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☐Original Article☐

CD34 Immunohistochemical Staining of Bone Marrow Biopsies in Myelodysplastic Syndromes

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Although it has been shown that the percentage of bone marrow blasts in myelodysplastic syndrome (MDS) constitute the only independent determinant of survival and progression to acute leukemia, the great variability in survival among patients with MDS of similar percentage of blasts has prompted us to investigate new objective, independent prognostic parameters for the selection of high-risk patients. It was suggested that CD34 antigen expression adversely affected the prognosis of acute myelogenous leukemia. However, no study has been published so far on clinical and prognostic significance of CD34 antigen expression in MDS. Bone marrow biopsies from 58 patients diagnosed as primary MDS were studied using QBEND/10, a monoclonal antibody which recognized the human progenitor CD34 antigen on routine aldehyde-fixed, paraffin-embedded samples. The high percentage of CD34-positive cells (above 3% of total bone marrow nucleated cells) was predominantly observed in cases with RAEB-T, CMML, and to a lesser degree in RAEB. But neither age, hemograms, bone marrow findings including percentage of blasts, ALIP, nor leukemic transformation correlated with the percentage of CD34-positive cells. The median actuarial survival time in the high positive group was significantly shorter (12.0 months) than that of the low group (30.0 months; $p=0.028$). The high CD34 aggregate (≥ 3) was selectively found in cases with RAEB, RAEB-T, and CMML. The percentage of bone marrow blasts ($p=0.007$) and ALIP ($p=0.030$) significantly correlated with number of CD34 aggregates. The median survival time in the high CD34 aggregate group was significantly shorter (11.0 months) compared to the low CD34 positive group (25.0 months; $p = 0.003$). On multivariate analysis, the positivity of CD34 cells ($p<0.05$) and the percentage of bone marrow blasts ($p<0.05$) revealed the significant independent parameters for prediction of survival. In conclusion, the CD34 immunostaining, which can be easily performed on routinely prepared bone marrow biopsies, was found to be a powerful independent prognostic parameters in MDS.

Key Words: Myelodysplastic syndrome, CD34, bone marrow biopsy, prognosis

The myelodysplastic syndromes (MDS) comprise wide variety of hematopoietic stem cell disorders characterized by ineffective hematopoiesis leading to blood cytopenias, and by a high risk of progression to acute myeloid leukemia (AML) (Oscier 1987; Ganser and Hoelzer 1992). They have been subdivided into five clinical entities on precise morphological criteria by French-American-British (FAB) Cooperative Group (Bennett *et al.* 1982). The most important prognostic value of this classification is the excess number of blast cells in

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blood and bone marrow. Nevertheless, there are considerable differences between individual patients within the same category as to survival and time to leukemic transformation (Weidorf *et al.* 1983; Foucar *et al.* 1985; Mufti *et al.* 1985; Sultan *et al.* 1987). A number of investigators have searched other biological characteristics with potential clinical relevance, including histologic examination of bone marrow trephine biopsies (Tricot *et al.* 1984), in vitro progenitor cultures (Spitzer *et al.* 1979; Verma *et al.* 1979), and cytogenetics (Geraedts *et al.* 1980; Benitez *et al.* 1985).

CD34 expression has been demonstrated selectively on hematopoietic progenitor cells, including colony-forming unit (CFU)-Blast, high proliferative (HPP)-CFC, long-term bone marrow culture-initiating cells (LTBMC-IC), CFU-granulocytic-macrophage (GM), and burst forming-unit erythroid (BFU-E) (Civin *et al.* 1984; Holyoake and Alcorn. 1994). It was suggested that CD34 expression adversely affected the prognosis of AML (Borowitz *et al.* 1989; Campos *et al.* 1989; Geller *et al.* 1990). CD34 expression of the aspirated bone marrow mononuclear cells has also been demonstrated in patients with MDS, predominantly in the refractory anemia with excess of blast (RAEB) or refractory anemia with excess of blast in transformation (RAEB-T) (Guyotat *et al.* 1990). Recent reports suggest that the growth abnormalities of progenitor cells in these disorders involve a defect in the capacity of CD34-positive cells to respond to stimulation with various hematopoietic growth factors (Sawada *et al.* 1993).

However, no study has been published so far on their clinical and prognostic significance of CD34 antigen expression in MDS. In the present study, we analyzed the prognostic relevances of CD34 expression of bone marrow progenitor cells in 58 patients with primary MDS using monoclonal antibody, anti-CD34 (QBEND10), on routine aldehyde-fixed, paraffin-embedded bone marrow biopsy samples.

MATERIALS AND METHODS

Patients

From January 1987 to December 1992, bone marrow biopsy specimens from 58 untreated patients with primary MDS according to FAB classification were studied. FAB subtypes were: refractory anemia (RA) 13, refractory anemia with ringed sideroblast (RARS) 3, RAEB 18, RAEB-T 16 and chronic myelomonocytic (CMML) 8. Evolution to overt leukemia was diagnosed when the proportions of marrow blasts were greater than 30%. Treat-

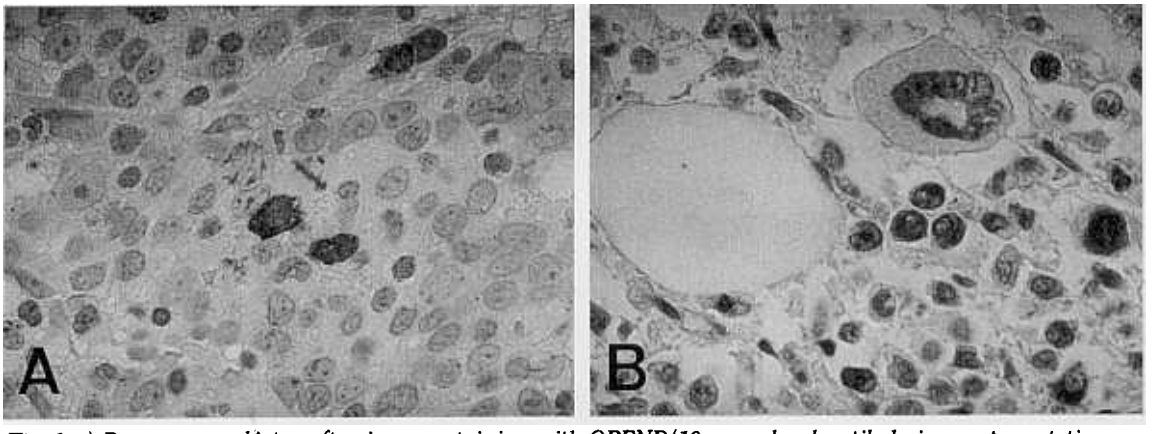

Fig. 1. *a) Bone marrow biopsy after immunostaining with QBEND/10 monoclonal antibody in a representative case showing CD34-positive mononuclear cells b) Aggregates of CD34-positive cells (LSAB staining, ×400).*

ment modality was based on age, general condition, degree of cytopenias, and evolution to overt leukemia. Of the 58 patients, 33 received supportive care only, 14 received low dose cytosine arabinoside, and 11 patients received standard-dose induction chemothrapy.

Bone marrow biopsy and CD34 immunohistochemical staining

Biopsies were obtained from posterior iliac crest with a Jamshidi needle. All samples were examined independently by two observers, who were unware of the patients' identification and FAB subtype. Characteristics recorded from each biopsy sample include: cellularity, quantitative megakaryopoiesis and erythropoiesis, dysmegakaryo-/dysmyelo- and dyserythropoiesis features, abnormal localization of immature progenitor cells (ALIPs).

The section of aldehyde-fixed paraffin-embedded biopsies sliced as 5 μm thickness were deparaffinized in xylene, graded ethanols, and incubated with phosphate bufferred saline containing 5% human decomplemented AB serum and 5% normal goat serum. Subsequently immunohistochemical staining with the QBEND/10 anti-CD34 mouse monoclonal antibody (IgG1; Serotec, England) as primary antibody which recognized the human progenitor CD34 antigen was done by universal labelled streptavidin-biotin (LSAB) kit (DAKO, Denmark). The number of positive cells reactive with the CD34 antibody, and the number of CD34 aggregates were scored by light microscopy after counting 500 total nucleated cells. The CD34 aggregate was defined as clusters of 3 or more CD34 positive cells (Fig. 1). It was seen that a greater number of patients had cells with no expression or only minimal expression of CD34 and the remaining cases had a wide distribution of CD34-positivity above 3%. So the threshold of 3% was used in our study, which was also justified biologically, because in a normal marrow, 1-2% of cells express CD34.

Statistical analysis

The Chi-square test or the Fisher exact test when appropriate were used for statistical analysis of the results. The correlation between the different parameters were assessed by linear regression analysis. Survival was plotted according to the Kaplan-Meier method and curves were compared by using the log-rank test. Further multivariate analysis by means of the proportional hazards regression method was used in order to identify the most significant independent prognostic factors (Cox 1972).

Table 1. Correlation of the percentage of CD34-positive cells with various parameters

	CD34-positive cells(%)*		P Value
	<3(n=34)	≥3(n=24)	
FAB subtype			
RA(n=13)	10(77%)	3(23%)	
RARS(n=3)	3(100%)	0	
RAEB(n=18)	12(67%)	6(33%)	
RAEB-T(n=16)	6(38%)	10(62%)	
CMML(n=8)	3(37%)	5(63%)	
Age(Yr)	48.1±4.5	47.5±6.2	NS
Hemogram			
Hgb(g/dl)	6.4±1.9	7.3±2.5	NS
Neutrophils(/μl)	950±78	1,887±143	NS
Platelets(/μl)	32,100±5,600	54,000±12,400	NS
Blast(%)	0±1	1±1	NS
Bone Marrow			
Cellularity(%)	76±8	80±9	NS
Blast(%)	6.2±2.9	14.2±4.3	NS
Cases with dysplasia(%)			
Erythroid	79	83	NS
Myeloid	59	92	NS
Megakaryocytic	85	83	NS
Trilineage	50	58	NS
ALIP-positive(%)	46±19	75±24	NS
Leukemic transformation(%)	27±9	41±14	NS
Survival(months)**	30.0	12.0	0.028

RA: Refractory anemia, RARS: Refractory anemia with ringed sideroblast, RAEB: Refractory anemia with excess of blast, RAEB-T: Refractory anemia with excess of blast in transformation, CMML: Chronic myelomonocytic leukemia ALIP: Abnormal localization of immature precursors

*Percent of CD34-positive cells among bone marrow nucleated cells

**Numerical data presented as median value

RESULTS

The percentage of CD34-positive cells in bone marrow nucleated cells in this study was divided into lower (<3%) and higher (≥3%) groups. Table 1 shows the FAB subtype, age, hemograms, bone marrow findings including abnormal localization of immature precursors (ALIP) phenomena and leukemic transformations in each group. Neither of age, hemograms, bone marrow findings including percentage of blasts, ALIP, nor leukemic transformation correlated with the CD34 expression determined as above 3% of total bone marrow nucleated cells. But the high percentage of CD34-positive cells was predominantly observed in cases with RAEB-T, CMML, and to a lesser degree in RAEB. The prognostic value of CD34 was first studied in univariate analysis. The median actuarial survival time in the high CD34 positivity group was significantly shorter (12.0 months) compared to that of the low CD34 positive group (30.0 months; p=0.028) (Table 1 & Fig. 2).

The CD34 aggregate in this study was also divided into lower (<3) and higher (≥3) aggregate groups. The high CD34 aggregate was

Table 2. Correlation of the number of aggregates of CD34-positive cells with various parameters

	CD34 Aggregate*		P Value
	<3(n=47)	≥3(n=11)	
FAB subtype			
RA(n=13)	13	0	
RARS(n=3)	3	0	
RAEB(n=18)	14	4	
RAEB-T(n=16)	11	5	
CMML(n=8)	6	2	
Age(Yr)	47.1±12.3	52.1±14.5	NS
Hemogram			
Hgb(g/dl)	6.4±2.1	7.2±3.4	NS
Neutrophils(/μl)	980±125	1,750±320	NS
Platelets(/μl)	32,100±1,100	84,000±2,900	NS
Blast(%)	0±1	1±1	NS
Bone Marrow			
Cellularity(%)	74±9	69±12	NS
Blast(%)	6.7±2.1	21.9±3.9	0.007
Cases with Dysplasia(%)			
Erythroid	83	73	NS
Myeloid	66	82	NS
Megakaryocytic	81	82	NS
Trilineage	57	45	NS
ALIP-positive(%)	51±7	89±9	0.030
Leukemic transformation(%)	29±19	52±20	NS
Survival(months)**	25.0	11.0	0.003

*CD34 aggregate was defined as clusters of 3 or more CD34-positive cells

**Values are expressed as median

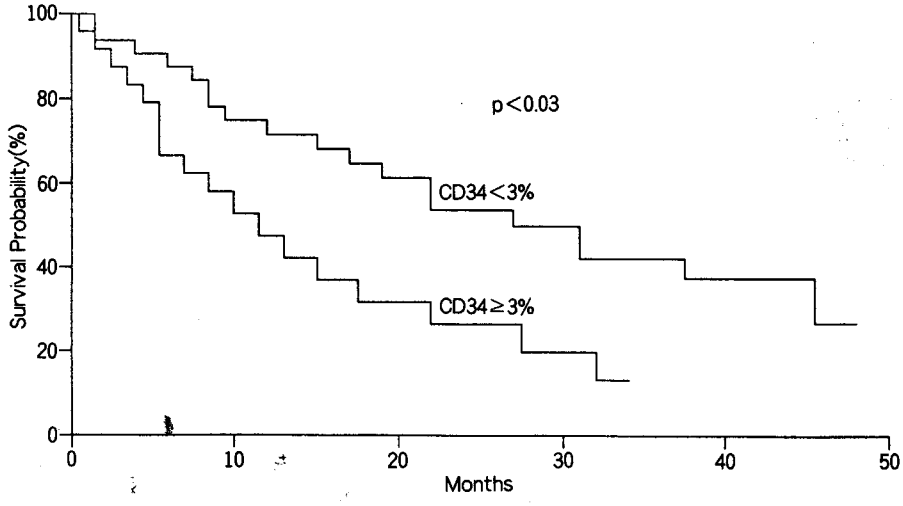

Fig. 2. *Survival curves of the patients with myelodysplastic syndrome according to the percentage of CD34 positive cells.*

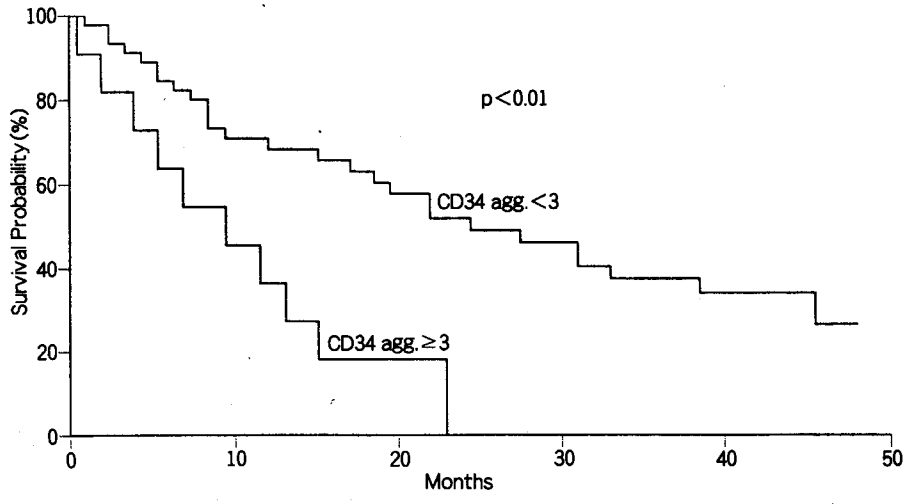

Fig. 3. Survival curves of the patients with myelodysplastic syndrome according to the number of CD34 aggregates.

Table 3. Multivariate analysis of survival prognostic factors

Variables	Coefficient/S.E.	p Value
Age(Yr)	1.5728	NS
Platelet(/l)	−1.1213	NS
Bone Marrow blast(%)	2.8925	<0.05
CD34 positivity*	2.0448	<0.05

*Percent of CD34+ cells and/or number of CD34 aggregates among bone marrow uncleated cells

selectively found in cases with RAEB, RAEB-T, and CMML. As the results, percentage of bone marrow blasts ($p=0.007$), and ALIP ($p=0.030$) significantly correlated with CD34 aggregates (≥3). The rate of leukemic transformation was found to be higher in the high aggregate group (55%) than in the low aggregate group (28%), but without significance (Table 2). The median survival time in the high CD34 aggregate group was significantly shorter (11.0 months) compared to the low CD34 positive group (25.0 months; $p=0.003$) (Table 2 & Fig. 3).

Because of the correlations between some prognostic factors, the respective influence of each of the following factors was assessed by multivariate analysis: age, platelet counts, percentage of blasts in bone marrow, CD34 positivity(percentage of CD34+ cells higher than 3% and/or the number of CD34 aggregates over three). Only the positivity of CD34 cells ($p<0.05$), and the percentage of bone marrow blasts ($p<0.05$) remained the significant and independent parameters for prediction of survival (Table 3).

DISCUSSION

The introduction of the FAB criteria for the diagnosis and classification of MDS has prompted the search for disease characteristics which would predict the natural history in an indivisdual patient (Weisdorf *et al.* 1983; Tricott *et al.* 1984; Jacobs *et al.* 1986; Sanz *et al.* 1989; Goasguen *et al.* 1990). There is, however, a marked variation in the reported survival between various studies within each FAB subtype. For example, the median survival of the patients with RA varies from 18 to 64 months and with RARS from 25 to 72 months (Economopoulos *et al.* 1987; Pierre *et al.* 1989). And although overall patients with

RAEB-T and to a lesser extent RAEB and CMML have a uniformly poor prognosis, there is, however, a subgroup in which the disease evolves slowly, sometimes over a period of several years (Noel and Solberg, 1992). Subdivision of this group by the percentage of blasts did not yield further prognostic information (Mufti *et al.* 1985; Tricot *et al.* 1984). So it should be stressed that a clinical, or biological heterogeneity exists within each of the subtypes of MDS, which is not reflected by the FAB classification.

Although several biological parameters have shown prognostic value in MDS, in particular progenitor cell cultures, labelling index of bone marrow precursors, karyotype, RAS mutation and bone marrow biopsy (Tricot *et al.* 1984; Shihaba-El-Deen *et al.* 1987; Rios *et al.* 1990; Greenberg 1991; Noel and Solberg. 1992), multivariate analyses have generally demonstrated that the percentage of bone marrow blasts constituted the only independent determinants of survival and progression to AML (Tricot *et al.* 1984; Wattel *et al.* 1993). The great variability in survival among patients with MDS of similar percentage of blasts has prompted us to investigate new objective, and independent prognostic parameters for the selection of high-risk patients who should benefit from new therapeutic approaches.

CD34 expression has been demonstrated selectively on hematopoietic progenitor cells, including CFU-Blast, HPP-CFC, LTBMC-IC, CFU-GM, and BFU-E (Civin *et al.* 1984; Holyoake and Alcorn. 1994). Recent reports have indicated that in a considerable proportion of AML cases, leukemic blasts expressed a CD34 surface antigen (Vaughan *et al.* 1988; Borowitz *et al.* 1989; Geller *et al.* 1990). According to the concept of "maturation arrest" in acute leukemia, it is conceivable that the CD34-positive (CD34+) represents "immature" biologic characteristics of leukemic blasts; a high frequency of CD34 expression may reflect the acute leukemia involving transformation of a primitive stem cell that undergoes limited differentiation. In addition, CD34+ AML was demonstrated to show distinct clinicopathologic features, including poor prognosis associated with a low rate of complete remission (Borowitz *et al.* 1989; Campos *et al.* 1989; Geller *et al.* 1990) and a relatively high frequency of chromosomal abnormalities (Borowitz *et al.* 1989; Geller *et al.* 1990). Therefore the evaluation of CD34 expression in MDS might be helpful to explain the biological heterogeneity by analysing abnormal hematopoietic clones. But the value of CD34 expression at diagnosis in patients with MDS has not been investigated extensively so much as in AML.

In this paper, CD34 expression in patients with MDS was evaluated by using an imunohistochemical staining of bone marrow biopsies. And the correlations of the pattern of CD34 expression with the clinical outcomes were analyzed. We observed that the high proportion of CD34+ cells among bone marrow nucleated cells and the high number of CD34 aggregates were predominantly observed in cases with RAEB, RAEB-T, and CMML. The median actuarial survival time in these high CD34 positivity group was significantly shorter compared to that of low CD34 positive group. The percentage of bone marrow blasts, and ALIP significantly correlated with number of CD34 aggregates. After multivariate analysis, the positivity of CD34 cells, and the percentage of bone marrow blasts remained as significant and independent parameters for prediction of survival.

It is not clear why CD34 expression in MDS adversely affects patient outcome. It does not seem to be associated with high probability of leukemic transformation according to our study. It was reported that in approximately 50% of patients with MDS the purified CD34+ cells did not respond to a stimulation of any kind of lineage-specific colony-stimulating factors such as GM-CSF, G-CSF, or interleukin-3 (Sawada *et al.* 1993). And the expression of CD34 was associated with low colony number and high cluster numbers, which probably represented the growth pattern of more immature cells without differentiation capacities (Guyotat *et al.* 1990). These findings suggest that CD34 expression in MDS is related to ineffective hematopoiesis including early intramedullary programmed cell death in the early hematopoietic stem cell level in MDS.

A study on the correlation between CD34 expression and biological characteristics, clinical outcomes in MDS was performed previously (Guyotat *et al.* 1990). But that study was performed by analysing phenotypes of aspirated bone marrow mononuclear cells harvested from patients with MDS (Guyotat, 1990). By contrast we used CD34 immunohistochemical staining of bone marrow biopsy specimens. By this method, it was not necessary to isolate mononuclear cells by ficolling, and it could provide further invaluable information about abnormal localization and cluster formation of CD34+ cells in the marrow space.

In conclusion, CD34 immunostaining, which can be easily performed on routinely prepared bone marrow biopsies, was found to be a powerful prognostic parameter in MDS. In addition, it further expands the usefulness of the histochemical examination of bone marrow biopsies in MDS. With advances in the treatment of these diseases, it is to be anticipated that the presence of CD34+ cells may influence the type of therapy chosen to treat subgroups of patients with MDS.

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