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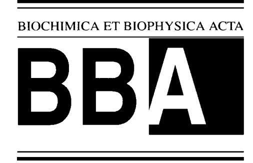

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Review

HEMPAS

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Abstract

Congenital dyserythropoietic anemia type II or HEMPAS (hereditary erythroblastic multinuclearity with positive acidified serum lysis test) is a genetic anemia in humans caused by a glycosylation deficiency. Erythrocyte membrane glycoproteins, such as band 3 and band 4.5, which are normally glycosylated with polylactosamines lack these carbohydrates in HEMPAS. Polylactosamines accumulate as glycolipids in HEMPAS erythrocytes. Analysis of *N*-glycans from HEMPAS erythrocyte membranes revealed a series of incompletely processed *N*-glycan structures, indicating defective glycosylation at *N*-acetylglucosaminyltransferase II (GnT-II) and/or α-mannosidase II (MII) steps. Genetic analysis has identified two cases from England in which the MII gene is defective. Mutant mice in which the MII gene was inactivated by homologous recombination resulted in a HEMPAS-like phenotype. On the other hand, linkage analysis of HEMPAS cases from southern Italy excluded MII and GnT-II as the causative gene, but identified a gene on chromosome 20q11. HEMPAS is therefore genetically heterogeneous. Regardless of which gene is defective, HEMPAS is characterized by incomplete processing of *N*-glycans. The study of HEMPAS will identify hitherto unknown factors affecting *N*-glycan synthesis. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Hereditary erythroblastic multinuclearity with positive acidified serum lysis test (HEMPAS); Glycosylation deficiency; Erythrocyte membrane glycoproteins; MII gene; GnT-II gene; Chromosome 20q11

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1. Introduction

Congenital dyserythropoietic anemia (CDA) is a group of inherited disorders characterized by an ineffective erythropoiesis, bone marrow erythroid multinuclearity, and secondary tissue siderosis [1–4]. In 1968, Heimpel and Wendt classified CDA into three types (Table 1) [2]. CDA type II is the most common CDA and is also called HEMPAS, since Crookston characterized this disease as hereditary erythroblastic multinuclearity with positive acidified serum lysis test [3]. This chapter covers only CDA type II (CDA-II, CDAN2 or HEMPAS), but those interested in other types of CDAs are encouraged to read references cited [5–8].

As described below, HEMPAS is a biochemically homogeneous entity characterized by incomplete processing of *N*-glycans [9–11]. Genetically, however, HEMPAS is heterogeneous [12]. Clinical variations may be linked to genetic heterogeneity. This reviewr describes symptoms and biochemical and genetic characterizations of HEMPAS disease and discusses unsolved problems and future prospects regarding this disease.

2. Occurrence

HEMPAS is an autosomal recessive anemia in humans. Parents and some siblings of HEMPAS patients are clinically normal but are heterozygous carriers of the defective gene. Both sexes are equally affected. Conservative numbers of patients reported in the literature are 130 [8], although as many as 300 cases may exist [11]. HEMPAS patients have been found on all continents and in a variety of races. As is the case with other rare recessive genetic diseases, HEMPAS patients are found more often in areas where people have a high incidence of intermarriage.

Literature on HEMPAS suggests that the disease is often asymptomatic and is therefore not as rare as hitherto believed. This disease may become clinically apparent when another hereditary or acquired disease occurs. For example, deficiencies in glutathione reductase, acetylcholine esterase, and adenyl cyclase have been reported in association with HEMPAS [13]. HEMPAS patients are associated with glucose-6-phosphate dehydrogenase deficiency [14,15], abnormal hemoglobin [16], genetically determined a β-lipoprotein anemia [17], or ectodermal dysplasia [18]. HEMPAS cases accompanying hairy cell leukemia [19], acquired tocopherol deficiency [4,20], and acquired aplastic crisis caused by infection with parvovirus or other pathogens [21] indicate that incidence of acquired disease can result in the manifestation of HEMPAS.

Table 1
Three types of CDAs

Features	Type I	Type II	Type III
No. of patients [7]	70	130	40
Anemia	Mild to moderate	Mild to severe	Mild to moderate
Bone marrow	~5% binucleated chromatin bridge	10–40% bi- and multinucleated	10–40% multinucleated gigantoblasts
Inheritance	Recessive	Recessive	Dominant
Primary defect	Unknown	Defect in *N*-glycan synthesis	Unknown
Defective genes and chromosome localization[a]	CDAN1 (15q15.1–3) [54]	MII (5q25) [43,44]; CDAN2 (20q11.2) [47]; others [11]	CDAN3 (15q21–25) [55]

[a]Each protein encoded by CDAN1, CDAN2 and CDAN3 genes has not been identified.

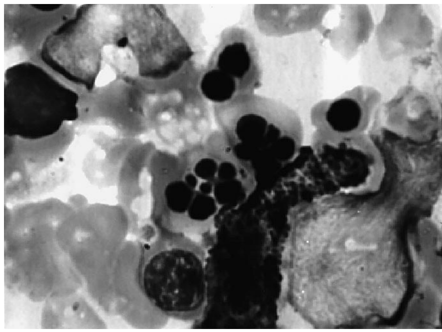

Fig. 1. Bone marrow aspirate from a HEMPAS patient showing multinucleated erythroblasts.

3. Symptoms

HEMPAS patients suffer from a life-long anemia. Jaundice is common. In most cases the magnitude of anemia is mild; however there are severely affected patients who require constant care and frequent transfusions.

The most striking diagnostic feature of HEMPAS is abnormal multinucleated erythroblasts in the bone marrow [1–5]. While immature erythroblasts appear normal, 5–40% of mature erythroblasts are multinucleated (Fig. 1). Multinuclearity may be caused by a failure of cell division. Erythrocytes released to the peripheral blood are short-lived: 7–34 days in HEMPAS versus 100 days in normal adults. The spleen may be the principal site of erythrocyte destruction, leading to splenomegaly in HEMPAS. Surgical removal of the spleen (splenectomy) often alleviates the anemia.

Also common are liver hemosiderosis and cirrhosis [22–24]. Although hemosiderosis is seen in other ane-

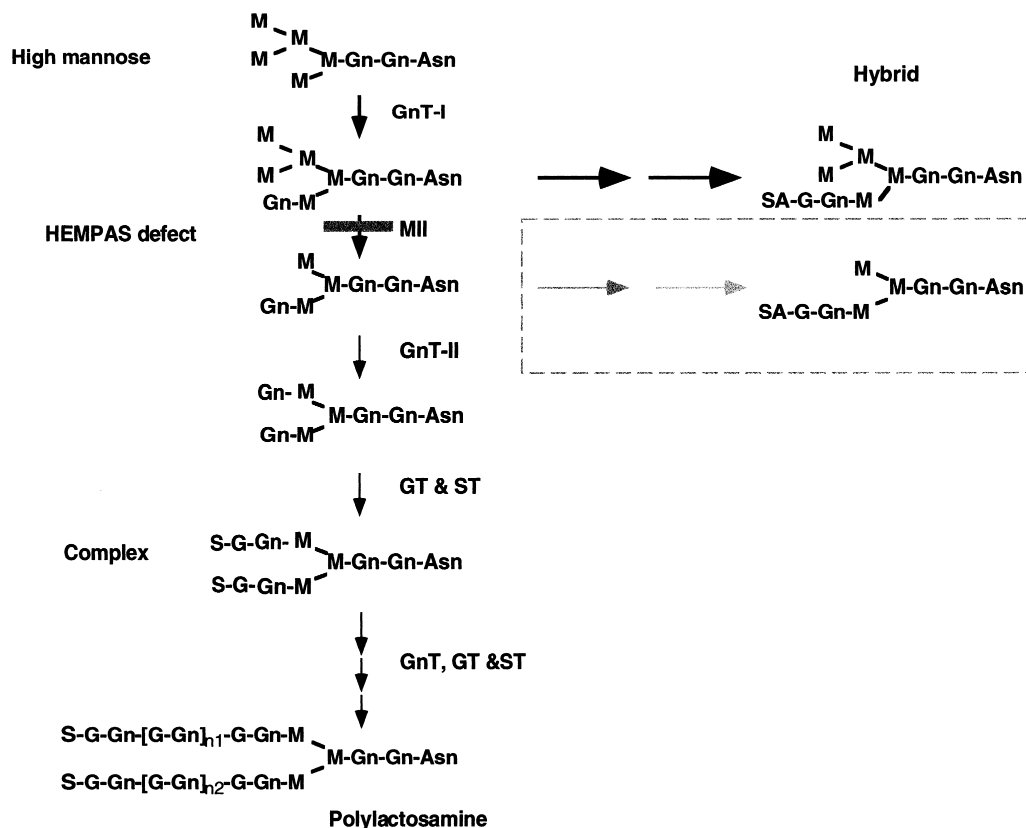

Fig. 2. Biosynthesis of *N*-glycans and HEMPAS defect. In the Golgi, high mannose oligosaccharides attached to a nascent glycoprotein are processed to trimannosyl oligosaccharide by MII. Trimannosyl oligosaccharide normally receives GlcNAc (Gn) due to GnT-II action and is processed to a complex type oligosaccharide or further elongated to polylactosamines. In HEMPAS, a genetic defect of MII results in blockage of *N*-glycan processing at the MII step, leading to the accumulation of pentamannosyl hybrid type oligosaccharide. However, in some HEMPAS patients, accumulation of trimannosyl hybrid type oligosaccharide (boxed area in the figure) is detected, suggesting an as yet unknown genetic defect at a locus other than the MII gene disrupts glycosylation.

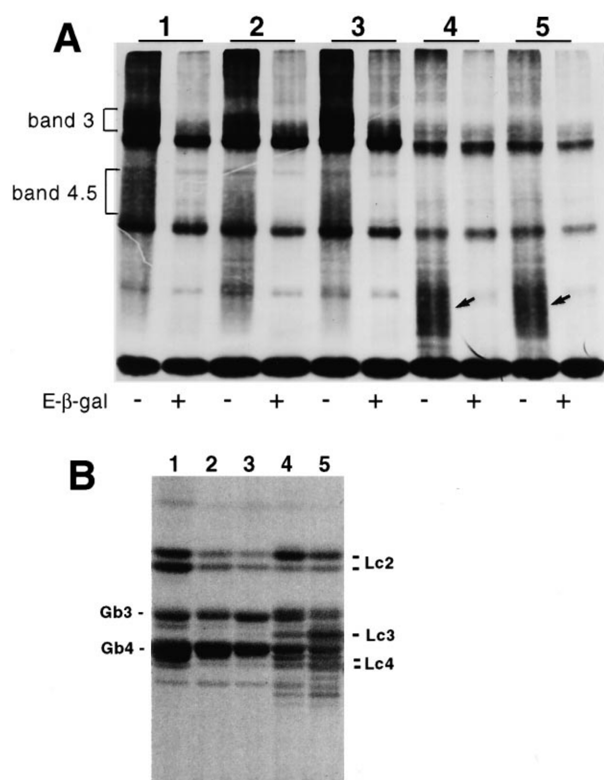

Fig. 3. Abnormal glycosylation of glycoproteins and glycolipids in HEMPAS erythrocyte membranes. (A) Fluorogram of erythrocyte membrane components resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Galactose and *N*-acetylgalactosamine residues on the erythrocyte surface were labeled by the galactose oxidase-Na[B^3H]$_4$ method. Surface labeled cells were then treated with endo-β-galactosidase. In normal erythrocytes, band 3 and band 4.5 glycoproteins are labeled (left in 1) and the labels disappear after endo-β-galactosidase treatment (right lane in 1) because these glycoproteins are glycosylated by polylactosamines. Lanes 2 and 3 are parents of the HEMPAS patients. Parents are clinically normal, but they are heterozygous for the HEMPAS defect. Note that the parents showed a profile similar to that of normal. In contrast, two HEMPAS children (lanes 4 and 5) showed an abnormal profile: band 3 and band 4.5 are not glycosylated, while polylactosamines appear in the low molecular weight region (shown by arrows). These polylactosamines characteristic to HEMPAS are polylactosaminyl ceramide. Thus it appears that a genetic factor in HEMPAS blocks the formation of polylactosamines on the glycoprotein acceptors, and shifts these carbohydrates to the lipid acceptors. (B) Thin-layer chromatogram showing glycolipids from normal and HEMPAS erythrocyte membranes. The samples were prepared from the same HEMPAS family presented in A. Major glycolipids in normal erythrocytes (lane 1) are globo tri- and tetraosylceramides (Gb3, Gb4). Parents of HEMPAS children who are heterozygous for the HEMPAS defect show a pattern almost identical to the normal. In contrast, glycolipids from HEMPAS patients show an accumulation of the lacto-series glycolipids (Lc3, Lc4).

mias, the magnitude of hemosiderosis is remarkable although the patient's circulation does not show iron overload [23]. Some HEMPAS patients need to have blood withdrawn in order to prevent hemosiderosis and cirrhosis.

The incidence of diabetes is high among HEMPAS patients. The cause of diabetes is an interesting question as incomplete glycosylation (see below) may result in exposure of otherwise cryptic antigens resulting in autoimmunity against pancreas cells. A transient autoimmune reaction to erythrocyte RhD antigen was also reported in one HEMPAS case [25].

Severe cases of HEMPAS show mental and sensory abnormalities [3,4]. It is not clear at this time whether the neuronal symptoms are directly caused by a glycosylation defect. As reported by Schachter [26–28], glycosylation deficiency syndrome (CDGS) type II, which is caused by a GnT-II defect, shows severe neuronal abnormalities. As GnT-II catalyzes the step following MII in *N*-glycan synthesis (Fig. 2), the occurrence of neuronal abnormalities in some HEMPAS patients suggests that phenotypes appearing in extreme cases of HEMPAS may resemble CDGS type II.

4. Glycosylation defect in HEMPAS

Pioneering studies by Heimpel [2], Crookston and Vervirgen [3,4] suggested that HEMPAS is caused by abnormal organization of erythrocyte membranes. In 1975, Joseph and Gockerman [29,30] found an abnormality in the glycolipid profile of HEMPAS erythrocyte membranes. Their analysis showed an increase in lacto-*N*-triaosylceramide and lacto-*N*-tetraosylceramide in HEMPAS. Such an abnormal glycolipid profile has been consistently detected in later studies [31,32] (Fig. 3). On the other hand, in 1977 Anselstetter reported that band 3 glycoprotein from HEMPAS erythrocytes migrates slightly faster than the normal band 3 [33]. Such a shift in the apparent molecular mass of band 3 glycoprotein has been detected in all HEMPAS cases, whereas it has not been found in the erythrocytes from patients with other hematological disorders [34–38]. Careful proteolytic analysis of HEMPAS band 3 excluded the possibility of peptide deletion, and the data suggested underglycosylation of HEMPAS band 3 [34].

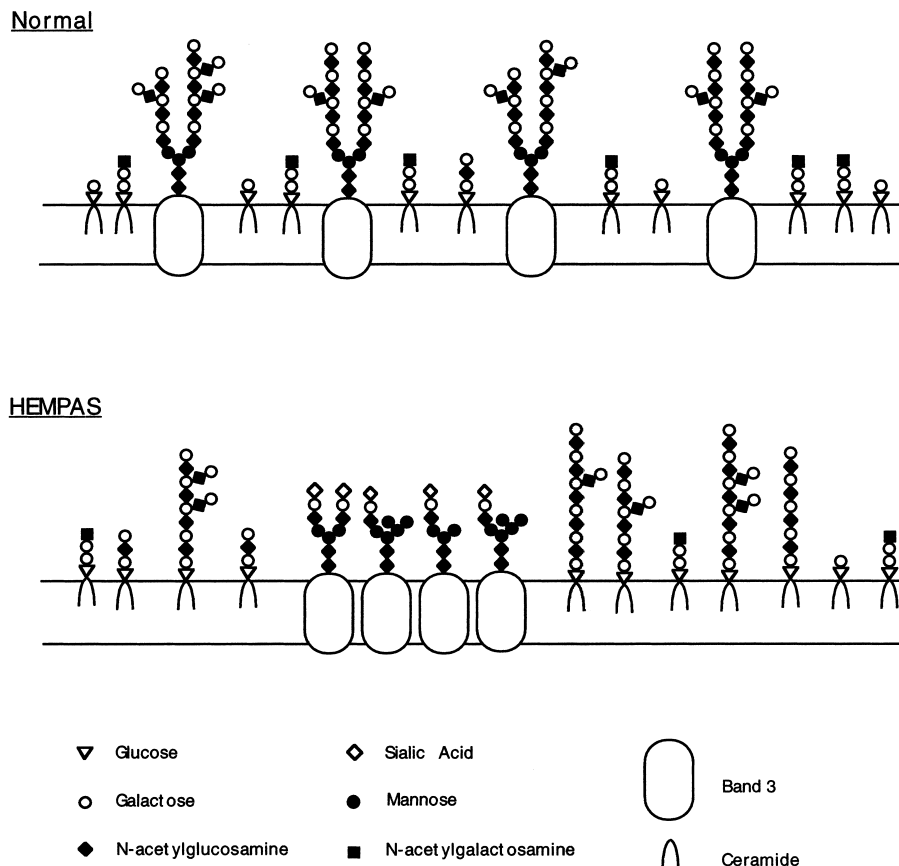

Fig. 4. Erythrocyte membrane glycoproteins and glycolipids in normal and HEMPAS. In normal erythrocyte, glycoproteins, such as band 3, are glycosylated by polylactosamines. Although polylactosaminyl ceramides are present in small quantity in normal erythrocytes, the majority of erythrocyte glycolipids belong to the globo-series with short carbohydrate chains. In HEMPAS, glycoproteins lack polylactosamines whereas lacto-series glycolipids including polylactosaminylceramides are accumulated.

Surface labeling and endo-β-galactosidase treatment analysis demonstrated that both band 3 and band 4.5 glycoproteins, which normally are glycosylated with polylactosamines, virtually lack polylactosamines in HEMPAS [37] (Fig. 3). Absence of polylactosamines in HEMPAS erythrocyte glycoproteins was also demonstrated by Western blot using tomato lectin [27]. Furthermore, in HEMPAS erythrocytes polylactosamines accumulate as polylactosaminyl ceramides [32,37,39]. Thus it appears that a genetic factor in HEMPAS blocks the glycosylation of glycoprotein acceptors and shifts polylactosamines to lipid acceptors, resulting in an increase in the lacto-series glycolipids including polylactosaminyl ceramides (Figs. 3 and 4).

Structural analysis of HEMPAS band 3 carbohydrates showed the presence of the so-called hybrid type oligosaccharides in addition to complex type oligosaccharides [40–42]. However, it was difficult to predict at which step the glycosylation stops solely by determining the *N*-glycan profiles of HEMPAS band 3. Nevertheless, the results suggested disruption of *N*-glycan synthesis near the *N*-acetylglucosaminyltransferase II (GnT-II) and α-mannosidase II (MII) steps (Fig. 2). These analyses also showed that the glycosylation defect is leaky in HEMPAS and suggested the existence of an isozyme of MII, which eventually led to identification of a gene encoding an MII-like enzyme, α-mannosidase IIx (MIIx) [43].

Analysis of *N*-glycan structures of transferrin and α_1-acid glycoprotein from HEMPAS patients sera revealed that these glycoproteins have hybrid and high mannose type oligosaccharides [44]. Because these serum glycoproteins are synthesized in hepatocytes, it is evident that the glycosylation defect is not restricted to the erythroid lineage. This evidence is

surprising because serum glycoproteins need to be fully sialylated, otherwise asialo- and agalacto-glycoproteins are recognized by hepatic lectin and are cleared from the circulation. In fact, a significant part of transferrin purified from HEMPAS patient sera was quickly cleared from the circulation by the liver upon injection into a rat. The quantity of incompletely processed serum glycoproteins which should be digested in the lysosomes in the liver is overwhelming in HEMPAS patients. It seems unavoidable that HEMPAS patients develop cirrhosis.

5. Primary defect and genetic heterogeneity of HEMPAS

Clinical variations of HEMPAS suggested that this disease is a genetically heterogeneous collection, and recent analysis has indeed indicated that this is the case.

Patient GC was the first HEMPAS case identified as being defective in the MII gene. GC showed accumulation of hybrid type oligosaccharides with the core structure Gn1M5Gn2, the substrate for the Golgi *N*-glycan processing enzyme MII. An enzyme activity assay showed a significant reduction in membrane bound MII activity. Northern analysis revealed that lymphoblasts from GC express the MII transcript at less than 10% of normal levels [42]. Recently another HEMPAS case (LF) having mutations in the coding region of the MII gene has been identified. LF is a compound heterozygous patient: one allele has a deletion and the other has an insertion (T. Akama et al., unpublished). Both the deletion and insertion occur at exon-intron junctions, suggesting that mutations in the LF MII gene lead to alternative splicing of each allele. GC and LF are of British and Irish descents, respectively.

While we could identify HEMPAS cases with defective MII genes, the gene defect of HEMPAS patients from southern Italy remained undetermined. Northern analysis or reverse transcriptase–polymerase chain reaction followed by sequencing of GnT-II and MII genes failed to show a mutation in many HEMPAS cases (Fukuda and Schachter, unpublished). On the other hand, Iolascon's group employed linkage analysis using sets of microsatellite markers flanking MII, MIIx and GnT-II genes. Their data excluded linkage to all three genes in HEMPAS cases from southern Italy [45]. Furthermore, they identified the HEMPAS causative gene (CDAN2) on chromosome 20q11.2 [46]. It is predicted that a YAC clone containing the identified region will eventually lead to identification of the protein encoded by the CDAN2 gene.

In addition to MII and CDAN2 defects, linkage analysis showed that two families from southern Italy did not have defects in MII, MIIx, GnTII or CDAN2 [12].

The question remains how a defect in a gene encoding a protein other than a glycosylation enzyme results in disruption of *N*-glycan synthesis at the GnT-II and MII steps. One possibility is that the gene encodes a transcription factor regulating expression of GnT-II or MII [11,45,46]. However, Northern blot analysis did not show reduction of GnT-II or MII transcripts in southern Italy HEMPAS cases (Fukuda, unpublished). An alternative possibility is that a defect exists in a Golgi or cytoplasmic protein regulating membrane trafficking and that this defect affects glycosylation. Biochemical similarities among HEMPAS patients suggest that different gene products affect the same biochemical pathway. Further studies on HEMPAS will provide new information about factors affecting *N*-glycan biosynthesis.

6. Animal model of HEMPAS

Recent advances in transgenic animal technology allow the creation of mutant animals that mimic human genetic diseases. Using this technology, a mutant mouse in which the MII gene is inactivated by homologous recombination has been created [47]. MII null mice appear normal at birth and grow to adulthood. There are no obvious deformities or life threatening defects in these mice. MII null mice, however, show various signs of anemia and remarkable splenomegaly. The peripheral blood contains immature erythrocytes or reticulocytes. Lectin blots of erythrocyte membrane proteins confirmed a lack of complex type *N*-glycans in MII null mice, presumably due to the shift of *N*-glycan synthesis to high mannose type and hybrid type oligosaccharides. Structural analysis using spleen cells and fibroblasts indicated a reduced amount of complex type *N*-gly-

cans in MII null mice, suggesting the presence of an alternate pathway which bypasses the MII step. The mutant erythrocytes agglutinate strongly with anti-i antibodies, a condition which resembles HEMPAS erythrocytes. An increase in lacto-series glycolipids, characteristic of HEMPAS erythrocytes, was also seen in MII null mice.

There are subtle differences between the phenotypes of MII null mice [47] and the symptoms of HEMPAS [1–4]. For example, MII mutant mice do not show multinucleated erythroblasts. Diabetes, gall stones and neuronal anomalies were also not detected in MII null mice.

Studies of gene knock-out mice by others indicated that phenotypes appearing in a mutant mouse are not always identical to what is seen in a human disease. This is because of significant differences between human and mouse [48,49]. The lack of multinucleated erythroblasts in MII null mice could be due to quantitative differences in polylactosamines in erythroblasts between humans and mice. Besides species difference, it is known that the genetic background of mice affects phenotypes observed in mutants. Since HEMPAS cases show clinical heterogeneity [3,4] and there are many cases associated with other genetic diseases [11,14–18], it is likely that the MII defect could produce a severe phenotype if additional defects co-existed in MII null mice. Considering species differences and factors potentially affecting the phenotypes in the mutant mice, dyserythropoiesis exhibited by MII null mice supports the hypothesis that defective *N*-glycan synthesis at the MII step results in HEMPAS.

7. Unsolved problems and future prospects

Phenotypes appearing in MII null mice support the link between genetic defects of MII and HEMPAS [47]. However, cases in which the primary defect is found in the MII gene appear to be limited to the HEMPAS patients from England. Majority of HEMPAS patients from southern Italy are caused by a mutation in the CDAN2 gene on chromosome 20q11. The nature of the protein encoded by CDAN2 is unknown. In addition, there are some cases which exclude all these candidate genes.

Besides typical HEMPAS cases, variants are known [50–53]. These variants may be extreme cases caused by various mutations of the same gene. Because symptoms of HEMPAS are more severe when other genetic and acquired illnesses are present, it is possible that undefined genetic factors affect HEMPAS, giving variant phenotypes. It is also possible that genes other than MII and CDAN2 are involved in the variants [11].

In our earlier studies, GnT-II was proposed to be defective in some HEMPAS cases [41]. However, later genetic analysis failed to identify a GnT-II defect in HEMPAS. It became clear that the GnT-II defect results in CDGS type II but not HEMPAS [27,28]. We cannot, however, exclude the possibility that the HEMPAS gene defect indirectly affects GnT-II activity, which causes incomplete blockage of *N*-glycan processing and results in phenotypes similar to those seen in MII gene defects. The evidence that polylactosamines are formed in CDGS type II erythrocyte glycoproteins seems paradoxical since in some HEMPAS patients the erythrocyte membranes accumulated the same trimannosyl hybrid oligosaccharide as that due to the GnT-II defect. It seems that factors other than enzymes and acceptor oligosaccharide structures are involved in polylactosamine synthesis. The study of HEMPAS will identify hitherto unknown factors affecting *N*-glycan and polylactosamine synthesis.

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