FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 44, No. 1, 2006
pp. 49-52

Prognostic significance of Ki67-negative blast cell clone in the high risk group of children treated for acute myeloid leukaemia

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Abstract: The aim of this study was to demonstrate the value of immunocytochemical staining of Ki67 antigen expression in blast cells of children with acute myeloid leukemia (AML) and to evaluate its correlation with treatment failure. The material included bone marrow specimens obtained during induction treatment from 46 children treated for AML between 1998-2003. Immunocytochemical staining for Ki67 was based on the ABC technique. Expression of Ki67 antigen on day 0 of induction treatment was confirmed in all patients. The percentage of immunopositive blasts ranged from 88.4% to 99.8% (mean 91.8%). On day 15, according to chemotherapy response, patients were divided into two groups: G1 - 36 children who responded to induction treatment and reached remission (blast level 5%, low risk group) and G2 - 10 patients who did not meet remission criterion (blast level > 5%) and were assigned to the high risk (HR) group. Out of 10 children assigned to this group, Ki67 expression in blast cells was confirmed in 4 cases. The fraction of immunopositive blasts ranged from 78.4% to 88.6%. In the other 6 cases, blasts were Ki67-negative. In 12-month period after beginning the treatment, 18 cases of treatment failure (including 7 deceases) were observed in both groups. Five deaths, observed in the HR group, concerned the patients characterized by Ki67-negative blasts. The results indicate a possible correlation between the Ki67-immunonegative blast pattern on day 15 of treatment induction and early decease of AML children assigned to HR group. (www.cm-uj.krakow.pl/FHC)

Key words: Ki67 - Acute myeloid leukemia - Prognosis

Introduction

Acute myeloid leukemia (AML) constitutes up to 15% of all leukemias in children [16]. Definition of AML encompasses a number of proliferative diseases developing secondary to neoplastic transformation of non-lymphocytic and poorly differentiated bone marrow cells. The current classification was established in 1976 by a French-American-British (FAB) team [2]. Modified in 1985 by supplementation with erythroleukemia and megakaryocytic leukemia [3, 4], it is based first of all on morphological criteria related to neoplastically transformed hematopoietic cells.

In children with AML, among other factors, the original leukocytosis in peripheral blood 50 G/l can be regarded as unfavourable [20]. Patient age < 2 yr, forms M4 and M5 [19] or genetic abnormalities are also used to be listed [21, 24]. In the Polish Pediatric Leukemia/Lymphoma Study Group, patients are included to the high risk group if the M5 subtype is preliminarily diagnosed or when leukemic blasts constitute > 5% nucleated cells of bone marrow on day 15 after commencing the treatment (AML-98 regimen) [5, 6]. In our observations, high risk group criteria are met by around 20% of patients [7]. Although the hematological remission is obtained in 70%-85% of those patients on day 29, follow-up survival without relapses and decease can be obtained in no more than 50% of patients [9]. This may indicate that the high histological and clinical malignancy in this group has not yet been counteracted with an appropriate therapeutic protocol.

In earlier study performed in a group of children with acute lymphoblastic leukaemia, we have found that the presence of Ki67-positive blast cells before the start of the treatment represents a potential index of favourable prognosis and can assist qualification of the patients to risk groups [14].

Ki67 antigen expression appears to occur in the middle of the G_1 phase of the cell cycle. It increases

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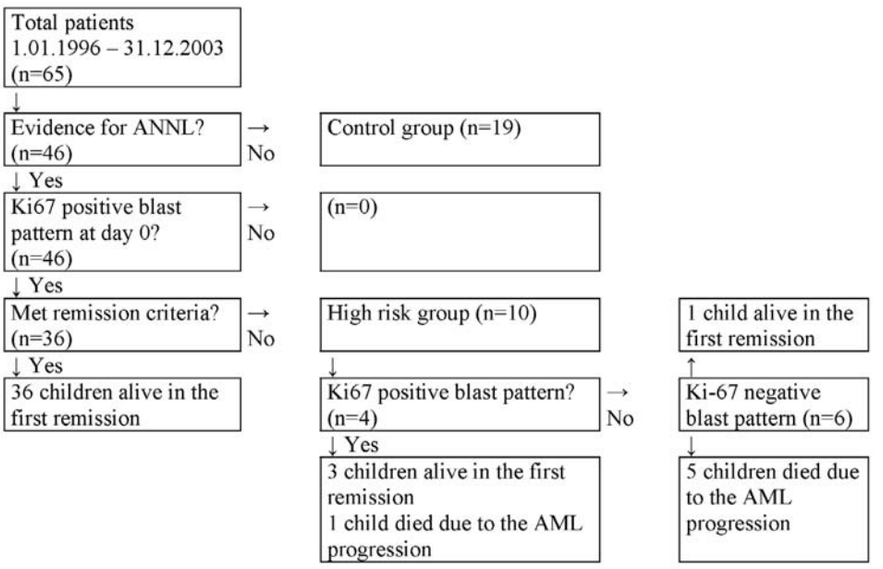

Fig. 1. Diagrammatic representation of the sample outcome.

during the S and G_2 phases to reach a peak during mitosis [23]. The Ki67 molecule becomes rapidly hydrolysed soon after the end of the M phase. Together with proliferating cell nuclear antigen (PCNA), the Ki67 antigen is one of the most sensitive markers of undifferentiated cell proliferation [1, 10, 23]. It is absent during the G_0 phase - the stage at which neoplastic cells are totally unresponsive to chemotherapy [23].

In line with the abovementioned findings, the aim of the present report was to demonstrate the value of Ki67 antigen expression in blast cells of patients with AML during chemotherapy regimen and to evaluate its correlation with early treatment failure.

Materials and methods

Material used in this study consisted of bone marrow samples obtained from children treated at the Department of Pediatric Oncology, Hematology and Transplantology, Poznań University of Medical Sciences, between 1998 and 2003. The research protocol was approved by the local Ethical Commission of the University. Sixty-five children referred to our Department were included in the investigation. Bone marrow biopsies were performed in all of them and evidence for neoplasia was found in 46 cases. The remaining 19 children, all of whom presented only one enlarged lymph node, served as the control group. In this group, histological examination of the enlarged node indicated an inflammatory response only. Subsequent observation of the children for around 12 months in the Outpatient Clinic for Hyperplastic Diseases, detected no clinical traits of a neoplastic disease.

Four children were diagnosed as AML type M0, 8 as AML M1, 26 as AML M2, 4 as AML M3 and 4 as AML M4. There were no patients diagnosed with AML M5, M6 and M7 types. The relevant data are presented in Table 1.

Bone marrow, according to ANLL-98 protocol, was sampled on days 0, 15 and 29 after starting the treatment. Its aspiration was carried out under intravenous anesthesia (pethidine 1mg/kg b.w., midazolam 0.1mg/kg b.w.). The samples were collected from the posterior superior iliac spine. The bone marrow smears were fixed in 96% ethanol (30 min, room temperature) within 24 hours of sampling and kept at -80˚C until immunophenotyping was performed.

Ki67 antigen detection involved an immunocytochemical procedure with the use of mouse monoclonal antibodies against human Ki67 (Dako, M 7187) and StreptABComplex/HRP technique amplified by the use of biotinylated tyramine (Dako Catalysed Signal Amplification System, Peroxidase, K 1500). Heat-induced antigen demasking pre-treatment was carried out (Target Retrieval Solution, Dako S 1699) [15, 17, 18]. The endogenous peroxidase activity was blocked by 10 min preincubation in 10% hydrogen peroxide. The smears were then treated with anti-Ki67 antibodies, diluted 1:500, for 12 h at 4˚C. Incubation with the secondary antibody (biotinylated goat anti-mouse, Dako E 0433, diluted 1:300) was performed at room temperature for 60 minutes. This was followed by the incubation with diaminobenzidine (DAB, Dako S 3000). The number of immunopositive blasts, as compared to the total number of blast cells, was established under a light microscope (magnification × 100). Quantitative analysis was carried out using MultiScan® computer software.

Immunocytochemical staining of the following antigens was used to verify leukemic and normal cells: HLA-DR, CD13, CD14, CD15, CD33, CD34, CD41 and CD61 [9]. The diagnosis of AML remission was confirmed by blood morphology (clinical remission), microscopic analysis of subsequent bone marrow samples (May-Grünwald-Giemsa staining, hematologic remission) and the second immunophenotyping of bone marrow nucleated cells (immunological remission).

In every case of Ki67 immunocytochemical detection, each laboratory test was performed at least twice. In addition, each examination of bone marrow was accompanied by positive and negative controls [8, 13]. All results which were doubtful, nonspecific, or difficult to interpret were excluded from the study.

Since the studied group was relatively small, the statistical analysis was based on the Fisher exact test.

Results

Expression of Ki67 antigen on day 0 of initial treatment was confirmed in all patients with AML. The percentage

Table 1. Clinical characteristics and data on disease course in patients with acute myeloid leukaemia (AML)

Study group	AML type (markers)	Number of patients	Age (yr)	Risk group	
				HR (%)	LR (%)
1	M0 (HLA-DR, CD33)	4	11.3 1.2	0 (0)	4 (100)
2	M1 (HLA-DR, CD13, CD33)	8	10.8 5.2	4 (50)	4 (50)
3	M2 (HLA-DR, CD13, CD33, CD15, CD34)	26	9.8 4.6	5 (19)	21 (81)
4	M3 (CD13, CD33, CD15)	4	8.9 4.8	1 (25)	3 (75)
5	M4 (HLA-DR, CD13, CD14, CD15, CD33)	4	9.4 6.7	0 (0)	4 (100)

of immunopositive blasts ranged from 88.4% to 99.8% (mean of 91.8% blast cells) and did not significantly differ between AML subtypes. Ki67 was absent (< 5% nucleated cells) in the normal hematopoietic cells of 19 children in the control group.

The AML remission was diagnosed in 36 children (78.3%, low risk group) on day 15 (mean 3.2% leukemic cells). In 10 children (21.7%, high risk group) disease progression was diagnosed (4 cases of AML type M1, 5 of M2 and 1 of M3, respectively) with a poor response to cytoreducing treatment on days 15 and 29 of treatment with cytosine arabinoside, idarubicin and etoposide (a reduction of the bone marrow blast level by less than 5%).

In 4 out of 10 children assigned to the high risk group, Ki67 expression in blast cells was confirmed on day 15 (40.0%). The fraction of immunopositive blasts ranged from 78.4% to 88.6%. In the remaining 6 cases (60.0%), blasts were Ki67-negative.

During the observation period (12 months), 18 cases of early leukemia treatment failure were noted. In the low risk group one child died due to *Pseudomonas aeruginosa* infection. In 7 cases, the subsequent bone marrow examination was performed due to relapse of the disease. It revealed Ki67-positive blasts (mean 88.5%) in 3 children and Ki67-immunonegative blast pattern in the other 4 children. The treatment protocol was changed to a more aggressive Ida-FLAG program [11] followed by bone marrow transplantation and, except of one Ki67 immunonegative patient, these children are still alive in their second remission. In the high risk group, 6 children died due to progression of the disease resulting from the pan-drug resistance. Apart from one child who was Ki67-positive on day 15, all other patients revealed an immunonegative Ki67 blast pattern on that day of treatment. The detailed information is presented in Figure 1.

The probability of the first remission was significantly higher in the low risk group patients (p=0.0001), but independent from the initial Ki67 expression.

The distribution of mortality in high risk group patients was Ki67-dependent (regardless of AML type). Statistical analysis of these children revealed that the probability of permanent leukemia remission is significantly higher in patients with Ki67-immunopositive blast pattern on day 15 (p=0.0194). It has been also shown that the risk of death following leukemia progression in patients with Ki67-immunonegative blast pattern treated according to only ANLL-98 programme is significantly higher (p= 0.0194) as compared to the patients who were Ki67-negative but were treated according to Ida-FLAG scheme.

Discussion

Ki67 antigen, which is regarded as a marker of proliferative cells, has been identified as a free peptide in the nucleus or complexed with DNA [12]. Despite the optimal fixation and the amplified antigen detection, some neoplastic cells in the G_1 and G_2 phases fail to reveal immunocytochemical expression of the marker. In the experimental study on cell populations with surface phenotyping of human T-cell leukemia (Molt-4 human leukemia cells), the cell cycle was arrested at G_1 and G_2 phases using 10 nM 12-0-tetra-decanoylphorbol-13-acetate. When applied to cell cultures, it reduced Ki67 expression to nondetectable levels, if standard immunocytochemical methods were employed [24]. Thus, some cells with a relatively long G_1 phase gradually lose their ability to express Ki67 during G_1 and G_2. There is, therefore, a population of G_1 Ki67 negative blasts which can still proliferate.

The above observations correspond to results of the present study. In the majority of the high risk group children in whom Ki67-immunonegative blast pattern has been detected on day 15 of induction regimen, the treatment has ended in a failure.

It is interesting that the high percentage of Ki67-positive blasts has been found in bone marrow before treatment in all the children, regardless of AML type or future complications. It seems to indicate that originally all neoplastic cells in AML may exhibit high sensitivity to chemotherapy. However, in the course of the undertaken induction therapy the originally homogenous population of blasts becomes selected into Ki67-positive and Ki67-negative cells. At this stage of treatment, the most important day is the day 15, on which the potential to reach stable remission may become determined.

The present protocol of AML treatment (ANLL-98) does not introduce Ida-FLAG regimen to children who did not respond to induction treatment on day 15. It becomes recommended when the AML relapse is recognised. In our study, seven early relapses were noted in low risk patients. Although in 4 of them Ki67-negative clone of blast cells was selected, 3 children have reached the second remission and after bone marrow transplantation they are still alive in their second remission. It should be emphasized that such a remission was reached only by the single child of high risk group who was Ki67-negative on day 15 and had undergone the AML-98, no-Ida-FLAG, protocol.

Without doubt, oncological treatment may be effective only when it is adjusted to aggressiveness of the disease. Therefore, recognition of risk factors is of key importance for designing the effective therapy in any oncological disease. In this aspect, the AML status is less favourable than that of ALL. This reflects less frequent manifestation of AML and its higher pathogenetic variability. The selection of Ki67-negative clone of blasts cells seems to be a potent unfavourable factor of childhood myeloid leukemia. However, introduction of Ida-FLAG protocol to all children who are Ki67-negative on day 15 needs a further multi-centre investigation.

In conclusion, Ki67 immunonegative blast pattern on day 15 of the induction treatment in acute myeloid leukemia may represent a new index of unfavourable prognosis in the high risk group children. The obtained results may also justify routine determination of Ki67 antigen before and during the treatment regimen of acute myeloid leukemia.

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Received: June 29, 2005
Accepted after revision: July 18, 2005