Heterozygosity of CDAN II (HEMPAS) gene may be detected by the analysis of erythrocyte membrane glycoconjugates from healthy carriers

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Background and Objectives. Congenital dyserythropoietic anemia (CDA) type I, II, and III, is associated with abnormalities of erythrocyte membrane glycoconjugates that are most pronounced in type II CDA or hereditary eryhtroblastic multinuclearity with a positive acidified-serum test (HEMPAS). The abnormalities consist in hypoglycosylation of polylactoaminoglycans linked to proteins (as in band 3 glycoprotein) and ceramides (known under the name of polyglycosylceramides) as well as in accumulation of some oligoglycosylceramides: lactotriaosylceramide, neolactotetraosylceramide, and sometimes globotetraosylceramide. Glycophorin A is partially unglycosylated with respect to O-linked glycans. Types I and II of the disease are inherited in an autosomal recessive fashion. The aim of the present study was to investigate a possibility that heterozygosity with respect to CDAN2 gene in healthy carriers could be detected by analysis of erythrocyte membrane glycoconjugates.

Design and Methods. We examined a family which consisted of heterozygous parents and their two sons, one of whom was afflicted with CDA II (proband) while the other was healthy. In all family members the glycosylation status of band 3 glycoprotein, polyglycosylceramides and glycophorin A was evaluated from their carbohydrate molar composition. In addition we determined erythrocyte membrane contents of oligo- and polyglycosylceramides, and agglutinability of erythrocytes by anti-i antibody.

Results. We found that the heterozygous parents showed, but about 50% less pronounced, most of the typical abnormalities of erythrocyte membrane glycoconjugates that were present in the proband. These abnormalities included: hypoglycosylation of band 3, accumulation and hypoglycosylation of polyglycosylceramides, and accumulation of lactotriaosylceramide. The level of neolactotetraosylceramide in the erythrocyte membranes of the parents was, however, normal. Globotetraosylceramide content was elevated in erythrocytes from the proband and, surprisingly, even more so in the parents. Glycophorin A in the proband was only slightly abnormal. Erythrocytes from both the parents and the proband expressed increased agglutinability with anti-i antibody. All glycoconjugates examined were normal in erythrocytes from the healthy son.

Interpretation and Conclusions. Individuals heterozygous with respect to CDAN2 gene can be identified through determination of the carbohydrate molar composition of band 3 and polyglycosylceramides as well as by an elevated erythrocyte content of polyglycosylceramides. In the parents these abnormalities show dosage effects. Determination of the carbohydrate molar composition of glycophorin A and of oligoglycosylceramides seems to be less promising. These findings indicate that the analysis of erythrocyte membrane glycoconjugates may be a valuable addition to the repertoire of methods used in studies on the genetics of CDA.

Key words: CDA II, glycoconjugate abnormalities, heterozygosity.

Congenital dyserythropoietic anemias (CDA) are a group of rare heritable diseases that are characterized by a certain degree of erythroblastic multinuclearity and a greatly increased ineffective erythropoiesis.[1-3] Three major types of the disease, CDA I, II, and III, are known.[3] Genes for type I and type II are transmitted in an autosomal recessive fashion and have been localized to chro-

mosome 15q15.1-15.3[4] and chromosome 20q11.2,[5] respectively. Inheritance of type III of the disease is autosomal dominant and the disease gene has been localized to chromosome 15q22.[6,7] The structures and functions of the disease genes have not so far been elucidated and, thus, their localization was determined by employing a painstaking linkage analysis. Glycoconjugate abnormalities of erythrocyte membrane glycoconjugates in CDA erythrocytes were initially recognized only in type II CDA.[1] They consist of underglycosylation of an anion transporter also known as band 3 glycoprotein, and accumulation of certain glycosphingolipids including lactotriaosylceramide, neolactotetraosylceramide and polyglycosylceramides. More recently, similar though less pronounced abnormalities were also found in type I and III though in type III they affect almost exclusively glycosphingolipids.[8-10] Apart from band 3 and glycosphingolipids the abnormalities in type I and II involve partial unglycosylation of O-linked chains in glycophorin A (GPA).[9,10] Polyglycosylceramides are hypoglycosylated in all three types of the disease.[10] Yet another abnormality affects globotetraosylceramide which may be elevated in erythrocytes of only some patients with either CDA type I[10] or CDA type II.[11]

In the present study we investigated the issue of whether glycoconjugate abnormalities in CDA type II could be discernible in heterozygotes. Apart from theoretical implications, a positive result of such a study would be of a practical value because it would enable researchers to follow the inheritance of CDA in heterozygous carriers of the disease. Also, any conclusions arrived at through DNA linkage analysis could be confirmed through analysis of erythrocyte membrane glycoconjugates.

Design and Methods

A single family consisting of the proband with CDA II (P), his healthy brother (B) as well as his healthy father (F) and mother (M) were examined. We found no evidence of consanguinity between the parents through analysis of 14 polymorphic loci including 10 STRs and 4 VNTRs. The diagnosis of CDA type II in P was based on a number of criteria including congenital anemia, bilirubinemia, high ineffective erythropoiesis, and the presence of bi- and trinuclear erythroblasts in the bone marrow (12% of the total). In addition, the erythroblasts exhibited characteristic peripheral cisternae as revealed by electron microscopy. Typical glycoconjugate abnormalities were present in erythrocyte membranes (see under Results). The Ham test

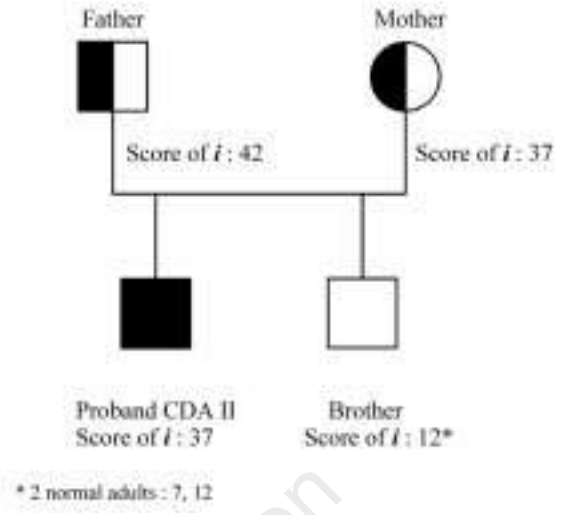

Figure 1. The pedigree of the family under study and agglutinability scores of erythrocytes with anti-i antibody.

was positive. Agglutinability of erythrocytes from P by mouse monoclonal anti-i antibody (from Gamma Biologicals BV, Leiderdorp, The Netherlands) was increased. The parents, on the other hand, were healthy and their red cell indices normal (F: RBC, 5×10^{12}/Hb 14.7 g/dL, HCT 43.4, MCV, 96 fL, MCH 32.7 pg, MCHC 33.9g/dL; M: RBC 4.4×10^{12}Hb 13.3 g/dL, HCT 38.4, MCV 87.1 fL, MCH 30.2 pg, MCHC 34.6 g/dL. Their spleens were not palpable on physical examination. Erythrocyte glycoconjugates were examined using the same methods as in previous studies. Thus, carbohydrate molar composition of band 3 glycoprotein was determined according to Zdebska and Koscielak[12] while that of GPA by the modified method.[9] Glycosphingolipids and polyglycosylceramides were isolated according to Zdebska *et al.*[13] and Miller-Podraza *et al.*,[14] respectively. Carbohydrates in hydrolyses of glycoconjugates were determined by high pH anion exchange chromatography with pulsed amperometric detection using a Dionex system and a PA-1 column.[12] Amino acids were determined by the fluorescamine method.[15] Sphingosine was quantified according to Higgins.[16]

Results

The pedigree of the family investigated is shown in Figure 1 which also shows agglutinability scores of erythrocytes with anti-i antibody. It is evident that only in B was the reaction strength of i antigen normal. In other family members it was significantly increased. Lack of a dosage effect in the

Table 1. Carbohydrate molar composition of erythrocyte band 3*.

	Fuc	GalNAc	GlcNAc	Gal	Man	NeuAc	Total CHO**
				mol/mol			
Proband	1.2±0.1	0.6±0.1	5.6±0.3	4.2±0.2	3.1±0.1	1.6±0.03	16.3±0.3
Father	1.3±0.05	0.7±0.1	9.9±0.2	7.7±0.3	3.1±0.2	1.5±0.2	24.2±0.2
Mother	1.8±0.2	1.2±0.2	10.0±0.2	7.6±0.3	3.1±0.3	1.0±0.03	24.7±0.7
Healthy brother	1.7	0.9	13.8	14.3	3.0	0.8	34.5
Normal adults, n=9	2.4±0.5	1.2±0.5	14.7±0.8	14.1±1.4	2.8±0.3	0.8±0.5	35.9±1.1

**To show the precision of our technique we present mean results of triplicate determinations in P, F, and M together with the standard deviation calculated for each sugar analyzed. Means for the control group are based on single determinations. **CHO, carbohydrates.*

Table 2. Carbohydrate molar composition of glycophorin A.

	Fuc	GalNAc	GlcNAc	Gal	Man	NeuAc	Total CHO
			mol/mol				
Proband	1.7	10.1	5.8	15.7	2.9	19.6	55.8
Father	2.6	10.6	5.6	20.0	3.0	23.8	65.2
Mother	2.1	12.9	5.6	22.3	2.9	20.4	66.2
Healthy brother	2.7	12.4	7.1	22.8	3.0	21.2	69.2
Normal adults, n=9	2.9	12.2	6.8	23.2	3.0	21.2	69.2
± SD	0.6	1.2	0.8	1.1	0.2	1.2	1.8

heterozygous parents may be attributed to a low accuracy of the agglutination test. The results of glycoconjugate analysis are shown in Tables 1-4. Band 3 in the proband was severely hypoglycosylated to a similar extent as to that in the previously examined patient with CDA II[8] (see Table 1). Both parents exhibited a moderate hypoglycosylation of band 3 that, in quantitative terms, equaled about 50% of the amount found in the proband. Thus, band 3 in the heterozygous parents showed a dosage effect. This was reflected in electrophoretic mobility of the glycoprotein from F but not from M (Figure 2). Therefore, the electrophoretic mobility of band 3 as an index of heterozygosity of CDAN2 gene should be treated with caution. The proband, unlike the formerly described case of CDA type II[9] showed only a moderate deficiency of carbohydrates in GPA. Consequently this deficiency was even smaller in GPAs from the parents and made carriership diagnosis difficult (Table 2). For example, the GalNAc content of GPA from F was quite similar to that from P but GalNAc in GPA from M was normal. Among oligoglycosylceramides (Table 3) lactotriaosylceramide in F and M was clearly elevated but the content of neolactotetraosylceramide was surprisingly normal. In the proband and in all other patients with CDA type II so far examined by us[8,10,11] and other researchers[1] contents of both glycosphingolipids were high.

Polyglycosylceramides contain a polylactosaminoglycan similar in structure to that in band 3 but attached not to the protein but to ceramide.[17] In patients with CDA types I-III polyglycosylceramides in erythrocyte membranes are elevated and at the same time hypoglycosylated.[10] This was clearly seen not only in the proband but, with about 50% lesser magnitude, also in both parents. Thus, polyglycosylceramides in heterozygotes show a dosage effect with respect to both the content in erythrocyte membranes and the degree of hypoglycosylation. Interestingly, globotetraosylceramide was high in the red cells of the proband and those of his parents but not in the erythrocytes from his healthy brother.

Discussion

The results show that polylactosaminoglycans of band 3 and of polyglycosylceramides in the heterozygous parents of the proband were hypoglycosylated and exhibited a dosage effect that could be effectively used to trace the disease gene in heterozygotes. The same conclusion applies to the content of polyglycosylceramides in erythrocyte membranes. Other glycoconjugate abnormalities were less predictable. For instance, the carbohydrate deficiency in GPA from the proband was less pronounced than in that from a previously described patient.[9] Therefore, the determination of carbohy-

Table 3. Contents of oligoglycosylceramides in erythrocyte membranes.

	GlcCer	LacCer	Lc3Cer	Gb3Cer	Gb4Cer	nLc4Cer
	nmol/mg membrane protein					
Proband	1.5	12.8	12.8	7.2	18.5	3.3
Father	0.6	4.6	2.1	3.1	20.4	0.005
Mother	0.7	7.1	1.2	3.4	20.4	0.005
Healthy brother	1.1	4.1	0.1	2.3	14.4	0.006
Normal adults, n=15	0.8	3.4	0.1	3.1	11.6	0.006
± SD	0.07	0.2	0.05	0.3	1.2	0.004

Table 4. Content (a, nmol / 100 mg membrane protein) and total carbohydrates, (b, mol / mol) of polyglycosylceramides.

	a	b
Proband	108.1	15.1
Father	68.9	20.5
Mother	50.1	18.3
Healthy brother	37.9	27.9
Normal adults, n=9	35.6	33.8
±SD	3.1	2.6

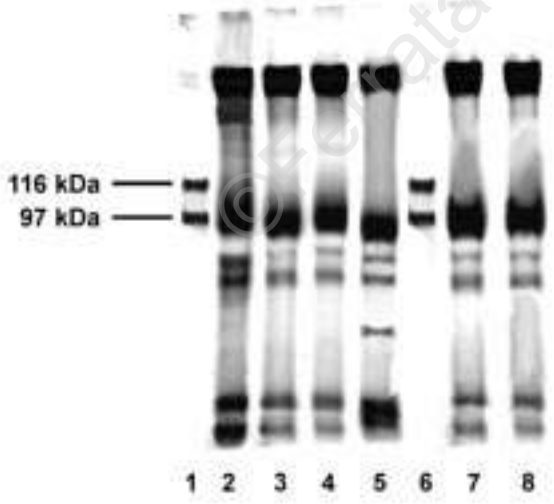

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of erythrocytes membranes performed as described in ref. #12. Lanes 1 and 6, standards of phosphorylase b (97 kDa) and β-galactosidase (116 kDa); lane 2, healthy brother; lane 3, father; lane 4, mother; lane 5, proband; lanes 7 and 8, normal adults. Stained with Coomassie blue.

drate molar composition of this glycoprotein is not well suited to carriership diagnosis in the family investigated. Lactotriaosylceramide was highly elevated in erythrocytes from the parents but neolactotetraosylceramide was not. Globotetraosylceramide content was higher in erythrocytes from the parents than in those from the proband. Thus, the hypoglycosylation of polyglycosaminoglycans attached through N-glycosidic linkage to band 3 protein or by O-glycosidic linkage to ceramide as well as the content of polyglycosylceramides in erythrocyte membranes are the most stable markers of the CDA gene in heterozygotes. The numerical value of each marker in either the F or M was more than 4.0-10.0 times the standard deviation from the appropriate mean value in normal adults. When the values of all four markers together, including lactotriaosylceramide, are taken into account the probability that they arose by chance is almost nil. So far the transmission of CDA II gene through generations has been followed using linkage analysis of microsatellite markers on the long arm of chromosome 20.[5] It is our opinion that the techniques we propose may be a valuable addition to the repertoire of methods employed in studies of the genetics of CDA, especially since some families do not show the linkage to the established microsatellite markers.[20] Another asset of our technique is that it shows the presence of the CDAN2 gene unequivocally and is not susceptible to recombination events. Admittedly we report on only a single family but the increased expression of i antigen in erythrocytes from CDAN2 carriers, as shown previously,[18,19] suggests that our findings should have a wider application. We may draw this conclusion because i activity of erythrocytes depends on hypoglycosylated, linear polylactosaminoglycans[17] that, in a form of polyglycosylceramides, are elevated in erythrocytes from CDA II subjects.[10,11] This is exactly what we found in erythrocytes from the heterozygous parents: increased expression of i antigen and elevated and hypoglycosylated polyglycosylceramides. It would be interesting, however, to examine more families, preferably those with a different genetic background.[5,20]

Abbreviations and formulae: Gb3, globotriaosylceramide, Galα1-4Galβ1-4GlcCer; Gb4, globotetraosylceramide, GalNAcβ1-3Galα1-4Galβ1-4GlcCer; LacCer, lactosylceramide, Galβ1-4GlcCer; Lc3Cer, lactotriaosylceramide, GlcNAc β1-3Gal β1-4GlcCer; nLc4Cer, neolactotetraosylceramide, Gal β1-4GlcNAcβ1-3Galβ1-4GlcCer; CHO, carbohydrates; SD, standard deviation.

Contributions and Acknowledgments

JK: conception and design, interpretation of data, writing of the paper; EZ: performed the analysis of glycoconjugates; EM-C: MD: spotted the family investigated; BW: performed electron microscopy of bone marrow erythroblasts; RP: performed analysis of relatedness.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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Peer Review Outcomes

What is known on this topic

Congenital dyserythropoietic anemia type II is considered a recessive autosomal disoder on the basis of clinical observations. The gene has not been identified yet, so that there is no reliable means of identifying heterozygous individuals.

What this study adds

A combinantion of phenotypic microstigmata (hypoglycosylation of band 3 and polyglycosylceramides) may allow identification of individuals who are heterozygous for congenital dyserythropoietic anemia type II.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received September 18, 2001; accepted November 26, 2001.

Potential implications for clinical practice

Should these preliminary observations be confirmed, evaluation of glycosylation of band 3 and polyglycosylceramides might be used as a means of identifying individuals who are heterozygous for congenital dyserythropoietic anemia type II. However, this approach requires highly specific expertise, and there is no doubt that ascertaining the heterozygotes will be much simpler using molecular approaches once the CDAN2 gene will be identified.

Mario Cazzola, Editor-in-Chief