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

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Supplementary appendix

Supplementary Materials and methods

Patients, controls and treatment

The study included 203 adult patients recruited between 1999 and 2012 in four Spanish institutions who were diagnosed of *de novo* AML with normal karyotype. Eligible patients were treated with intensive chemotherapy in which induction consisted of a combination of anthracycline plus cytarabine with or without etoposide following the PETHEMA and CETLAM AML trials. On achievement of complete remission (CR) patients proceeded to consolidation therapy and eligible cases were selected for autologous or allogeneic stem cell transplantation. Leukemia subtypes were classified according to both the French–American–British (FAB)[1] and the World Health Organization (WHO) criteria.[2] In addition, bone marrow samples 6 from healthy volunteers were used as normal controls. The Institutional Ethics Committee for Clinical Research approved this study. Written informed consent in accordance with the recommendations of the Declaration of Human Rights, the Conference of Helsinki and institutional regulations was obtained from all patients

CIP2A expression analysis

Total RNA was isolated from bone marrow (BM) samples using TRIzol reagent (Invitrogen, Carlsbad, CA). *CIP2A* quantitative expression was determined using the Taqman gene expression assay Hs00405413_m1 (Applied Biosystems, Foster City, CA) and all data were normalized with *GADPH* control gene expression. Fifty ng of cDNA were amplified in a 25 mL reaction containing 1x Taqman gene expression assay (Applied Biosystems) and 1x Taqman Universal PCR Master Mix (Applied Biosystems). The PCR program consisted of an initial denaturation at 95 ^{o}C for 10 min followed by 40 cycles of denaturation at 95 ^{o}C for 15 sec and annealing-extension at 60 ^{o}C for 1 min. The $2^{-(\Delta Ct)}$ method was used to quantify the relative expression of *CIP2A*. ΔCt = Ct (*CIP2A*) - Ct (*GADPH*). Efficiency of the quantification

was assessed by standard curves made using commercial bone marrow samples obtained from healthy donors The amplification efficiency for *CIP2A* was 1.98 and 2 for *GADPH*, indicating that the $2^{-\Delta Ct}$ method is applicable. Only those data with a Ct value <25 for the control gene *GADPH* were included in the evaluation. The experiment was repeated three times.

Cell cultures and transfection

EOL-1, HL-60, Kasumi-1, MV4-11, HEL, KG-1, KYO-1, MEG-01 and K562 cells were maintained in RPMI-1640 (Invitrogen) with 10% fetal bovine serum (FBS); KU-812 and MOLM13 in RPMI-1640 with 20% FBS; OCI-AML2 in alpha-MEM (Invitrogen) with 20% FBS, UT-7 in alpha-MEM with 20% FBS and 5 ng/mL GM-CSF; MUTZ-3 in 80% alpha-MEM with 20% FBS and 10 ng/mL GM-CSF; F36P and TF-1 in RPMI-1640 with 20% FBS and 10 ng/mL GM-CSF. Cell lines were grown at 37ºC in a 5% CO_2 atmosphere. Media were supplemented with penicillin G (100 U/mL), and streptomycin (0.1 mg/mL). For transfection experiments the Nucleofector System was used: solution V and protocol X-005 for HEL; solution V and protocol X-001 for TF-1, solution V and protocol T-019 for HL-60 (Amaxa), with 75 nM CIP2A or scramble siRNA.[9]

Cell viability

Cell proliferation was determined by trypan blue. Cells transfected with control or *CIP2A* siRNA were planted into six-well plates ($1x10^5$ cells/well) in triplicate and incubated for 72 h. The number of cells in each well was counted every 24 h, in triplicate with the Nucleocounter (Biogen), and the results confirmed by Trypan Blue method. The cell growth curve was generated accordingly.

Colony formation assay

Cells were plated in triplicate in six-well plates with agarose (Pronadisa) (top layer 0.3%; bottom layer 0.6%) and incubated until colonies were visible (10 days). The colonies formed were stained by adding 500µL of 5 mg/mL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide, Sigma) reagent during 4h at 37ºC. Colonies were fixed by overnight incubation with dimethyl sulfoxide (DMSO) at 37°C.

Cell differentiation

Cells were analyzed for surface expression of hematopoietic markers by real time expression and CD41 staining (FITC) (Becton Dickinson) and analyzed by using FACScalibur (Becton Dickinson). The primers used for the *CD41* detections were: *CD41* forward: 5´ ACCCAGTGCAGCTCACCTT 3´; *CD41* reverse: 5´ ACCACGATGGCCACTCTC 3´.

Western blot analysis

Protein extracts were isolated using TRIzol Reagent (Invitrogen) following the manufacturer's indications, clarified (12,000xg, 15 min, 4ºC), denatured and subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Antibodies used were STAT5, pSTAT5, AKT, pAKT(T308), ERK1/2, pERK1/2, (Cell Signaling), CIP2A and PP2Ac (Millipore), pPP2A (Epitome) and mouse monoclonal ACTIN (Sigma). Proteins were detected with the appropriate secondary antibodies by chemiluminescence (ECL kit, GE Healthcare).

PP2A phosphatase assays

PP2A assays were performed with cell lysates (50 µg) using a PP2A immunoprecipitation phosphatase assay kit (Millipore) following manufacturer's instructions. The amount of immunoprecipitated PP2A was assessed by western blots.

Statistical methods

The chi-square test, with Yates' correction if necessary, was used to analyze differences in the distribution of categorical variables between patient subsets. The Mann-Whitney's U test was used to analyze continuous variables for data that failed the normality test. For comparison, unadjusted time-to-event analyses were performed using the Kaplan-Meier estimate, log-rank tests and their generalizations. Overall survival (OS) was calculated from

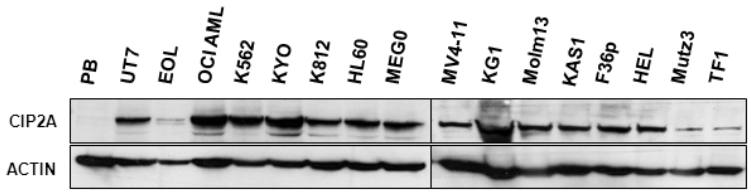
the time of diagnosis to the time of death. Disease–free survival (DFS) was calculated from the time of CR to the time of relapse or death, and relapse-free survival (RFS) from the date of CR to the time of relapse. Characteristics selected for inclusion in the multivariate analysis, using the Cox proportional hazards model, were those for which there was some indication of a significant association in univariate analysis ($P < 0.05$) and, if available, those for which prior studies had suggested a possible relationship. *CIP2A* was transformed in categorical variable for survival analysis using the *CIP2A* percentile 75 expression (*CIP2A*^{high} expressers presented higher values than the p75 and *CIP2A*^{low} expressers presented equal or lower values than the p75). Computations were performed using SPSS v12.0 statistical package (Chicago, IL). All P values reported are two-sided and a P value < 0.05 was considered significant.

Supplementary Tables

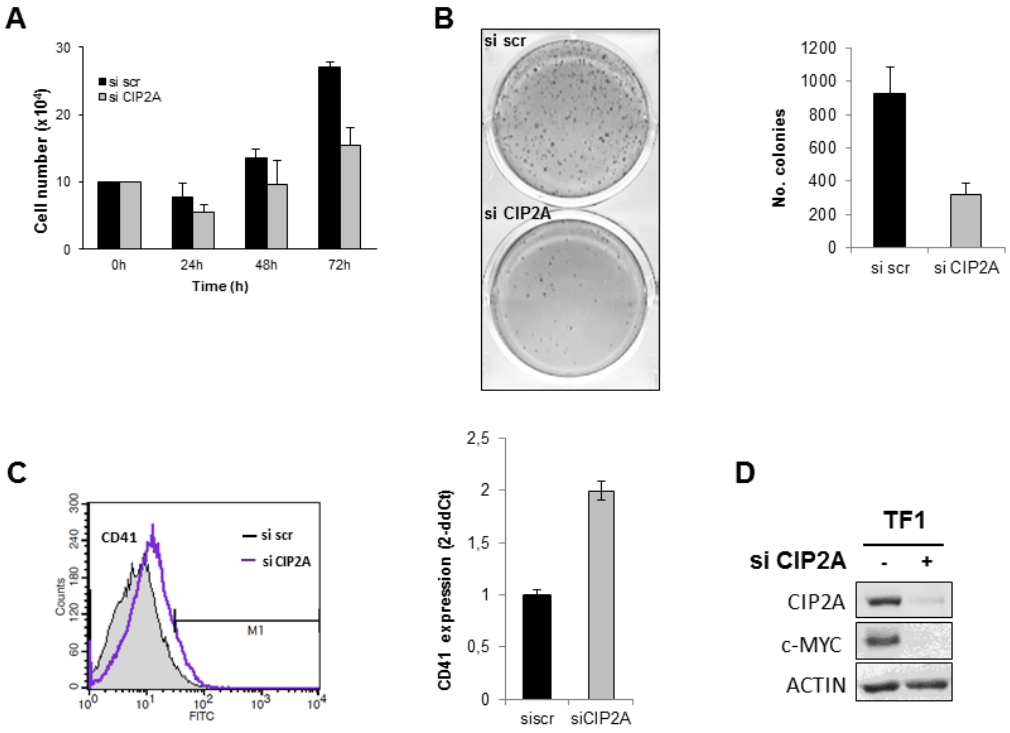
Supplementary Table 1. Clinical and genetic characteristics of NK-AML patients according to *CIP2A* expression

	CIP2A categorical expression				
	CIP2A^{low}		***CIP2A^{high}***		
Characteristic	**Median (range)**	**No. (%)**	**Median (range)**	**No. (%)**	***P***
Overall		152 (74.8)		51 (25)	
Age, years	55 (30-80)		58 (15-82)		
≤ 65		122 (80)		35 (69)	ns
> 65		30 (20)		16 (31)	
Gender					
Male		74 (49)		29 (57)	ns
Female		78 (51)		22 (43)	
WBC count (× 10^9/L)	15.2 (1-342)		10.6 (1.2-324)		
≤ 50		113 (75)		36 (76)	ns
> 50		37 (25)		11 (24)	
Missing values		2		4	
FAB					
M0		15 (10)		6 (12)	
M1		33 (22)		14 (29)	ns
M2		34 (23)		10 (21)	
M4		34 (23)		7 (15)	
M5		29 (20)		8 (17)	
M6		3 (2)		3 (6)	
Missing values		4		3	
Molecular markers					
***FLT3*-ITD**					
Yes		42 (28)		15 (31)	ns
No		107 (72)		33 (69)	
Missing values		3		3	
NPM1					
Yes		68 (48)		13 (30)	**0.021**
No		73 (52)		31 (70)	
Missing values		11		7	

Supplementary Figures



Supplementary Figure 1. CIP2A protein levels are high in AML cell lines. Western blot analysis showing CIP2A expression levels in 16 myeloid leukemia cell lines compared to peripheral blood samples of a normal donor. Actin was used as loading control.



Supplementary Figure 2. *CIP2A* depletion reduces cell proliferation and MYC stability and induces differentiation in the TF1 cell line. Graphic representation of (A) cell viability and (B) clonogenic growth in soft-agar. (C) FACS analysis (left) and real time expression (right) of the CD41 differentiation marker. (D) Western blot of CIP2A levels and c-MYC detection after *CIP2A* knockdown.

References

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