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The presence of CD56/CD16 in T-cell acute lymphoblastic leukaemia correlates with the expression of cytotoxic molecules and is associated with worse response to treatment

Leandro F. F. Dalmazzo, Rafael H. Jácomo, André F. Marinato, Lorena L. Figueiredo-Pontes, Renato L. G. Cunha, Aglair B. Garcia, Eduardo M. Rego and Roberto P. Falcão

Department of Internal Medicine, Haematology Division, and Centre for Cell Based Therapy, Medical School of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

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Correspondence: Prof. Roberto P. Falcão, Department of Internal Medicine, Hematology Division, Medical School of Ribeirão Preto, University of São Paulo, Av. Bandeirantes, 3900, 14048-900, Ribeirão Preto, São Paulo, Brazil.
E-mail: rpfalcao@fmrp.usp.br

Summary

Some cases of T-cell acute lymphoblastic leukaemia (ALL) express markers found in natural-killer (NK) cells, such as CD56 and CD16. Out of 84 T-cell ALL cases diagnosed at our Institution, CD56 and/or CD16 was detected in 24 (28·5%), which we designated T/NK-ALL group. Clinical features, laboratory characteristics, survival and expression of cytotoxic molecules were compared in T/NK-ALL and T-ALL patients. Significant differences were observed regarding age (24·9 vs. 16·4 years in T/NK-ALL and T-ALL, respectively, $P = 0{\cdot}006$) and platelet counts (177×10^9/l vs. 75×10^9/l in T/NK-ALL and T-ALL, respectively, $P = 0{\cdot}03$). Immunophenotypic analysis demonstrated that CD34, CD45RA and CD33 were more expressed in T/NK-ALL patients, whereas CD8 and terminal deoxynucleotidyl transferase were more expressed in T-ALL patients ($P < 0{\cdot}05$). The mean overall survival (863 vs. 1869 d, $P = 0{\cdot}02$) and disease-free survival (855 vs. 2095 d, $P = 0{\cdot}002$) were shorter in patients expressing CD56/CD16. However, multivariate analysis identified CD56/CD16 as an independent prognostic factor only for DFS. Cytotoxic molecules were highly expressed in T/NK-ALL compared to T-ALL. Perforin, granzyme B and TIA-1 were detected in 12/17, 4/17 and 7/24 T/NK-ALL patients and in 1/20, 0/20 and 1/20 T-ALL respectively ($P < 0{\cdot}001$, $P = 0{\cdot}036$ and $P = 0{\cdot}054$). Therefore, the presence of CD56/CD16 was associated with distinct clinical features and expression of cytotoxic molecules in the blasts.

Keywords: T-cell acute lymphoblastic leukaemia, CD56, CD16, cytotoxic enzymes, survival.

T-cell acute lymphoblastic leukaemia (ALL) represents about 25% of the ALL cases (Bassan *et al*, 2004) and its overall incidence in Brazil is 12·5 cases in 10^6 people-year (25·5 cases/10^6 for children and 6·2 cases/10^6 for adults) (Rego *et al*, 1996). Although many clinical and laboratory features have been reported as associated with adverse treatment outcome, several of them have lost predictive value with the intensification of therapy in the last years. Previous results imply that age, white blood cell (WBC) count at presentation, initial response to therapy and cytogenetics are strong predictors of outcome (Hoelzer *et al*, 1988). On the other hand, the value of the immunophenotype for prognosis is controversial. Ravandi *et al* (2002) analyzed 200 ALL patients and reported that CD56 expression was associated with a higher incidence of central nervous system (CNS) disease at diagnosis. In addition, another study reported that the expression of CD56 molecule was shown to be the only independent prognostic factor for achieving complete remission in a group of 30 T-ALL patients treated with the PETHEMA93 and PETHEMA96 protocols (Montero *et al*, 2003).

CD56 and CD16 are predominantly expressed in natural killer (NK) cells and a minor subset of T lymphocytes with cytotoxic activity (Edelman & Crossing, 1991; Hibett & Hogarth, 1994). These are both used as phenotypic markers in some haematological malignancies, particularly in lymphoproliferative diseases of large granular lymphocytes (LGL). CD56 is also expressed in rare cases of acute myeloid leukaemia (AML), nasal lymphomas associated with Epstein–Barr virus infection, other lymphomas and multiple myeloma (Suzuki *et al*, 1997; Ely & Knowles, 2002).

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Cytotoxic molecules are usually expressed in NK cells, cytotoxic T-cells (Takeuchi *et al*, 1992; Medley *et al*, 1996) and some haematological neoplasms, such as LGL leukaemia, NK-cell leukaemia/lymphoma (Mori *et al*, 2000), hepatosplenic T-cell lymphoma (Cooke *et al*, 1996), subcutaneous panniculitic T-cell lymphoma (Gonzales *et al*, 1991), anaplastic large cell lymphoma (Krenacs *et al*, 1997). The major cytotoxic molecules are perforin, granzyme B and T-cell intracellular antigen-1 (TIA-1). In the cytotoxic process, perforin, in the presence of calcium, polymerizes into transmembrane tubules to form pores, allowing granzyme B and TIA-1 to enter the target cells, activating apoptosis-related proteins (Felgar *et al*, 1997; Yamashita *et al*, 1998). Perforin and granzyme B are only expressed in activated cytotoxic cells. In contrast, TIA-1 is expressed regardless of the activation status of the cell. The expression of these cytotoxic molecules has also been described in some cases of B cell lymphomas, such as Hodgkin lymphoma (Krenacs *et al*, 1997). TIA-1 was also detected in some cases of hairy cell leukaemia (Mori *et al*, 2004). The aim of this study was to compare clinical features, response to treatment, laboratory characteristics and expression of cytotoxic molecules in T-cell ALL patients with and without the expression of CD56 and/or CD16.

Materials and methods

Patients

From January 2000 to December 2006, 84 cases of T-cell ALL were diagnosed at Ribeirão Preto's Clinical Hospital, University of São Paulo, according to classical criteria established by the World Health Organization. The clinical and laboratory records of these patients were reviewed, and pathological materials, such as frozen bone marrow aspirates and smears, were available for further investigations. Clinical data were available for 49 patients, which were treated with protocols involving high doses of cytarabine and methotrexate, associated with anthracyclines and cyclophosphamide: 14 patients received the HyperCVAD regimen (cyclophosphamide, vincristine, adriamycin, and dexamethasone; Garcia-Manero & Kantarjian, 2000), 15 were treated with the German Multicentre Study Group for ALL (GMALL) protocol (Gökbuget *et al*, 2000), 17 received the Brazilian Cooperative Childhood ALL (GBTLI) protocol (Brandalise *et al*, 1993) and three patients died before initiation of the therapy. Only two patients were submitted to allogeneic bone marrow transplantation, and both died after engraftment of infectious complications.

Among the 84 patients included in this study, 24 had more than 20% of blasts that expressed CD56 and/or CD16 (21 patients expressing only CD56 and three patients expressing only CD16; no patients expressed both CD56 and CD16 simultaneously). Blasts of the other 60 patients expressed none of these markers. The total T-cell ALL population could then be divided into two groups for comparison: one group defined as T/NK-ALL (that expressed CD56/CD16) and the other called T-ALL (where neither CD56 nor CD16 were demonstrated).

Morphology and immunophenotyping

Patients' bone marrow smears underwent Leishman and myeloperoxidase (MPO) staining. The presence of 20% or more lymphoblasts in a 500 cell count was diagnostic of ALL. Immunophenotyping of the blasts was performed by flow cytometry using a broad panel of fluorochrome-conjugated monoclonal antibodies (MoAbs), which included: anti-CD2 (fluorescein sitothiocyanate, FITC), CD3 (peridnin chlorophyll, PerCP), CD5 (phycoerythrin, PE), CD7 (PE), CD1 (FITC), CD4 (FITC), CD8 (PE), CD34 (PE), CD45 (allophycocyanin, APC), CD45RA (FITC), CD45RO (PE), TCR$\alpha\beta$ (FITC), TCR$\gamma\delta$ (PE), terminal deoxynucleotidyl transferase (TdT) (FITC), CD16 (FITC), CD56 (phycoerythrin-cyanin 5, PECy5), CD19 (APC), CD10 (FITC), HLA-DR (PE), CD13 (PE) and CD33 (FITC) (BD Biosciences, San Jose, CA, USA). A positive reaction to a given antibody was defined as a minimum threshold of 20% positive blasts to the respective antigen (Bain *et al*, 2002). Analysis of T-cell antigens was performed using the following combinations: (i) CD34/CD3/CD45, (ii) TdT/cCD3/CD45, (iii) CD4/CD8/CD3/CD45, (iv) CD16/CD56/CD3/CD45. Mononuclear cells were isolated by Ficoll Hypaque density gradient centrifugation (Sigma-Aldrich, St Louis, MO, USA) and then incubated with the MoAbs for a four-color fluorescent analysis on a FACScalibur flow cytometer (BD Biosciences). To analyze intracellular antigens, before incubation with MoAbs, blasts were permeabilized using the FIX & PERM® kit according to manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA). Multiparameter analysis of gated cells was performed using the CELL QUEST version 3.2 software (BD Biosciences).

Cytotoxic molecules analysis

Intracellular enzymes perforin and granzyme B were examined utilizing MoAbs (BD Bioscieneces) in a FACScalibur flow cytometer. The analysis of these molecules was performed in CD3 plus CD16/CD56 gated blasts in the T/NK-ALL group and in CD3 gated blasts in the T-ALL group.

TIA-1 was studied by cytochemistry utilizing a general alkaline phosphatase antialkaline phosphatase (APAAP) complex in smears of bone marrow aspirates obtained by usual diagnostic procedures. The smears were sequentially incubated with mouse anti-TIA-1 monoclonal antibody (Abcam, Cambridge, UK) followed by polyclonal rabbit anti-mouse (RAM) antibody and mouse APAAP (Dako, Carpinteria, CA) as the secondary and tertiary steps respectively. All antibodies were incubated for 30 min. The alkaline phosphatase-labelled cells were visualized by a red color reaction utilizing New Fuchsin, naphtol-AS-biphosphate and levamisole. Finally, the slides were counterstained

with haematoxylin. Positive cells were counted in a total of 200 cells, by optical microscopy.

Statistical analysis

Chi-square test, Student's *t*-test and Fisher's exact test were employed to compare differences between the groups. Disease-free survival (DFS) and overall survival (OS) were estimated by the Kaplan–Meier method. Differences among groups were compared by log-rank test. Multivariate analysis utilizing Cox regression was used to identify variables affecting survival. We considered α as 0·05. All the statistical tests were performed by the Statistical Package for the Social Sciences (SPSS) v. 13.0 software (SPSS Inc, Chicago, IL, USA).

Results

Clinical features

Eighty-four T-cell ALL patients were diagnosed between January 2000 and December 2006. From these, 24 (28·5%) had leukaemic cells that expressed CD56 and/or CD16. Clinical data were available for 49 patients. The major clinical characteristics are described in Table I. The two groups of patients were similar regarding gender distribution, presence of mediastinal mass, lymphadenopathy, hepatomegaly, splenomegaly, CNS involvement, hemoglobin value, WBC count and percentage of blasts in bone marrow and peripheral blood. There was a significant difference regarding age (mean value of 16·4 years in T group and 24·9 in T/NK group, $P = 0{\cdot}006$) and platelets count at diagnosis (mean value of 75×10^9/l in T group and 177×10^9/l in T/NK, $P = 0{\cdot}03$).

Table I. Clinical characteristics at diagnosis of T/NK and T-ALL patients.

	T-ALL group	T/NK-ALL group	*P*-value
Age, years	16·4	24·9	0·006*#
Sex (M/F)	46/14	20/4	0·50§
Mediastinal mass (%)	16/29 (55)	11/19 (57)	0·85§
Lymphadenopathy (%)	24/30 (80)	13/19 (68)	0·35§
Splenomegaly (%)	25/30 (83)	14/19 (73)	0·41§
Hepatomegaly (%)	24/30 (80)	13/19 (68)	0·35§
CNS involvement (%)	4/30 (13)	2/19 (10)	0·77§
Hemoglobin, g/l	102	99	0·68#
WBC count, $\times 10^9$/l	124·0	62·0	0·09#
Blasts in PB (%)	63	51	0·31#
Platelets, $\times 10^9$/l	75·0	177·0	0·03*#
Blasts in BM (%)	88	74	0·05#

CNS, central nervous system; WBC, white blood cell; PB, peripheral blood; BM, bone marrow.
Values represent mean.
#Student's *t*-test.
§Chi-square test.
*Significant *P*-value (<0·05).

Survival analysis

Figure 1 shows the DFS and OS curves for the two groups of T-cell ALL patients. The mean DFS was 2095 d in the T-ALL group compared to 855 d in T/NK-ALL group ($P = 0{\cdot}002$). The mean OS for the T group was 1869 d, whereas for the T/NK group it was 863 d ($P = 0{\cdot}02$).

We performed multivariate analysis for age, WBC count, treatment received (HyperCVAD *versus* GMALL *versus* GBTLI) and presence of CD56 and/or CD16. In this model, the classification into T/NK-ALL group was an independent risk parameter when considering DFS ($P = 0{\cdot}019$). Age, WBC and treatment received were not independent risk factors for DFS ($P = 0{\cdot}256$, $P = 0{\cdot}261$ and $P = 0{\cdot}059$ respectively). In contrast, considering OS, only age could be identified as an independent prognostic factor ($P = 0{\cdot}04$), whereas WBC, treatment received and the presence of CD56 and/or CD16 could not ($P = 0{\cdot}062$, $P = 0{\cdot}187$ and $P = 0{\cdot}247$ respectively).

Immunophenotype

The immunophenotypic features could be evaluated in all the 84 T-ALL patients (Table II). The findings in both groups were mostly similar, except for the expression of CD8, TdT, CD34, CD45RA and CD33. TdT and CD8 were more frequently expressed in the T-ALL group [CD8 in 6 out of 23 T/NK patients and in 37/59 T-ALL cases ($P = 0{\cdot}003$); TdT in 16/23 T/NK and in 52/57 T-ALL ($P = 0{\cdot}01$)] whereas CD34, CD45RA and CD33 were more often expressed in the T/NK-ALL group [CD34 in 15/24 T/NK-ALL and in 22/59 T-ALL ($P = 0{\cdot}03$); CD45RA in 18/21 T/NK and in 26/49 T-ALL ($P = 0{\cdot}01$); CD33 in 7/23 T/NK and in 4/52 T-ALL cases ($P = 0{\cdot}01$)]. There were no differences in the expression of others markers, such as CD2, cCD3, sCD3, CD5, CD7, CD1, CD4, CD45, CD45RO, TCR$\alpha\beta$, TCR$\gamma\delta$, CD19, CD10, HLA-DR and CD13. Of note, the immunophenotypic profile of the 35 patients in which clinical data was not available did not differ from those in the 49 patients in which clinical characteristics were analyzed.

Cytotoxic molecules

The analysis of perforin and granzyme B expressions was performed in 37 patients, while TIA-1 was evaluated in 44 patients. The presence of the cytotoxic molecules was considered positive when the percentage of blasts expressing the marker exceeded a cut-off value equal to mean plus two standard deviations of the T-ALL group values (Fig 2).

Analysis of these intracellular enzymes demonstrated higher expression of perforin and granzyme B in the T/NK-group ($P < 0{\cdot}05$), but no significant differences between the groups were noted when evaluating TIA-1 expression ($P = 0{\cdot}054$). Perforin was elevated in 12 of 17 T/NK patients and in only one of 20 patients in the T group ($P < 0{\cdot}001$); granzyme B was elevated in four of 17 T/NK patients and in no T-ALL patients ($P = 0{\cdot}036$); TIA-1 was elevated in seven of 24 T/NK-ALL

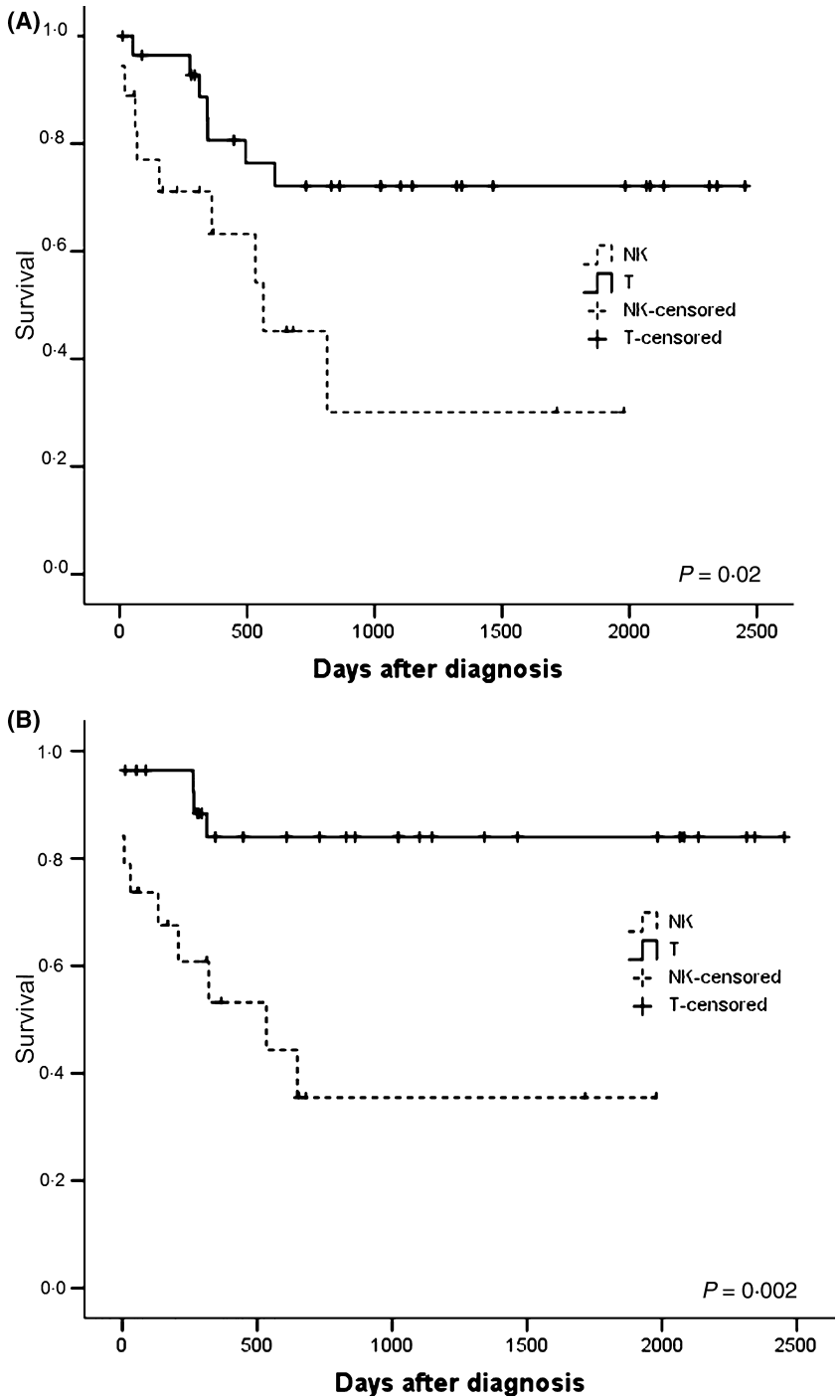

Fig 1. Kaplan-Meier analysis of overall survival (A) and disease-free survival (B) according to ALL group. The mean OS for the T group was 1869 d, whereas for the T/NK group it was 863 days ($P = 0{\cdot}02$). The mean DFS was 2095 d in the T-ALL group against 855 d in the T/NK-ALL group ($P = 0{\cdot}002$).

patients, while only one of 20 T-ALL patient expressed this molecule ($P = 0{\cdot}054$) (Table III).

Discussion

T-cell ALL classically expresses the lineage specific marker CD3 (on the cell surface or in the cytoplasm). Others T-cell markers include CD1a, CD2, CD4, CD5, CD7 and CD8, but none of these are absolutely lineage-specific. The expression of CD10 is quite common (25% of the cases) and non-specific (Greaves *et al*, 1981). CD34, myeloid antigens and NK-cell markers can also be expressed. The immunophenotype-based scoring system proposed by the European Group for the Immunological characterization of Leukaemia (EGIL) classified T-cell ALL

Table II. Immunophenotypic features of T/NK and T-ALL patients.

	T-ALL group, $n = 60$ (%)	T/NK-ALL group, $n = 24$ (%)	*P*-value
CD2	50/57 (87)	18/24 (75)	0·15
cCD3	60/60 (100)	24/24 (100)	NA
sCD3	14/20 (70)	40/62 (64)	0·53
CD5	58/60 (96)	22/23 (95)	0·82
CD7	57/57 (100)	24/24 (100)	NA
CD1	24/55 (43)	5/21 (23)	0·11
CD4	31/59 (52)	7/23 (30)	0·07
CD8	37/59 (62)	6/23 (26)	0·003*
TdT	52/57 (91)	16/23 (69)	0·01*
CD34	22/59 (37)	15/24 (62)	0·03*
CD45	58/58 (100)	22/22 (100)	NA
CD45RA	26/49 (53)	18/21 (85)	0·01*
CD45RO	27/48 (56)	7/21 (33)	0·08
TCRαβ	29/60 (48)	13/24 (54)	0·62
TCRγδ	8/58 (13)	3/22 (13)	0·98
CD19	12/59 (20)	5/23 (21)	0·88
CD10	23/59 (38)	8/23 (34)	0·72
HLA-DR	6/55 (10)	5/23 (21)	0·21
CD13	9/52 (17)	7/22 (31)	0·16
CD33	4/52 (7)	7/23 (30)	0·01*

cCD3, cytoplasmatic CD3; sCD3, surface CD3; TdT, terminal deoxynucleotidyl transferase.
Values are expressed as numbers of positive/examined cases for each marker.
*Significant *P*-value (<0·05), chi-square test.

according to each maturation stage (Bene *et al*, 1995). Some subtypes have been associated with worse prognosis in T-cell ALL, such as the pro-T and perhaps the mature T phenotypes (Garand *et al*, 1993).

Failure to respond to leukaemia treatment may depend on various factors, which are not fully understood. CD56 and CD16 are commonly present in NK cells and T cytotoxic lymphocytes and were also previously described in some T-ALL cases. It was observed that CD56 could be prognostically important in some types of AML and T-ALL (Raspadori *et al*, 2002; Montero *et al*, 2003). Montero *et al* (2003) demonstrated, in a series of 30 consecutives T-cell ALL patients, that the presence of CD56 was the only independent prognostic factor for achieving complete response to treatment. In that analysis, they found CD56 in a small subset of T-cell ALL patients (four of 30). Whether CD56/CD16 can distinguish a specific subtype of T-ALL is not clear.

We could demonstrate some clinical and laboratory differences in T-ALL presentation. Patients were older in the T/NK-ALL group when compared to the T-ALL group and presented with higher platelets counts. Other clinical characteristics were not found to be different considering the two groups, and the numbers found were similar to that observed in the literature (Bassan *et al*, 2004).

The most important clinical finding observed in T/NK-ALL patients was related to the survival analysis. We demonstrated

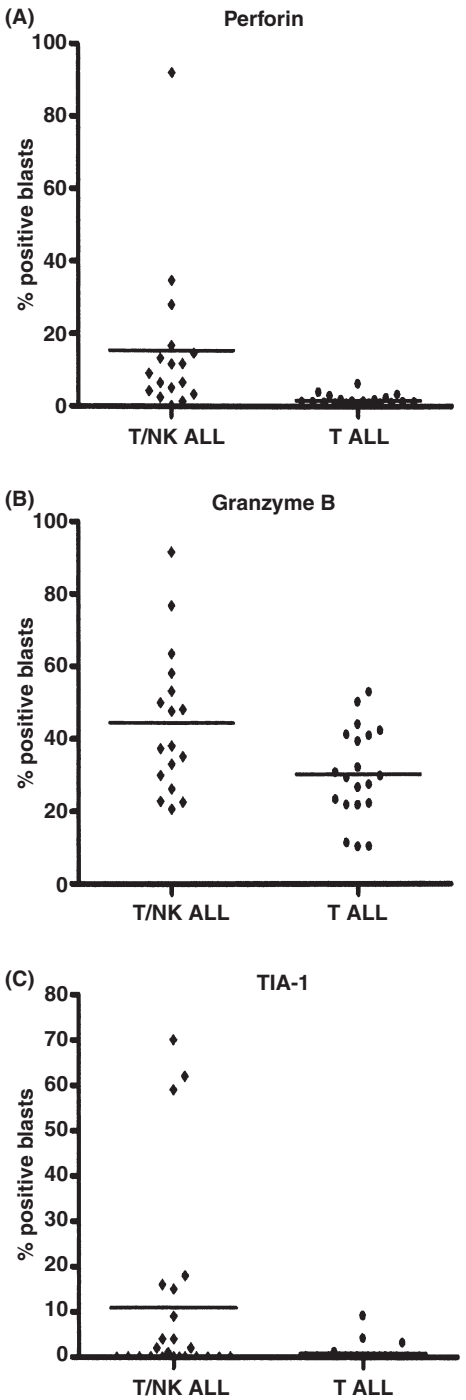

Fig 2. Cytotoxic molecules positivity on T/NK-ALL and T-ALL blasts. The expression of perforin and granzyme B was analyzed by flow cytometry and T-cell intracellular antigen-1 (TIA-1) was accessed by cytochemistry. (A) Mean expression of perforin was 15·4% and 1·6% in T/NK-ALL and T-ALL, respectively; a cut-off value of 4·4% (mean + 2 standard deviations in T-ALL group) was adopted to consider positivity for perforin. (B) For granzyme B, the means were 44·5% and 30·3% for T/NK and T-ALL, respectively; the cut-off value for positivity was 55·5%. (C) For TIA-1, the means of T/NK and T groups were 10·9% and 0·9%, respectively; positivity was considered based on a cut-off value of 5·3%.

Table III. Expression of cytotoxic molecules in T/NK and T-ALL patients.

	T-ALL group	T/NK-ALL group	*P*-value
Perforin	1/20	12/17	<0·001*
Granzyme B	0/20	4/17	0·036*
TIA-1	1/20	7/24	0·054

Values are expressed as numbers of positive/examined cases for each marker.
*Significant *P*-value (<0·05), Fisher's exact test.

that T-cell ALL patients that expressed CD56 and/or CD16 had a worse prognosis, especially regarding response to therapy. In our series, the mean duration of OS and DFS was shorter in patients CD56/CD26 positives (863 against 1869 d, $P = 0{\cdot}02$, and 855 against 2095 d, $P = 0{\cdot}002$ respectively). Regarding others features, such as age, WBC count and treatment performed, multivariate analysis identified the presence of CD56/CD16 as the only independent prognostic characteristic when considering DFS analysis, which suggests poorer response to therapy. However, only age was found to be an independent prognostic factor for OS. The mechanism by which this classification can affect survival remains to be investigated. Although cytogenetic features are considered relevant prognostic factors in acute leukaemias, this data was not available in our series.

Immunophenotypic analysis demonstrated some peculiarities in ALL patients classified as T/NK. Leukaemic cells of these patients had higher expression of CD34, CD45RA and CD33 whereas TdT and CD8 were detected to a lesser extent when compared to T-ALL blasts. Suzuki *et al* (1997) described seven cases of acute leukaemia with hybrid myeloid/NK origin, morphological features resembling lymphoblasts and expression of CD34, CD56, CD7 and CD33. These cases could not be characterized as truly T-cell ALL. Scott *et al* (1994) reported a more mature subtype of myeloid/NK acute leukaemia, being CD34-HLA-CD33$^+$CD56$^+$. However, our CD56/CD16 positive cases always co-expressed CD3, either in the cytoplasm or on the cell surface, thus characterizing the disease as a truly T-cell ALL.

Usually present in components of the innate immune system, the cytotoxic molecules perforin and granzyme B were more frequently expressed in the T/NK-ALL group, suggesting that these blasts originated from a cytotoxic lymphoid lineage. The expression of CD56/CD16 correlated with the expression of these enzymes in the T/NK group, denoting a peculiar disease, regarding phenotype and function. Cytotoxic molecules are generally present in LGL, which are T-CD8 lymphocytes (CD3$^+$/CD56$^-$) and NK cells (CD3$^-$/CD56$^+$). The co-expression of CD56 and CD3 is only seen in a small number of normal peripheral blood cells, and LGL neoplasias with the same phenotype have been reported (Sokol & Loughran, 2006). It is likely that these cells represent the normal counterparts of the T/NK-ALL that we described. Alternatively, it is possible that the leukaemic cells arose from normal T lymphocytes (CD3$^+$ and CD56/16$^-$), with later upregulation of CD56/16.

In conclusion, our findings demonstrated that the presence of CD56 and/or CD16 in the surface of blasts from T-cell ALL patients can predict worse outcome and differentiates a specific subtype of T-cell acute leukaemia. This T/NK-ALL subtype has clinical and laboratory peculiarities and the different expression of cytotoxic molecules suggests another origin and function of the cells.

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