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RESEARCH ARTICLE

Genetically Heterogeneous Selective Intestinal Malabsorption of Vitamin B_{12}: Founder Effects, Consanguinity, and High Clinical Awareness Explain Aggregations in Scandinavia and the Middle East

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Selective intestinal malabsorption of vitamin B_{12} causing juvenile megaloblastic anemia (MGA; MIM# 261100) is a recessively inherited disorder that is believed to be rare except for notable clusters of cases in Finland, Norway, and the Eastern Mediterranean region. The disease can be caused by mutations in either the cubilin (*CUBN*; *MGA1*; MIM# 602997) or the amnionless (*AMN*; MIM# 605799) gene. To explain the peculiar geographical distribution, we hypothesized that mutations in one of the genes would mainly be responsible for the disease in Scandinavia, and mutations in the other gene in the Mediterranean region. We studied 42 sibships and found all cases in Finland to be due to *CUBN* (three different mutations) and all cases in Norway to be due to *AMN* (two different mutations), while in Turkey, Israel, and Saudi Arabia, there were two different *AMN* mutations and three different *CUBN* mutations. Haplotype evidence excluded both *CUBN* and *AMN* conclusively in five families and tentatively in three families, suggesting the presence of at least one more gene locus that can cause MGA. We conclude that the Scandinavian cases are typical examples of enrichment by founder effects, while in the Mediterranean region high degrees of consanguinity expose rare mutations in both genes. We suggest that in both regions, physician awareness of this disease causes it to be more readily diagnosed than elsewhere; thus, it may well be more common worldwide than previously thought. Hum Mutat 23:327–333, 2004

KEY WORDS: vitamin B_{12}, malabsorption of; Imerslund-Gräsbeck syndrome; *MGA1*; *CUBN*, cubilin; *AMN*, amnionless; mutational origin; Scandinavian; Middle-Eastern; Arab

DATABASES:
CUBN – OMIM: 602997, 261100 (MGA); GenBank: NM_001081.2
AMN – OMIM: 605799; GenBank: NM_030943.1

INTRODUCTION

Megaloblastic anemia (MGA; MIM# 261100) is considered to be a rare condition. Some 250 cases have been diagnosed in all [Gräsbeck, 1997]. A notable cluster occurs in two Scandinavian countries: Norway [Imerslund, 1960] and Finland [Gräsbeck et al., 1960]. Another cluster occurs in the eastern Mediterranean region in Turkey [Altay et al., 1995; Celep et al., 1996], Kuwait [Ismail et al., 1997], Israel [Ben Bassat et al., 1969], and Saudi Arabia [Abdelaal and Ahmed, 1991; Salameh et al., 1991]. Outside of these regions, only scattered cases have been described, e.g., in South Africa [Stones and Ferreira, 1999], Tunisia [Ben Meriem et al., 1993], France [Flechelles et al., 1997], the United States

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of America [Mackenzie et al., 1972], Taiwan [Lin et al., 1994], and elsewhere. As there was no obvious simple explanation for this peculiar geographical distribution, we set out to clarify it. A likely reason appeared to relate to the recently established fact that the disease can be caused by mutations in two different genes, cubilin (*CUBN*, MIM# 602997) [Aminoff et al., 1999] and amnionless (*AMN*, MIM# 605799) [Tanner et al., 2003]. Hence, we hypothesized that a founder mutation in one gene might be responsible for the clustering in Scandinavia, while a founder mutation in the other gene might cause the high incidence in the Mediterranean region. The proteins encoded by the two genes form a complex designated cubam, which is essential for vitamin B_{12} uptake in the gut, renal protein reabsorption, and early embryogenesis [Fyfe et al., 2004]. The two genes in no way resemble each other and are located on different chromosomes—*CUBN* on chromosome 10 (known as locus *MGA1*) and *AMN* on chromosome 14—suggesting that mutations in them are likely to be independent of each other. We show here that our initial hypothesis was wrong and present another hypothesis to explain the findings. Moreover, by exclusion of both *CUBN* and *AMN,* we show that there is at least one more gene locus involved in MGA.

MATERIALS AND METHODS

Patient Samples

DNA samples were obtained after informed consent with prior Institutional Review Board approval according to the Declaration of Helsinki. The diagnosis of selective intestinal malabsorption of vitamin B_{12} was made based on established criteria [Broch et al., 1984; Wahlstedt-Fröberg et al., 2003].

Mutation Analysis

We amplified individual exons of *CUBN* (GenBank RefSeq: NM_001081.2) and *AMN* (GenBank RefSeq: NM_030943.1) from genomic DNA by PCR and analyzed them by DNA sequencing, restriction digestion, or single strand conformation polymorphism (SSCP). Sequencing was performed on an ABI PRISM® 3700 DNA analyzer (Applied Biosystems, Foster City, CA). PCR and SSCP conditions and primer sequences are available upon request. All nucleotide numbering is relative to the adenine in the first ATG start codon of *AMN* (GenBank RefSeq: NM_030943.1) or *CUBN* (GenBank RefSeq: NM_001081.2), while the amino acid residue numbering is relative to the first methionine deduced from these cDNA sequences according to standard nomenclature.

Genotype Analysis

For the *AMN* gene, we used flanking CA-repeat markers *D14S985* (2 Mb proximal), *D14S272* (111 kb proximal), *D14S293* (57 kb distal), and *D14S292* (1.2 Mb distal), four single nucleotide polymorphisms (SNPs; c.-87S/c.-74Y/c.-27Y/c.-23S) upstream of the ATG in exon 1, one SNP in intron 10 (c.1169+42S), and one SNP in exon 12 (c.1362+38S) for haplotype analysis. For the *CUBN* gene, we genotyped flanking CA-repeat markers *D10S1653* (~1.2 Mb distal) and *D10S548* (~1.3 Mb proximal), as well as intragenic CA-repeat markers *CUBN M2* (intron 28) and *CUBN M3* (intron 66). The CA-repeat markers were analyzed on the ABI PRISM® 3700 DNA analyzer using FAM-labeled primers and the Genotyper program (Applied Biosystems, Foster City, CA) while the SNPs were sequenced. PCR conditions and primer sequences are available from the authors.

RESULTS

We collected and analyzed a total of 42 MGA sibships from Finland (20), Norway (4), Turkey (10), Israel (2), the United States (2), Kuwait (2), Saudi Arabia (1), and Belgium (1) (Table 1). Genomic DNA from peripheral blood leukocytes or Epstein-Barr virus transformed cells was first examined for *AMN* mutations by SSCP [Tanner et al., 1998]. All migration variants and a region upstream of exon 1—encompassing four common SNPs (see Materials and Methods section)—were DNA sequenced. We found a total of six different *AMN* mutations in 4 out of 4 Norwegian, 3 out of 10 Turkish, 1 out of 2 Israeli, 1 out of 2 American, and one heterozygous frameshift mutation in the single Belgian index patient (10 out of 42) (Tables 1 and 2). Notably, two of the mutations were found in Scandinavia, two in the Eastern Mediterranean region, and two elsewhere.

Three different *CUBN* mutations accounted for all 20 Finnish MGA sibships (Tables 1 and 2). We genotyped the remaining 12 non-Scandinavian families for flanking and intragenic polymorphic CA-repeat markers for the *CUBN* gene (see Materials and Methods section) to evaluate if *CUBN* could be suggested or excluded. Haplotypes suggested *CUBN* as a prime candidate for two Turkish sibships and the single large Saudi family. A combination of SSCP and DNA sequencing of all 67 exons revealed different nonsense mutations (*CUBN* c.250C>T, Q84X and c.1951C>T, R651X) in the two Turkish families and the missense mutation *CUBN* c.434G>A, G145E in the large Saudi sibship with seven distantly related male patients (Fig. 1). Only the core haplotype defined by the three intragenic markers *CUBN M2* (intron 28), *CUBN M3* (intron 66), and the mutation in exon 5 was conserved in all seven Saudi cases, while the telomeric maker *D10S1653* was recombined twice and the centromeric marker *D10S548* was recombined three times among these seven cases. While genealogical evidence showed the mutation to have occurred at least six generations ago (Fig. 1) [Abdelaal and Ahmed, 1991], these recombinations suggest a considerable age for this mutation. The missense change completely cosegregated with the phenotype and was not found among 40 grandparents of Centre d'Etude du Polymorphisme Humain families, 30 European volunteers, and 50 unrelated samples from the same tribal background from the Western part of Saudi Arabia [Abdelaal and Ahmed, 1991]. Amino acid residue G145 is 100% conserved among human, dog, and rat cubilin and is located in the first EGF-like domain providing evidence for its functional importance. Thus, altogether six different *CUBN* mutations accounted for 23 out of 42 sibships (Table 2). Particularly, three of the mutations were found in Scandinavia, and three in the Mediterranean region.

Of 42 MGA families, 33 had been explained by *CUBN* or *AMN* (Table 1). In all of the remaining nine families,

TABLE 1. MGA Families Studied

Family	Origin	Mutation[a]
AMN mutations (10; 23.8%)		
Fam A	Norwegian	*AMN* c.14delG; G5fs
Fam C	Norwegian	*AMN* c.14delG; G5fs
Fam D	Norwegian	*AMN* c.14delG; G5fs
Fam K	Norwegian	*AMN* c.122C>T; T41I
Fam M	Sephardic Jewish	*AMN* c.208-2A>G; skip Ex4
Fam AK	USA	*AMN* c.683_730del48; Q228_L243del16
Fam CT	Turkish	*AMN* c.208-2A>G; skip Ex4
Fam ET	Turkish	*AMN* c.208-2A>G; skip Ex4
Fam FT	Turkish	*AMN* c.208-1G>C; splice site
Belgium 1	Belgian	*AMN* c.1253_1254insA; L419fs & unknown
CUBN mutations (*MGA1*: 23; 54.8%)		
18 P1297L families	Finnish	*CUBN* c.3890C>T; P1297L
Finnish compound family	Finnish	*CUBN* c.3890C>T; P1297L & c.1230+1G>A; splice site
Finnish splice mutation family	Finnish	*CUBN* c.3300-439C>G; splicing
Fam SA	Bedouin	*CUBN* c.434G>A; G145E
Fam DT	Turkish	*CUBN* c.250C>T; Q84X
Fam KT	Turkish	*CUBN* c.1951C>T; R651X
Unknown mutations (9; 21.4%)		
Fam 1	Kuwaiti	*AMN*+*CUBN* excluded
Fam 2	Kuwaiti	*AMN*+*CUBN* excluded
Fam AT	Turkish	*AMN*+*CUBN* excluded
Fam HT	Turkish	*AMN*+*CUBN* excluded
Fam IT	Turkish	*AMN*+*CUBN* excluded
Fam GT	Turkish	*AMN*+*CUBN* unlikely, parents 3rd cousins
4655-2590	Ashkenazi Jewish	*AMN*+*CUBN* unlikely, parental relationship unknown
Fam NY	Guinea-Bissau (USA)	*AMN*+*CUBN* unlikely, ostensibly unrelated
Fam BT	Turkish	Probably *AMN*
Total: 42		

[a]See Table 2 footnotes regarding nomenclature.

TABLE 2. MGA mutations in *AMN* and *CUBN*

cDNA[a]	Exon/intron	Protein prediction[b]	Frequency among 42 MGA families
		Amnionless	
AMN			
c.14delG	Exon 1	G5fs	3 (Norwegian founder mutation)
c.122C>T	Exon 2	T41I	1 (Norwegian, consanguinity)
c.208-2A>G	Intron 3	Skipping of exon 4; fs	3 (Turkish [2]; Israeli [1] founder mutation)
c.208-1G>C	Intron 3	Splice site	1 (Turkish, consanguinity)
c.683_730del48	Exon 7	Q228_L243del16	1 (American, consanguinity)
c.1253_1254insA	Exon 11	L419fs	1 (Belgian, heterozygous)
		Cubilin	
CUBN			
c.250C>T	Exon 2	Q84X	1 (Turkish, consanguinity)
c.434G>A	Exon 5	G145E	1 (Saudi Arabian, consanguinity)
c.1230+1G>A	Intron 11	Splice site	1 (Finnish, compound with P1297L)
c.1951C>T	Exon 16	R651X	1 (Turkish, consanguinity)
c.3300-439C>G[c]	Intron 23	Complex splicing; fs	1 (Finnish, consanguinity)
c.3890C>T	Exon 27	P1297L	18 (Finnish founder mutation)

[a]Numbering relative to adenine in the first ATG start codon of *AMN* (GenBank RefSeq: NM_030943.1) or *CUBN* (GenBank RefSeq: NM_001081.2).
[b]Numbering relative to the first methionine deduced from the cDNA sequences.
[c]Originally described by Aminoff et al. [1999] as IVS-intraCUB6 C→G (FM2).

no *AMN* mutations had been found by exon-by-exon sequencing; however, this does not definitely exclude the gene in all. Therefore, and because of the daunting task of searching for mutations in the 67 exons of *CUBN*, we proceeded to construct haplotypes for the *AMN* and *CUBN* regions in all nine families. Analyses showing different haplotypes in affected sibs or identical haplotypes in affected and unaffected sibs excluded both *AMN* and *CUBN* in five families (Fig. 2). In three further families (one Turkish, one Israeli, one American), both genes were tentatively excluded based on heterozygous haplotypes in single affected individuals, but the conclusion is only valid under the assumption that the patients are homozygous for the presumptive mutation. In Family GT, the parents are consanguineous. In Case 4655-2590, we have no detailed information about the parents. In Family NY, the parents are apparently unrelated; however, they originate from the same geographic region in Africa. Furthermore, in Turkish Family BT our haplotype data supported *AMN* but we

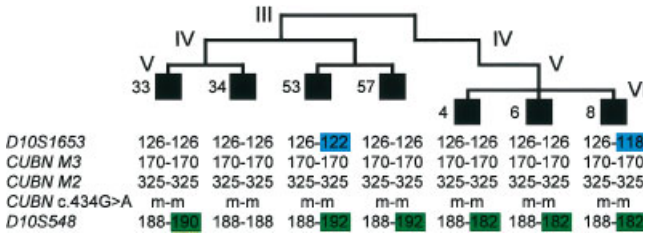

FIGURE 1. *CUBN* haplotypes in a Saudi Arabian family shown on a simplified pedigree of seven distantly related MGA patients belonging to a six-generation family in which most marriages are consanguineous. Only informative markers are shown in the order telomeric flanking marker *D10S1653* to centromeric flanking marker *D10S548* on the short arm of chromosome 10. The *CUBN* gene spans some 305 kb in this 3-Mb interval. The three intragenic markers are *CUBNM3* (in intron 66), *CUBNM2* (in intron 28), and the disease-causing missense mutation c.434G>A, G145E (in exon 5) where m indicates the presence of the mutation. Other alleles are shown as PCR amplicon sizes in bp. No intragenic recombinations were observed. Two recombinations had occurred on the telomeric side (blue color) and at least three recombinations had occurred on the centromeric side (green color). These recombinations tentatively support a more ancient origin of the mutation than six generations ago.

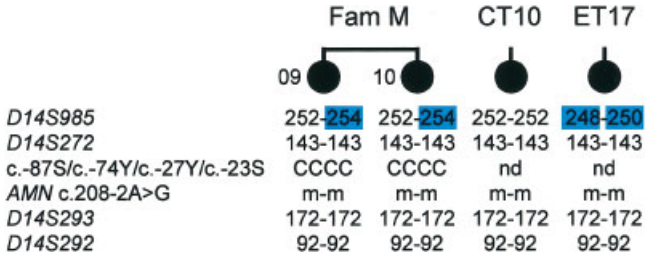

FIGURE 3. Haplotypes of four MGA patients having the *AMN* splice site mutation c.208–2A>G, skipping exon 4. The patients are Jewish Sephardic (M) and Turkish (CT; ET). Two markers on the centromeric side (*D14S985* and *D14S272*) and two markers on the telomeric side (*D14S293*, *D14S292*) flank the *AMN* gene with alleles shown by their PCR amplicon sizes in bp. The distance between *D14S985* and *D14S292* is 3.3 Mb. Alleles in two intragenic loci are shown. One is represented by four nucleotides in the region immediately upstream of exon 1 (see legend for Fig. 2). The mutation itself in intron 3 is shown as m. Haplotype identity supports a common origin of the mutation, because these markers are fully informative in Family M, and are heterozygous in at least one parent of families CT and ET. The ancestral haplotype is altered for the most distant centromeric marker, *D14S985*.

have not found any mutation in the coding region, splice sites, or by sequencing of 500 bp upstream of exon 1 as well as introns 1 and 2. Lack of RNA in this case prevented us from testing *AMN* gene splicing and expression. These findings provide preliminary evidence for the existence of at least one more disease-causing gene. We are conducting a genome wide scan in search of linkage to further loci.

DISCUSSION

In a recent report, Bonafé et al. [2003] described a cluster of three different *DLL3* mutations causing

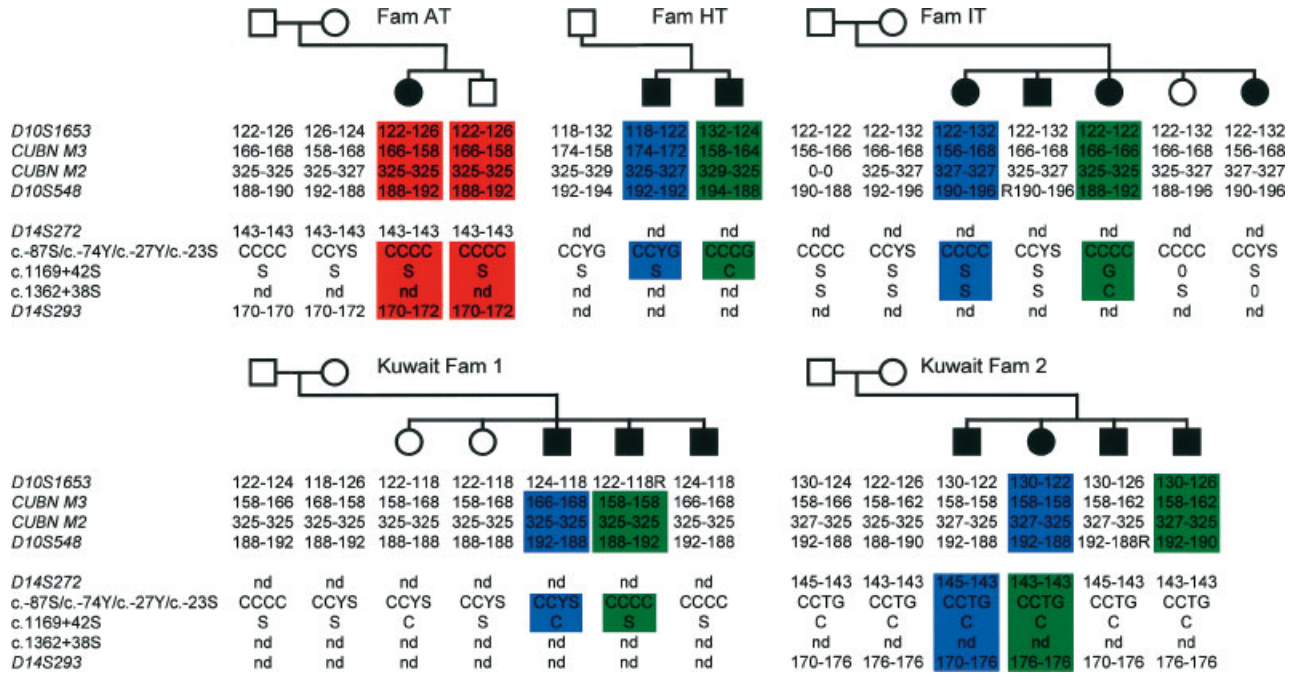

FIGURE 2. *CUBN* and *AMN* haplotypes among five families in which both genes appear to be excluded as causative of the disease. The three families on top are from Turkey, the two families at the bottom from Kuwait. *CUBN* haplotypes are shown as described in the legend of Figure 1. *AMN* haplotypes are shown centromeric to telomeric on the long arm of chromosome 14. The *AMN* gene spans 8.5 kb in the 176-kb interval between *D14S272* and *D14S293*. Three intragenic loci are shown; the first consists of polymorphisms affecting four different nucleotides in the region 87 to 23 nt upstream of the start codon in exon 1. In 39 of 80 individuals sequenced, at least one of these SNPs was informative (heterozygosity ~50%). Identical haplotypes in an affected and unaffected sib are shown in red, whereas examples of different haplotypes in affected sibs are shown in green and blue. These results suggest that *CUBN* and *AMN* are not involved in these five families.

autosomal recessive spondylocostal dysostosis (SCDO1; MIM# 277300) in a small Swiss village. A founder mutation had spread by genetic drift and by its relatively frequent presence revealed two rare disease alleles by compounding that might have gone undetected otherwise. The same scenario applies to the Finnish MGA families [Gräsbeck et al., 1960] with 18 out of 20 being homozygous for the *CUBN* c.3890C>T, P1297L missense change. The unique *CUBN* c.1230+1G>A splice-site mutation was exposed through compound heterozygosity with the common founder mutation while the detection of the *CUBN* c.3300-439C>G splice mutation in a single family probably was a rare chance event (originally described by Aminoff et al. [1999] as IVS-intraCUB6 C>G, FM2). Similarly, in Norway one founder mutation (*AMN* c.14delG) accounted for three MGA families all belonging to the original series of cases studied by Imerslund [1960]. In both Finland and Norway, physicians have a high degree of awareness of the syndrome that was first described in these countries [Gräsbeck et al., 1960; Imerslund, 1960]. Moreover, later publications from the same countries have reminded the medical profession of this condition [Gräsbeck, 1972; Furuhjelm and Nevanlinna, 1973; Broch et al., 1984; Norio, 2003].

In the Middle East, the situation is different. One founder mutation that may be very old (*AMN* c.208-2A>G, skipping exon 4, fs) was found in one Tunisian Jewish family living in Israel and in two Turkish families. While the Jewish family is of Sephardic origin, we were not able to ascertain any particular ethnic background of the two Turkish families with the same mutation, but we assume that during the long history of the Ottoman Empire, this mutation might have drifted from Tunisia to Turkey or the reverse. While the central *AMN* marker haplotypes (*D14S272*, *D14S293*, four SNPs, and the mutation) and distal *D14S292* were the same in all four patients, proximal *D14S985* showed three recombinations: one in Family M and two in Family ET (Fig. 3). Taking into account that all these markers are fully informative in both parents of Family M and are heterozygous in at least one parent of families CT and ET, this pattern did testify to the common origin of the mutation, but was too ambiguous to suggest whether it might have originated in Turkey or Tunisia. Furthermore, three *CUBN* mutations and one unique *AMN* mutation were found among three Turkish and one Saudi MGA families, while at least three more Turkish and two Kuwaiti sibships must carry mutations other than cubam (Table 1). This allows us to hypothesize that the "Mediterranean cluster" was mainly the result of frequent combinations of consanguineous marriages and high clinical awareness rather than major founder effects. Several publications about the disease have been published from Turkey [Yetgin et al., 1978, 1983; Altay et al., 1995; Celep et al., 1996], Israel [Ben Bassat et al., 1969], and some Arab countries [Abdelaal and Ahmed, 1991; Salameh et al., 1991; Ismail et al., 1997], where clinical awareness of the disease is obvious.

Overall, founder effects and genetic drift in combination with the historical awareness by a few clinical experts sufficiently explained the alleged higher incidence in the two regions. With the clear definition at the molecular level that is now possible, we expect to test more diagnosed MGA cases worldwide. Recently diagnosed cases in Belgium and the USA (this report) suggest that MGA might not be as clustered and perhaps not even as rare as previously thought. Consanguineous marriages are frequent in many populations, and improved clinical awareness in combination with available molecular diagnosis will undoubtedly lead to more patients being detected. With the genomic DNA screening strategy—analyzing *AMN* first followed by *CUBN* genotyping and screening—we achieved an overall 78% mutation detection for MGA and determined that ~12% must result from gene mutations in at least one more locus. About 10% of the MGA sibships known to us remained molecularly uncategorized.

The heterogeneous nature of the detected cubam mutations does not allow for robust genotype–phenotype correlations at this point. We are not aware of any clinical differences between patients in whom the disease is caused by *CUBN* vs. *AMN*, nor do there appear to be clinical features that are mutation specific. However, clinical variability has been reported [Dugué et al., 1999] and could be related to a yet unknown gene. Further, it is interesting to note that the two nonsense mutations (Fig. 4) in exons 2 (c.250C>T, Q84X) and 16 (c.1951C>T, R651X) of *CUBN* presented with exactly the same phenotype as the Saudi c.434G>A, G145E missense mutation in exon 5 that affects the first EGF-like repeat and the Finnish c.3890C>T in exon 27, P1297L missense, that resides in CUB domain 8 [Aminoff et al., 1999; Kristiansen et al., 2000]. This is somewhat surprising as early truncation of cubilin probably removes all functions of this multifunctional receptor [Christensen and Birn, 2002] either by nonsense-mediated mRNA decay (NMD) [Wagner and Lykke-Andersen, 2002] or at the protein level, while missense mutations might be compatible with residual function (Fig. 4). The two *CUBN* splice-site mutations produce aberrant transcripts leading to various premature termination codons that might invoke NMD as well. We conclude therefore that loss-of-function mutations in cubilin invariably lead to selective intestinal malabsorption of vitamin B_{12} in humans.

As to the functional consequences of the *AMN* mutations, several intriguing questions remain only partially answered. Based on previous observations, homozygous knockout of mouse *Amn* is lethal by day 10 of gestation [Kalantry et al., 2001]. In contrast, homozygous null mutations in the human *AMN* gene result in the MGA phenotype in otherwise normal individuals [Tanner et al., 2003]. The previously detected *AMN* mutations were all in the 5′ part of the gene (Fig. 4) and alternative transcription and translation initiation sites allowed the carboxy terminal part of amnionless to be generated. Hence, we postulated that in patients with missense or null mutations in the 5′ part of

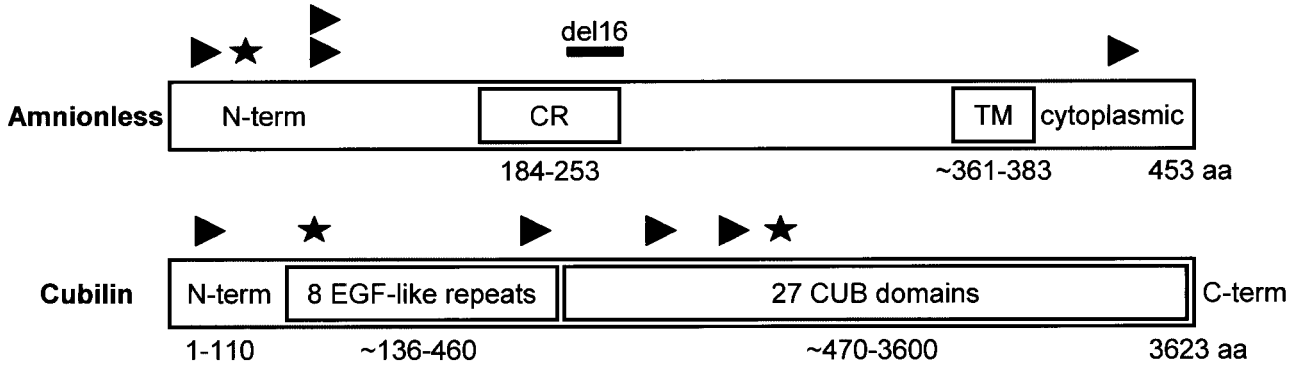

FIGURE 4. **Amnionless and cubilin protein structures according to Kalantry et al. [2001] and Christensen and Birn [2002], respectively. Symbols above the proteins show the location of the six different mutations in each classified as premature termination codon (arrowheads) due to frameshift or stop mutations, missense mutation (stars) or in-frame deletion (del). The mutations are arranged following the order in Table 2. Amnionless is 453 amino acids (aa) long with a cysteine-rich (CR) domain and a transmembrane (TM) domain [Kalantry et al., 2001], while cubilin consists of 3623 aa with eight epidermal growth factor-like repeats followed by 27 CUB domains [Christensen and Birn, 2002]. The extent of the domains is indicated below. The previously published *AMN* mutations are all in the N-terminal part of amnionless [Tanner et al., 2003], while two novel mutations described in this report affect either the CR domain or the cytoplasmic tail. Four of the *CUBN* mutations create premature termination codons while the two missense changes affect either the first EGF-like repeat or CUB domain 8.**

the gene the amino terminal part of amnionless was dispensable for embryonic development [Tanner et al., 2003]. With the addition of the present mutations further downstream in the gene, especially the c.683_730del48 in exon 7, Q228_L243del16 affecting the cysteine-rich domain (amino acids 184-253) and the c.1253_1254insA in exon 11, L419fs that affects the cytoplasmic tail (Fig. 4), this hypothesis is less likely to explain the findings. It is now becoming clear that *AMN* mutations lead to MGA because of disrupted interactions with cubilin [Fyfe et al., 2004]. Cubilin and amnionless may perform a critical function in mouse embryonic development that is different or redundant in humans and therefore causes the relatively mild MGA phenotype. These hypotheses can probably best be investigated by animal modeling. Experiments in our laboratory are presently aimed at knocking-in the naturally occurring human *AMN* mutations into mouse *Amn* in order to determine if the phenotype in mice, too, might be MGA-like or whether they produce the embryonic lethal condition known as amnionless.

In conclusion, MGA is caused by various mutations in at least three genes and founder effects, consanguinity, and high clinical awareness explain reported clusters in Scandinavia and the Middle East.

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