_{ass}= 7.7 nM^{-1} compared to B_{max}= 7.7 fmol/ mg creatinine and K_{ass}= 3.5 nM^{-1} for the control sample. (B) Detection of the Rec-IF-Cbl activity by Superose 6 gel filtration chromatography (column 1x30 cm) in culture medium of confluent tubular renal cells and Caco-2 cells. The Rec-IF-Cbl, IF-Cbl and free Cbl were detected by gamma-counting, using IF-[57Co]CNCbl as a tracer and eluted at retention time of 23, 43 and 53 min according to the procedure described by Guéant *et al.*[4] Briefly, 0.5 ml of culture medium was incubated with 0.4 pmol of tracer in the presence of 1 mmol/L $CaCl_2$ and eluted in Tris-HCl buffer at a flow rate at 0.8 mL/min, with one fraction collected *per* minutes. No Rec-IF-Cbl peak was observed when replacing $CaCl_2$ by 10 μmol/L EDTA. Inset: top left, renal tubular cells isolated from fresh urine of control subjects and cultured until confluence; top right, immunofluorescence detection of amnionless (in red); bottom left, immunofluorescence detection of cubilin (in green); bottom right, confocal examination showing co-localization of amnionless and cubilin. Calibration bars = 20 μm.

pound heterozygous for these 2 mutations. The mother YMo was a 208-2G heterozygous carrier and the father was a c.742C>T heterozygous carrier. The asymptomatic daughter inherited only one mutated allele from her father and was a c.742C>T heterozygous carrier (Figure 2). Thus, each one of these 2 mutations, c.208-2A>G and c.742C>T was not deleterious when found in a heterozygous carrier. In contrast, the compound heterozygosity associating c.742C>T, p.Gln248X and c.208-2A>G caused disease, as this was the case with the 2 siblings, YM and YS. The genetic lesion yielded a disease with similar phenotypes in both cases, with proteinuria and early disease onset. Mild proteinuria is not always present in Imerslund-Gräsbeck disease and patients without proteinuria have a tendency to present cobalamin deficiency symptoms later in life.[17]

Up till now, most reported IGS cases were caused by a *CUBN* founder mutation in Finland and by a founder *AMN* mutation in Norway, whereas in Middle Eastern countries, mutations were identified on both genes.[7,10,17] In a previous case with English-Israeli parentage and *AMN* compound mutations, the first mutation was expected to truncate the protein and the second (c.701G>T, C234F) to alter the residual function of cubam.[18] It has been suggested that AMN is a 'moonlighting' protein that fulfils 2 functions, one in embryonic development, proved in mice and the other in Cbl absorption, demonstrated in humans.[8,19] In humans, homozygous mutations affecting exons 1-4 of human *AMN* lead to selective malabsorption of Cbl and no embryonic effect. As a consequence of these mutations, translation initiation occurs downstream and produces truncated AMN proteins in HEK293 transfected cells.[10,19] It has been hypothesized that these truncated AMN products were produced for maintaining a proper embryonic development in mice.[8,19] The same group reconsidered this hypothesis after reporting IGS cases with mutations in exons 7 and 11 which were inconsistent with the production of truncated proteins.[10] It seems, therefore, that the role of *AMN* in embryonic human development, if any, is less critical than that in mice.[10] However, these studies and our report did not investigate the splicing and translation products in intestinal or renal biopsies from patients. In our case, the premature stop codons may produce either a truncated protein or a nonsense-mediated decay. In any case, the truncated protein or mRNA degradation can lead to a loss of function of *AMN* responsible for the IGS phenotype.

In a previous study, we had showed that the dramatic decrease of the IF-Cbl receptor reported in ileal biopsies of IGS patients was similarly observed in concentrated urine.[4,20] In the present report, we observed also a dramatic

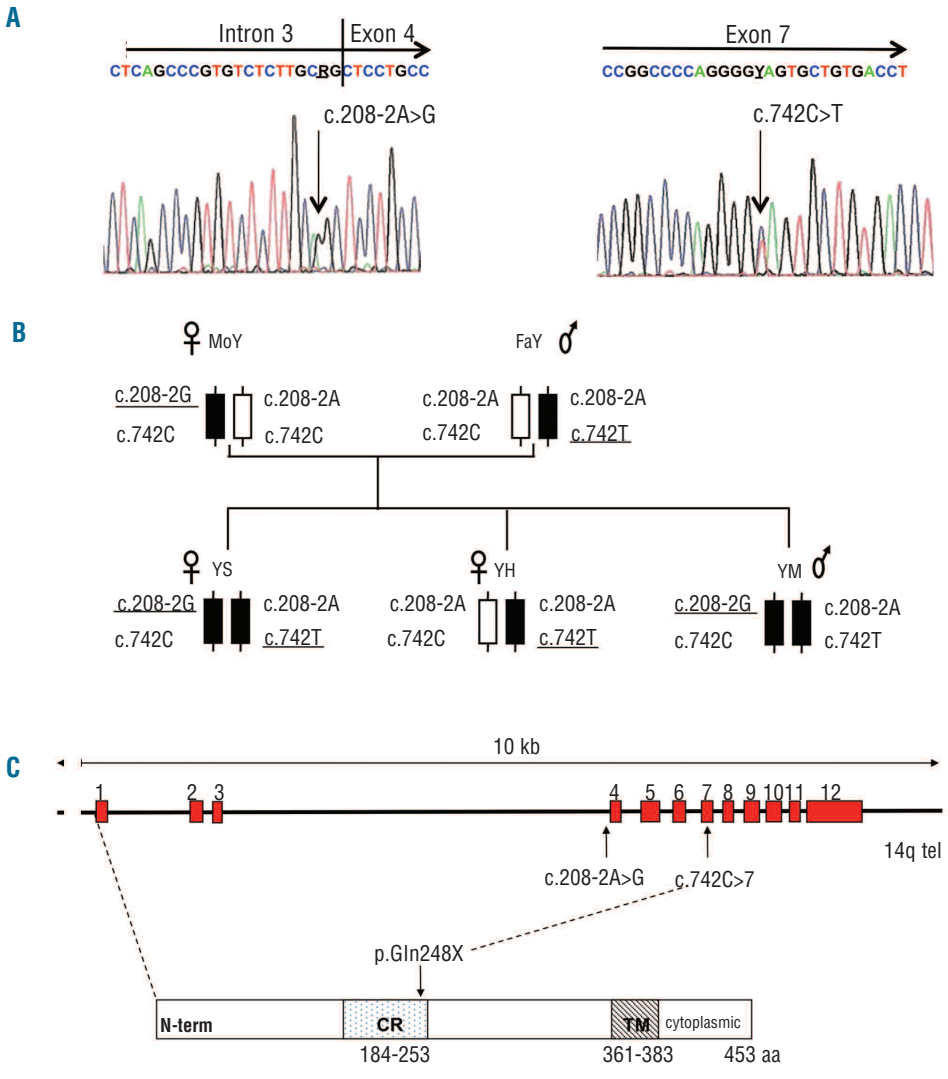

Figure 2. (A) DNA sequencing chromatograms of the amnionless gene showing the 2 heterozygous mutations identified in the 2 probands with Imerslund-Gräsbeck disease, YM and YS. (B) Amnionless mutation profile of all family members. The mother (MoY) and the father (FaY) are heterozygous carriers for c.208-2G and c.742T, respectively, and bear no pathological signs. The 2 siblings with Imerslund-Gräsbeck disease, YS and YM, are compound heterozygous. YH inherited only one deleterious allele from her father: she is an asymptomatic heterozygous c.742T carrier. Deleterious nucleotides are underlined. (C) Location of mutations in *AMN* gene map and predicted consequence of c.742T in protein translation.

decrease in IF-Cbl receptor in concentrated urine from patients compared to control urine (Figure 1). This was not due to a saturation of the receptor by excreted proteins, since the first step of the assay procedure includes an EDTA washing of the receptor adsorbed to phenyl-Sepharose in order to remove the endogenous protein ligands. The receptor had a normal affinity for IF-Cbl, a result that was consistent with the absence of mutations in the *CUBN* gene. Amnionless is essential for the cell-surface expression of cubilin by influencing its brush border targeting and its half-life, in opossum kidney cells, and rat and rabbit kidney.[21] We cultured tubular epithelial renal cells isolated from fresh urine of control subjects and we showed the cell expression of cubilin and amnionless in confluent cells and the release of the IF-Cbl receptor in the culture medium (Figure 1). We observed the same result in confluent Caco2 (Figure 1), a human colon carcinoma that resembles enterocytes and expresses the IF-Cbl receptor in the apical side.[22] This apical release is probably produced by a physiological cleavage, as observed for other membrane receptors. Taken together, these data suggest that the decreased IF-Cbl receptor activity in urine corresponded to a decreased luminal expression of cubilin in the renal tubule, as a consequence of a lack of interaction between mutated amnionless and cubilin. This interdependency of cubilin and amnionless in cubilin brush-border expression has also been shown in a spontaneous Imerslund-Gräsbeck canine model with a mutated AMN homolog.[23] Our cases with a compound mutation in *AMN* and no mutation in *CUBN* provide the first evidence in humans that cubilin depends on AMN for its correct expression in the epithelium of the proximal tubule. An inverse situation was recently reported where the altered interdependency of both proteins was due to a *CUBN* mutation in exon 23 and no mutation in *AMN*.[24]

In conclusion, we report a case of IGS with compound heterozygous mutations on *AMN* which indicates that amnionless is essential for the luminal expression of cubilin in humans.

Authorship and Disclosures

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