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Thymic Immunophenotype, and Expression of CD4 and Myeloid Antigens is Associated with Outcome in Adult Patients with T–Cell Acute Lymphoblastic Leukemia

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Abstract:

Background: In adult patients with T–cell acute lymphoblastic leukemia (T–aALL), age, WBC count and cytogenetics are used for prognostic stratification. We report the prognostic significance of immunophenotype in T–aALL patients.

Methods: We analyzed 27 T–aALL patients treated at the Leukemia-BMT Program of British Columbia between 1989 and 2010 using a standardized protocol for diagnosis, treatment and follow up. The immunophenotyping raw data was re-analyzed to record positivity (≥20% blasts positive), number of blasts positive, and intensity and homogeneity of expression. Thymic phenotype (TP) and expression of myeloid antigens (My+) were defined as any one of CD1a+ or dual expression of CD4 and CD8, and any one of CD13+, CD33+ or CD117+ respectively.

Results: Twenty-two (81%) T–aALL patients achieved complete remission (CR); of these, 7(32%) relapsed within 5-22 months (median 15 months). The relapse-free survival (RFS) and overall survival (OS) were 1 -119 (median 18) months and 1-119 (median 25) months respectively. Frequency of CD1a+, CD4+, TP+ and My+ was 58%, 58%, 66% and 50% respectively. Expression of T-cell antigens CD1a, CD4, and TP+ status were favorably associated with outcome: CD1a+ status with OS (p=0.017), CD4+ status with RFS (p=0.015) and OS (p=0.005), TP+ status with CR (p=0.028) and OS (p=0.024). My+ status was adversely associated with CR (p=0.013) and OS (p=0.026).

Conclusions: In T–aALL patients, CD1a+, CD4+ and TP+ are favorably, and My+ status are adversely associated with outcome. The percentage of positive blasts and intensity and uniformity of staining for different antigens shows wide variations.

Keywords: Lymphoblastic leukemia; Antigens; Immunophenotyping

Introduction

T–cell acute lymphoblastic leukemia of adults [1] (T–aALL) constitutes 25% of all adult ALL patients [2]. In general, ALL in adults is distinct disease from its pediatric counterpart. Overall cure rates are 40-50% for adults, vs 85-90% in children [3]. Mutations of NOTCH and FBXW7 predict favorable outcome in children with T–ALL, but not in adults [4]. T–aALL is a heterogeneous disease with high rate of complete remission, but frequent relapses and survival varying from a month to several decades [1,5]. Proven prognostic factors include age, sex, tumor load, cytogenetics, immunophenotype at diagnosis [2], detection of MRD by immunophenotyping [2,6] or molecular means.

Immunophenotyping [1,2,5,7-9] is routinely used for diagnosis, and for assessment of minimal residual disease (MRD) of adult ALL patients. In addition to lineage assignment, immunophenotyping enables sub classification, either as pro–T, pre–T, cortical–T and mature T–cell [9], or as pre-thymic, thymic and mature T–cell [2]. Immunophenotype is increasingly used for assessment of MRD [10], which is an independent prognostic factor. Immunophenotyping identifies the high-risk (HR) early T–cell precursor (ETP) phenotype in pediatric [11-14] as well as adult [15] T–ALL patients. Vitale et al. [9] reported that the complete remission (CR) rate for ALL patients expressing any one of CD13, CD33, CD34 or MDR was inferior to those lacking them (57 vs 96%, p=0.001). Perbellini and Scupoli [7] reported that expression of any one of CD13 or CD33 was associated with lower CR (74 vs. 94%, p=0.04). Marks et al. [8] reported that absence of CD13 was associated with superior survival. Hallmarks of thymic phenotype (TP) are expression of CD1a and dual expression of CD4 and CD8. Of these, expression of CD1a has been associated with better survival [8]. We report the detailed immunophenotype analysis, and its association with treatment response and survival in protocolised group of T–aALL patients at a single institution.

Patients and Methods

Patients

All 171 adult ALL patients treated at the Leukemia Bone Marrow Transplant Program (L/BMT) of BC at Vancouver General Hospital (VGH) from 1989 to 2010 were included in this study. Of these 10 were excluded from further analysis because of re-classification of

leukemia (n=7) or incomplete immunophenotyping data (n=3). From the remaining 161 adult ALL patients 27 had T–cell ALL. Every patient followed an institution specific protocol for laboratory testing, multi agent treatment, and follow up. The laboratory testing included a complete blood count, bone marrow aspirate and biopsy for morphology, flow cytometric immunophenotyping complemented with immunohistochemistry as necessary, and conventional karyotyping. The diagnosis of acute leukemia and its classification was based initially on French-American-British system [16,17] and later on the World Health Organization scheme [18,19]. The patients were stratified into standard or high risk groups based on WBC count ($\geq 100\times10^9$/L), karyotype (complex karyotype, hypodiploidy, aberrations involving 11q23 and t (1;19), and inability to achieve CR after the first course of chemotherapy [20]. Patients with incomplete immunophenotyping data, those re-classified after review and those who did not get therapy on our in-house protocol (ALL89-1A) were excluded from further analysis.

Treatment

All patients were treated with a consistent chemotherapy (our in house ALL-89 01 protocol). Phase I induction consisted of daunorubicin 60 mg/m² intravenously (IV) on days 1-3; vincristine 1.4 mg/m² (maximum dose 2.0 mg) IV on days 1, 8, 15 and 22; L-asparaginase 10,000 units IV on days 17-28 and prednisone 60 mg/m²/day orally on days 1-28. The L-asparaginase was deleted for patients older than 50 years of age as well as for those patients who were to receive stem cell transplantation. Methotrexate 12 mg was given intrathecally as soon as platelets are $\geq 50\times10^9$/L and no blasts are found on blood film. Patients who achieved a complete morphologic remission to induction phase I, went ahead for Phase II induction. If persistent leukemia is present additional treatment was given. Risk stratification as per the above criteria was completed by this time and all standard-risk (SR) patients receive further chemotherapy as follows:

Phase II of the induction regimen began on day 29 and was postponed in patients with delayed hematologic recovery. It consisted of cyclophosphamide 650 mg/m² IV on days 1, 14 and 28; cytarabine 75 mg/m² IV per day on days 1-4, 8-11, 15-18 and 22-25; 6-Mercaptopurine 60 mg/m² orally days 1-28; methotrexate 12 intrathecally days 1, 8, 15, 22; leucovorin 5 mg P.O days 2-4, 9-11, 16-18, 23-25. Patients who were not to receive allogeneic stem cell transplantation would also receive cranial irradiation 1800 cGy in 9X 200 cGy fractions during this period.

The consolidation regimen consisted of five phases. Phases I, II, IV and V were identical and consisted of cytarabine 75 mg/m² IV and teniposide 60 mg/m² IV both given on days 1-5. Phase III consolidation was started 4 weeks after consolidation II and consisted of intensification with dexamethasone 10 mg/m² PO days 1-28; vincristine 1.5 mg/m² days 1, 8, 15 and 22; daunorubicine 25 mg/m² on days 1, 8, 15 and 22; cyclophosphamide 650 mg/m² IV day 29; cytarabine 75 mg/m² IV on days 31-34 and 38-41 and thioguanine 60 mg/m² PO on days 29-42. Patients completing the five courses of consolidation were continued on maintenance chemotherapy treatment including 6-mercaptopurine 75 mg/m² PO per day and methotrexate 20 mg/m² PO once per week for a total of two and a half years dated from completion of Phase II induction.

The cut-off date for follow up was June 2010. Following survival parameters were recorded: achievement of CR, occurrence of relapse, RFS (defined as interval between morphologic or molecular CR and relapse or the last follow up date) and OS (defined as interval between diagnosis and last follow up date or date of death from any cause).

Ethics approval

The study was approved by the institutional review ethic board of British Columbia Cancer Agency and the University of British Columbia.

Flow cytometry

ACD or EDTA anticoagulated blood and/or bone marrow specimens were processed and stained using pre-lysing technique as previously described [21,22] using a variable combination of following antibodies: CD1a, CD2, sCD3, cCD3, CD4, CD5, CD7, CD8, CD10, CD11b, CD11c, CD13, CD14, CD15, CD16, CD19, CD20, cCD22, CD33, CD34, CD36, CD45, CD56, CD61, CD64, CD71, cCD79a, CD117, CD123, HLA-DR, cMPO and nTdT. The specimens were analyzed using either BD FACSCanto II or FACSCalibur (both from BD Biosciences, Mississauga, ON, Canada) or Epics Profile II (Coulter, Mississauga, ON, Canada) flow cytometers. The immunophenotyping data was re-analyzed in patients where raw data was available, and re-interpreted when the plots were available. The debris and cell aggregates were gated out using forward scatter (FS) / side scatter (SS) and FS-height / FS-area plots respectively. The blasts were gated in using FS/SS, CD45/SS and/or CD34/SS plots. The expression was recorded as positive or negative using a combination of isotype negative control, and comparing with built in positive/negative populations. The specimen was considered positive if ≥20% of the gated blast events expressed the antigen. TP, ETP, and Myeloid+ (My +) patterns were defined as CD1a+ or CD4+/CD8+, partialCD5+ (<75%) and CD1a– and CD8–, and CD13+ or CD33+ or CD117+ respectively. The pattern and intensity of fluorescence were recorded as homogeneous or heterogeneous, and as negative, dim, normal or bright respectively, by comparison with built in normal populations or isotype controls. Re-analysis and re-interpretation of the data was done by three investigators. Initial concordance study showed >90% agreement.

Statistical analysis

For categorical parameters, we used two-tailed Chi-square test and Fisher's exact test. For continuous variables, we used unpaired t-test or Mann-Whitney test where the data did not follow normality. Statistical analysis was performed using GraphPad Prism software (GraphPad, v5.0a, La Jolla, CA) and all p-values were recorded as two-tailed.

Results

A total of 171 adult patients with the diagnosis of ALL were retrieved from the L/BMT Program and VGH medical records between 1989 and 2010. Of these 10 were excluded from further analysis because of re-classification of leukemia (n=7) or incomplete immunophenotyping data (n=3). Of the remaining 161 adult ALL patients, 134 (83%) had B–cell ALL and 27 (17%) had T–cell ALL.

Demographic, clinical and laboratory features of T–aALL patients

The median age of 27 patients with T–aALL was 28 years (range 17-66 years). There were 21 males and six females. There were 26 Caucasians, one from the South Asian Indian subcontinent, and no

Asians. Nine (33%) patients had splenomegaly, 11 (42%) had mediastinal mass and 13 (50%) had lymphadenopathy.

Age (median, range)	**28 (17-66)**
<60 years	25
≥60 years	2
Sex (M/F)	21/6
Ethnicity (Caucasian / Indian; South Asian /Asian)	26/1/0
Organomegaly	
Splenomegaly	9 (35%)
Mediastinal mass	11 (41%)
Lymphadenopathy	13 (50%)
CNS involvement	2 (7%)
Laboratory Data	
WBCs×10^9/L (median, range)	19 (0.9-285.6)
Abs neutrophils ×10^9/L (median, range)	0.9 (0.0-15.2)
Blood blasts ×10^9/L (median, range)	15.6 (0.1-282.7)
Hemoglobin G/L (median, range)	102 (34-149)
Platelets×10^9/L (median, range)	40 (8-466)
Bone marrow blasts (%, median, range)	90 (60-100)
Karyotype	
Complex	3 (15%)
Ph+ / BCR-ABL	0
Normal	10 (37%)
Amitotic	7 (26%)
Others	6 (22%)
Hyperdiploidy	1 (4%)
Risk stratification	
Standard risk	20
High risk	7

Table 1: Clinical and Laboratory Features of Adult T–ALL Patients

Five (19%) patients had leukopenia at presentation, four (15%) had normal WBC counts, 16 (59%) had leukocytosis of ≤100×10^9/L, and two (7%) had leukocytosis of >100×10^9/L. Thirteen (48%) patients had neutropenia, while three (11%) had neutrophilia. All patients had blasts in blood in varying numbers. Eleven (41%) patients were anemic (hemoglobin ≤ 100 g/L), 20 (74%) were thrombocytopenic (platelet count <150×10^9/L). while two (7%) had pancytopenia. The blood LDH levels were normal in two patients; in the remainder they ranged from 309-12370 U/L (median 814 U/L, normal range 90-240 U/L). The INR was mildly elevated in two patients, at 1.3 and 1.4. PTT was normal in all patients. The fibrinogen was low in one patient. The blast percentage in bone marrow ranged from 60-100% (median 90%).

Antigen / phenotype	**Positive†, % (n)**	**Partial expression††, n**
T–cell antigens		
cCD3 (cytoplasmic)	100 (22/22)	3
sCD3 (surface)	32 (8/25)	17
CD7	100 (22/22)	4
CD5	93 (25/27)	9
CD2	70 (16/23)	10
CD1a	57 (8/14)	7
CD4	58 (14/24)	14
CD8	71 (17/24)	12
CD4+/CD8+	58 (14/24)	NA†††
ETP phenotype*	15 (2/14)	NA†††
TP phenotype**	66 (16/24)	NA†††
Other lymphocyte antigens		
CD10	32 (7/22)	16
CD19	0/21	21
CD20	0/21	21
CD79a	0/13	13
CD22	0/12	12
CD56	16 (3/19)	17
TdT	80 (20/25)	12
Myeloid antigens		
CD13	23 (5/22)	21
CD33	33 (7/21)	18
CD117	18 (3/17)	17
My+***	48 (11/23)	NA†††
CD14, 64, 61	0/19, 0/13, 0/12	19, 13, 12
Other antigens		
CD45	100 (21/21)	0
CD34	41 (9/22)	14
HLADR	20 (4/21)	21

† ≥20% blasts positive; ††<75% blasts positive; †††Not applicable; *11 CD1a–, CD8–, <75% CD5+, and positivity for at least one of CD13 or CD33 or CD117 in blasts; ** CD1a+ or coexpression of CD4 and CD8; *** CD13+ or CD33+ or CD117+

Table 2: Immunophenotype of Adult T–ALL Patients

CSF contained leukemic blasts in two patients at presentation. The conventional cytogenetics failed to provide enough mitotic cells in seven patients (26%) and was normal in 10 (37%). Of the remaining 10, three had complex karyotype (≥ 5 cytogenetic abnormalities), one

had hyperdiploidy and six had miscellaneous cytogenetic abnormalities, not classifiable under HR or SR (Table 1).

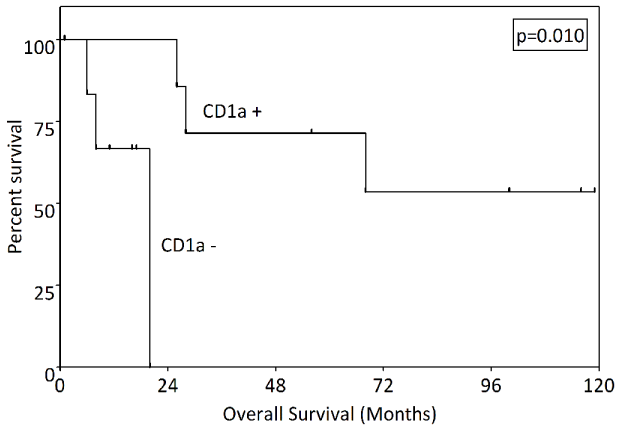

Figure 1: Kaplan Meier plots showing superior OS in adult T-ALL patients expressing CD1a antigen

Response to treatment and course of disease

Twenty-two (81%) patients achieved CR after the first course of chemotherapy, and seven (32%) of them relapsed within 5-22 months (median 15 months). Six patients received allogeneic SCT, four in first CR, one during first relapse after showing resistance to chemotherapy, and one during second CR. Overall survival ranged from 1-119 months (median 25 months). Twelve patients (44%) died within 6-68 months of diagnosis (median 11 months), four died without achieving CR, seven died in relapse and one died of a metastatic neuroendocrine tumor. There was no correlation of conventional prognostic factors age, sex, and WBC count at diagnosis with attainment of CR, occurrence of relapse, RFS, OS and immunophenotype.

Immunophenotypic profile of T–aALL

Immunophenotyping showed the presence of surface or cytoplasmic CD3 and absence of MPO, CD19 and CD20 in all cases, thus confirming the diagnosis of T–ALL. The frequency of expression of T–cell antigens, in decreasing order, was cCD3 = CD7>CD5>CD2>CD8>CD4 = dual expression of CD4 and CD8 = CD1a>sCD3. Frequency of expression of myeloid antigens was My +>CD33>CD13>CD117. The only B–cell antigen expressed was CD10, in 32% of patients.

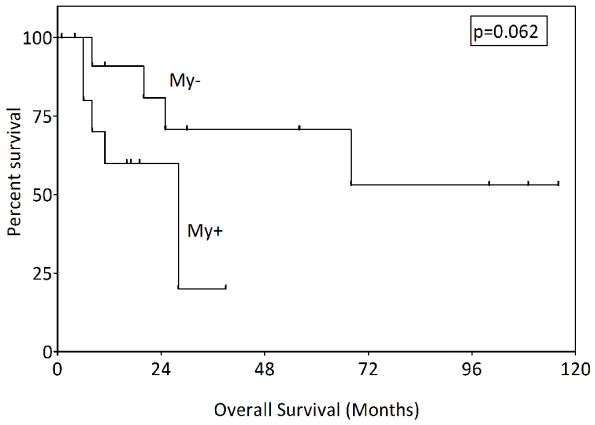

Figure 2: Kaplan Meier plots showing inferior OS in adult T-ALL patients with My+ status (expression of any one of CD13, CD33 or CD117 antigens)

TdT, CD34 and HLADR were expressed in 80%, 41% and 18% respectively. The number of blasts expressing antigens varied from 20-100%; the number of patients showing partial expression, defined as <75%, was variable, but each marker had a few patients with partial expression.

	CD1a+	CD4+/CD8+	TP*	CD4+	My+**
Achievement of CR	NS† Sensitivity 75% Specificity 100%	NS†	16/16(100%) vs 5/8(63%), p=0.0277	14/14(100%) vs 7/10 (70%), p=0.0593 Sensitivity 66% Specificity 100%	6/11(55%) vs 12/12 (100%), p=0.0137 Sensitivity 66% Specificity 100%
Occurrence of relapse	NS†	NS†	NS†	NS†	NS†
RFS (median, months)	NS†	43 vs 16, p=0.0146	NS†	43 vs 16, p=0.0146	NS†
OS (median, months)	62 vs 13.5, p=0.0166	43 vs 13.5, p=0.0052	29 vs 13.5, p=0.024	43 vs 13.5, p=0.0052	16 vs 27.5, p=0.0263
* CD1a+ or coexpression of CD4 and CD8; **CD13+ or CD33+ or CD117+; †Not significant					

Table 3: Association of Clinical and Immunophenotpic Features with Survival in Adults with T–ALL

The intensity of expression of markers was also variable, from negative to bright, in most patients. The pattern of expression was heterogeneous in most but not all patients (Table 2). Blasts with TP and ETP pattern were seen in 66% and 15% of patients respectively. The TP profile correlated with mediastinal enlargement (p=0.0064), absence of HLADR (p=0.0421), of myeloid antigens CD13 (p=0.0096), CD33 (p=0.0181) and My+ status (CD13 or CD33 or CD117, $p \leq 0.0001$).

Association of Immunophenotypic profile with outcome

CR: 22/27 patients achieved CR after first course of chemotherapy. Achievement of CR was directly associated with TP+ (p=0.0277) and CD4+ status (p=0.0593, sensitivity 66%, specificity 100%), and inversely associated with My+ status (p=0.0137, sensitivity 66%, specificity 100%). Four of five patients who did not achieve CR were CD34+, compared to one CD34+ patients in those who achieved CR. However, the difference is not statistically significant (p=0.1353) due to small sample size (Table 3).

Relapse: Occurrence of relapse was not associated with any immunophenotype features.

RFS: CD4+ status and dual expression of CD4 and CD8 were associated with longer RFS (p=0.0146 for both).

OS: CD1a+ (Figure 1), CD4+, dual expression of CD4 & CD8, and TP+ status were associated with longer OS (p=0.0166, p=0.0052, p=0.0052 and 0.024 respectively), while My+ status (Figure 2) was associated with shorter OS (p=0.0263). The percentage of positive blasts for any marker, and intensity and pattern of expression did not correlate with clinical outcome.

Discussion

In this study we report association of immunophenotypic features with clinical outcome in adult patients with T–ALL. The strengths of this study are: 1. Standardized protocol-driven diagnosis, management and follow up algorithm, 2. Focus on adult T–ALL patients, who have distinct clinical and biological features compared to the pediatric group [23,24] and, 3. Re-analysis of the raw listmode data files, recording of the percentage of blasts expressing the antigen, intensity and pattern of staining, and analysis of its clinical relevance. The weaknesses of this study are 1. Age bias, due to our focus on "transplantable" patients: 25/27 patients were <60 years of age, and the oldest patient was 66 years, 2. Small sample size and 3. Retrospective nature of the study,

Our finding of frequency of expression of different lymphoid antigens in T–aALL patients is similar to Czuczman et al. [25], who reported frequency of expression of CD1a, CD2, sCD3, CD4, CD5, CD7, CD8, CD10, CD19 or CD20 in 63%, 70%, 41%, 61%, 84%, 93%, 55%, 16%, 0% and 0% of T–aALL patients respectively. Our finding of frequency of expression of myeloid antigens (CD13, 33, 117 and My+ status, 23%, 33%, 18% and 48% respectively) is similar to Perbellini and Scupoli [7], (CD13, 33, any one of them 22%, 21%, 33% respectively), but not Czuczman et al. [25] (CD13 and CD33 25% and 2% respectively), Vitale, et al. [9] (CD13, CD33 and any one of them 16%, 10%, 24% respectively) and Marks, et al. [8] (CD13, CD33 51% and 30% respectively). These differences may be related to technical variables like specimen used, blood vs. bone marrow, clone of antibody used, and also clinical bias, viz. younger patients in our series. Wide variability in the percentage of blasts expressing antigens, and intensity and homogeneity of expression are important finding. Such information would assist in planning targeted antibody therapies, focusing on those antigens whose expression is seen in 100% of blasts, the intensity is bright, the staining is homogeneous and does not change following chemotherapy [26-28].

Our finding of favorable association of expression of CD4+ status with CR, RFS and OS and that of TP+ status with CR and OS have not been reported before. Our designation of the TP pattern (CD1a+ or dual expression of CD4 and CD8 is somewhat similar to the "thymic phenotype" (CD5+/–, CD2+/–, sCD3+/–, CD4+/–, CD8+/–, CD1a+) reported by Baak et al. [2], who also found this to have favorable prognosis. We confirm the favorable association of CD1a+ status with overall survival reported by Baak et al. [2] and Mark, et al. [8] We did not find any immunophenotypic features associated with increased incidence of relapse. This is at variance with Marks, et al. [8] who reported CD1a– status correlated with a higher incidence of relapse at a 5 year cut-off (p=0.02). The favorable prognostic significance of CD1a and CD4 expressions could be related to the fact that they are acquired late during the T-cell development, during intra-thymic development. Expression of these antigens indicates "late T-cell precursor phenotype", in contrast to the "early T-cell precursor phenotype" [15].

We also confirm the adverse impact of My+ status on CR rate as reported by Perbellini and Scupoli [7] and on OS, as reported by Mark et al. [8], who found fewer patients in this group with >60 month survival.

Absence of patients of ethnic Asian patients in our study group is perplexing, considering the significant ethnic Asian population in British Columbia. In contrast to T-ALL group, the B–ALL group in our database comprised of 8% (11/134) ethnic Asians. Ethnic Asians comprised approximately 8% of British Columbia's population in 1991, 11% in 2001 and 27% in 2009 [29,30]. This may be just a sampling error due to small sample size. Or it may indicate that Asians have lower incidence of T–ALL in their adult ALL patients; Tong, et al. [31] reported that 7% of 113 Chinese adult ALL patients were T–ALL type, compared to our finding of 17% of 161 patients and 21% and 25% other adult ALL cohorts [2,7].

Conclusion / Summary

In T–aALL patients, CD4+ status was associated with higher CR rate and longer RFS and OS, TP+ status with higher CR rate and longer OS, CD1a+ status with longer OS, and My+ status with lower CR rate and shorter OS. Based on the results of this small study we conclude that immunophenotype is a significant prognostic indicator in T–aALL patients.

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Competing Interest

All authors declare no competing or conflicting interests.

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