Exclusion of Three Candidate Genes as Determinants of Congenital Dyserythropoietic Anemia Type II (CDA-II)

By Achille Iolascon, Emanuele Miraglia del Giudice, Silverio Perrotta, Matteo Granatiero, Leopoldo Zelante, and Paolo Gasparini

Congenital dyserythropoietic anemia type II (CDA-II) is the most common form of inherited dyserythropoiesis. Previous studies have shown that the anion transporter (band 3) is narrower and migrates faster on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); this aspect was related to insufficient glycosylation. Biochemical data support the hypothesis that this disease is due to a deficiency of N-acetylgalactosaminyltransferase II (GnT II) or α-Mannosidase II (α-Man II), which represent the key to glycosylation. In addition, a third candidate gene is α-Man Ix, which shows a strong homology with α-Man II. The knowledge of the chromosomal localization of these putative genes allowed us to perform a linkage study using three sets of microsatellite markers flanking the candidate genes.

Six families with two or more affected children were enrolled in this study. The data obtained exclude linkage to all three candidate genes. In consideration of the biochemical data (reduction of enzymatic activity) of the same enzymes, our results suggest the hypothesis that a defect in an unknown transcriptional factor is involved in CDA-II.

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Southern region of the candidate genes were used. Candidate genes and their chromosomal location were \( \alpha \)-Man II, which is mapped to chromosome 5 (5q21.2-2.2); \( \alpha \)-Man IIx isozyme mapped to chromosome 15q25.1-2.2 and human GnT II, which is mapped to the long arm of chromosome 14 (14q21). To speed up the analysis, four unrelated families containing at least two CDA II individuals and two kindreds with three affected patients were selected and included in the study. No consanguinity was established in any case. Thus, a total of 14 affected individuals were included in the study. The main clinical and biochemical aspects regarding six patients belonging to five different kindreds have been reported elsewhere (see subjects CE, DO, GE, RO II.1, RO II.2, and VA in Alloisio et al\(^{10}\)). The other eight patients were found to have anemia of variable degrees, jaundice, and mild splenomegaly. Bone marrow observation showed the presence of erythroid hyperplasia with binuclearity and multinuclearity in approximately 10% to 40% of more mature erythroblasts. Erythrocyte protein membrane analysis was performed using SDS-PAGE.\(^{10}\) Western bloting was performed after SDS-PAGE. A monoclonal antibody against a synthetic peptide of rat glucose-regulated protein (GRP)-78 (Stress Gen Biotechnologies Corp, Victoria, British Columbia, Canada) and a polyclonal rabbit antibody against bovine protein disulfide isomerase (PDI; Stress Gen Biotechnologies Corp) were used as previously reported.\(^{20}\)

High molecular weight DNA was extracted from peripheral blood leukocytes using an automatic DNA extractor (ABI DNA PURE) according to the protocols of the manufacturers (Perkin Elmer, Foster City, CA). Fluorochrome-labeled primers were prepared by chemically attaching a fluorescent dye to the 5' end of each forward oligonucleotide primer employing the fluorescent amide reagent, 6-FAM amide. Names and primer sequences for detecting D5S433, D5S2051, D5S421, D14S978, D14S281, D14S1064, D15S205, and D15S152 alleles were previously described.\(^{21,22}\) Polymerase chain reactions (PCR) were prepared using a BIOMEK 1000 robot station (Beckman Instrumentation, Fullerton, CA) and performed on an automated Thermal cycler (Perkin Elmer, Norwalk, CT) using conditions previously described for each microsatellite marker. After PCR amplification, 5 \( \mu \)L of each sample was precipitated by the addition of 400 \( \mu \)L of 100% ethanol. After centrifugation, dried pellets were resuspended in 5 \( \mu \)L of deionized water. Multiplexing was performed before loading the gel, mixing 1 \( \mu \)L of each sample before ethanol precipitation. Acrylamide gel electrophoresis was performed in 8 mol/L urea containing 1 \( \times \) Tris-Borate-EDTA (TBE), with the length and acrylamide percentage changing depending on the resolution required. For all gels, the acrylamide/bisacrylamide (N,N'-methylene-bis-acrylamide) ratio was 19:1 (wt/vol) for a cross-linking concentration of 5\% (wt/vol) with 24-cm well-to-read distances. Data were collected using the GeneScan Data Collection program version 1.1 and analyzed using the GeneScan Data Analysis software version 1.1, 1.2, or 1.2.1b1 (Applied Biosystems Inc, Foster City, CA). Correct allele size was assigned using the Genotyper software. Linkage was assessed by analysis of allele segregation of the eight different polymorphic markers (D5S433, D5S2051, D5S421, D14S978, D14S281, D14S1064, D15S205, and D15S152). Pairwise linkage analysis was performed using MLINK and ILINK from the FASTLINK program package (version 2.2).\(^{10}\) Gene frequency was based on disease prevalence (\( \Theta = .001 \)), and full penetrance was also assumed. Calculations were performed at recombination fractions (\( \Theta \)) of 0, 0.05, 0.10, 0.20, 0.30, and 0.40 between each marker and the disease locus. The HOMOG program was used to test for nonallelic heterogeneity.

**RESULTS**

The clinical features as well as the characteristic morphological abnormalities of the bone marrow allowed us to perform CDA II diagnosis in all of the patients investigated. Diagnosis was further supported by the presence on SDS-PAGE of a band 3 with a narrower aspect and a faster migration. The presence of GRP 78 and PDI in the erythrocyte membrane, in agreement with findings observed in the patients previously reported,\(^{10}\) was shown on Western blotting (not shown).

Results of linkage analysis performed between CDA-II phenotype in the six index families versus specific chromosomal regions containing the candidate genes are reported in Table 1.

Microsatellite markers flanking the GnT-II, \( \alpha \)-Man II, and \( \alpha \)-Man IIx genes always gave negative lod scores at recombination frequencies of 0.0, 0.01, and 0.05. Only marker D5S421 showed a positive but not significant lod score of 0.04 at a recombination frequency of 0.1. Anyway, in this case the two other markers defining the same chromosomal region gave negative lod scores at the same recombination frequency. In most of the cases the affected members of each of the six families shared different alleles showing the absence of cosegregation within each family. An example of the results in the two most representative families is given in Fig 1.

Negative results obtained indicated that most likely none of the investigated regions contains the gene involved in CDA-II.

**Table 1. Pairwise Iod Scores Between CDA-II and Chromosome Markers**

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Recombination Frequencies</th>
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<tr>
<td></td>
<td>0</td>
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<tr>
<td><strong>Chromosome 15 (( \alpha )-Man II)</strong></td>
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</tr>
<tr>
<td>D15S205</td>
<td>.00</td>
</tr>
<tr>
<td>D15S152</td>
<td>.00</td>
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<tr>
<td><strong>Chromosome 5 (( \alpha )-Man II x)</strong></td>
<td></td>
</tr>
<tr>
<td>D5S433</td>
<td>.00</td>
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<tr>
<td>D5S2051</td>
<td>.00</td>
</tr>
<tr>
<td>D5S421</td>
<td>.00</td>
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<tr>
<td><strong>Chromosome 14 (GnT-II)</strong></td>
<td></td>
</tr>
<tr>
<td>D14S978</td>
<td>.00</td>
</tr>
<tr>
<td>D14S281</td>
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<tr>
<td>D14S1064</td>
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**EXCLUSION OF CANDIDATE GENES IN CDA II**

**FIG 1. Example of allele segregation for the markers under study in two representative CDA II families (CDAGE and CDACE):**

The solid squares and circles indicate the affected individuals. The microsatellite markers are tightly linked to the following candidate genes: α-Man II, mapping on chromosome 5 (5q2.1-2.2); α-Man IX isozyme, mapping on chromosome 15q25; and human GnT II, mapping on chromosome 14q21. The above-mentioned markers are listed according to the order established in Weissenbach et al.21 and Gypay et al.22

**DISCUSSION**

CDA-II is a rare recessive disorder often characterized by a mild clinical phenotype. Clinical findings usually do not appear in the neonatal period. For this reason, the possible existence of tissue-specific or developmental stage-specific isozymes should be considered.13,24 A number of associations with other RBC-inherited disorders have been reported and it appears to be clear that the association could worsen clinical findings.25,26 The latter could account for the clinical heterogeneity of this disease.

The Ham’s test positivity, the increased agglutinability anti-i, and the SDS-PAGE aspect of band 3 strongly suggest the existence of a glycosylation defect. The formation of such unusual oligosaccharides corresponds to reduced activity of GnT-II14 in some cases and with low α-Man II in others.15 Fukuda et al17 described a variant of CDA-II characterized by the absence of polyalactosamines either on protein or on lipids. In this case, a quantitative defect of galactosyltransferase was established.

The glycosylation defect causes hypoglycosylation of band 3; the latter, in turn, causes abnormal clustering of these molecules only during the intracytoplasmic life of this molecule,27 whereas the effect on the anion transport is not well established.28,29 Mapping and cloning of cDNA encoding for GnT-II and α-Man II allows for the molecular studies.18,19 Low expression of α-Man II was shown by Northern blotting and no abnormal mRNA species are detected.15 Sequencing of the coding sequence suggested that a mutation in the promoter region of α-Man II gene results in insufficient transcription.15 Using a previously isolated murine cDNA clone as a probe, Misago et al19 isolated cDNA clones encompassing the human α-Man II cDNA open reading frame and initiated isolation of human genomic clones. This gene maps at 5q21-22 region and codes a protein of 1144 amino acid residues. A related gene was recently isolated that encodes a truncated protein with 796 amino acid residues and maps at 15q25. The human GnT II is a single exon gene that contains a 1341-bp open reading frame encoding a 447 amino acid protein. This gene was mapped by fluorescence in situ hybridization to chromosome band 14q21.20 Localization of the putative genes and the availability of polymorphic marker loci in these regions allow a linkage study in families with multiple affected siblings.

Our data on linkage analysis and allele segregation showed that there was no linkage between CDA-II phenotypes and the chromosomal regions containing the three candidate genes. These results showed for the first time that the molecular lesion(s) causing CDA-II is not primarily located in α-Man II and GnT II genes.

However, biochemical assays showed a lowered activity of these two enzymatic activities in CDA-II RBCs. In addition, Northern analysis showed in some of our kindreds a reduction of α-Man II and GnT II mRNAs in the same patients, whereas this finding was not detected in the corresponding obligate carriers (A. D’Agostaro, personal communication, September, 1995 and Iolascon et al18). These data clearly exclude these genes as candidates and suggest at the same time that the disease is most likely due to a defect of a transcriptional factor regulating both α-Man II and GnT II.

A genome-wide search by means of a large number of CDA II families could allow for mapping of this disease gene. This knowledge will help significantly in our understanding of its pathogenesis and will be essential in the design of a rational therapy.

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