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Immunophenotyping parameters as prognostic factors in T-acute leukemia patients

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The main aim of this study represents the extension of our studies using multiparametric flow cytometry analysis for exact definition of membrane and intracellular (cytoplasmic and nuclear) markers of acute leukemia cells of T-phenotype. The study of blasts of each patient with all available monoclonal antibodies targeted to T-cell differential antigens and against possible marker coexistence from different lineages has been performed. The main aim was concerned to more proper T-ALL diagnosis and stage definition and identification of the prognostic factors and the useful markers for the follow-up of T-ALL in remission. New knowledge of the T-cell maturation stages of hematopoietic cells in bone marrow and thymus has been applied, as each T-acute leukemia clone is representative of one blocked stage through maturation.

We evaluated 44 patients with T-ALL by multiparameter flow cytometry. Patients with more favorable prognosis (i. e. those of cortical stage) could have been already differentiated at diagnosis from those, allocated to pro-T stage, with very immature phenotypes and of an unfavorable clinical course. These patients had very distinctive immunophenotes, CD1a and CD8 markers completely negative, CD7 and cCD3 positive; CD5 was weakly expressed and myeloid markers CD33 and CD13 were coexpressed, or immature markers CD34, HLA-DR were coexpressed, together with myeloid markers CD13 and CD33 of weak positivity. The patients were either completely unresponsive to therapy or because of persistent MRD during continuation therapy, indicated for allogeneic hematopoietic stem-cell transplant. The results have been discussed with similar the most relevant immunophenotypic results of others and mainly with gene-expressing profiling associated with a significantly worse clinical outcome.

Key words: T-acute lymphoblastic leukemia, T-cell differentiation antigens, T-cell maturation sequence, prognostic factors of follow-up of T-ALL patients, multiparameter flow cytometry

T-cell acute lymphoblastic leukemia (T-ALL) is a malignant clonal expansion of immature T-cells that accounts for 10-15% of childhood and 25% of adult ALL cases. With wider use of intensive chemotherapy, the prognosis for childhood T-ALL has improved remarkably; nearly 75-80% of patients can currently be cured [1, 2]. In adults, the long-term survival rate only reaches 30-40% [1, 3]. Further gains of treatment outcome will require methods to identify patients who continue to fail on contemporary protocols, so that alternative therapy can be introduced as early as possible.

The T-cell differentiation begins in the bone marrow (BM) and is completed in the thymus. Normal T-cell differentiation proceeds through sequential stages defined according to the expression of CD4 and CD8, from double negative to double positive in the thymic cortex, to single positive thymocytes in the medulla. Double-positive cells temporary express CD1a and CD10 (cortical thymocytes). Thymocyte maturation encompasses sequential rearrangement and expression of the TCR chains, and cytoplasmic followed by surface CD3 expression. Subgroups of T-cell lymphoblastic neoplasms have been defined by immunophenotyping, and can be matched (even though not unambiguously) to the various stages of normal thymocyte development [4]. Gene expression profiling and cytogenetics have identified corresponding molecular-cytogenetic sugroups, based on the aberrant expression of T-cell-specific transcription factors and the associated genetic rearrangements. This classification seems to have some prognostic significance [2].

The EGIL classification [4] recognizes the four differentiation stages of the neoplastic clone: pro-T, pre-T, cortical and mature T-ALL (Table 1). The cortical stage has been associated with a better outcome [5].

Efforts in outcome have been made to identify additional antigens of prognostic significance that may drive therapeutic decisions. FISHER et al. [6] showed that CD56, whose expression is correlated with an inferior outcome in acute myeloid leukemia (AML) and aggressive lymphoma, is associated in T-ALL with a lower remission rate and a higher percentage of resistant disease. Other antigens that have been associated with outcome include

CD2 [7], CD34 [8] and the coexpression of myeloid markers. The myeloid antigen CD33 is also expressed in a small proportion of T-ALL cases, though at a low level of expression [5, 9].

More recent studies have provided insights into the genetic abnormalities underlying T-ALL development, some of which seem to correlate well with prognosis [10, 11, 12, 13].

COUSTAN-SMITH et al. [8] described lymphoblasts with an early T- precursor (ETP) phenotype, which have ETP-related-gene-expression signature or its associated distinctive immunophenotype ($CD1^-$, $CD8^-$, $CD5^{weak}$ with stem cells or myeloid markers). Patients with this form of leukemia had high risk of remission failure or hematological relapses. ETP-ALL is a distinct, previously unrecognized pathobiological entity that confers a poor prognosis with use of standard intensive therapy. Its early recognition, by use of the gene expression and distinct immunophenotypic criteria, is essential for the development of an effective clinical management strategy.

The main aim of this study was the identification of prognostic factors and useful markers for the follow-up of T-ALL patients in remission. The further aspect was to assess the coexsistence of the blast´ surface or cytoplasm markers from different lineages, which is one of the most common characteristics used to identify these malignant cells when they become very scarce (minimal residual disease – MRD).

Materials and methods

The principal source of biological material utilized for research program was obtained from clinical samples delivered to the laboratory for phenotypic diagnosis.

Table 1. Immunological classification of T-lineage ALL according to the EGIL proposal

	cCD3	CD7	CD2,CD5, CD8	CD1a	CD3+/ CD1a-
T-I (Pro-T)	+	+	-	-	-
T-II (Pre-T)	+	+	+	-	-
T-III (cortical)	+	+	+	+	-
T-IV (mature)	+	+	+	-	+

Cervical thymus tissue from 14 year old female was sent to our laboratory to exclude malignancy. Single cell population was obtained by mechanical homogenization of the tissue and has been used as a control for immunophenotyped T-ALL samples. Heparin anicoagulated bone marrow and/or peripheral blood samples from children and adults (33 were males and 11 females) with mean age 16.7 (from 2 – 55 years) and with age distribution <18 years 30 patients, >19 years 14 patients.

Multiparameter flow cytometry was performed using a lysed-whole-blood technique without isolation of cells on density gradient, using a commercially available red cell lysing solution. Percentages of all cell types were determined based on total events of all BM cells. The cells labeling and membrane fixation/permeabilization procedures have been done by standard methods; cell staining was performed as in our previous study [14]. Monoclonal antibodies targeting membrane antigens directly conjugated by 4 fluorochromes (fluorescein isothiocyanate [FITC], phycoerythrin [PE], R-phycoerythrin-texas red [ECD], phycoerythrincyanin

Table 2. Monoclonal antibodies used for immunophenotyping T-acute leukemia

	FITC	PE	ECD	PC5
T-ALL	CD2, CD3, CD4, CD8, CD10, CD7, CD5, CD34, TCRγδ	CD1a, CD3, CD5,CD7, CD8, CD4, TCRαβ, CD34	CD45, CD3	CD45, CD7, CD34

Table 3. Immunophenotyping of different T-ALL subtypes

T-ALL type	CD3	CD7	CD4	CD8	CD4/ CD8	CD1	CD2	CD5	TCRαβ	TCRγδ	CD10	cCD3	nTdT	CD34	HLA DR	CD19	CD13	CD33
	marker expression													marker coexpression				
pro-T	0/2	2/2	0/2	0/2	0/2	0/2	0/2	1/2	0/2	0/2	0/2	2/2	NT	1/2	1/2		2/2	2/2
pre-T	8/10	10/10	4/10	8/10	0/10	0/7	7/9	9/9	1/5	0/5	6/10	4/6	1/1	6/10	4/10	1	1	
cortical	14/26	25/26	23/26	23/26	17/25	19/23	17/22	21/21	5/18	2/18	17/26	15/15	16/16	3/24	0/14			
mature	1/1	1/1	0/1	1/1	0/1	0/1	1/1	1/1	1/1	0/1	0/1	NT	NT	0/1	0/1			
NHL	3/5	5/5	4/5	4/5	2/5	1/4	2/4	5/5	0/3	1/3	2/4	2/2	1/1	2/4	0/1			

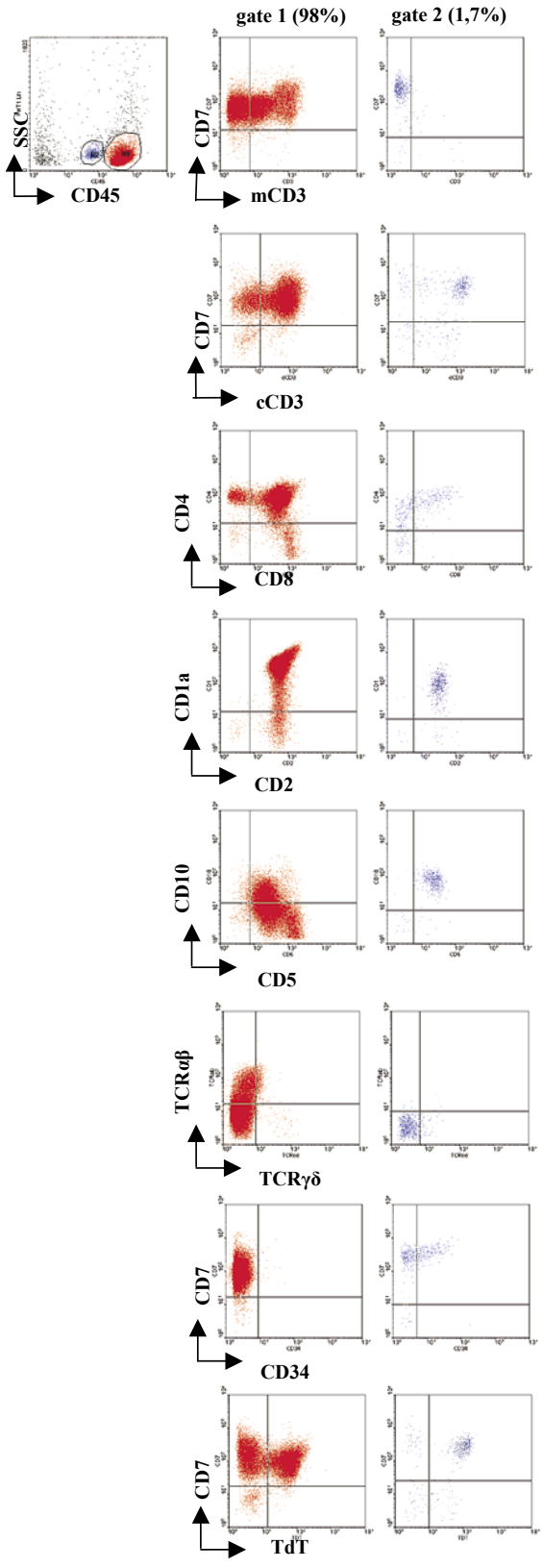

Figure 1 Immunophenotype of normal thymus cells from 14 years old female.

5 [PC5]) have been used. For the identification of T-cell precursors and leukemia cells of T-lineage the main quadruple combinations of monoclonal antibodies were selected from those given in Table 1. To detect aberrant phenotypes in leukemia blasts some other combinations of monoclonal antibodies were used according to our experience. Antigen expression on BM cells was systematically analyzed by flow cytometry EPICS ALTRA equipped by EXPO32 program for analysis (both from Beckman Coulter). At diagnosis, 30 000 events were evaluated and leukemic blasts were identified by their light-scattering properties and gated on CD45.

Results

The most common phenotypic pattern of examined samples of T-cell origin (thymus and T-ALL cases) was the coexistence of terminal deoxynucleosid transferase (TdT) with CD7 and/or cytoplasmic (c)CD3. Traditional criteria for aberrancy have included T-cell predominance, T-cell subset restriction, considerable antigen deletion, coexpression or loss of both CD4 and CD8 and expression of antigens not normally present on T cells (Table 3). For comparison of T-ALL cases and their correct allocation to the classification stages according to EGIL [4], flow cytometric analysis of the normal thymocytes is given in Fig.1. Dot plots of thymus tissue from 14 year old female sent to our laboratory to exclude malignancy are given separately for two gated populations, larger one (gate 1, red color), defining more mature cortical phenotype and a smaller, more immature population (gate 2, blue color) with expression of CD34 and without expression of TCRαβ, γδ.

The diagnoses of 44 patients analyzed by multiparameter flow cytometry for T-ALL populations of both, children and adults are summarized in Table 3. Two patients (4.5%), have been allocated into the most immature T-I, pro-T stage, ten patients belonged to T-II, pre-T stage (22.7%). The most frequent was cortical, T-III stage, with 26 (59.1%) patients. Into the mature, T-IV stage one patient was allocated (2.2%). 5 cases of T-lymphoma (11.3%) were immunophenotyped simultaneously. More common T-ALL subtype is present in T-III, cortical stage, with the most characteristic markers CD1a and CD4/CD8-double positivity. An example is given in Fig. 2. In two of studied patients, both adults, allocated to T-I, pro-T stage, very immature phenotypes could be observed. In both, CD1a and CD8 were completely negative, CD7 and cCD3 positive; in the 1st one CD5 was weakly expressed and CD33 and CD13 were lightly positive (Fig. 3). In the 2nd patient CD34, HLA-DR markers were positive and CD13 and CD33 were weakly expressed. This cell-marker profile (i. e., the pro-T immunophenotype) clearly differred from that of most normal thymocytes (Fig. 1).Both patients were adult males, 21 and 28 year-old, with worse clinical outcome. The 1st one was completely unresponsive to therapy and died on day 13 after diagnosis. In the 2nd patient because of persistent MRD during continuation therapy allogeneic hematopoietic stem-cell transplant has been indicated and patient is still in remission.

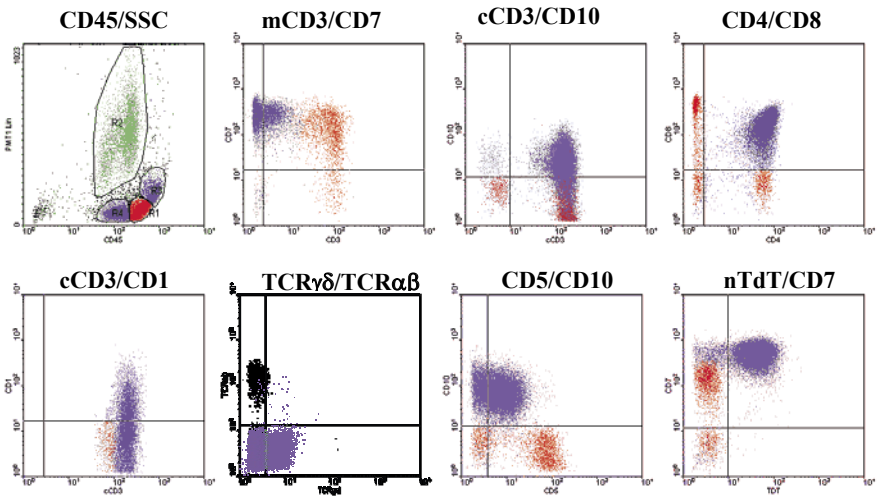

Figure 2 Dot-plots of T-cell markers of cortical stage of T-ALL. Violet cells are pathological gate and red cells are residual normal lymphocytes.

Expression of antigens not normally present on T cells was found in 22 cases (50%) of 44 T-ALL studied patients. Mostly coexpressed antigens CD34, HLA-DR, CD19, CD13 and CD33 were observed (Table 3).

Discussion

By immunophenotyping, the lymphoblasts in T-ALL usually express TdT, and can be stratified into different stages of thymocyte development according to the expression of the T-cell receptor (TCR) components and the other antigens linked to normal T-cell differentiation, reflecting the immunophenotypic properties of their putative normal cellular counterparts [15]. However, because of asynchronous and aberrant antigen expression in neoplastic lymphoblasts, many cases of T-ALL/LBL cannot be matched unambiguously to a thymic differentiation stage.

Prognostic factors for patients with T-ALL have been unreliable in early studies [16, 17], with only marginal differences in outcome for subgroups defined by cell-marker expression, patients with T-ALL have been treated uniformly in all major study protocols. Prednisone response [18] and, more recently, MRD [19, 20] are often used as prognostic factors. However, diagnosis of early T-cell- precursor (ETP) ALL, have been described by COUSTAN-SMITH et al. [8] very recently, seems to be strong predictor of treatment outcome.

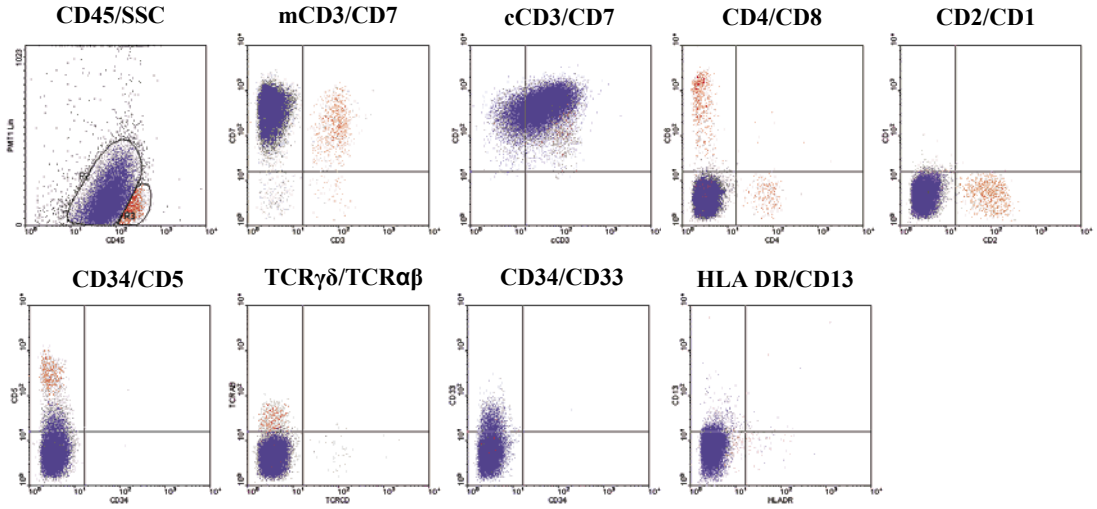

Figure 3 Dot-plots of immature, pro-T stage of T-ALL. Violet cells are pathological gate and red cells are residual normal lymphocytes.

These authors have identified a unique biological subtype of children leukemia, early T-progenitor (ETP)-ALL, which is associated with a high risk of remission induction failure or relapse in patients treated with contemporary protocols of intensive chemotherapy for ALL. ETP-ALL cases have characteristic gene-expression profiles, an increased number and size of genomic lesions, denoting genomic instability and distinct cell surface features that readily enable diagnosis. In their study 30 cases (12.6%) from their 239 T-ALL patients expressed an uncommon ETP phenotype, CD1a and CD8 negative, $CD5^{weak}$ (with <75% positive lymphoblasts), expression of at least one stem-cell-associated or myeloid-associated antigens. These findings suggest that the target cell for clonal expansion is an early immigrant from the bone marrow to the thymus, a well expressing abundant T-lineage, stem cell, and myeloid-associated transcripts, possesing both, lymphoid and myeloid developmental potential [21, 22, 23, 24]. This subset of thymocytes that retain stem-cell-like features would respond poorly to lymphoid-cell directed therapy [8].

We evaluated by multiparameter flow cytometry 44 patients with T-ALL, and in two of them (4.5%), both adults, allocated to pro-T stage, the very immature phenotypes could be observed at diagnosis. In both patients, CD1a and CD8 were completely negative, CD7 and cCD3 positive; in the 1st one CD5 was weakly expressed and CD33 and CD13 were lightly positive, and in the 2nd patient CD34, HLA-DR were expressed, together with CD13 and CD33 of weak positivity. Both patients were adult males, 21 and 28 year-old, with worse clinical outcome. The 1st one was completely unresponsive to therapy and died on day 13 after diagnosis, unresponsive to chemotherapy. In the 2nd patient because of persistent MRD during continuation therapy allogeneic hematopoietic stem-cell transplant has been indicated and patient is still in remission. These phenotypes were similar to ETP-ALL pattern described by COUSTAN-SMITH [8], which has been recognized at diagnosis.

COUSTAN-SMITH et al. [8] simultaneously performed gene-expression profiling analysis of murine ETPs and showed that ETP-ALL cases have characteristic gene-expression profiles. The set of genes upregulated in ETPs was highly enriched in ETP-ALL, whereas the set of downregulated genes was highly enriched in typical (non-ETP) T-ALL. Cases of ETP-ALL had higher expression of *LMO1, LYL1* and *ERG* oncogene than those of typical T-ALL, whereas expression of *TAL1*, *TLX1*, and *LMO2* was not significantly different between ETP-ALL and typical T-ALL.

Constitutive overexpression of *LYL1*+ transcription factors is characteristic in cases with early thymocyte phenotype, expressing CD34 and sometimes myeloid markers [7]. The *HOX11*+, *HOX11 L2*+ and *HOXA*+ signatures correspond to early cortical thymocytes with more favorable outcome (5, 25).

Other antigens that have been associated with outcome include CD2 [7], CD34 [8] and the coexpression of myeloid markers. The myeloid antigen CD33 is also expressed in a small proportion of T-ALL cases, though at a low level of expression.

In our study the expression of antigens not normally present on T cells was found in 22 patients (50 %) from 44 T-ALL studied patients. Mostly coexpressed antigens CD34, HLA-DR, CD19, CD13 and CD33 were observed (Table 3).

As a novel finding, the overlapping areas between AML and T-ALL have been described recently. WOUTERS et al. [26] found a subset of AML cases whose genomic profile was similar to that of CEBPA mutated patients, characterized by the expression of a set of T-lineage genes, *Notch1* mutations. These results suggested that CEBPA hypermethylation together with Notcht1 mutation may lead to a mixed T-lineage/myeloid lineage scenario. Overall, these results indicate that CEBPA is involved in lineage orientation and that its deregulation may be responsible for mixed genotypes; moreover, they also indicate the presence of overlapping areas between distinct diseases.

The analysis of our pro-T ALL adult patients and that of patients with similar phenotypes of ETP-ALL in children [8] have shown an early strong predictor of outcome. The high risk of remission failure or subsequent relapse for patients, if treated with standard intensive chemotherapy indicates the need for alternative approaches to treatment. Extensive research has allowed a molecular profile of T-ALL to be defined. Some lesions have been well characterized, while others require further research. Multiparameter flow cytometry was found to be utilized for prognostication.

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