



Geographic distribution of CDA-II: did a founder effect operate in Southern Italy?

ACHILLE IOLASCON,* VERONICA SERVEDIO,* RUGGIERO CARBONE,* ANGELA TOTARO,[°] MASSIMO CARELLA,[°] SILVERIO PERROTTA,[#] SUNITHA N. WICKRAMASINGHE,[®] JEAN DELAUNAY,[^] HERMAN HEIMPEL,[§] PAOLO GASPARINI[°]

*Dipartimento di Biomedicina dell' Età Evolutiva, Università degli Studi di Bari, Italy and Centro Interuniversitario per lo Studio delle Malattie Ereditarie, CISME, Italy; [°]Servizio di Genetica Medica, IRCCS Casa Sollievo Sofferenza, San Giovanni Rotondo, Italy; Dipartimento di Pediatria, Seconda Università degli Studi di Napoli, Naples, Italy; [#]Department of Haematology, Imperial College School of Medicine, St Mary's Campus, Norfolk Place, London W2 1PG, United Kingdom;

[^]Service d'Hématologie, Hôpital de Bicêtre, Assistance Publique-Hôpitaux de Paris, INSERM U 473; Le Kremlin-Bicêtre, France; [§]Medizinische Universitätsklinik und Poliklinik, Ulm, Germany

ABSTRACT

Background and Objectives. Congenital dyserythropoietic anemia type II (CDA-II) is an autosomal recessive condition, whose manifestations range from mild to moderate. Its exact prevalence is unknown. Based on a recently established International Registry of CDA-II (64 unrelated kindreds), a high frequency of CDA II families living in South Italy became evident.

Design and Methods. The aim of this study was to define the haplotypes of the CDA II kindreds living in Southern Italy based on markers D20S884, D20S863, RPN, D20S841 and D20S908. These markers map to 20q11.2, within the interval of the CDAN2 gene that is responsible for CDA II. Next, we looked at these markers in kindreds from other regions of Italy and from other countries, with special attention to families having ancestors in Southern Italy.

Results. Evaluation of the geographic distribution of the ancestry of Italian CDA-II patients clearly demonstrated the unusually high incidence of this condition in Southern Italy. Our statistical calculations and linkage disequilibrium data also clearly demonstrate a strong association of the markers of chromosome 20 with the disease locus in our sample. Almost all the regions defined by the markers here used is in disequilibrium with the disease. Combining the data from the Italian sample together with those obtained from the non-Italian ones, we can restrict the area of highest disequilibrium to that defined by markers D20S863-D20S908.

Interpretation and Conclusions. Despite the presence of this linkage disequilibrium the search for a common haplotype failed. This could suggest that the mutation was very old or that it occurred more than once on different genetic backgrounds.

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Key words: congenital erythropoietic anemia, epidemiology, CDAN2, haplotype, founder effect

Correspondence: Achille Iolascon, M.D., Dipartimento di Biomedicina dell'Età Evolutiva, Piazza G. Cesare 11, 70124 Bari, Italy. Phone: international +39-080-5478923 - Fax: international +39-080-5592290 - E-mail: a.iolascon@bioetaev.uniba.it

Congenital dyserythropoietic anemia type II (CDA II or HEMPAS) (MIM224100) is an autosomal recessive condition. The earliest case was described by Sansone¹ in Genoa, Italy, but it was not until the late 1960s that the disease was recognized as an entity.²⁻⁴ It represents the most frequent form of congenital dyserythropoietic anemia (CDA).^{5,6} The clinical presentation is remarkably homogeneous, except in a few instances.⁷ It includes normocytic or microcytic anemia of different severity, chronic or intermittent jaundice, splenomegaly, a normal or insufficiently increased reticulocyte count, and complement-mediated lysis of the patients' red cells by acidified sera from about 40% of normal ABO-compatible donors. Transfusional needs are occasional and splenectomy can have some beneficial effects on the anemia in almost all patients.⁸ Cholelithiasis and secondary hemosiderosis are frequent complications. Between 10 and 40% of erythroblasts in the bone marrow are bi- and multinucleated and some erythroblasts show karyorrhexis. Electron microscopy demonstrates the presence of a double membrane (peripheral cisternae) running parallel to and beneath the plasma membrane in a substantial proportion of intermediate and late stage erythroblasts.^{9,10} These hematologic abnormalities appear to be secondary to a glycosylation defect.¹¹

CDA II has been reported in more than 200 patients. Although the reported cases show a wide geographic distribution, the majority are from Europe. Furthermore, most of the European cases are Italians or of Italian ancestry. Among 28 families with CDA II detected in residents of Germany, 10 are from the Mediterranean including 3 of Italian origin.¹² This observation excludes recruitment bias as the dominant explanation for the large numbers of CDA II-families observed in Italy, but rather suggests that there is an unusually high incidence of one particular CDAN2 allele in Italy.

Recently we recruited a panel of CDA II families, characterized them carefully and used them to map the CDAN2 gene by linkage analysis. The panel consisted of a homogeneous group of 14 families, 13 of which were of Italian origin. We first excluded three candidate genes (α -mannosidase II, α -mannosidase

x, and N-acetylglucosaminyltransferase II)(MANA, MANAx, Gnt-II),¹³ involved in glycosylation or deglycosylation, and obtained conclusive evidence for linkage of the CDA II to microsatellite markers on the long arm of chromosome 20 (20q11.2).¹⁴ Specifically, evidence for allelic association with marker D20S863 was detected. This data also suggested that a founder effect may exist in CDA-II.

The aim of the present study was two-fold. Firstly, to assess the geographic distribution of CDA-II further by studying the families enrolled in the recently established International Registry of CDA-II and, secondly, to use these families to explore the possibility of a founder effect. For this purpose, we conducted a genetic linkage analysis in these families using five microsatellite markers mapping within the CDAN2 interval. Admixture analysis showed that 90% of these families had linkage to the CDAN2 locus. Haplotype analysis in CDAN2-linked families did not show evidence for a strong founder effect on the world scale.

Design and Methods

Patients

We investigated 81 CDA II patients (mean age: 17.7 years; range 3-36 years) from 64 unrelated families: 44 (56 affected members) originated from Italy and 20 (25 affected members) came from various areas in the world. The composition of the families was as follows: 25 families had a single affected patient; whereas 13 (9 Italian and 4 from abroad) were multiplex and 26 families had one affected and other unaffected children.

The patients were enrolled in the *International Registry of CDAs* (set up in 1996). The diagnosis was based on bone marrow examination in all cases, and the acidified serum lysis test or erythrocyte ghost protein analysis in 75% of cases. There was an increased number of binucleate erythroid precursors in all patients. Whenever studied, red cell membrane band 3 was thinner than normal and also migrated slightly faster upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE); this abnormality is highly specific for CDA-II.⁶

Genetic analysis

High molecular weight DNA was extracted from peripheral blood leukocytes using an automatic DNA extractor (ABI DNA PURE; Applied Biosystems Inc., Foster City, CA, USA), according to the protocols of the manufacturer. Fluorochrome-labeled primers were prepared by chemically attaching a fluorescent dye to the 5'-end of each forward oligonucleotide primer employing fluorescent 6-FAM amidite. Names and primer sequences for analyzing markers D20S884, D20S863, RPN, D20S841 and D20S908 have been previously described.¹⁴ The investigated region spans approximately 3 cm. Polymerase chain reaction (PCR) was carried out on an automated thermal cycler (Perkin Elmer, Norwalk, CT, USA) using conditions described before for each microsatellite marker. Following PCR amplification 5 µL of each sample was precipitated by the addition of 400 µL of 100% ethanol. After centrifugation dried pellets were re-suspended in 5 µL of deionized water. Multiplex-

ing was done before loading the gel mixing 1 µL of each sample prior to ethanol precipitation. Acrylamide gel electrophoresis was performed in 8 M urea containing 1x 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 (w/w) for a cross-linking concentration of 5%. Reactions were analyzed either on 4.75% or 6% (w/v) gel with 24 cm well-to-read distances. Data were collected using the GeneScan Data Collection program version 1.1 and analyzed using GeneScan Data Analysis software version 1.1, 1.2 or 1.2.1b1 (Applied Biosystems Inc., Foster City, CA, USA). Correct allele size was assigned using Genotyper™ software.

Statistical analysis

Chi-square values were calculated to evaluate the significance of the distribution of the different alleles for each marker. The measure of strength of association between alleles was the standardized disequilibrium coefficient (delta), defined as:

$$\Delta = ad - bc / ((a+b)(c+d)(a+c)(b+d))e^{1/2}$$

where a, b, c, and d are frequencies of ++, +-, -+ and -- genotypes, and relative risks or odds ratio. The number of alleles was down-coded to two considering the allele with the highest difference among normal and affected chromosomes.

Results

The residences of CDA II patients were spread widely throughout the Italian territory: 33 CDA II members lived in Southern Italy. However, when family histories



Figure 1. Distribution of the Italian kindreds affected by CDA II.

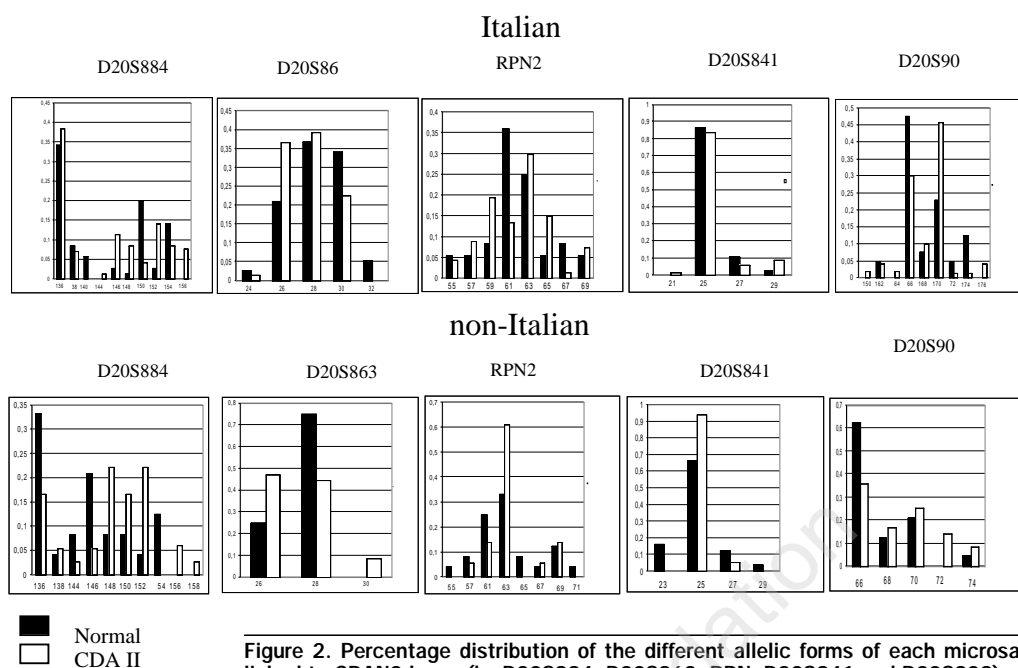


Figure 2. Percentage distribution of the different allelic forms of each microsatellite linked to CDAN2 locus (by D20S884, D20S863, RPN, D20S841 and D20S908), providing a comparison of the distribution in the affected and unaffected chromosomes.

were taken and the exact origin of the grandparents determined, it became evident that the majority of ancestors came from Southern Italy: 7 kindreds had CDA II members residing elsewhere in Italy and knowledge of ancestors who had lived in Southern Italy (Figure 1). The non-Italian kindreds originated from many parts of the world (13 from Europe (outside Italy), 3 from Asia, 2 from America and 2 from New Zealand). The number of non-Italian cases notified to the International CDA-II registry is small and, consequently, the registry may not provide reliable information on the prevalence of CDA-II in any particular country. Nonetheless, it is unlikely that the actual incidence of CDA II in these countries matches the high incidence observed in Italy.

Analysis of microsatellites linked to the CDAN2 locus was performed by D20S884, D20S863, RPN, D20S841 and D20S908. For families with CDAN2 allele linkage, the distribution of each microsatellite is shown in Figure 2, which also provides a comparison between distribution in the affected and unaffected chromosomes. For the Italian sample, statistical analysis demonstrated a very significantly different distribution ($p < 0.001$) of alleles at markers D20S884, RPN and D20S908 (Table 1). In contrast, in non-Italian samples, the most significantly different distribution of alleles between normal and affected chromosomes was detected with marker D20S841 ($p = 0.003$) (Table 1). Δ disequilibrium coefficients were calculated. For the Italian sample, the highest value was detected with markers RPN and D20S908 ($\Delta = 0.27$) whereas statistically insignificant results were obtained with marker D20S841. This marker showed the highest delta coefficient (0.36) in the non-Italian sample in which statistically significant

results were also obtained with marker D20S863 ($\Delta = 0.316$).

The search for an ancestral haplotype obtained in multiplex families by the association of RPN with D20S863 and D20S841 markers failed ($p = 0.7$) and no specific haplotype was identified among affected chromosomes as compared to normal chromosomes (Figure 3). Nevertheless, although not statistically significant, a preferential association of two haplotypes with the disease was noted for the haplotypes defined by the markers RPN and D20S841.

Table 1. Statistical assessment of the significance of the difference in microsatellite allele distribution within Italian or non-Italian CDA-II families and a normal population. Delta represents the disequilibrium coefficient for the alleles shown in parentheses.

| | D20S884 | D20S863 | RPN | D20S841 | D20S908 |
|--------------------|---------|---------|--------|---------|---------|
| <i>Italian</i> | | | | | |
| χ^2 | 43.01 | 13.035 | 35.420 | 5.473 | 27.57 |
| p | 0.000 | 0.008 | 0.000 | 0.186 | 0.000 |
| Δ | .24 | .18 | .27 | nd | .27 |
| | (a150) | (a226) | (a161) | | (a170) |
| <i>Non-Italian</i> | | | | | |
| χ^2 | 19.54 | 10.008 | 13.04 | 14.61 | 11.08 |
| p | 0.012 | 0.007 | 0.055 | 0.003 | 0.026 |
| Δ | nd | .32 | .28 | .36 | .26 |
| | | (a228) | (a163) | (a125) | (a166) |

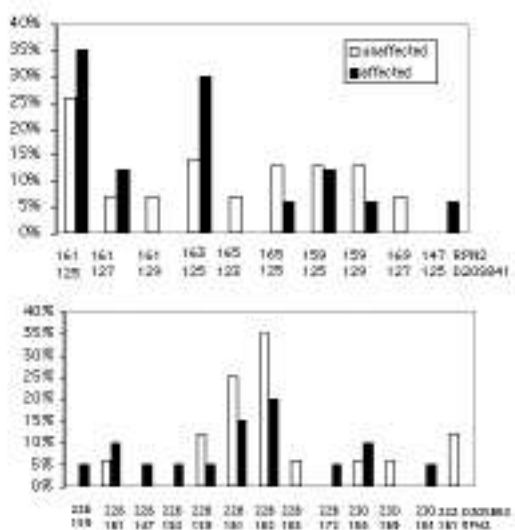


Figure 3. Percentage distribution of the haplotypes generated by the association of the markers D20S863 and RPN (top); RPN and D20S841 (bottom).

Discussion

CDA-II appears to be more frequent in Italy than in other European countries and again to be more frequent in Europe, in particular in individuals of Mediterranean origin, than in other continents. Of course, the overall sample is too scanty and the magnitude of recruitment biases is unknown, so that only a limited number of questions may be addressed at this time. Whether a founder effect might exist in Italy is one such question, based on the unusually high incidence of CDA-II in this country. One may conceive that a particular CDAN2 mutation arose in Southern Italy, or was introduced therein, and that endogamy earned it some incidence generation after generation, irrespective of a hypothetical selective advantage (in the heterozygous state) which, presumably, would not have had time to operate over the period of interest. Eventually, the northward migrations allowed the deleterious allele to spread over the rest of the country. We have no clue as to the times the different steps of such a plausible history took place.

A founder effect requires the demonstration of a linkage of CDA-II to one particular haplotype. The fact that we found a preferential association of CDA-II with alleles but not with specific haplotype does not support the hypothesis of a founder effect.

Previous studies of the first 14 kindreds (13 from Italian patients) showed a strong linkage disequilibrium with microsatellite 863, approximately 75% of the affected individuals carrying the same allele. Present data on a larger series of patients (81 affected members from 64 kindreds) showed a lower linkage disequilibrium with the proportion of affected individuals carrying the same allele reduced to 40%. Our

statistical calculations and linkage disequilibrium data also clearly demonstrate a strong association of the four other markers of chromosome 20 with the disease locus in our sample. Almost all the region defined by the markers here used is in disequilibrium with the disease. Combining the data from the Italian sample together with those obtained for other countries, we can restrict the area of highest disequilibrium to that defined by markers D20S863-D20S908. Despite the presence of this linkage disequilibrium the search for a common haplotype failed. This could suggest that the mutation was very old or that it occurred more than once on different genetic backgrounds.

In conclusion, the analysis of the geographic distribution of CDA-II cases showed a concentration of this disease in Southern Italy and in Mediterranean countries in general. However molecular epidemiology approach showed that a founder effect does not exist.

Funding

This paper was supported by MURST 40% and Telethon grants to A.I. (project E-645).

Acknowledgments

The Authors are particularly grateful to the chairmen of the Centers participating to the recruitment of patients: U. Ramenghi (Torino); C. Camaschella (Torino); M.D. Cappellini (Milano); P. Scartezzini (Genova); G. Barosi (Pavia); R. Galanello (Cagliari); D. Gallisai (Sassari); P. Rigano (Palermo); L. Felici (Pesaro); P. Izzo (Bari); G. Schilirò (Catania); P. Sticca (Como); B. Nobili, E. Miraglia del Giudice (Napoli); J. Vives-Corrans (Barcelona); G. Tchernia (Paris); S. Eber (Zurich); F. Gilsanz (Madrid); V. Premetis (Athens); H. Toriello (USA); S. Aftimos (New Zealand). Furthermore the Authors thank the pediatricians affiliated to the AIEOP and ESPHI.

Contribution of each author: AI is the chairman of the International Registry, he designed the research, collected the data and wrote the manuscript; VS, AT and MC performed the DNA extraction and haplotyping; RC analyzed the clinical data of the Registry; SP performed the SDS PAGE for the biochemical validation of several cases; SNW and HH collected cases in their countries and validated the morphological diagnoses; JD wrote the manuscript and collaborated in data analysis; PG performed the genetic data management and collaborated in revision of the manuscript.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received 3 November 1999; accepted January 31, 2000.

Potential Implications for clinical practice

- ◆ Congenital dyserythropoietic anemia type II does not appear to be restricted to any ethnic group.

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