Cytometric evaluation of transferrin receptor 1 (CD71) in childhood acute lymphoblastic leukemia

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Abstract: Transferrin receptor 1 (CD71) is a transmembrane glycoprotein responsible for cellular iron uptake. Higher expression of CD71 has been identified as a negative prognostic marker for numerous solid tumor types and for some lymphomas. The aim of this study was to evaluate CD71 expression on acute lymphoblastic leukemia (ALL) cells and to follow its possible clinical correlations. Sixty one patients, aged 1–17 years and diagnosed with ALL, were enrolled in the study. CD71 expression was analyzed on the bone marrow blastic cells by flow cytometry. CD71 expression on the leukemic blasts was diversified; in most patients, all blastic cells showed expression of CD71, but levels of expression varied. CD71 expression was statistically higher on T-lineage leukemias. Within the B lineage ALL, a significant difference in CD71 expression existed between precursor B ALL and mature B-ALL, which showed higher CD71 expression. CD71 expression positively correlated with Hgb concentration at diagnosis. Initial risk group assessment and therapy response were not correlated with CD71 expression, although disease free and overall survival times tended to be shorter in patients with B-lineage leukemias with initial high CD71 expression. (Folia Histochemica et Cytobiologica 2012, Vol. 50, No. 2, 304–311)

Key words: transferrin receptor 1, CD71, acute lymphoblastic leukemia

Introduction

The transferrin receptor 1 (TfR1), designated CD71, is a type II transmembrane homodimeric glycoprotein (180 kDa) responsible mainly for cellular iron uptake via internalization of iron loaded transferrin [1, 2]. This makes iron available for heme synthesis and for many metabolic processes necessary for cell growth and proliferation, including DNA synthesis, electron transport, nitrogen fixation and oxygen sensing [1, 2]. CD71 is expressed at high levels on cells with high iron demand, such as maturing erythroid cells and placental trophoblast cells, and on cells with a high proliferation rate, such as cells of the basal epidermis and intestinal epithelium [2–4]. Activated peripheral blood mononuclear cells express high levels of TfR [2]. This may be attributable to the increased need for iron as a cofactor of ribonucleotide reductase, which activity supplies substrates for DNA synthesis [5]. CD71 overexpression has been reported in many neoplasms, including solid tumors such as lung [6, 7], bladder [8], colon [9], pancreas [10] and breast cancer [11, 12], gliomas [13] as well as hematological malignancies [5, 14–16]. Higher expression of CD71 has been identified as a negative prognostic marker for numerous solid tumor types [10, 12] as well as for non-Hodgkin’s lymphoma [4, 17, 18]. Antibodies targeting CD71 have been introduced as an experimental adjuvant anticancer therapy [19].
There is still very little information on the possible importance of CD71 in childhood acute lymphoblastic leukemias (ALL), and the data that is available is conflicting [17, 20, 21].

The aim of this study was to evaluate CD71 expression on acute lymphoblastic leukemia cells and to follow its possible clinical correlations.

Material and methods

Patients. Sixty-one patients diagnosed with ALL in the Department of Pediatrics, Hematology, Oncology and Endocrinology, Medical University of Gdansk, from whom a surplus bone marrow (BM) was available after the diagnostic biopsy, were enrolled into this study between 2005 and 2009. The only inclusion criteria were diagnosis of acute lymphoblastic leukemia and material availability.

There were 27 girls and 34 boys, median age 5.3 years (from 1 to 17.6 years); with no significant differences between the genders. The bone marrows were routinely assessed by the hematological diagnostic laboratory and the leukemic blasts were classified according to their morphology (following French-American-British classification: L1–L3) and immunophenotype.

55 patients were diagnosed with B-lineage ALL, five patients had T-lineage ALL, and one patient biphenotypic T/B ALL. Immunophenotyping: 45 (74%) patients had common ALL, three (5%) proB, three (5%) prepreB, two (3%) preB, two (3%) mature B-ALL, four (6.5%) T-cell ALL, and one (1.5%) preT ALL.

All BMs were cultured in order to obtain karyotype; chromosomes were obtained in 38 patients (62%) and 29 were found to have chromosomal abnormalities, including 17 cases of hyperdiploidy and 12 cases of structural abnormalities. Bcr-abl mutation was investigated by fluorescence in-situ hybridization (FISH) in 49 patients, and was found positive in three.

Clinical data was retrospectively obtained from the routine clinical evaluations. Risk group qualification was based on the ALLIC 2000 criteria and was performed after 33 days of treatment. Patients were categorized as standard (SR), intermediate (IR), or high risk (HR). Patients who had died before the risk group assessment were, for the purposes of statistical analyses, allotted to the high risk group.

Organ involvement. Central nervous system involvement was defined as organ margin palpable more than 1 cm below the appropriate costal margin.

Hematological abnormalities. Initial peripheral blood (PB) morphology was routinely evaluated with a Sysmex XE 2100 analyzer. White blood cell (WBC), hemoglobin (Hgb) and platelet (PLT) count were analyzed. Hyperleukocytosis was defined as PB white blood cell count (WBC) > 50,000/µl.

Biochemistry. Initial PB serum uric acid (UA) and lactic dehydrogenase (LDH) were measured.

Treatment response. Treatment response was evaluated according to the ALLIC 2000 protocol: initial steroid response was evaluated based on the absolute count of blasts cells per µl in the PB on the 8th day of the treatment protocol (Day 8 blastosis). Induction therapy response was assessed by microscopic analysis of the bone marrow specimens collected on the 15th and 33rd days of the chemotherapy protocol (respectively Day 15 BM and Day 33 BM). Remission pattern was denoted M1 (< 5% blasts cells in the BM, complete remission), M2 (5–25% blast cells, partial remission), or M3 (> 25% blast cells, no remission).

Disease-free survival (DFS) and overall survival (OS). DFS was defined as survival without leukemia relapse; for OS, deaths from all causes were included. All clinical evaluations are summarized in Figure 1.

Sample preparation. BM was obtained by aspiration biopsy; preservative free heparin was used as an anticoagulant. All biopsies were performed prior to treatment implementation. Bone marrow was first immunophenotyped to select markers for further blasts' staining. Mononuclear cells were isolated by density gradient centrifugation on Histopaque 1077 (Sigma–Aldrich). Interphase cells were collected and washed twice in RPMI medium (Sigma–Aldrich). Aliquots of 0.3 × 10⁶ cells/50 µl were used for further staining. From the separation onwards, all staining/washing steps were performed at 4°C.

Antigen expression. CD71 surface expression was measured on bone marrow blasts. Staining to identify blasts cells (either CD19/CD10 or CD19/CD34 for B-lineage ALLs or CD7/CD34 for T-lineage ALLs) was chosen based on the routine bone marrow immunophenotyping performed for diagnostic purposes. The percentage of blasts cells in our bone marrow samples varied from 45% to 100% with a median of 90%. Bone marrow mononuclear cells were stained with the following antibody conjugates: anti-CD71 FITC/anti-CD19 (or anti-CD10) PE/anti-CD34 (or anti-CD19) PE-Cy5 or anti-CD71 FITC/anti-CD7 PE/anti-CD34 PE-Cy5 (all antibodies were from BD Biosciences). 5 µl of each monoclonal antibody was added to the appropriate tubes and incubated for 30 min. Finally, the cells were washed and analyzed by flow cytometry. Each sample was run with an appropriate isotype control.
Flow cytometry analysis. Samples were analyzed by flow cytometry using a Becton Dickinson FACScan instrument equipped with a 488-nm argon laser. Five data parameters were assessed: linear forward and side scatter (FSC, SSC), FL-1(FITC), FL-2(PE) and FL-3 (PE-Cy5). CellQuest (Becton Dickinson) and FlowJo (ThreeStar) softwares were used for data analysis. Mean fluorescence intensity (MFI) was used as a measure of antigen surface expression. For individual samples, an anti-CD71 FITC δMFI was calculated: \(\delta\text{MFI} = \text{MFI of the population of interest} – \text{MFI of the appropriate isotype control (Figure 2)}. \) MFI is expressed in arbitrary values.

Statistical analysis. Statistica 8.0 PL (StatSoft) software was used for all of the analyses. The statistical significance of the results was analyzed by means of Mann–Whitney U test and Kolmogorow–Smirnoff. Spearman rank test was used to assess the correlation between the variables. Differences were considered of statistical significance if the p value was < 0.05. Disease free survival time and overall survival time measured from diagnosis were estimated according to the method of Kaplan–Meier and compared between groups by means of log-rank test.

The study was approved by the Local Ethics Committee; the patients and/or their parents (legal guardians) gave informed consent to participate in the study.

Results

CD 71 expression found on the leukemic blasts was diversified. In 56/61 patients (92%), all blastic cells showed expression of CD71 with \(\delta\text{MFI} \) median of 13 (ranging from 2 to 284), in four cases CD71 was found on a subpopulation of 17–24% blasts, only one leukaemia did not show expression of CD71 (Figure 2). CD71 expression did not differ between sexes — \(\delta\text{MFI} \) median 10.6 for girls and \(\delta\text{MFI} \) median 15.6 for boys, nor did it show a correlation with patient age (Rs = 0.023).

Immunophenotype

CD71 expression was statistically higher on T-lineage leukemias, with \(\delta\text{MFI} \) median for B-lineage ALL of 12.4 (range 2–100) and 27.9 for T-lineage ALLs (range 14–284) (\(p = 0.01\)) (Figure 3).
Within the T-lineage group, the lowest MFI was noted in the only pre-T leukemia, while T-ALLs all showed higher MFIs. Within the B-lineage group, the highest MFI was seen on the mature B and pro B blastic cells.

Within the B lineage ALL, a significant difference in CD71 expression existed between precursor B ALL and mature B-ALL showing higher CD71 expression levels (p = 0.02) (Figure 4).

Chromosomal abnormalities

The presence of chromosomal abnormalities did not correlate with CD71 expression. Patients with chromosomal aberrations showed median CD71 expression of 15 (range 2–118) vs. 15.9 (range 6–284) in patients with normal karyotype of the leukemic blasts (p = 0.49). Hyperdiploidy was also not related to CD71 expression (median 10.5 in the hyperdiploidy group vs. 15.9 in non-hyperdiploid blasts; p = 0.23), nor was bcr-abl presence (32.1 vs. 13.6; p = 0.17).

Initial laboratory and clinical parameters; initial organ involvement

Patients with initial hyperleukocytosis (n = 11) showed markedly higher CD71 expression on the blastic cells with median δMFI = 25.7 compared to the non-hyperleukocytosis patients (n = 50) with δMFI = 11.8 (p = 0.007336), although it was due to the prevalence of hyperleukocytosis in T-lineage leukemias. When only B-lineage ALLs were analyzed, the difference, although present (δMFI = 11.5 for patients with hyperleukocytosis [n = 6] and 15.4 for the remaining patients), was not statistically significant (p = 0.26).

δMFI CD71 positively correlated with Hgb concentration at diagnosis (Rs = 0.31), PLT count...
(Rs = 0.35) and with UA (Rs = 0.30) and LDH (Rs = 0.26) concentrations. Correlation with Hgb was stronger in the group of ALLs with lower CD71 expression (dMFI < 35, n = 53) (Figure 5).

CD71 expression on the blastic cells did not differ between patients with extramedullary organ involvement (Table 1).

### Therapy response and risk groups

CD71 expression did not differ statistically between the clinically evaluated risk groups. Therapy response was assessed based on several parameters: initial response to the steroid therapy expressed as a PB blastosis on the 8th day of treatment, bone marrow analysis on the 15th and 33rd day of treatment. None of these parameters showed correlation with CD71 expression (Table 2).

### Disease-free survival (DFS) and overall survival (OS)

There were no relapses or deaths in the T-lineage ALL groups. The B-lineage group was divided into two groups based on the MFI expression — MFI < median (i.e. MFI < 13) and MFI > median (MFI > 13). The 5 year DFS and 5 year OS probabilities were lower in the MFI > 13 group (DFS 0.64 vs. 0.91, Figure 6 and OS 0.81 vs. 0.97, Figure 7) The mortality rate in the B-lineage ALL with CD71 MFI < 13 was 4% (1/25), while in the MFI > 13 group 20% (4/20) patients had died by the end of the observation time.

### Discussion

In normal human tissues, CD71 is found predominantly in the basal epidermis, endocrine pancreas, hepatocytes, Kupffer cells, testes, and the pituitary...
gland; it has also been detected on activated lymphocytes, natural killer cells, monocytes and macrophages [15, 22–25]. In hematopoiesis, CD71 is not present on a pluripotent stem cell, next it is detectable on erythroid progenitors, and again it is not detected on mature cells of the erythroid line [26]. Myeloid line bone marrow precursors are variably CD71 positive: dividing cells on the level of blast to myelocytes are all CD71 positive, while non-dividing late precursors (from metamyelocytes) are predominantly CD71 negative [26–28]. Quiescent PB lymphocytes, of both T and B lineages, lack CD71 which is produced in them after stimulation [2].

In contrast to a limited pattern of expression in normal tissues, CD71 is reported to be widely present on neoplastic cells [2, 15]. Yeh et al. found increased transferrin binding in peripheral blood mononuclear cells from patients with leukemia, lymphoma and myeloma [29]. In B-cell chronic lymphocytic leukemia, CD71 expression is heterogenous, with some cases showing high expression [30–32]. Both B and T lineage non-Hodgkin’s lymphomas have been reported to express CD71, with the expression level correlating with the lymphoma grading, though not with patient survival [18, 33]. Transferrin receptor is overexpressed in adult T-cell leukemia, a neoplasm related to human T-lymphotropic virus type 1 (HTLV-1) with a poor prognosis [34, 35]. It is also overexpressed in acute myeloid leukemia [14]. Investigation of CD71 expression and importance, although ongoing in many adult malignancies, did not draw much attention in childhood leukemias.

ALL in children has thus far been evaluated in terms of CD71 expression by two authors only [21, 17]. Koehler et al. investigated CD71 expression in childhood ALL by flow cytometric immunophenotyping, and found that 92% of T-lineage ALLs and 32% of B-lineage ALLs were CD71 positive [21]. Das Gupta et al. performed an immunohistochemical study of CD71 in ALLs and showed 62% of T-lineage ALLs were CD71 positive, compared to 10.9% of positive common ALLs [17]. One more paper addressed CD71 expression in ex vivo ALL specimens from adults [20]. Glasova et al. reported CD71 expression on a few common ALLs with less than 10% of positive cells in the PB. Ten cases of T-ALL analyzed in this study showed a higher percentage of CD71 positive cells: from 13% to 75% [20].

Our study showed CD71 is present on blasts of almost all ALL cases, the expression being uniform within one case but significantly varied between cases. T-lineage ALLs showed higher CD71 expression than B-lineage leukemias, which is in concordance with the previously published data. Surface CD71 expression has been proven to be a downstream marker of Notch mediated PDK1 activation [20, 36, 37] and further proposed as a downstream marker of mTOR activity [38]. It seems that activity of survival pathway PI3K/PDK1/Akt/mTOR is linked with CD71 expression. In this context, high expression of CD71 on T-cell malignancies is not surprising as the importance of Notch signaling for T cell development has been well established [37]. Gain of function mutations of NOTCH [39, 40] as well as inactivating mutations of the suppressor PTEN and increased activity of PI3K/Akt pathway have been frequently identified in T cell ALL [41].

We demonstrated CD71 expression on most B-lineage ALLs including common ALL. Other authors report low frequency of CD71 positives in common ALL. This discrepancy could be explained by a different analysis approach whereby leukemias were classified as CD71 positive or negative only. Analysis of mean florescence intensity of CD71 staining on blasts allowed for identification of patients expressing smaller amounts of TfR1, which would be excluded by standard positive-negative analysis (Figure 2) [42]. Analysis of CD71 as a quantitative feature reveals more correlations with clinical data, such as Hgb and PLT. Like Das Gupta et al., we also found correlation of CD71 with initial hyperleukocytosis, but it was due to high frequency of hyperleukocytosis in T-lineage ALLs. The group analyzed by Das Gupta contained a relatively higher proportion of T-ALL cases, which is characteristic for the Indian subcontinent. The involvement of Notch and PI3K/Akt signaling in the development of some B-lineage acute lymphoblastic leukemia cases is emerging in the literature [39, 40, 43]. Diversity of CD71 expression among lymphoblastic leukemias originating from the B-lineage observed in this study might indicate different levels of PI3K/Akt/mTOR pathway activity in this group [44].
We also attempted an analysis of CD71 putative prognostic value in precursor-B ALL and found less favorable outcomes in patients with higher CD71 expression. Though the observation did not reach statistical significance, taking into account a relatively small number of events in our group and a short time of the follow-up, it seems noteworthy. Kohler et al. showed no relation between CD71 positivity and the EFS in a large group of patients. It is difficult to compare our data with Kohler’s, as two decades ago EFS oscillated around 70% after three years, while in our material in the low CD71 group it exceeds 90% in a five-year perspective. Different protocols, improved supportive therapy, and improved outcomes of bone marrow transplantations have improved survival rates in all centers, making direct comparisons between old and new data impossible. Our group consisted of all the patients with ALL from whom bone marrow specimens were available over a period of four years in a single center with the same treatment protocol. The number of events noted allowed for statistical EFS and OS analysis only for B-lineage ALLs. We are continuing the follow-up of the patients included in this study.

In recent years, increasing effort has been focused in pediatric oncology on identifying molecular features of malignant cells in order to establish new risk factors, especially in the clinical low risk groups. The role of TfR1 requires further investigation in this context.

Acknowledgements

This study was supported by Polish Ministry of Science and Higher Education grant No 2 P05E 103 30.

References


Submitted: 24 October, 2010
Accepted after reviews: 18 September, 2011