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Research paper

A seven-color panel including CD34 and TdT could be applied in > 97% patients with T cell lymphoblastic leukemia for minimal residual disease detection independent of the initial phenotype

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ABSTRACT

A seven-color panel was used to detect minimal residual disease (MRD) in T cell acute lymphoblastic leukemia (T-ALL) via flow cytometry (FCM). Its availability and clinical significance were studied in T-ALL patients with newly diagnosed (n = 64), relapsed (n = 48) and morphologically complete remission (n = 103). The following four features were used to identify immature cCD3+ T cells: CD34+, TdT+, but mCD3-/dim+, and CD45dim+. Among these features, either TdT or CD34 expression was the most useful and were found in 93.8% of patients at diagnosis and 86.7% of patients who relapsed. Although some of the immature markers had disappeared in 23 of 59 cases after therapy, only one case presented with a false negative MRD. Of the 74 consecutive patients who underwent allogeneic hematopoietic stem cell transplantation (allo-HSCT), MRD-positive patients showed a higher relapse rate, a higher cumulative incidence of relapse at 4 years and a shorter median relapse-free survival than MRD-negative patients at post-HSCT(72.7% vs 17.3%, P = 0.000; 100% vs 19.9%, P < 0.0001; and 16 months vs undefined, P < 0.0001). We demonstrated that this panel could be applied to > 97% of T-ALL patients to detect MRD and predict relapse after allo-HSCT even in the absence of the initial immunophenotype.

1. Introduction

T cell acute lymphoblastic leukemia (T-ALL) accounts for approximately 15% and 25% of newly diagnosed ALL cases in children and adults, respectively [1,2]. In contrast to B cell acute lymphoblastic leukemia (B-ALL), T-ALL is associated with a higher frequency of relapse and less favourable outcomes. Minimal residual disease (MRD) may persist after treatment and eventually lead to clinical relapse.

Over the past 2–3 decades, much effort has been invested to develop accurate and sensitive methods that can detect MRD. In parallel with polymerase chain reaction (PCR)-based molecular techniques, flow cytometry (FCM) has been explored as a less labour-intensive and faster MRD technique. Its sensitivity had even reached 10^{-4}, especially with the availability of 6- to 10-color cytometers. Many studies have demonstrated that detecting MRD by FCM is a powerful predictor of clinical outcomes in ALL [3–8]; however, all these studies focused on B-ALL patients [8–11], and there are relatively few T-ALL cases because of its low incidence [12].

Normally, T cell development and differentiation occur in thymus in four stages [13]. The first stage is thymus seeding progenitors (TSPs) that express CD34 and CD7. The second stage is early thymic progenitors (ETPs) that begin to express CD99 and Terminal deoxynucleotidyl Transferase (TdT) followed by the gradual expression of CD45, CD2, CD5 and cytoplasmic CD3 (cCD3). The third stage is precursor T cells, which are characterized by the loss of CD34, and the expression of CD1a and membrane CD3 (mCD3); precursor T cells eventually differentiate into CD4 and CD8 double-positive immature T cells. In the last stage, T cells become mature T cells that are CD1a-negative and express either CD4 or CD8. These cells leave the thymus and translocate to the bone marrow (BM). In most T-ALL cases, immature T cells may resemble the various differentiation stages of T cells in the thymus. Additionally, immature T cells often manifest as many aberrant phenotypes, including asynchronous antigen expression (e.g., mCD3+/CD2dim+), lineage infidelity (e.g. CD33+) and abnormal expression intensity of CD7, CD2 and CD5. However, these aberrant phenotypes can also be seen in mature T cell neoplasms; thus, detection of only those phenotypes cannot indicate immature T cells are present if the patient's immunophenotype is unknown at diagnosis. Therefore, it

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is critical to confirm the presence of immature T cells to detect MRD in T-ALL [14].

Ectopic (thymus-associated) antigen expression is the most common leukemia-associated phenotype in T-ALL [15], which normally is either absent or expressed at levels lower than 10^{-4} in the BM and peripheral blood (PB) [16–19]. Therefore, observation of the CD34 + CD7 + or cCD3 + TdT + phenotypes, particularly at frequencies greater than 10^{-4}, were was considered MRD in most early reports [15]. However, Roshal M. et al. [20] reported that immature markers, particularly TdT and CD99, were dramatically decreased and that CD34 and CD10 expression on leukemic blasts was unstable during therapy. Their study suggested that MRD should not be monitored using only immature markers. Based on our clinical experience, CD34- and TdT-positive T cells are frequently observed in MRD-positive (MRD +) samples from patients with T-ALL after therapy.

To validate the feasibility, stability and clinical prognostic significance of seven-color panel, samples from patients with newly diagnosed, relapsed and remitted T-ALL were analyzed.

2. Materials and methods

2.1. Specimen collection

During the period between April 2009 and August 2016, 133 patients with T-ALL and a median age of 22 years (from 1 to 74 years), including 25 children aged 1 to 14 years, were retrospectively analyzed. Within this group, 64 patients with newly diagnosed T-ALL, 48 with relapsed T-ALL (24 cases had an initial phenotype) and 103 who achieved morphologically complete remission(CR) (43 patients had an initial phenotype) were included. Seventy-five patients aged less than 55 years underwent allogeneic hematopoietic stem cell transplantation (allo-HSCT) and were categorized into low risk (n = 39), intermediate risk (n = 30) and high risk groups (n = 5) according to the MRC UKALL XII/ECOG E2993 study [21,22], with a median follow-up of 24 months after HSCT (range: 2–60 months). MRD was examined in all patients. Five patients prior to HSCT and one patient after HSCT exhibited greater than 5% blast cells by morphology, and therefore 70 pre-HSCT patients and 74 post-HSCT patients were divided into MRD + and MRD- groups and analysed. BM samples from 15 healthy volunteers were collected as controls. Informed consent was obtained from all patients and volunteers.

2.2. Seven-color antibody panel

A seven-color reagent cocktail was created according to preliminary analysis of the antigen profile of newly diagnosed T-ALL patients. TdT-FITC (clone TdT-6, Life technologies, Frederick, MD, USA) and CD7-APC (clone eBio124-1D1, eBioscience, San Diego, CA, USA) were included. CD34-PerCP-Cy5.5 (clone 581) and CD5-PE-Cy7 (clone UCHT2) were obtained from Biolegend, San Diego, CA, USA. All other reagents were purchased from BD Biosciences, San Jose, CA, USA, including cCD3-PE (clone UCHT1), mCD3-APC-H7 (clone SK7) and CD45-V500 (clone HI30). CD99-PE (clone TU12, BD Biosciences) was added in August 2015 to form an eight-color panel; simultaneously, cCD3-PE was replaced with cCD3 V450 (clone UCHT1, BD Biosciences).

2.3. Sample preparation

First, 100 µl of BM containing up to 2×10^6 cells were incubated with a membrane reagent cocktail according to manufacturer's recommendations followed by an incubation at room temperature in the dark for 15 min. Next, 2 ml of 1 × FACS lysis solution (BD Biosciences, San Jose, CA, USA) were added and incubated in the dark at room temperature for 15 min followed by a single wash with phosphate-buffered saline. Then, samples were processed using a Fix&Perm Cell Permeabilization kit (Invitrogen, Carlsbad, CA, USA). Nuclear TdT and cCD3 were stained using the same membrane reagent procedure described above. Finally, events were acquired on a MACSQuant Analyzer (Miltenyi, Cologne, Germany) that was calibrated daily with quality control beads to ensure consistent performance, and up to 750,000 events were collected per sample.

2.4. Data analysis

Data were analyzed with Kaluza Analysis software (Beckman Coulter, Fullerton, CA, USA). Nucleated cells were gated by excluding low forward scatter (FSC) and low side scatter (SSC) debris. T cells were identified by cCD3 expression and low FSC/SSC. Immature T cells were identified by the expression of CD34 and TdT, CD45 or mCD3 dim expression in cCD3 + T cells.

2.5. Definition

The mean percentage of cCD3 + TdT + and cCD3 + CD34 + cells was confirmed in healthy volunteers, and the cut-off value was calculated based on the mean value plus three standard deviations. The calculated values were 0.008% and 0.001%, respectively. The expression of each antigen was described using one of the following situations: negative (-), which was defined as < 20% of abnormal blasts expressing the antigen, and positive (+), which was defined as > 20% of leukemic blasts expressing the antigen; low expression(low +), which was defined as > 20% and < 60% of blasts, and high expression(high +), which was defined as > 60% of blasts showing expression; bright expression (bri +), which was defined as a fluorescence intensity brighter than that observed in normal T cells, and dim expression (dim +), which was defined as weaker than that in normal T cells. The following two conditions were defined as MRD-positive (MRD +): 1) higher frequencies of CD34 + cCD3 + and TdT + cCD3 + T cells than their respective cut-off values, and 2) any percentage of abnormal immature T cells. All other cases were defined as MRD-negative (MRD-).

2.6. Real-time quantitative polymerase chain reaction (Q-PCR)

The SIL-TAL1 fusion transcript and WT1 gene expression were detected using TaqMan Q-PCR technology [23]. ABL1 was selected as the control gene to compensate for variations in the quality and quantity of the RNA and cDNA. The primers and probe for WT1 and ABL1 were based on a Europe Against Cancer Program report [24,25].

2.7. Statistical analysis

The SPSS 19.0 software package and GraphPad Prism 5.0 were used for data analysis. Different proportions of abnormal antigen expression in patients with newly diagnosed and relapsed T-ALL and the relapse rates of the MRD + and MRD- groups were analyzed using the Chi-square test. Differences in intervals from MRD detection to relapse between the MRD + and MRD- groups were analyzed by the nonparametric test for continuous variables. The probability of relapse following MRD detection was estimated using the cumulative incidence procedure and was applied to the follow-up up to the end of September 2016. Cumulative relapse probabilities were compared using the log-rank test. P values < 0.05 were considered statistically significant.

3. Results

3.1. Abnormal antigen expression

Table 1 presents a detailed description of all the antigens included in seven-color panel that were used to analyze patients with newly diagnosed and relapsed T-ALL. First, the most common leukemia-associated phenotype was immature antigen expression of either TdT or CD34, which was observed in 93.8% (60/64) of patients at diagnosis