CIP2A high expression is a poor prognostic factor in normal karyotype acute myeloid leukemia

Acute myeloid leukemia (AML) comprises a biologically and clinically heterogeneous group of aggressive disorders that occur as a consequence of a wide variety of genetic and epigenetic abnormalities in hematopoietic progenitors. Despite significant advances in the understanding of AML biology, overall survival remains poor due chiefly to the high rate of relapse after achieving complete remission, as well as primary induction chemotherapy failure. In fact, apart from the exceptions represented by a few subgroups, such as younger patients with more favorable genetic outcome, improvements in the treatment of AML have been slow.[1,2] Therefore, it is necessary to develop new therapies aimed at molecular targets to improve clinical outcome in AML.

Protein phosphatase 2A (PP2A) is a major serine/threonine phosphatase that negatively regulates numerous signal transduction pathways, and functions as a tumor suppressor in several cancers.[3] Our group reported that PP2A inhibition is a common event in AML, and that restoration of PP2A activity induces cell growth arrest and caspase-dependent apoptosis, suggesting that PP2A inactivation plays a crucial role in AML, and confirming its potential as a therapeutic target in hematologic malignancies.[4] Moreover, we found the PP2A endogenous inhibitor *SET* over-expressed in 28% of AML patients, where it is associated with short overall survival (OS).[5] Here, we study the prevalence and the prognostic significance of the high expression of *CIP2A*, another specific endogenous inhibitor of PP2A, in AML.

The CIP2A protein mainly performs its role in malignant cellular growth by inhibiting PP2A activity toward the oncogenic transcription factor c-MYC,[6] an oncoprotein that plays an important role in AML.[7] *CIP2A* is expressed in very few normal tissues but it is over-expressed in most human cancer types, where it is often associated with a clinically aggressive behavior.[6] Nevertheless, its role in AML is unknown. To further clarify the significance of *CIP2A* high expression in AML, we first investigated its prevalence using quantitative real time RT-PCR (QRT-PCR) in a series of 203 normal karyotype AML patients (NK-AML) at diagnosis. Detailed information about the molecular and statistical analyses performed is provided in the *Online Supplementary Appendix*. *CIP2A* $2^{-\Delta Ct}$ median expression in AML was 0.0044 (range 0.00012-0.59) while in bone marrow (BM) samples from healthy donors the median expression was 0.0055 (range 0.0027-0.0070). To define *CIP2A* high (*CIP2A*^{high}) expression levels, a cut-off value of 0.0072 was chosen (corresponding to CIP2A mean expression in

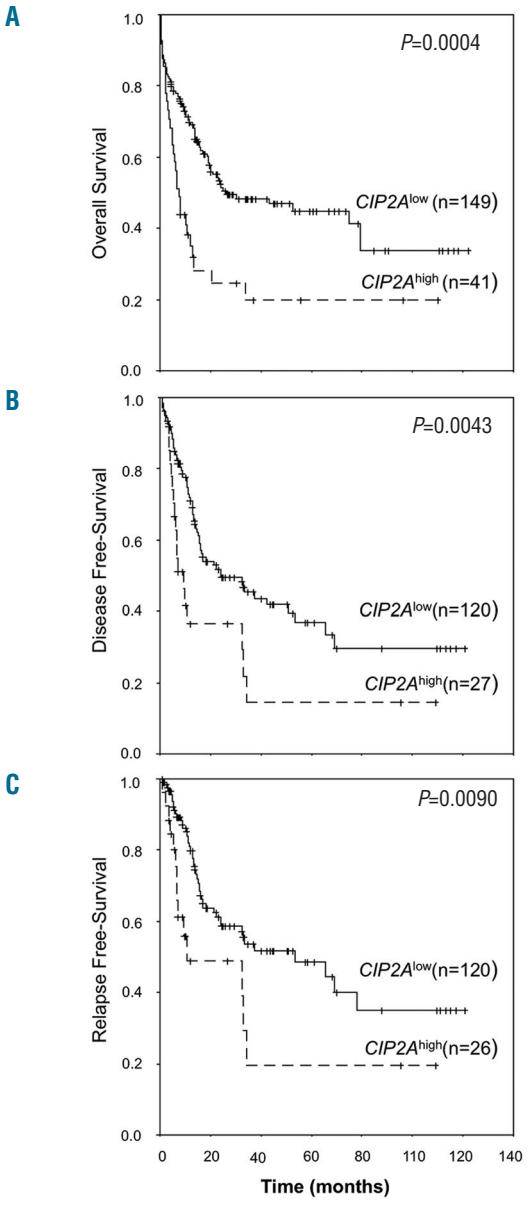

Figure 1. CIP2A^{high} expression is a poor prognostic factor in normal karyotype acute myeloid leukemia patients. Kaplan-Meier curve for (A) overall survival, for (B) disease-free survival and for (C) relapse-free survival in AML patients according to *CIP2A* expression.

Table 1. Multivariate analysis for overall survival, disease-free survival and relapse-free survival in the entire cohort and in young patients.

	OS		DFS		RFS	
Parameter	HR	*P*	HR	*P*	HR	*P*
All patients						
Age >65 years	2.423	0.001	1.920	0.042	2.497	0.018
FLT3-ITD	1.654	0.038	–	–	–	–
*NPM1*mut/*FLT3*wt	–	–	0.560	0.036	–	–
CIP2A^{high}	2.017	0.013	2.488	0.003	2.650	0.010
Patients under 65 years						
FLT3-ITD	1.828	0.044	1.882	0.037	2.006	0.047
*NPM1*mut*FLT3*wt	0.530	0.033	0.423	0.004	–	–
CIP2A^{high}	2.130	0.013	2.600	0.003	2.859	0.010

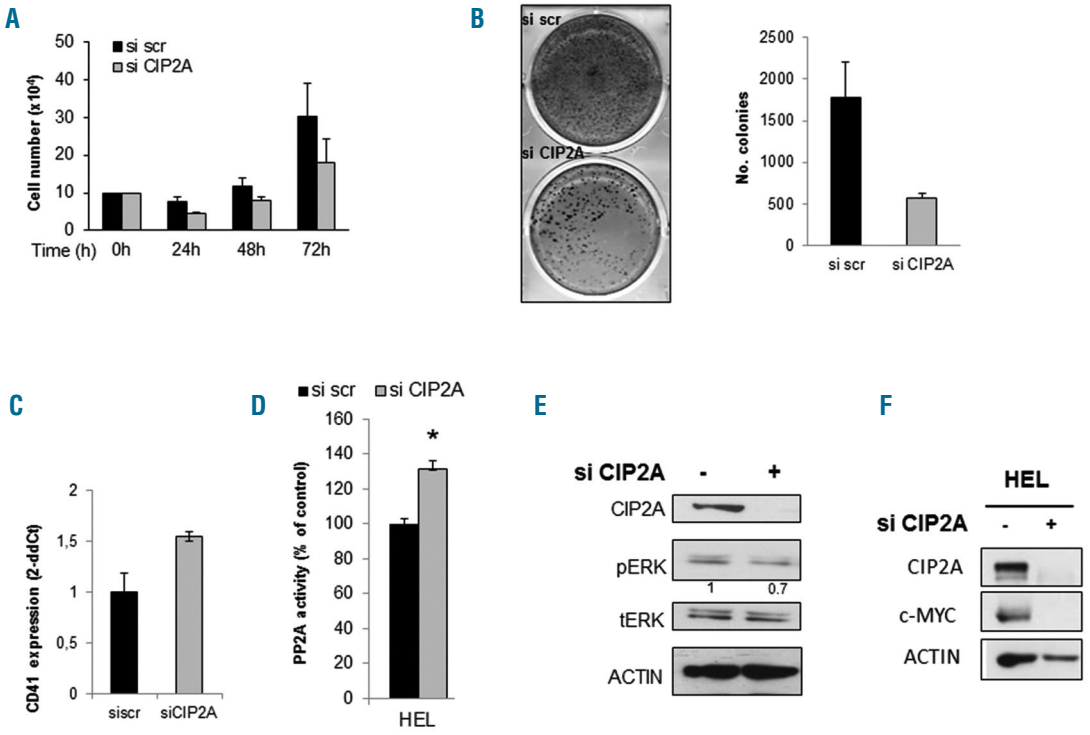

Figure 2. *CIP2A* depletion reduces cell proliferation and c-MYC stability in AML cells. Graphic representation of HEL cell line (A) viability; (B) clonogenic growth in soft-agar; and (C) CD41 expression as a marker differentiation, after *CIP2A* knockdown. PP2A phosphatase activity was determined by (D) free phosphatase quantification with phosphatase kit assay, and by (E) western blot of phosphorylated residues of ERK (Threonine202/Tyrosine 204). (F) Western blot of CIP2A and c-MYC after *CIP2A* knockdown.

normal BM+2SD and 75th percentile in AML patients). Surprisingly, several AML cases had low levels of CIP2A, even below normal BM. Additional studies are needed to clarify the meaning of this interesting finding. *CIP2A*^{high} expression showed no significant differences regarding clinical characteristics. However, correlations with molecular markers revealed a significant inverse association between *CIP2A* and *NPM1*. Patients with higher CIP2A expression showed a reduced incidence of *NPM1* mutations (*NPM1* mut) (30% *vs.* 48%; *P*=0.021). Patients' characteristics classified according to CIP2A expression are shown in *Online Supplementary Table S1*. To date, only one study has analyzed *CIP2A* expression in AML.[8] In this study, Wang *et al.* using conventional RT-PCR found that 77.4% of cases with AML expressed *CIP2A* (55 of 84), and confirmed their results at protein level. They classified the patients according to the French-American-British (FAB), but they provided no quantitative data. Thus, for the first time, we show *CIP2A*^{high} expression in a large cohort of NK-AML patients using QRT-PCR.

We next analyzed the prognostic significance of *CIP2A* expression in 190 cases treated with intensive chemotherapy (missing follow-up data, n=13). There were no significant differences in the rate of complete remission (CR) (75% *vs.* 83%), resistance (7% *vs.* 5%) or death during induction therapy (12% *vs.* 17%) between patients with *CIP2A*^{high} or *CIP2A*^{low} expression. The median follow-up time for survival was 20 months (range 1-122 months), and the estimated probability of overall survival (OS) at three years was 41%±4. Univariate analysis revealed that patients with *CIP2A*^{high} expression presented a significantly shorter OS compared with those with *CIP2A*^{low} expression (27% *vs.* 50%; *P*=0.0004) (Figure 1A). The median follow-up time in 147 AML patients who achieved CR was 21 months (range 0.9-120 months). Disease-free survival (DFS) at three years was 40%±4 and relapse-free survival (RFS) was 48%±5. Univariate analysis showed that patients with *CIP2A*^{high} expression presented a significantly shorter DFS (30% *vs.* 47%; *P*=0.0043) and RFS (46% *vs.* 60%; *P*=0.0090) compared with those with *CIP2A*^{low} expression (Figure 1B and C). Similar results for OS, DFS and RFS were obtained in the subgroup of patients younger than 65 years (32% *vs.* 58%, *P*=0.0017; 30% *vs.* 53%, *P*=0.0062; and 50% *vs.* 66%, *P*=0.0218, respectively).

Cox multivariate analysis included age, sex, white blood cell count, percentage of BM blasts, *FLT3*-ITD, *NPM1* mutations and *CIP2A* expression. For OS, age over 65 years (*P*=0.001; HR=2.423), *FLT3*-ITD (*P*=0.038; HR=1.654) and *CIP2A*^{high} expression (*P*=0.013; HR=2.017) were independent prognostic factors. For DFS, age over 65 years (*P*=0.042; HR=1.920), *NPM1*mut without *FLT3*-ITD (*P*=0.036; HR=0.560) and *CIP2A*^{high} expression (*P*=0.003; HR=2.488) were independent prognostic factors. For RFS age over 65 years (*P*=0.018; HR=2.497) and *CIP2A*^{high} expression (*P*=0.010; HR=2.650) were independent prognostic factors (Table 1). When only AML patients younger than 65 years were considered, *FLT3*-ITD and *CIP2A*^{high} expression were independently associated with a worse outcome for OS, DFS and RFS. *NPM1*mut without *FLT3*-ITD were associated with better clinical outcome for OS and DFS (Table 1). The clinical relevance of *CIP2A*^{high} expression as a prognostic marker has been established in various solid and hematologic cancers, including gastric, bladder, ovarian, tongue, hepatocellular, colon, non-small cell lung carcinoma, and chronic myeloid leukemia;[6] however, the prognostic impact of this molecular marker in AML was unknown. Therefore, our results show for the first time that *CIP2A*^{high} expression is an independent poor prognostic factor in NK-AML. The

adverse prognosis for patients with *CIP2A*^{high} expression was particularly significant in younger patients without *NPM1* mutations. This interesting finding could be relevant because molecular markers may define subgroups of patients and help in clinical decision-making.

We next evaluated the functional role of *CIP2A* in AML. Western blot showed that high CIP2A protein level is a recurrent event in AML cell lines (*Online Supplementary Figure S1*). Afterwards, *CIP2A* was down-regulated using a specific siRNA as previously described[9] in the AML HEL and TF1 cell lines. As previously reported in the HL-60 cell line,[8] *CIP2A* knockdown in these cell lines decreased cell proliferation and clonogenic growth, together with c-MYC protein stability (Figure 2 and *Online Supplementary Figure S2*). c-MYC is frequently activated in AML and plays an important role in leukemogenesis induction.[7] Our results indicate that *CIP2A*^{high} expression could contribute to the leukemogenic role of c-MYC in AML. Moreover, it has been reported that myeloid progenitor cells expressing *c-MYC* in bone marrow transplantation-transduction assays possess an intrinsic mechanism of resistance to c-MYC-induced apoptosis.[10] Although we detected no changes in apoptosis when *CIP2A* was down-regulated (*data not shown*), we observed an increase of differentiation to the megakaryocytic lineage (Figure 2 and *Online Supplementary Figure S2*). Finally, as expected, *CIP2A* knockdown increased PP2A activity by nearly 30%, together with a reduction of the inactivated form of PP2A catalytic subunit (PP2Ac) in HEL cells, decreasing the activity of ERK, a PP2A target (Figure 2). Two recent studies reported that patients with CIP2A negative tumors respond significantly better to cancer therapies.[6] In this regard, we and others have previously shown that PP2A activating drugs represent very promising anti-cancer molecules that can be used alone or in association with either kinase inhibitors or traditional chemotherapy in myeloid leukemias.[11,12] The 5-year survival rate of NK-AML patients varies from 24% to 42%, suggesting a genetic diversity within this subgroup.

Mutational and wide-genomic studies are proving very useful to refine prognosis in this AML subgroup, and it has been reported that the screening of at least ten genes: *ASXL1, CEBPA, DNMT3A, FLT3, IDH1, IDH2, MLL, NPM1, PHF6*, and *TET2*, would discriminate intermediate-risk AML patients into robust, clinically relevant risk groups.[13,14] However, as indicated above, the challenge in this aggressive disease is to provide potential therapeutic targets. Given this, our results identify a subgroup of patients who might benefit from future therapies with PP2A activators.[3]

In conclusion, we show that *CIP2A*^{high} expression is a recurrent event in AML where it represents a marker of reduced overall survival and a poor prognostic factor, as previously reported in other tumors. Moreover, *CIP2A* depletion down-regulates c-MYC, leading to a reduction of cell proliferation, and supporting the positive relationship between *CIP2A* and this oncogenic transcription factor in AML. Our results confirm that *CIP2A* behaves as an oncoprotein in AML, and suggest that it could represent a novel therapeutic target in AML.

Eva Barragán,[1] María Carmen Chillón,[2] Remedios Castelló-Cros,[3] Nerea Marcotegui,[3] María Isabel Prieto,[2] Montserrat Hoyos,[4] Raffaella Pippa,[3] Marta Llop,[1] Amaia Etxabe,[3] José Cervera,[1] Gabriela Rodríguez,[5] Ismael Buño,[5] José Rifón,[6] Jorge Sierra,[4] Marcos González,[2] María J. Calasanz,[7] Miguel A. Sanz,[1] and María D. Odero[3,7]

[1]Clinical Laboratory and Department of Hematology, University Hospital La Fe, Valencia; [2]Department of Hematology, University Hospital of Salamanca, Salamanca Institute for Biomedical Research (IBSAL); [3]Program of Hematology-Oncology, CIMA, University of Navarra, Pamplona; [4]Department of Hematology, Hospital Santa Creu i Sant Pau, Barcelona; [5]Department of Hematology, Gregorio Marañón Institute for Health Research, Madrid; [6]Department of Hematology, Clinical University of Navarra, Pamplona;and [7]Department of Biochemistry and Genetics, University of Navarra, Pamplona, Spain

EB and MCC contributed equally to this work.

Correspondence: barragan_eva@gva.es
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