Cup-like acute myeloid leukemia: new disease or artificial phenomenon?

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ABSTRACT

We investigated cup-like nuclear morphology of acute myeloid leukemia blasts in 266 randomly selected patients and its association with hematologic findings, disease markers and outcome data. Cup-like acute myeloid leukemia was diagnosed in 55 patients (21%). It was associated with female sex, high white blood cell and blast cell counts, normal karyotype, and low CD34 and HLA-DR expression. Mutations of *FLT3*, *NPM1* or both were detected in 84.9% compared with 58.1% in cases without this morphology (p=0.001). There was no influence on response to treatment or survival. Therefore, cup-like nuclear morphology is an indicator of normal karyotype and should guide more specific molecular analyses.

Key words: acute myeloid leukemia, morphology, cup-like, *FLT3*, *NPM1*.

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Introduction

There is an increasing role for molecular and cytogenetic findings in acute myelogenous leukemia (AML). In some categories, a correlation with morphologic features can be found, e.g. AML with inv(16), acute promyelocytic leukemia. More recently, several novel abnormalities have been identified, e.g. mutations of *FLT3*, *CEBPA*, *NPM1* and *RUNX1*, some of which are predominantly found in patients with normal karyotype.[1] Although an association with certain morphologic features has been described (e.g. FAB M5 morphology and *FLT3* mutations), no highly specific morphology has been identified so far. Two recent studies[2,3] reported cases of AML showing a distinctive, cup-like nuclear indentation in myeloid blast cells. This was associated with a lack of CD34 expression, a higher frequency of normal cytogenetics, and mutations in *NPM1* and *FLT3* genes. Although a significantly higher rate of complete remissions was found in one of the studies, no impact on survival could be identified. We have previously reported on the prevalence and impact of mutations in *FLT3* and/or *NPM1* genes in patients with AML.[4,5] A high mutant-to-wild-type (wt) ratio (>0.78) of the *FLT3* internal tandem duplication (ITD) was an independent prognostic factor for shorter overall and disease-free survival. By contrast, single *NPM1* mutations were associated with significantly better outcome. Because of this strong impact of *FLT3* and *NPM1* mutations on patients' outcome, we re-evaluated the association of cup-like nuclear morphology with clinical and molecular features as well as outcome in AML patients.

Design and Methods

The data reported comes from the multicenter AML96 protocol of the Deutsche Studieninitiative Leukämie (DSIL). Detailed descriptions of this study have been previously reported.[4-6] All patients received two cycles of standard induction chemotherapy. In patients under 60 years of age, postinduction treatment was stratified according to cytogenetic risk groups (low, intermediate, high risk) and included conventional chemotherapy (intermediate *vs.* high dose cytarabine), autologous or allogeneic stem cell transplantation. Patients with acute promyelocytic leukemia (AML FAB M3) were treated in another trial and therefore are not included in the present analysis. Complete remission (CR) was defined as the presence of less than 5% of blast cells in standardized bone marrow

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aspirate after the second induction cycle. The study was approved by the Institutional Review Board (IRB) of Dresden University Hospital.

All investigated samples were obtained at the time of diagnosis. Smears of peripheral blood (PB) and/or bone marrow (BM) from 266 randomly selected patients (male, n=148; female, n=118; median age 57 years) were re-evaluated for blast cell morphology. Two-hundred blast cells in PB and BM each were differentiated by two consecutive investigators (step 1) to determine the percentage of cells with cup-like nuclear invagination (spanning at least 25% of the nuclear diameter). The slides were then seen by two other investigators (step 2) who assigned the AML to a cup-like positive or a cup-like negative cohort without knowing the results from step 1. A comparison of the results from steps 1 and 2 was used to define cut-off (step 3). Both cohorts (cup-like positive *vs.* cup-like negative defined by cut-off value) were then analyzed for FAB-type, number of PB and BM blasts, CD34 and HLA-DR expression, karyotype abnormalities, molecular aberrations, as well as response and survival data. Antigen expression was detected by a standard indirect immunofluorescence method as previously described.[7] Cytogenetic analyses were performed after routine preparation of fresh material using G-banding staining and fluorescence *in situ* hybridization (FISH). Molecular evaluation focused on mutations in *FLT3* gene (internal tandem duplication, ITD; mutation in tyrosine kinase domain, TKD), *NPM1* gene, and partial tandem duplication (PTD) mutations of the mixed lineage leukemia (*MLL*) gene. The techniques have recently been described in detail.[4,5,8,9] In addition, electron microscopy was performed in cases with high numbers of cup-like blasts.

Results and Discussion

The typical morphology of blasts with cup-like nuclei is demonstrated in Figure 1. Electron microscopy revealed that the morphologic phenomenon is caused by an accumulation of cytoplasmatic organelles rather than by primary nuclear changes (*Figure 2, online supplement*). The median percentage of blast cells with cup-like nuclei was higher in PB compared with BM (2.0% *vs.* 0.5%). In both cohorts, the percentage of cup-like cells correlated with the total number of blasts (PB, r=0.339; BM, r=0.263, $p<0.01$). Based on the results presented in Table 1 we used a cut-off of 5% to define cup-like positive AML (n=55) in the further analysis. FAB types M1 and M2 represented 62% of patients with cup-like AML. However, the frequency of cup-like positive cases among the monocytic (M4, M4Eo, M5a, M5b) and non-monocytic leukemias (M0, M1, M2, M6, M7, RAEB-T) was similar (20/89, 22.5% *vs.* 35/177, 19.8%; $p=0.632$). Table 2 summarizes the results of flow cytometric and genetic analyses. Cup-like nuclear morphology was associated with lower expression of CD34 as well as HLA-DR. However, this

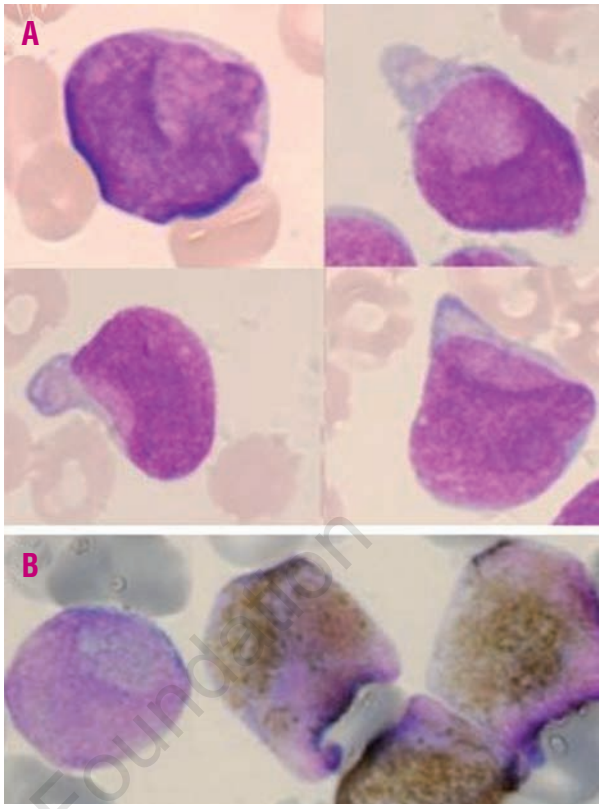

Figure 1. Typical morphology of myeloid blasts with cup-like nuclear invagination in AML. (A) The "cup" seems to be a part of the nucleus in Pappenheim staining, however it is positive for myeloperoxidase (MPO) in reactive blasts. (B) MPO-positive and MPO-negative cup-like blast cells in a patient with AML FAB M1.

was due to highly significant differences among the non-monocytic leukemias, while an impact of nuclear morphology was not found to affect the expression of these antigens in the monocytic entities (*data not shown*). Cup-like morphology was significantly associated with normal karyotype, and molecular aberrations were also more often found in this cohort. In fact, in cup-like AML, *FLT3* (ITD or TKD) mutations, *NPM1* mutations or both were detected in 84.9% of cases, compared with 58.1% in the cup-like negative AML ($p=0.001$). We also analyzed the frequency of these mutations in monocytic leukemias (FAB M4, M4Eo, M5a, M5b) and non-monocytic entities (FAB M0, M1, M2, M6, M7, RAEB-T). In monocytic AML (with cup-like nuclear morphology), the frequency of *FLT3* mutations, *NPM1* mutations, or both were 30%, 25%, and 30% compared with 21.2%, 3.0%, and 60.6% in the non-monocytic leukemias. Mutations in *FLT3* and *NPM1* genes were significantly more frequent in cup-like AML compared with cases without this morphology among the non-monocytic FAB types (Table 1). By contrast, nuclear morphology had no impact on the frequency of these mutations among the monocytic leukemias (cup-like positive *vs.* cup-like negative: *FLT3* mutations, 60% *vs.* 58%, $p=1.000$; *NPM1* mutations, 55% *vs.* 46%, $p=0.611$). There was no statistical difference in the number of patients with *MLL-PTD* mutations between cup-

like positive and negative diseases, either in the entire cohort or in the subgroups.

The rate of complete responses in cup-like positive and cup-like negative AML were 49.1% *vs.* 54.0% respectively (p=0.546) for the entire group and 40.0% *vs.* 53.5% respectively (p=0.187) in the non-monocytic entities. The median overall survival for the patients with cup-like positive and cup-like negative AML was 8.4 and 12.2 months (p=0.510). There was also no difference in median disease-free survival between the two cohorts of patients (p=0.739). In addition, we analyzed the subgroups of young patients <60 years, patients with *FLT3* or *NPM1* mutation, patients with *FLT3* and *NPM1* mutation, and patients with *FLT3* ratio >0.8. However, cup-like nuclear morphology was not shown to have any impact on response rate or survival parameters.

At least for the experienced hematologist, cells with cup-like nuclear morphology are very easy to identify. Therefore we used a cut-off of ≥5% blast cells with typical morphology in blood and/or bone marrow for the definition of cup-like AML for the statistical analyses, which was lower than in the studies by Kussik *et al.*[2] and Chen *et al.*[3] The phenomenon seemed to be more pronounced in the peripheral blood and this was in line with the higher median percentage of cup-like positive blast cells in this group. Whether there really are more cup-like cells in the PB, or whether the phenomenon is induced by preparing the smears, remains hypothetical. Cellularity in the blood is very much lower than in the marrow and the specific morphology might therefore be easier to identify. A major finding from this series is that cup-like nuclear blast cell morphology is not restricted to acute myeloblastic leukemias as was suggested by other authors.[2,3] In terms of HLA-DR expression, data are controversial. Kussik *et al.*[2] reported a complete or partial loss of HLA-DR expression in cup-like AML. By contrast, cup-like AML was associated with a significantly increased expression of this antigen compared with a control group in the study by Chen *et al.*[3] In our series, an association of nuclear morphology and HLA-DR was found only in the non-monocytic FAB types, not in the monocytic leukemias. The prognostic relevance of mutations in *FLT3* and *NPM1* genes has been proved in several trials.[1] If not associated with *FLT3* -ITD mutations, mutant *NPM1* identifies a subgroup of patients with favourable prognosis, especially in AML with normal karyotype. By contrast, the negative prognostic impact of mutant *FLT3* dominates in

Table 1. Number of cases with ≥2.5%, ≥5.0% and ≥10% cup-like blasts in PB and/or BM (step 1); results from identification of cup-like AML by subjective decision only (step 2, cases with divergent decision were discussed between the investigators); cut-off was defined on the basis of comparison of the results from steps 1 and 2.

STEP 1 – Investigator 1 and 2 (differential count)

Median [%] of cup-like blasts	*Number of cases*		
	PB	*BM*	*PB and/or BM*
≥2.5%	83	33	93
≥5.0%	53	16	55
≥10.0%	26	6	27

STEP 2 – Investigator 3 and 4 (subjective decision)

	independent	*interactive*
corresponding: n=245 (92.2%)		
divergent: n=21 (7.8%)	I-3 pos , I-4 neg: n=18 (6.8%)	neg: n=16 (CLB ≥5%: n=2) pos: n= 2 (both CLB <5%)
	I-3 neg, I-4 pos: n=3 (1.1%)	pos: n= 3 (CLB ≥5%: n=1)

STEP 3 – Definition of cut-off

Median [%] of cup-like blasts	*Proportion of cases which where identified as "cup-like AML" in step 2*	
	PB	*BM*
≥2.5%	61/83 (73%)	24/33 (76%)
≥5.0%	49/53 (92%)	16/16 (100%)
≥10.0%	26/26 (100%)	6/6 (100%)

PB: peripheral blood; BM: bone marrow; I-3, I-4: Investigator 3 and 4; CLB: cup-like blast.

Table 2. Comparison of patients with cup-like positive and cup-like negative AML in terms of epidemiologic, hematologic, cytogenetic and molecular parameters. The left of the table shows the results for the entire cohort including all FAB-types, whereas a second analysis (right of the table) was restricted to patients with non-monocytic leukemias (FAB M0, M1, M2, M6, M7, RAEB-T)

	All FAB-types			*Non-monocytic leukemias*		
	Cup-like negative AML (n=211)	*Cup-like positive AML (n=55)*	*p value*	*Cup-like negative AML (n=142)*	*Cup-like positive AML (n=35)*	*p value*
Median age [years]	58	56	0.448	57	58	0.881
Male/Female [n]	123/88	25/30	0.096	85/57	14/21	0.038
Median WBC at diagnosis [x10^9/L]	19.1	77.4	<0.001	10.0	61.0	<0.001
Median BM blasts at diagnosis [%]	65.0	78.0	<0.001	61.5	82.5	<0.001
Median number of CD34+ blasts [%]	27.0	7.0	0.001	39.0	16.0	0.003
Median number of HLA-DR+ blasts [%]	64.0	66.5	0.443	62.0	24.0	0.012
FLT3 mutation (ITD or TKD) [%]	49.3	72.7	0.002	45.1	80.0	<0.001
FLT3-ITD mutation [%]	46.4	70.9	0.001	42.3	77.1	<0.001
FLT3-ITD ratio (mutant/wt) >0.8 [%]	14.2	41.5	<0.0001	13.1	42.4	<0.001
NPM1 mutation [%]	33.3	60.4	<0.001	26.9	63.6	<0.001
FLT3+NPM1 mutation [%]	25.3	49.1	0.001	19.2	57.6	<0.001
MLL-PTD mutation [%]	5.4	14.0	0.058	6.5	12.5	0.269
Cytogenetic abnormalities [%]	39.5	18.5	0.004	41.1	17.1	0.01

patients carrying both aberrations.[5] In our study, about 70%, 60%, and 50% of cases with cup-like positive AML were positive for *FLT3* -ITD mutation, *NPM1* mutation, or both, respectively. Therefore, the detection of characteristics similar to those described for the *FLT3* and *NPM1* positive diseases is not surprising (high WBC and blast cell count at diagnosis, predominance in females and patients with normal karyotype, low expression of CD34 on blasts). Mutations of *FLT3* and *NPM1* are known to be more frequent in monocytic leukemias. However, a comparison of the frequency of these aberrations in patients with cup-like AML showed no difference between the monocytic and non-monocytic FAB types. Furthermore, the higher rate of *FLT3* and/or *NPM1* mutations in cup-like AML was mainly due to differences between cup-like-positive and -negative cases among the non-monocytic FAB-types, whereas the impact of nuclear morphology on the frequency of these mutations among myelomonocytic and monocytic leukemias was less pronounced. Therefore, cup-like nuclear morphology is indicative, but not specific, for the presence of potential risk-relevant mutations, particular in non-monocytic AML. Although a higher rate of complete remissions was found by Chen *et al.*,[3] we could not show that cup-like morphology had any impact on response to treatment or survival either in the entire cohort or in subgroups. It may be that the number of patients even in our study is too small to detect differences in outcome. However, given the high frequency of molecular aberrations with very different prognostic consequences in cup-like AML (*FLT3* and *NPM1*), it seems very unlikely that morphology should represent an independent predictive marker. Even more exciting is the question as to which biologic links exist between molecular aberrations and distinct nuclear morphology. A recently published model from HL-60 cells[10] has shown that the changes in the nuclear shape during granulopoiesis involve factors that increase the flexibility of the nuclear envelope, augment connections to the underlying heterochromatin, and promote distortions imposed by the cytoskeleton. As demonstrated by ultra structural investigation, as well as by myeloperoxidase staining, the *cup* is filled in with cytoplasm and organelles. Due to the nucleocytoplasmatic shuttling properties of *NPM1*, a mutation in this gene can be considered as a causative event. While the bulk of physiological *NPM1* protein resides in the nucleolus, mutant *NPM1* protein is mainly detectable in the cytoplasm.[5,11]

Altogether, our study supports data showing a close association between the distinctive cup-like nuclear morphology in AML blasts, specific clinical features and a high frequency of molecular aberrations with prognostic impact. This morphology can be, therefore, an indicator of normal karyotype and specific analyses of *FLT3* and *NPM1* mutations.

Authorship and Disclosures

FK and US performed all the morphological investigations, RF and GB performed the ultrastructural study, FK, CT and UO wrote the manuscript, UO and RR performed the flow cytometric analyses, BM performed the cytogenetic analyses, SS was responsible for the statistics, MS and GE were responsible for the patient treatment and carefully checked the manuscript, and CT was the principal investigator who contributed the idea for the study and checked all the data. The authors reported no potential conflicts of interest.

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