The morphological diagnosis of congenital dyserythropoietic anemia: results of a quantitative analysis of peripheral blood and bone marrow cells

The congenital dyserythropoietic anemias comprise a group of very rare hereditary disorders characterized by ineffective erythropoiesis and by distinct morphological abnormalities of the erythroblasts in the bone marrow. The classification proposed in 1968[1] is still used today.[2,3] Morphological analysis is the first step in the diagnosis of all types of congenital dyserythropoietic anemia, to be followed by confirmatory tests. The morphological hallmarks of the erythroblasts are not per se specific, but may also be observed as single abnormalities in other disorders of erythropoiesis. We, therefore, attempted to evaluate the quantity of the characteristic morphological abnormalities in patients with CDAI and CDA II.

Smears of peripheral blood and aspirated bone marrow were obtained from the German Registry on Congenital Dyserythropoietic Anemias. All specimens were originally used for diagnostic purposes. Patients were asked for informed consent for their use for scientific research, in agreement with the decision of the ethical committee of the University of Ulm. All patients were analyzed using a unique patient's number (CDA-UPN) that does not permit recourse of identifying data. Specimens were stained by May-Grünwald Giemsa stain: 1,000 cells were examined for abnormalities. Occurrence of basophilic stippled cells or nucleated erythrocytes in the peripheral blood was listed but not quantified.The relative fraction of erythroblasts was expressed as the ratio of erythroblasts to granulopoietic cells. Macrophages were examined for birefringence under polarized light. The Mann-Whitney – U-test was used to compare cases and controls.

The diagnosis was based on parameters described previously.[4,5] Confirmation of the diagnosis of CDA I required a mutation of the *CDAN1*-gene and/ or typical aberrations seen by electron microscopy.[2,6] Confirmation of the diagnosis of CDA II required a mutation of the *SEC23B*-gene or at least one of the following parameters: positive acid serum lysis test with ABO-compatible sera,[7] a typical abnormality of band 3 shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE),[8,9] or a discontinuous double membrane in mature erythroblasts seen by electron microscopy.[2] Specimens of 19 (5 males, 14 females) and 36 patients (21 males, 15 females) with CDAI and CDA II, respectively, were available.

Specimens of 10 patients with distinct erythroid hyperplasia were selected as controls (6 with hemolytic anemia, 2with myeloid dysplastic syndromes, one with thalassemia intermedia, and one with bleeding and iron deficiency).

Peripheral blood showed distinct anisopoikilocytosis in all cases, with grade 2 (25–50% poikilocytes) in 19 (34%), and grade 3 (> 50%) in 36 (66%) patients. There was no difference between types I and II (P=0.34). A few mature erythroblasts were seen in specimens of 31 and basophilic stippled red cells in 36 cases. These changes are highly sensitive but in no way specific. All types of poikilocytes such as ovalocytes, microspherocytes, tear drop cells or irregularly contracted cells are seen. In CDA I, large irregularly shaped macrocytes are seen and may suggest megaloblastic disease. The variegated appearance as described permits the exclusion of hereditary spherocytosis, an erroneous diagnosis that was often made before the diagnosis of CDA I or II was recognized.[4,5] Mature erythroblasts may also be found in other types of severe anemia, but rarely in moderate anemias with a hemoglobin concentration of 90–110 g/L characteristic for most patients with congenital dyserythropoietic anemias.[2,3]

Marrow hypercellularity and distinct erythropoietic hyperplasia were present in all patients. The relative frequency of the pertinent abnormalities of erythroblasts, as compared to controls, is shown in Tables 1 and 2. Highly significant differences were found for binucleated cells, abnormalities of chromatin structures, chromatin bridges between erythroblasts, incompletely divided and large polyploid cells (*See Online Supplementary Appendix*).

Pseudo-Gaucher cells containing birefringent needles were seen in 23 (63%) cases with CDA II. Also, in 3 patients with CDA I but not in controls some birefringent material in macrophages was detected.

In clinical practice, evidence of any type of congenital dyserythropoietic anemias is primarily based on the morphology of peripheral blood and bone marrow, although confirmation of the diagnosis of the two most frequent types CDA I and CDA II is based on more refined tests. These tests are expensive and available in only a few spe-

Table 1. Erythroblasts in specimens of patients with CDA I (n =19) and controls (n = 10). Data except E:G ratio are given in % of all erythroblasts.

	CDA I			Controls			
	Median	Range	95%.confidence	Median	Range	95%.confidence	*P*
Age	28	0.5-51	21-34	26	1-68	8-43	0.8
E:G ratio	2.1	0.9-3.6	1.8-2.5	3.6	1.7-5.4	2.1-3.9	0.3
All nuclear abnormalities	26	21-39	26-32	6.9	1.8-16	3.6-10	<0.0001
Binucleated	5.5	2.4-10	4.7-6.8	0.9	0.3-4.3	0.4-2	<0.0001
Multinucleated	0.2	0-0.6	0.1-0.23	0.1	0-0.4	0-0.2	0.147
Incompletely divided nuclei	2.2	1-5	2.1-3.4	0.5	0.1-1.3	0.3-0.8	<0.001
Chromatin bridges	3.0	1.4-7.9	2.7-4.4	0.14	0-1.3	0.2-0.4	<0.001
Abnormal chromatin structure	8.8	3.5-17	6.8-11	0.2	0-0.7	0-0.4	< 0.0001
Polyploid cells	0.8	0.2-2.0	0.6-1.0	0.1	0-0.6	0-0.2	0.0193
Karyorrhexis	1.0	0-11	0.5-2.7	1.1	0.2-2	0.6-1.6	0.6965
Basophilic stippling	3.7	0.1-7.6	2.5-4.8	0.48	0-6.8	0-3.4	0.0034

Table 2. Erythroblasts in specimens of patients with CDA II (n =36) and controls (n = 10). Data except E:G ratio are given in % of all erythroblasts.

	CDA I			Controls			
	Median	Range	95%.confidence	Median	Range	95%.confidence	*P*
Age	27	0.2-78	19-34	26	1-68	8-43	0.8
E:G ratio	4.2	1.7-10	3.5-4.9	3.6	1.7-5.4	2.1-3.9	0.9
All nuclear abnormalities	22	14-33	20-24	6.9	1.8-16	3.6-10	<0.0001
Binucleated	11	4.6-20	10-13	0.9	0.3-4.3	0.4-2	<0.0001
Multinucleated	1.1	0.30-6.1	0.79-1.5	0.1	0-0.4	0-0.2	0.147
Incompletely divided nuclei	1.0	0.0-2.2	0.87-1.3	0.5	0.1-1.3	0.3-0.8	<0.001
Karyorrhexis	2.90	0.19-8.1	2.3-3.5	1.1	0.2-2	0.6-1.6	0.6965
Basophilic stippling	1.0	0.0-8.2	1.2-2.9	0.48	0-6.8	0-3.4	0.0034

cialized laboratories. The correct diagnosis of congenital anemias is often delayed.[4,5] Many hematologists or clinical pathologists have never seen a case of CDA and do not recognize the well known morphological abnormalities or because, by misinterpretation of clinical and laboratory findings, a bone marrow biopsy is not performed. On the other hand, the diagnosis of a congenital dyserythropoietic anemia is often erroneously suspected, since the observer overvalues the presence of abnormalities that can be seen in the CDAs but also in other more common red cell disorders.

If an appropriate technique of preparation of bone marrow is used, hypercellularity can be recognized, and is always seen in histobiopsies (*data not shown*). The relative frequency of red cell precursors in the bone marrow is increased, with a mean E:G ratio of 4 and 8 times the normal in CDA I and CDA II. Previous experience in normal adults showed a range of 0.2 -1.0, corresponding to data on 50 and 67 adults published by Bain[10] and den Ottolander,[11] respectively. These investigators found a range of 0.2-0.9 (mean 0.42, 95% confidence limits 0.2-0.9) and a range of 0.24-0.80 (mean 0.46, 95% confidence limits 0.42-1.2), respectively.

The diagnosis of CDA I can be made with high specificity from morphological analysis by light microscopy alone, when aberrations of the erythroblast nuclei as listed in Table 1 are present in more than 20% of these cells. Abnormal chromatin structure and the fine chromatin bridges are the most specific changes. They are not seen in normal bone marrow, but are occasionally observed in single cells in patients with myelodysplastic syndromes or erythroleukemia. In CDA II, the most specific finding is the presence of binucleated cells with equal size of two nuclei. As shown in Table 2, the fraction of such binucleated cells is usually more than 10% of all erythroid precursors. If the fraction of binucleated erythroblasts is related to the compartment of late polychromatic and mature oxyphilic erythroblasts, the 95% confidence interval is 13-16%. No more than 2% of binucleated cells may be found in normal individuals[10] or a variety of red cell disorders with erythroid hyperplasia. If more than 10% of typical binucleated erythroblasts are seen, together with more than 2% of cells with karyorrhexis, the diagnosis of CDA II is almost confirmed, and confirmatory tests such as sequencing the *SEC23B* gene are indicated. Pseudo-Gaucher macrophages containing birefringent needles are found in the majority of cases, but in contrast to previous belief do not permit the differential diagnosis as compared with CDA I.

In conclusion, the diagnosis of CDA I and CDA II can be made with high reliability from diligent analysis of peripheral blood and technically appropriate specimens of aspirated bone marrow. However, the results of this study clearly demonstrate that their specificity for the diagnosis of a congenital dyserythropoietic anemia relies not on their presence but rather on the mosaic of morphological aberrations and the frequency of their occurrence.

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References

1. Heimpel H, Wendt F. Congenital dyserythropoietic anemia with karyorrhexis and multinuclearity of erythroblasts. Helv Med Acta. 1968;34(2):103-15.
2. Wickramasinghe SN. Congenital dyserythropoietic anemias: clinical features, haematological morphology and new biochemical data. Blood Rev. 1998;12(3):178-200.
3. Heimpel H, Iolascon A. Congenital dyserythropoietic anemia. In: Beaumont C, Beris Ph, Beuzard Y, Brugnara C, editors. Disorders of homeostasis, erythrocytes, erythropoiesis. 2 ed. Paris: European School of Haematology; 2009. p. 178-201.
4. Heimpel H, Anselstetter V, Chrobak L, Denecke J, Einsiedler B, Gallmeier K, et al. Congenital dyserythropoietic anemia type II: epidemiology, clinical appearance, and prognosis based on long-term observation. Blood. 2003;102(13):4576-81.
5. Heimpel H, Schwarz K, Ebnöther M, Goede JS, Heydrich D, Kamp T, et al. Congenital dyserythropoietic anemia type I (CDA I): Molecular genetics, clinical appearance and prognosis based

on long-term observation. Blood. 2006;107(1):334-40.
6. Heimpel H, Forteza-Vila J, Queisser W, Spiertz E. Electron and light microscopic study of the erythropoiesis of patients with congenital dyserythropoietic anemia. Blood. 1971;37(3):299-310.
7. Crookston JH, Crookston MC, Burnie KL, Francombe WH, Dacie JV, Davis JA, et al. Hereditary erythroblastic multinuclearity associated with a positive acidified-serum test: a type of congenital dyserythropoietic anemia. Br J Haematol. 1969;17(1):11-26.
8. Anselstetter V, Horstmann K, Heimpel H. Congenital dyserythropoetic anaemia, types I and II: Aberrant pattern of erythrocyte membrane proteins in CDA II, as revealed by two-dimensional polyacrylamide gel electrophoresis. Br J Haematol. 1977;35(2):209-15.
9. Delaunay J. Genetic disorders of the red cell membrane. Crit Rev Oncol Hematol. 1995;19(2):79-110.
10. Bain BJ. The bone marrow aspirate of healthy subjects. Br J Haematol. 1996;94(1):206-9.
11. Den Ottolander GJ. The bone marrow aspirate of healthy subjects. Br J Haematol. 1996;95(3):574-5.

Methylation patterns in CD34 positive chronic myeloid leukemia blast crisis cells

Treatment results of chronic myeloid leukemia blast crisis are unsatisfactory, even with second generation tyrosine kinase inhibitors and allogeneic stem cell transplantation. Loss of tumor suppressor gene activity is one of the events that is associated with progression to blast crisis. Next to genetic alterations, this may be related to hypermethylation of the promoter regions of these genes. Demethylating drugs, like decitabine and 5-azacytidine, have recently become available for clinical purposes and may act by reversing abnormal methylation of chronic myeloid leukemia blast crisis. To estimate their potential value for treatment of chronic myeloid leukemia blast crisis, we investigated the methylation status of the promoters of tumor suppressor genes in clinical samples, using methylation specific multiplex ligation-dependent probe amplification (MS-MLPA), a technique that has been demonstrated to give reliable results, comparable to classical sodium bisulphite sequencing based assays or methylation specific polymerase chain reaction (MSP).[1-3] Methylation specific multiplex ligation-dependent probe amplification enables simultaneous assessment of a large number of chronic myeloid leukemia blast crisis, requires low amounts of DNA (unlike MSP) and is relatively inexpensive.

After informed consent, DNA was isolated from peripheral blood and bone marrow CD34 positive cells (selected by AutoMACS, Miltenyi Biotec, Bergisch Gladbach, Germany) of 19 blast crisis patients (Table 1) and 15 newly diagnosed, untreated chronic myeloid leukemia patients as controls. MS-MLPA was subsequently performed as described previously using probe mixes ME001-Tumor suppressor-1 and ME002-Tumor suppressor-2, which include probes targeted to the CpG islands within the promoter regions of 35 candidate tumor suppressor genes (Online Supplementary Table S1).[1,3]

Using this technique, we observed methylation of at least one tumor suppressor gene promoter CpG island in all 19 blast crisis samples, with a total number of 69 methylated genes. *CDH13*, *ESR1*, *IGSF4*, *MGMT* and *CDKN2B* were the genes that were most frequently

Table 1. Clinical characteristics and results of MS-MLPA. Only genes that were methylated in at least one patient sample are shown. Blanks indicate unmethylated promoter regions.

Pt n.	Age (yr)	Myeloid/ Lymphoid BC	Interval D/-BC (mos)	Previous T/	BRCA 1 1/2	CD 44 1/2	CDH 13 1/2	CDKN 2A 1/2	CDKN 2B	ESR 1 1/2	FHIT	GATA 5	IGSF 4 1/2	MGMT 1/2	MSH 6	PAX 5	PAX 6	RARB 11 1/2	STK 1	THBS 3	TIMP 73 1/2	TP 1	WT	total*
				probe set number																				
1	38	my	26	IM		U/M	M/M[2]		M	M/M	M	M	M/M	M/M			M	M/M	M	M	M	M/M		14
2	44	my	43	HU		U/M									M									2
3	17	my	3	HU			U/M							U/M				U/M						3
4	26	my	0	none					M												M	U/M		3
5	71	bi[3]	0	none		U/M	U/M			U/M	M		M/M	U/M										6
6	53	my	0	none			M/M		M				M/U										M	4
7	66	ly	4	IM			M/M			M/M			M/M					U/M				U/M		5
8	56	my	1	none			M/M															U/M		2
9	65	my	84	IM			U/M			U/M			M/U											3
10	46	my	156	IFN/LD-AraC			U/M			U/M														2
11	39	my	13	IM/ID-AraC						U/M				U/M			M							3
12	54	my	7	IM/ID-AraC; Ida/AraC			U/M			U/M			M/U	U/M										4
13	66	my	40	IM			U/M			U/M				U/M										3
14	59	my	46	HU/IFN			U/M									M								2
15	52	ly	0	none																		U/M		1
16	46	my	0	none			U/M	U/M																2
17	62	my	3	IM/ID-AraC	U/M		U/M		M															3
18	61	my	3	HU/IFN			U/M																	1
19	79	u	119	u			M/M		M		M	M	M					U/M						6
total					1	3	15	1	5	8	3	2	7	6	1	1	2	4	1	1	2	5	1	

D/: diagnosis; BC: blast crisis; U: unknown; T/: treatment; IM: imatinib; HU: hydroxyurea; IFN: interferon-alpha; LD-AraC: low-dose cytarabine; ID-AraC: intermediate dose cytarabine; Ida: idarubicin 1: blanks in this row indicate that only one probe set was used as shown in Online Supplementary Table S1. *2: M: methylated; U: unmethylated; 3: biphenotypic *: In case two probe sets for a single gene promoter were used, the gene was scored positive when at least one probe set showed methylation; if two CpG islands tested in a single gene promoter were methylated, then counted only once .*