Evaluation of focal adhesions as new therapeutic targets in acute myeloid leukemia

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1. Introduction

Acute myeloid leukemia (AML) is a neoplastic proliferation of immature cells (myeloblasts) which derive from an altered hematopoietic progenitor with unregulated maturation capacity. Excessive myeloblast proliferation displaces normal hematopoietic cells, inducing medullar failure. In addition, leukemic cells can infiltrate extramedullar tissues.

In the last two decades, knowledge on the biological basis of AML has substantially increased, particularly regarding its cytogenetic and molecular aspects. However, with the exception of acute promyelocytic leukemia, AML therapy has not substantially changed and most patients still die from their illness. About 70-80% of AML patients achieve complete remission after intensive induction chemotherapy. Unfortunately, 30 to 80%, depending on their age and cytogenetics, suffer relapse almost always involving the bone marrow. The level of acceptable toxicity in current polychemotherapy has reached its limit. Thus, novel, less toxic therapeutic strategies are needed to improve patient survival.

The molecular characterization of AML has yielded novel biomarkers with enhanced prognostic capacity. Despite the many alterations identified in this disease, their ability to predict clinical outcome is still limited. For this reason, there is a need to further pursue our understanding of the molecular pathophysiology of AML, in order to discover new prognostic markers and to identify new therapeutic targets. In AML, signaling through integrins and cytokine receptors is essential for leukemic cell survival and their overexpression correlates with poor prognosis. Activation of both receptor types induces signaling through focal adhesions. The main objectives of this project are the evaluation of the prognostic value of focal adhesion protein expression in human AML and the preclinical development of a new inhibitor of focal adhesion signaling, the pyrazoline compound, E7123.

Objectives

1. To evaluate the effect and mechanism of action of E7123, a new pyrazoline derivative, in vitro and in vivo, in a novel mouse model of acute myeloid leukemia (AML):

a) Evaluation of the alteration in focal adhesion signaling induced by E7123 in vitro in human AML cells.

b) Assessment of the in vitro antitumor effect on human AML cells by the combination of E7123 with Ara-C, daunorubicin or idarubicin

c) Analysis of the effect of focal adhesion protein overexpression on the antitumor activity and mechanism of action of E7123.

d) Establishment of a new animal model of AML in NOD/SCID mice and evaluation of E7123 antitumor effect and toxicity in vivo.

e) Evaluation of the effect of focal adhesion protein overexpression on E7123 antitumor effect in vivo.

2. To determine the prognostic value of the expression of the focal adhesion proteins FAK, Pyk2, p130Cas, Hef-1, Src and Lyn, in bone marrow samples of AML patients.

2. Results

Evaluation of the effect and mechanism of action of the E7123 compound in AML cell lines: We have demonstrated the involvement of focal adhesions in the antitumor mechanism action of E7123, a new pyrazoline compound patented by our group. This compound induces cell death in AML through the inhibition of the expression and/or phosphorylation of different proteins involved in focal adhesion signaling (FAK, p130Cas, Lyn, Paxillin). We have also demonstrated that E7123 induces the proteolysis of the p130Cas protein, generating a 31kDa fragment that is translocated to the nucleus where it regulates transcription. On the other hand, we have evaluated the antitumor effect of E7123 in combination with different

chemotherapeutic drugs currently in clinical use (Ara-C, idarubicin, daunorubicin). We have observed that all the combinations show an additive antitumor effect. These results imply that E7123 and the combined compounds act through different signaling pathways. Thus, their in vivo combination could improve antitumor activity against AML.

Effect of focal adhesion protein overexpression: In this project we have generated different cell lines that overexpress FAK or p130Cas proteins and evaluated their role in cellular behavior. We have demonstrated that overexpression of FAK and p130Cas increases cell proliferation in AML cell lines. Moreover, overexpression of FAK partially reverses the in vitro antitumor effect of Ara-C. On the other hand, we have also observed that FAK overexpression significantly increases the in vivo dissemination of leukemic cells, towards the bone marrow and spleen, as shown by the enhanced bioluminescence registered in these organs. However, this increase is not traduced in an alteration of mouse survival time.

Results in animal models: During this project, we set up a new AML animal model that allows the evaluation of new antitumor drugs. We performed several assays using different cell lines, injection techniques and mouse strains. We finally concluded that the bone marrow injection of NSG immunosuppressed mice with the AML cell line THP-1 generates an animal model with a dissemination pattern similar to that in patients (Figure 1A). This model has a 100% take rate and shows a survival time similar in all animals. Moreover, the bioluminescence emitted by the injected cells allows the in vivo follow up of their dissemination, facilitating the study of the antitumor activity of the evaluated compounds. In this model, we have demonstrated that treatment with Ara-C decreases cell dissemination and significantly increases mouse survival (Figure 1B). After setting up the animal model, we performed several in vivo assays aimed at evaluating the antitumor effect of the new compound E7123. In all the assays we observed that E7123 decreased leukemic cell dissemination, as determined by bioluminescence images

(Figure 1B). However, we did not observe a significant increase in mouse survival time. We think that this may be due to the high aggressiveness of the animal model. The short animal survival time did not allow the administration of enough number of drug doses to have an impact on their survival. Currently, we are setting up new animal models with longer survival time. We expect to use them to demonstrate not only E7123 inhibition of leukemic cell dissemination, but also its capacity to increase mouse survival time.

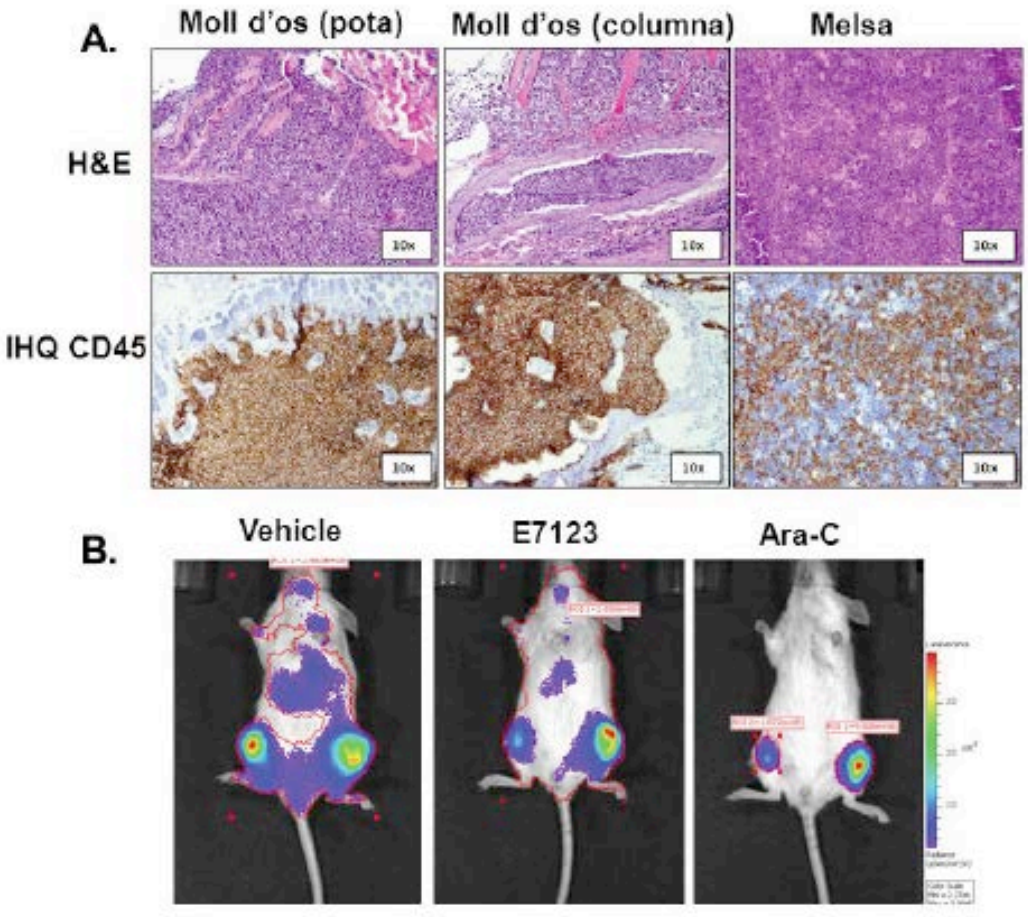

Figure 1. Animal model of AML. **A)** Image of H&E and IHC of CD45 from bone marrow and spleen showing the infiltration of leukemic cells. **B)** Image showing levels of bioluminescence of the control group mice, E7123 and Ara-C, 9 days after starting treatment. Treatment with E7123 or with Ara-C decreases dissemination of leukemic cells.

Evaluation of focal adhesion gene expression in bone marrow samples of AML patients: This part of the project has been performed co-ordinatedly in three

hospitals: Hospital Sant Pau (Barcelona), Hospital La Fe (Valencia) and Hospital Universitario (Salamanca). A total of 365 intermediate-risk AML patient samples were evaluated. We focused the study on this subgroup because it includes patients with widely different prognosis, being in the most urgent need of the establishment of new prognostic factors. We evaluated the expression of the following focal adhesion signaling genes: FAK, Pyk2, p130Cas, Hef-1, Src and Lyn. We studied the prognostic value of them all, regarding overall survival, disease-free survival and relapse rate (Figure 2). Both Kaplan Meier survival curves and additional univariate analyses showed that Pyk2, Hef-1 or Lyn overexpression significantly increased overall survival and disease-free survival in intermediate-risk AML patients. Patients that overexpressed Pyk2 or Hef-1 also showed a lower relapse rate. Moreover, we performed univariate analyses for the main clinical evolution variables determining that age (<50 years vs >50 years) and mutations of FLT3+NPM (FLT3wt+NPMmut as compared to other combinations) were significant prognostic factors. By performing multivariate analyses that included the variables that were significant in previous univariate analyses, we concluded that age, mutations in FLT3+NPM (FLT3wt+NPMmut vs other combinations) and overexpression of Hef-1 were maintained as independent prognostic factors for overall survival and disease-free survival. However, in the multivariate analysis, age, mutation in FLT3+NPM and overexpression of Pyk2 were independent prognostic factors for relapse rate. It is interesting to remark that we have identified one member of each of the three focal adhesion protein families (FAK, Cas and Src) studied as a new good prognostic factor in intermediate-risk AML patients. Thus, out of the two FAK family evaluated members (FAK and Pyk2) only Pyk2 has been identified as a prognostic factor in AML patients. In the Cas family we evaluated the p130Cas and Hef-1 genes and observed that only Hef-1 has prognostic value. Finally, in the Src family, while we evaluated the genes Src and Lyn, only Lyn had prognostic value. Thus, we can conclude that whereas all three focal adhesion gene families have prognostic value, only a single member of each family has been identified as a prognostic marker.

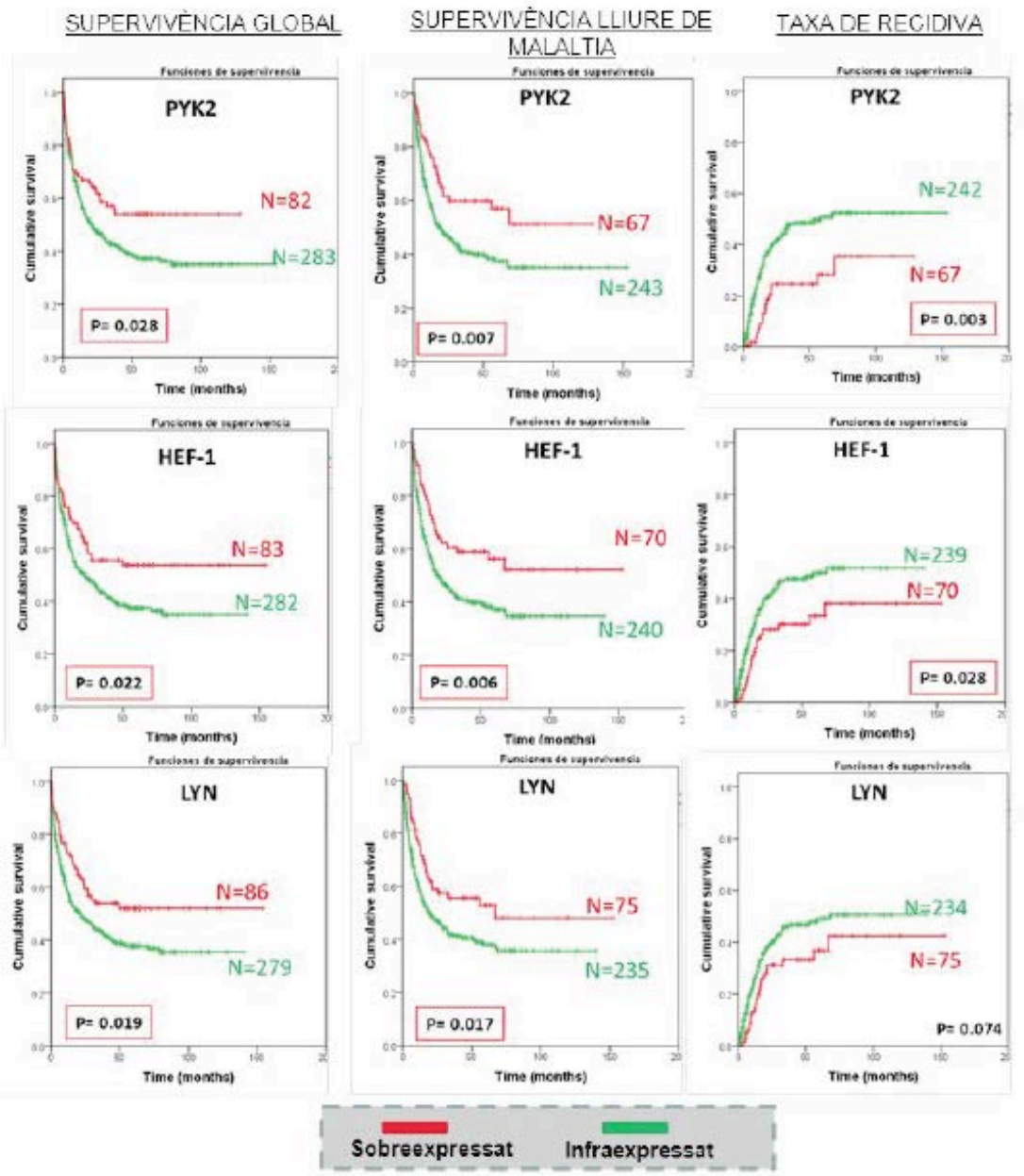

Figure 2. Survival curves (Kaplan Meier) for global survival, disease free survival and relapse rate for people who showed prognostic value (Pyk2, Hef-1 and Lyn). The *p* values were calculated with the Logf Rank test.

3. Relevance and possible implications

The results obtained in this project may have clinical repercussions for two main reasons:

a) **Demonstration of E7123 antitumor activity:** The results we have obtained so far do not allow us to conclude whether or not this new compound could be used in the future in the clinic. Two main data argue in favor of continuing the study of this compound. On the one hand, we have demonstrated that the combination of E7123 with some of the currently used drugs in the clinic has an additive effect. Therefore, these combinations may improve patient response. However, the in vitro results should be validated in vivo in order to determine the relevance of these observations. The fact that we have observed an additive effect indicates that E7123 has a mechanism of action different from the classical chemotherapeutic drugs (Ara-C, idarubicin, daunorubicin) and that it may act through alternative signaling pathways. On the other hand, in all the in vivo assays performed to evaluate E7123 antitumor effect, we have concluded that this compound decreases the dissemination of leukemic cells as observed by bioluminescence follow-up. In contrast, we have not been able to demonstrate that this compound induces a significant increase in mouse survival time, a required result for keeping on developing this compound. We think that this may be caused by the high aggressiveness of the animal model used, as its survival time was only two weeks. We are currently considering the evaluation of this compound in new animal models that we are developing with less aggressive cell lines, which would allow the administration of the compound during a longer time and the determination of its effect on survival. Thus, we still need to perform additional experiments to assess the clinical relevance of this study by determining the possibilities that E7123 could enter clinical assays.

b) **Identification of new prognostic factor in intermediate-risk AML patients:** The results we have obtained in evaluating the expression of the 6 focal adhesion genes may have an important clinical impact. We have analyzed the prognostic value of the genes FAK, Pyk2, p130Cas, Hef-1, Src and Lyn in intermediate-risk AML patients. This patient subgroup is not identified by any specific molecular alteration; on the contrary, it includes all patients that do not have the markers that characterize the low-risk or high-risk subgroups. For this reason, although

some molecular prognostic markers have already been identified, the intermediate-risk group includes patients with different clinical characteristics, showing widely different response to treatment and survival. For this reason, an important clinical need still remains, which is to find new markers that could help in identifying the patient subset that needs a more aggressive therapy and distinguishing them from those with better prognosis who may respond to a less intensive treatment. In this study, we have indentified a patient subgroup with favorable prognosis that is associated with Pyk2, Hef-1 or Lyn gene overexpression. Overexpression of these genes is associated with longer overall survival and disease-free survival. In addition, Pyk2 or Hef-1 overexpression is also associated with a lower relapse rate. These factors could help improve prognosis in AML patients by identifying an intermediate-risk subgroup with high probability of responding to therapy. The results show that these three focal adhesion genes (Pyk2, Hef-1 and Lyn) are involved in the regulation of migration and adhesion of normal hematopoietic cells. It is still necessary to validate these results in an independent series of AML patients in order to confirm these conclusions. For this reason, one of our next objectives is to obtain patient samples from a different center to perform this independent validation.

4. Publications

Hoyos M, Nomdedeu JF, Esteve J, Duarte R, Ribera JM, Llorente A, Escoda L, Bueno J, Tormo M, Gallardo D, de Llano MP, Martí JM, Aventín A, Mangues R, Brunet S, Sierra J. Core binding factor acute myeloid leukemia: the impact of age, leukocyte count, molecular findings, and minimal residual disease. Eur J Haematol. 2013 Sep;91(3):209-18. doi: 10.1111/ejh.12130. Epub 2013 Jul 25. PubMed PMID: 23646898.

Nomdedéu JF, Hoyos M, Carricondo M, Bussaglia E, Estivill C, Esteve J, Tormo M, Duarte R, Salamero O, de Llano MP, García A, Bargay J, Heras I, Martí-Tutusaus JM,

Llorente A, Ribera JM, Gallardo D, Aventin A, Brunet S, Sierra J; CETLAM Group. Bone marrow WT1 levels at diagnosis, post-induction and post-intensification in adult de novo AML. Leukemia. 2013 Nov;27(11):2157-64. doi: 10.1038/leu.2013.111. Epub 2013 Apr 15. PubMed PMID: 23584566.

Nomdedéu J, Hoyos M, Carricondo M, Esteve J, Bussaglia E, Estivill C, Ribera JM, Duarte R, Salamero O, Gallardo D, Pedro C, Aventin A, Brunet S, Sierra J. Adverse impact of IDH1 and IDH2 mutations in primary AML: experience of the Spanish CETLAM group. Leuk Res. 2012 Aug;36(8):990-7. doi: 10.1016/j.leukres.2012.03.019. Epub 2012 Apr 19. PubMed PMID: 22520341.