Chemotherapy Protocols

Seventy-five patients suitable for intensive treatments received chemotherapy induction regimens based on PETHEMA Spanish Cooperative group LAM99 (N=23) or LAM2007 (N=43) protocols. Briefly, they received cytosine-arabinoside (200 mg/m^2, days 1-7) and idarubicin (12 mg/m^2, days 1-3), and a second course with the same drugs and doses. Patients received one or two consolidation chemotherapy with high-dose cytosine-arabinoside 3 g/m^2 every 12 hours days 1-3, followed by autologous (N= 14) or allogeneic (N= 20) stem cell transplantation according to risk-profile. Promyelocytic acute leukemia patients were treated with an ATRA-based specific protocol LAP-PETHEMA2005 (N=9).

Primary samples and cell lines

Samples had been acquired during routine diagnostic assessments and were analyzed in accordance with the regulations and protocols approved by the University Hospital Reina Sofia. Informed consent had been obtained in accordance with the Declaration of Helsinki. Mononuclear cells (MNCs) of marrow aspirates from AML patients at diagnosis and healthy donors were isolated by Ficoll-Hypaque (Lymphoprep, Nycomed, Oslo, Norway) density gradient centrifugation after informed consent. For *in vitro* experiments, K562, HL-60 and MV4-11 leukemia cell lines (purchased from American tissue cell culture, ATCC) were routinely maintained in RPMI 1640 medium plus 10% of heat-inactivated fetal-calf serum (Both from BioWhittaker® Lonza, Switzerland) and antibiotics (100U/ml penicillin and 100µg/ml streptomycin, Sigma, St Louis, MO, USA).

Inhibition experiments

Inhibition experiments of PI3K or m-TOR pathways were performed in quadruplicates using Wortmannin (230 nM/mL), Ly294002 (25 µM/mL) and Rapamycin (50 nM/ml) (all from Sigma, St Louis, MO, USA). Briefly, 1x10^6 cells/ml were cultured in 24-wells plates with specific inhibitors during 12 hours with or without adding growth factors: Stem-cell factor (SCF) at 150nM and Flt-3 Ligand (FLT3-L) at 150 n/M (from R & D, Systems, MN, USA).

Preparation of cytosolic and nuclear protein extracts

Briefly, cell pellet from 5x10^6 cells was resuspended in 1ml of ice-cold extraction buffer 1 with protease inhibitor solution (10 min at 4° under rotation). Cells were then centrifuged (1000 x g, 10 min), the supernatant recovered as cytosolic proteins while pellet was resuspended in Extraction buffer 2 with protease inhibitor solution (30 min,

at 4º under rotation). Cells were again centrifuged (6000 x g, 10 min) and supernatant recovered as membrane proteins while pellet was resuspended in Benzonase® nuclease and mixed with extraction buffer 3 with protease inhibitor solution. After centrifuging (6800 x g, 10 min) supernatant was recovered as nuclear proteins. Protein concentrations from all compartmental fractions were quantified using the Bradford method using Protein Assay Kit (Bio-Rad, Madrid, Spain).

Western Blot

Proteins (50µg) were resolved by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Criterion XT-12% pre-cast gels (Bio-Rad, Madrid, Spain) and transferred onto a nitrocellulose membrane (Pall Corporation, Pensicola, Mexico) using a semi-dry transfer apparatus (Bio-Rad, Madrid, Spain), at 25v for 45 minutes. Membranes were first incubated with the following primary monoclonal antibodies: anti-survivin (6E4, 1:1000), total Akt (1:1000), and Akt-Ser473 (1:1000), (All from Cell Signaling Technology). Anti-survivin MnAb recognizes the common amino terminal included the dimer interface and the BIR domains. Survivin WT and splicing variants were clearly distinguished by their different molecular weights: 16.5 Kda for WT, 18 Kda for 2B and 14 Kda for Delta-Ex3. Specificity of each isoform was further confirmed by blocking peptide experiments (**Supplemental Figure 1**). Cytosolic and nuclear specific fractions were tested for purity using anti-β-actin (1:1000, Cell Signaling) and lamin-A (H-102, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) **(Supplemental Figure 2)**. Membranes were washed and incubated with horse radish peroxidase (HRP)-conjugated secondary anti-mouse-immunoglobulin G or anti-rabbit-immunoglobulin G (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Washing and incubation were performed using the ECL System (Amersham, GE heathcare). Finally, proteins were detected by chemiluminescence using in Chemigenius-2 device and quantified using Gene-Tools analysis software (both from Syngene, Cambridge, UK) using β-actin or laminin as loading control for cytoplasmic and nuclear extracts respectively. Briefly, tracks were located by specific molecular weights and Raw volumes of each track were obtained after background and overlapping corrections. These adjusted raw volumes values for each peak were expressed as normalized ratio with those raw volumes obtained from normal bone marrow samples processed in each experiment.

Analysis of G_0 quiescent and cell cycle

Hst was prepared at 1.6 µM in Hst buffer consisting of Hanks balanced salt solution (HBSS; BioWhittaker, Walkersville, MD) supplemented with 20 mM HEPES (BioWhittaker), 1 g/L glucose, 10% fetal calf serum (FCS; Hyclone, Logan, UT). PY was prepared in the same buffer to deliver a final concentration of 1 µg/mL. Test cells were washed twice in Hst buffer and resuspended in 1.5 mL 1.6 µM Hst solution (up to 5 × 10^6 cells) at 37°C for one hour at which time PY was added (without prior washing) to a final concentration of 1 µg/mL. Incubation was continued for another 45 minutes. Acquisition was performed in a FACS Vantage ^{SE} (BDIS) device with three-laser capability and UV tuned at 350 nm. As control to set-up regions, we used mononuclear peripheral blood cells from chronic lymphocytic leukemia patients, mostly residing in G_0 cycle fraction with very low PY RNA uptake. Cells were stained as described below but combined with surface FITC-conjugated CD19 instead of CD34. Analysis of percentages was carried out using CellQuest Software (BDIS).

Measurement of Apoptotic cells with DioC6.

To analyze changes in the mitochondrial transmembrane potential (ΔΨm), cells were incubated in PBS with 20 nmol/L 3,3¶-dihexyloxacarbocyanine iodide [DioC6(3);Sigma-Aldrich] for 20 minutes at 37ºC, washed with PBS, and 1 µg/ml propidium iodide (Sigma-Aldrich) were added followed by fluorescence-activated cell sorting analysis in a FACS Vantage $_{SE}$ (BDIS) device.

Statistical analysis

Normalized values of survivin WT and isoforms were categorized as positive if they were >1.0 (more than the expression in a normal marrow) or negative if they were ≤ 1 (equal-less than the expression in a normal marrow). For pSer473-Akt, positive was considered when normalized values were >1.0, with low expression ranging from 1.0 to <2.0 and high levels of pSer473-Akt if normalized value was ≥ 2.0. Correlation between survivin isoforms expression and with pSer473-Akt was assessed with linear regression analysis. χ^2 and Fisher's exact tests were used to compare survivin expression with various clinicopathologic parameters. The Mann-Whitney U test was chosen for nonparametric correlation of survivin expression with continuous variables. Response criteria were those reported by Cheson et al[22]. Relapse-free survival (RFS) was defined as the interval between the dates of achievement of a remission (Complete remission or CR with incomplete recovery, CRi) and relapse or death from any cause, whichever occurred first. Overall survival (OS) was defined as time from the

date of entry on treatment to the date of death from any cause or last-follow-up. The estimation of survival measures was performed using the Kaplan-Meier method and curves were compared by means of log-rank test. Confiance Intervals (CIs) were calculated according to Greenwood´s formula. The statistical independence between prognostic variables was evaluated by multivariate analysis using Cox's proportional hazards model with step-forward entry of all variables. Follow-up was updated at 1st May- 2012 which allows a minimum follow-up of 18 months for all patients.
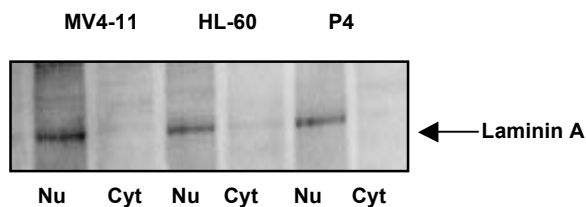
SUPPLEMENTAL FIGURES
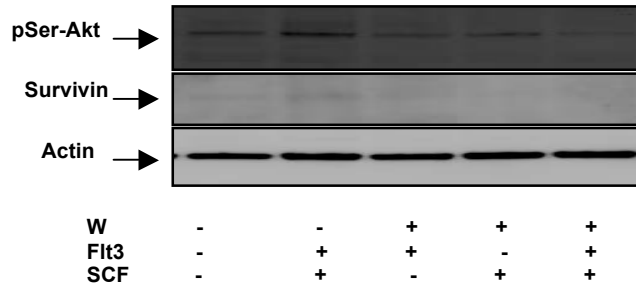
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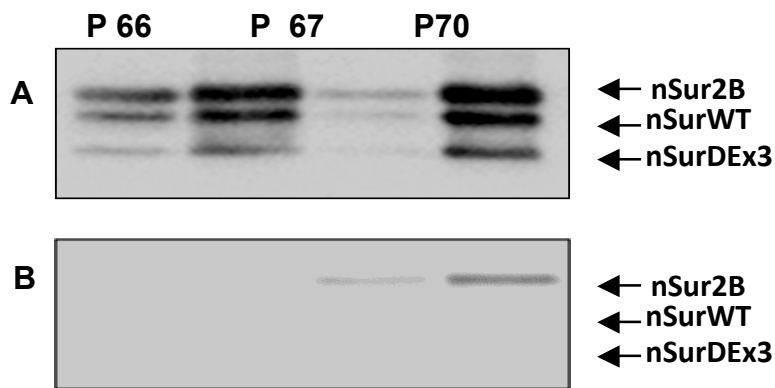
On line Supplementary Figure S1- Peptide competition assay of Western-blot analysis of Survin WT and isoforms. Western Blot using positive samples without (A) and with (B) blocking peptide SUR11-P.
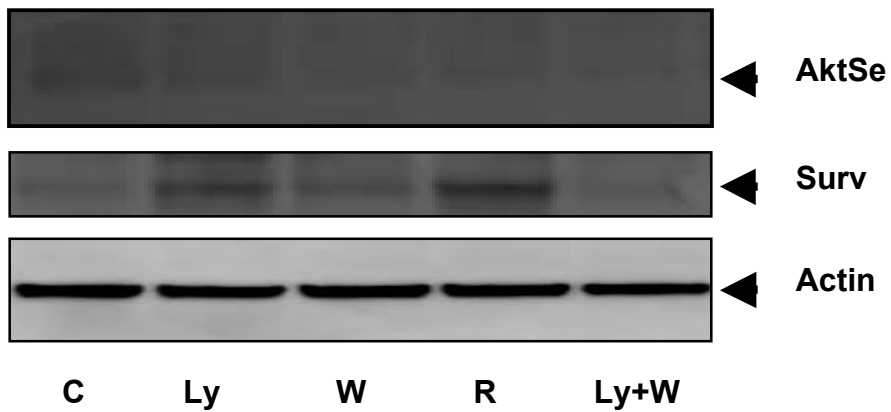
Supplemental Figure 2

On line Supplementary Figure S2.- Confirmation of nuclear and cytoplasmic protein extracts by Western-Blot for laminin-A positive in nucleus and negative in cytoplasm in cell-lines and samples.

Supplementary Figure 3

| pSer-Akt → | | | | | |
Survivin →					
Actin →					
W	-	-	+	+	+
Flt3	-	+	+	-	+
SCF	-	+	-	+	+

On line Supplementary Figure S3- Western-blot analysis of pSer473-Akt and CytSurWT after 24 hours culture of MV4-11 cell line with the PI3K inhibitor Wortmannin (W) and the combination of early-acting cytokines stem cell factor (SCF) and/or FLT-3 ligand (Flt3-L). Inhibition of CytSurWT expression by PI3k blocking was effective even in the presence of cytokines.

Supplemental Figure 4

AktSe

Surv

Actin

| **C** | **Ly** | **W** | **R** | **Ly+W** |

On line Supplementary Figure S4.- Western-blot analysis of pSer473-Akt and CytSurWT after 24 hours culture of HL-60 cell line with the PI3K inhibitors Ly294002 (Ly) and Wortmannin (W) and mTOR inhibitor Rapamycin (R)

Supplemental Figure 5
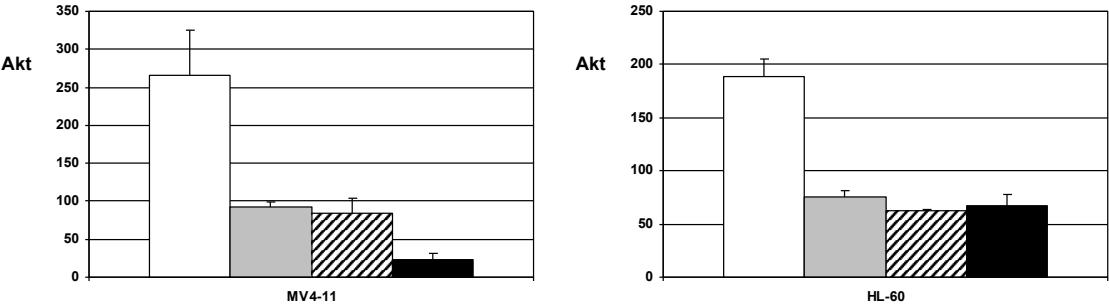
Supplementary Figure 5S.- Figure mean plus SD of raw volumes of Akt of quadruplicate experiments in MV4-11 and HL-60, Control white bars, Ly grey bars, n: grey shaded bars and Ly+Wort: black bars

Supplemental Figure 6
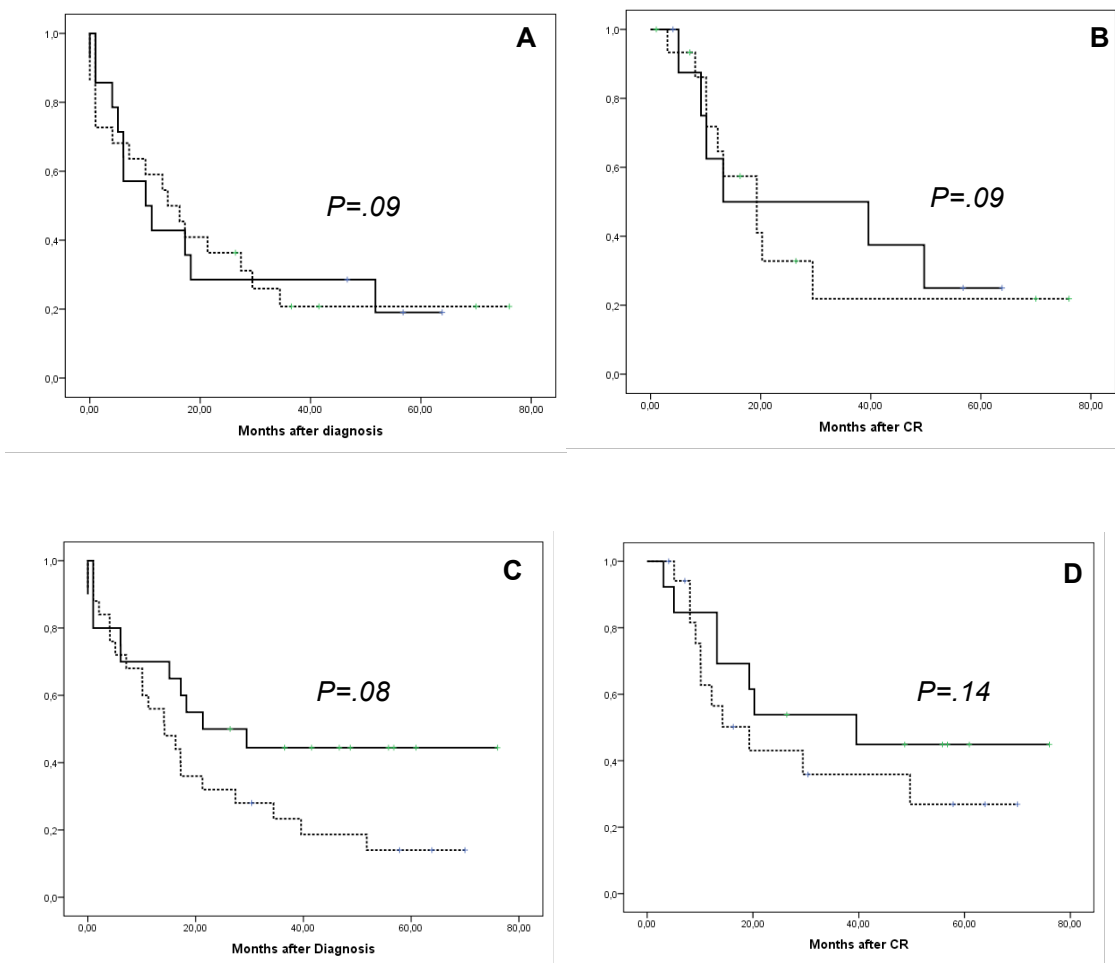
Supplemental Figure S6: Outcomes of AML patients according to SurWT subcellular localization. Overall survival was calculated with Kaplan-Meier curves for all 66 intensively treated patients and Relapse-Free-Survival were calculated for intensively treated patients achieving Complete Remission.
A: Kaplan-Meier curves for OS according to Sur2B cytoplasmic expression. **B**: Kaplan-Meier curves for RFS according Sur2B cytoplasmic expression. C: Kaplan-Meier curves for OS according to Delta-Ex3 nuclear expression and D: Kaplan-Meier curves for RFS according Sur2B survivin Delta-Ex3 nuclear expression. Continuous lines are positive for expression and dotted lines are negative for expression