High class II-associated invariant chain peptide expression on residual leukemic cells is associated with increased relapse risk in acute myeloid leukemia

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The presence of class II-associated invariant chain (CLIP) on leukemic cells is negatively associated with clinical outcome in untreated acute myeloid leukemia (AML). CLIP plays a role in the immune escape of leukemic cells, suggesting that it impairs the immunogenicity of minimal residual disease (MRD) cells causing a relapse. Here, we demonstrate that CLIP expression on leukemia-associated phenotype (LAP)-positive cells during follow-up is significantly correlated with a shortened relapse-free survival, even in those patients who are generally considered as MRDlow (0.01–0.1% LAP+ cells). Consequently, CLIP evaluation could be of additional value in the evaluation of MRD to predict a relapse of AML.

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1. Introduction

Although high-dose chemotherapy effectively reduces the tumor burden in most acute myeloid leukemia (AML) patients, some leukemic cells survive treatment and cause a relapse. Presence of aberrant immunophenotypic markers on malignant cells, the so-called leukemia-associated phenotype (LAP), provides a powerful tool to monitor minimal residual disease (MRD) [1,2]. By flow cytometric identification of LAP+ cells, information is gained about MRD frequency in the bone marrow (BM), which serves as a highly reliable predictor of relapse free survival (RFS) and overall survival (OS) after chemotherapy. One possible mechanism for the outgrowth of residual leukemic cells is their escape from immune surveillance due to a non-immunogenic phenotype [3]. During immune surveillance, presence of HLA class II on antigen-presenting cells (APC) is critical for optimal antitumor immunity by inducing a T helper cell response [4,5]. To enable loading and presentation of antigenic peptides, HLA class II molecules should release class II-associated Invariant chain peptide (CLIP) from their antigen-binding groove [6,7]. Besides professional APC, also leukemic cells have the capacity to express co-stimulatory and HLA class II molecules, serving as potent APC [8,9]. Previously, we showed that CLIP expression on primary HLA-DR+ leukemic cells interferes with effector T helper cell activation, indicating that it may serve as an immune escape mechanism in AML [10]. Additionally, high CLIP expression (>35%) on leukemic cells at diagnosis is associated with a shortened disease-free survival [8,11]. Here, we investigate the clinical impact of CLIP expression on LAP+ cells during follow-up in AML.

2. Materials and methods

2.1. Patient samples

For our study, we selected blood and BM samples from 50/412 AML patients who were admitted to our hospital between 2001 and 2010. From the total of...
412 patients, ~60% were eligible for both induction and consolidation therapy and reached complete remission (CR). HLA-DR negative acute leukemia (such as acute promyelocytic leukemia) and AML without a LAP were excluded. Also, in approximately one third of the remaining patients, CLIP could be analyzed and evaluated during follow-up, resulting in the final inclusion of 50 AML cases. Informed consent was obtained from all patients in accordance with theDeclaration of Helsinki.

Patients received chemotherapy according to the Dutch-Belgian cooperative trial group for Hematology Oncology (HOVON)-protocol (www.hovon.nl).

2.2. Immunophenotypical analysis

The presence of a LAP was determined as previously described [1,2]. In short, a panel of different monoclonal antibodies (moAb) was used to define a LAP on leukemic cells at diagnosis (Supplementary Table 1). BM was obtained after each treatment cycle or stem cell transplantation (SCT) and thereafter on a regular basis. The last available follow-up sample before relapse was used for MRD and CLIP analysis. During follow-up, 2 × 10^6 (at least 1 × 10^6) BM-derived WBC were labeled after red blood cell lysis with the moAb that defined the LAP, together with a primary marker (i.e. CD34 and/or CD117, CD45 and anti-CLIP (cercLIPI; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-HLA-DR moAb [28]. First, the total WBC compartment was characterized by CD45 expression and FSC. Next, aberrances were analyzed at diagnosis on primitive marker-positive cells with a low to intermediate SSC. In general, a single LAP marker was sufficient to analyze CLIP and HLA-DR expression on LAP cells; all samples were measured using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA); if an extra LAP-defining marker was necessary for CLIP and HLA-DR analysis on LAP cells, data files from different immunofluorescence stainings were merged using Infinicyt software (Cytognos, Salamanca, Spain). At least two markers (e.g. CD45 and CD34) and FSC/SSC were used as common markers. For detection of LAP^+ cells a cut-off of 0.01% was used. At least 200 LAP^+ cells were evaluated to ensure that 10% change in CLIP expression constituted at least 20 events. Presence of 0.1% or more LAP^+ cells (defined as LAP^+ cells but corrected for percentage LAP^+ cells at diagnosis [21]) was considered as MRD^high, while presence of 0.01–0.1% LAP^+ cells was regarded as MRD^low. The frequencies of CLIP-expressing cells within the LAP^+ population were determined as indicated in Supplementary Figure 1. A 35% cut-off was used to discriminate between patients with CLIP^high and CLIP^low expression [8]. Relative CLIP expression was determined by calculating the ratio between CLIP and HLA-DR expression based on both percentages and mean fluorescence intensity [10].

2.3. Statistical analysis

Relapse-free survival (RFS) was defined as the interval between the date of complete remission (CR) and relapse (respectively <5% and ≥5% leukemic cells in BM, as determined by cytomorphology). For survival data analysis, Kaplan Meier curves were compared by means of log-rank test; Cox univariate linear regression analysis was performed to evaluate the predictive value of LAP and CLIP percentages. p < 0.05 was considered significant.

3. Results and discussion

Previously, we reported that high MRD frequency is associated with poor prognosis and early relapse in AML [1,2]. This could be confirmed in our current cohort of 50 AML patients. Presence of MRD^high (>0.1% LAP^+ cells) was largely discriminative for a shortened RFS; all but one of the 13 MRD^high patients experienced a relapse compared to 14/37 patients considered MRD^low or MRD^neg (<0.1% LAP^+ cells; p < 0.001; data not shown). The median frequency of LAP^+ cells in the 26 patients who showed a relapse was 0.05% (range 0.00–4.53%) as compared to 0.03% (range 0.00–0.09%) in the 24 patients who remained in continuous remission (p = 0.03, n = 50, data not shown). There was no difference in distribution of the LAP in the MRD^low and MRD^high group.

Subsequently, we assessed the role of CLIP expression on LAP^+ cells regarding relapse occurrence during follow-up. In 5 patients, LAP^+ cells as defined at diagnosis were not detected during follow-up. Consequently, we were able to evaluate CLIP on LAP^+ cells in 45 patients; 24 experienced a relapse, while 21 remained in continuous CR (Fig. 1). Both the percentage of LAP^+ cells and the percentage of CLIP^+ cells within the LAP^+ population significantly predicted the occurrence of a relapse (p = 0.025 and p = 0.038, respectively; Cox univariate analysis, data not shown). Notably, high CLIP expression on LAP^+ cells was negatively associated with RFS (p = 0.001 [log rank]; Fig. 2(A)). No significant differences were found in leukocyte counts, percentages of blasts in the bone marrow, cytogenetic

Fig. 1. Schematic overview of the analyzed AML patient group with respect to leukemia associated phenotype (LAP), minimal residual disease (MRD), class II associated invariant chain peptide (CLIP) expression and occurrence of a relapse.

Fig. 2. The association of class II-associated invariant chain peptide (CLIP) on leukemia-associated phenotype (LAP)-positive cells with relapse-free survival (RFS). CLIP expression on LAP^+ cells was analyzed using a previously defined cut-off of 35%. Its impact on RFS was evaluated in all patients with LAP^+ cells, i.e. minimal residual disease-high (MRD^high) and low (MRD^low) (A) and in patients with 0.01–0.1% LAP^+ cells and thus considered MRD^low (B).
mechanism of leukemic cells. Moreover, in concordance with CLIP as a sole marker, the CLIP/DR ratio on leukemic cells at diagnosis was shown to be of importance in predicting RFS in AML [8]. For all cases in the present study, more than 90% of LAP+ cells were HLA-DR positive at diagnosis, however, the mean fluorescence did vary (MFI range 35–470). In 16 patients we analyzed both CLIP and HLA-DR expression on LAP+ cells during follow-up. A shortened RFS was found for patients with a high CLIP/DR ratio (CLIP/DR ratio cut off: 0.05; p = 0.03 [log rank], data not shown).

In the group regarded as MRDlow (0.01%–0.1% LAP+ cells), a minority of patients experienced a relapse (12 out of 32, Fig. 1). Interestingly, the presence of CLIP on LAP+ cells predicted a significantly shortened RFS in these MRDlow cases (p = 0.003 [log rank]; Fig. 2(B)). Flow cytometric detection of MRD is important for predicting a relapse in AML. However, for a few patients, the presence of MRD is not predictive although a relapse is experienced. In these cases, analysis of CLIP on LAP+ cells during follow-up will help to better define MRD and to identify patients who will likely develop a relapse. In addition, current treatment modalities may be utilized earlier and could benefit from future developments in immunomodulatory treatment to enhance the immunogenicity of MRD cells in these CLIP-positive high risk cases.

Remarkably, no correlation was found between CLIP expression on LAP+ cells during follow-up and CLIP expression in AML at diagnosis; from 11 patients with CLIPlow LAP+ cells during follow-up, 7 patients showed low CLIP expression (<35%) at diagnosis, while 4 patients had high CLIP expression (>35%; data not shown). In cases with CLIPlow expression at diagnosis, but CLIPhigh expression at relapse, it remains to be investigated whether the small group of CLIPhigh leukemic cells preferentially survived chemotherapy and escaped immune surveillance or residual leukemic cells acquired CLIP expression during follow-up.

In conclusion, the data presented here demonstrate the clinical impact of CLIP on LAP+ cells during follow-up in AML. Expression of CLIP on LAP+ cells resulted in poor survival, not only in MRD-positive AML patients, but also in patients with low MRD (<0.1% LAP+ cells). This may point to CLIP expression as a mechanism for MRD cells to escape immune surveillance and consequently a promising target for future research aiming at the prevention of a relapse.

Conflict of interest statement

All authors have no conflict of interest to declare.

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Authors’ contributions. MMvL and WvdA designed the research study, performed the research, analyzed the data and wrote the paper; TMW, GJS, GJO and AAvdL designed the research study, analyzed the data and helped write the paper; AK, MT and AZ helped perform the research; NF, MFC and SMH helped design the research study, perform the research and analyze the data. All authors approved the submitted and final version.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2014.03.014.

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