



Phosphorylation of GSK3 α / β correlates with activation of AKT and is prognostic for poor overall survival in acute myeloid leukemia patients



Peter P. Ruvolo^{a,*}, YiHua Qiu^a, Kevin R. Coombes^{b,d}, Nianxiang Zhang^b, E. Shannon Neeley^c, Vivian R. Ruvolo^a, Numsen Hail, Jr^a, Gautam Borthakur^a, Marina Konopleva^a, Michael Andreeff^a, Steven M. Kornblau^{a,*}

^a Department of Leukemia, University of Texas MD Anderson Cancer Center, Houston, TX 77030, United States

^b Bioinformatics and Computational Biology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, United States

^c Department of Statistics, Brigham Young University, Provo, UT, United States

^d Department of Biomedical Informatics, Ohio State University Medical Center, Columbus, OH 43210, United States

ARTICLE INFO

Article history:

Received 28 April 2015

Received in revised form 7 July 2015

Accepted 10 July 2015

Available online 23 July 2015

Keywords:

Leukemia
GSK3
AKT
PKC delta
RPPA
Signal transduction

ABSTRACT

Background: Acute myeloid leukemia (AML) patients with highly active AKT tend to do poorly. Cell cycle arrest and apoptosis are tightly regulated by AKT via phosphorylation of GSK3 α and β isoforms which inactivates these kinases. In the current study we examine the prognostic role of AKT mediated GSK3 phosphorylation in AML.

Methods: We analyzed GSK3 α / β phosphorylation by reverse phase protein analysis (RPPA) in a cohort of 511 acute myeloid leukemia (AML) patients. Levels of phosphorylated GSK3 were correlated with patient characteristics including survival and with expression of other proteins important in AML cell survival.

Results: High levels of p-GSK3 α / β correlated with adverse overall survival and a lower incidence of complete remission duration in patients with intermediate cytogenetics, but not in those with unfavorable cytogenetics. Intermediate cytogenetic patients with FLT3 mutation also fared better respectively when p-GSK3 α / β levels were lower. Phosphorylated GSK3 α / β expression was compared and contrasted with that of 229 related cell cycle arrest and/or apoptosis proteins. Consistent with p-GSK3 α / β as an indicator of AKT activation, RPPA revealed that p-GSK3 α / β positively correlated with phosphorylation of AKT, BAD, and P70S6K, and negatively correlated with β -catenin and FOXO3A. PKC δ also positively correlated with p-GSK3 α / β expression, suggesting crosstalk between the AKT and PKC signaling pathways in AML cells.

Conclusions: These findings suggest that AKT-mediated phosphorylation of GSK3 α / β may be beneficial to AML cell survival, and hence detrimental to the overall survival of AML patients. Intrinsically, p-GSK3 α / β may serve as an important adverse prognostic factor for a subset of AML patients.

© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Acute myeloid leukemia (AML) remains a highly fatal disease, so a better understanding of the signaling pathways that support leukemia cell growth and survival is necessary in order to develop improved therapies. Strategies designed to target signaling cascades have been suggested as a way to optimize AML therapy [1–8]. The activation of survival kinases such as AKT, PKC, and ERK has been shown to predict poor clinical outcome for patients with AML [8]. AKT is a key regulator of protein translation, transcription, cell proliferation, and apoptosis, and is emerging as a potentially important target for AML therapy [1,3,4,8].

AKT has been shown to phosphorylate and inactivate GSK3, which is a key regulator of differentiation, metabolism, apoptosis, autophagy, as well as tumorigenesis [9–16]. Two functional isoforms of GSK3 known as GSK3 α and GSK3 β are produced from separate genes, but share 97% amino acid homology [9–11]. GSK3 α and GSK3 β are unique among kinases in that they are constitutively active unless their activity is blocked by post-translational modification such as phosphorylation [11]. An appraisal of kinase consensus sequences revealed that GSK3 had more possible substrates than any other kinase [12,13], although many GSK3 substrates require a “priming” phosphorylation event independent of GSK3. The intricate regulation of GSK3 and its substrates likely reflects the importance of modulating GSK3 signaling pathways as these cascades are critical for cellular homeostasis.

A prime example of role of GSK3 role in tumorigenesis involves the regulation of the WNT/ β -catenin pathways [11,16]. GSK3 mediates degradation of β -catenin by phosphorylating the molecule and stabilizing the docking molecule (i.e., axin) required for its destruction [11,13,16]. Failure to properly regulate β -catenin can result in

* Corresponding authors at: Department of Leukemia, Unit 448, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, United States.
E-mail addresses: pruvolo@mdanderson.org (P.P. Ruvolo), skornblau@mdanderson.org (S.M. Kornblau).

aberrant expression of pro-oncogenic molecules such as c-MYC, Cyclin D1, and c-JUN [13]. Thus a more comprehensive, understanding of the AKT–GSK3 signaling axis and its role in leukemogenesis is critically needed.

In the current study, we examined the ability of GSK3 total protein expression level, and protein activation state, based on serine 9 and/or serine 21 phosphorylation status, to predict overall survival (OS) and remission duration (RD) in AML patients using reverse phase protein array (RPPA) methodology. As phosphorylation of these sites results in inactivation of the GSK3 kinases, high phosphorylation levels indicate lower activity of these kinase in the tumor cells. Results suggest that AML patients with blast cells with inactivated GSK3, highly phosphorylated, have inferior OS and RD. RPPA revealed correlation of phospho-GSK3 with a number of proteins including phosphorylated AKT,

phosphorylated PKC δ , FOXO3A, and β -catenin. These results suggest that the AKT/GSK3 axis is critical in AML, which may be of benefit to the development of therapeutic strategies in AML patients classified with intermediate cytogenetics.

2. Material and methods

2.1. Patient samples

Peripheral blood and bone marrow specimens were collected from 511 patients with newly diagnosed AML evaluated at The University of Texas M.D. Anderson Cancer Center (MDACC) between September 1999 and March 2007. Samples were acquired during routine diagnostic assessments in accordance with the regulations

Table 1
Patient characteristics of all AML patients in the study.

Demographics for GSKp219															
Raw numbers															
		Treated							Treated						
		All cases	Lowest 2/3rd	Highest 3rd	P	Test used			All cases	Lowest 2/3rd	Highest 3rd	P	Test used		
Number		511	341	170	NA	Chi square	Number		511	341	170	NA	Chi square		
Gender	M	291	203	88	0.1153		Gender	M	56.9%	59.5%	51.8%	0.1153			
	F	220	138	82				43.1%	40.5%	48.2%					
Age	Min	15.8	16.01	15.83		t-Test	Age	Min	15.8	16.01	15.83		F test		
	Max	87.23	87.23	86.8				87.23	87.23	86.8					
FAB	Median	65.7	65	65.21	0.76	Chi square	FAB	Median	65.7	65	65.21	0.76	Chi square		
	M0	29	20	9				<0.00001	M0	5.7%	5.9%			5.3%	<0.00001
	M1	55	39	16					M1	10.8%	11.4%			9.4%	
	M2	169	127	42					M2	33.1%	37.2%			24.7%	
	M4	119	66	53					M4	23.3%	19.4%			31.2%	
	M5	50	19	31					M5	9.8%	5.6%			18.2%	
	M6	27	21	6					M6	5.3%	6.2%			3.5%	
	M7	10	7	3					M7	2.0%	2.1%			1.8%	
WHO	RAEBT	33	29	4	0.1	Chi square	WHO	RAEBT	6.5%	8.5%	2.4%	0.1	Chi square		
	Unknown	19	13	6				3.7%	3.8%	3.5%					
	Abnormal cytogenetics	47	33	14				9.2%	9.7%	8.2%					
	Therapy related	73	49	24				14.3%	14.4%	14.1%					
Cytogenetics	Multilineage dysplasia	110	83	27	0.36	Chi square	Cytogenetics	Multilineage dysplasia	21.5%	24.3%	15.9%	0.36	Chi square		
	Not in others	281	176	105				55.0%	51.6%	61.8%					
	Favorable	34	26	8				6.7%	7.6%	4.7%					
	Intermediate	225	170	82				44.0%	49.9%	48.2%					
FLT3	Unfavorable	252	145	80	0.145	Chi square	FLT3	Unfavorable	49.3%	42.5%	47.1%	0.145	Chi square		
	ITD	83	49	34				16.7%	9.9%	6.9%					
	D835	25	17	8				5.0%	3.4%	1.6%					
NPM1	Both	9			0.92	Chi square	NPM1	Both				0.92	Chi square		
	4BP insert	55	179	89				0.76	16.3%	83.6%	81.7%			0.76	
	WT	283	35	20				83.7%	16.4%	18.3%					
Zubrod PS	ND	173	127	61		Chi square	Zubrod PS	ND	33.9%	37.2%	35.9%		Chi square		
	3 or 4	17	11	6				3.3%	3.2%	3.5%					
AHD	≥ 2 mo	204	143	61	0.23	Chi square	AHD	≥ 2 mo	39.9%	41.9%	35.9%	0.23	Chi square		
Infection	Yes	101	62	39	0.35	Chi square	Infection	Yes	19.8%	18.2%	22.9%	0.35	Chi square		
WBC	Median	8.8	6.9	19.25	0.02	F test	WBC	Median	8.8	6.9	19.3	0.02	F test		
Absolute blast count	Median	1.8	1.13	3.54	0.28	F test	Absolute blast count	Median	1.8	1.1	3.5	0.28	F test		
Platelet	Median	56.0	55	57	0.08	F test	Platelet	Median	56.0	55.0	57.0	0.08	F test		
Hemoglobin	Median	9.6	9.6	9.45	0.15	F test	Hemoglobin	Median	9.6	9.6	9.5	0.15	F test		
% marrow blasts	Median	46.0	44	57	0.0002	F test	% marrow blasts	Median	46.0	44.0	57.0	0.0002	F test		
% blood blasts	Median	19.0	18	20.5	0.3	F test	% blood blasts	Median	19.0	18.0	20.5	0.3	F test		
Response	CR	231	153	78	0.17	Chi square	Response	CR	56.5%	44.9%	45.9%	0.17	Chi square		
	Resistant	136	101	35				Resistant	33.3%	36.7%	26.1%				
	Fail	42	21	21				Fail	10.3%	7.6%	15.7%				
Relapse	Yes	151	93	58	0.09	Chi square	Relapse	Yes	65.4%	60.8%	74.4%	0.09	Chi square		
Alive	Yes	103	76	27	0.14	Chi square	Alive	Yes	20.2%	22.3%	15.9%	0.14	Chi square		
Overall survival	Median, weeks	40.85	44.14	35.14			Overall survival	Median, weeks	40.85	49.1					
Remission duration	Median, weeks	51.6	50	34.5			Remission duration	Median, weeks	51.6	52					

and protocols (Lab 01–473) approved by the Investigational Review Board of MDACC. Informed consent was obtained in accordance with the Declaration of Helsinki. Samples were analyzed under an Institutional Review Board approved laboratory protocol (Lab 05–0654). The patient characteristics and sample preparation are described in Table 1.

2.2. RPPA method

Proteomic profiling was done on samples from patients with AML using RPPA. The method and validation of the technique are fully described in previous publications [7,19]. Briefly, patient samples were printed in five serial dilutions onto slides along with normalization and expression controls. Slides were probed with strictly validated primary antibodies. Antibodies against total GSK3 α/β (Santa Cruz Biological, Catalog # 9331, primary dilution 1:200) and the serine 9 and serine 21 sites on GSK3 α/β (Cell Signaling, Catalog #9331, primary antibody dilution 1:200) were used. This same array was probed with 229 other antibodies as listed in Supplemental File 1. An IgG subtype specific secondary antibody (1:15,000 dilution) was used to amplify the signal and finally a stable dye is precipitated. The stained slides were analyzed using the MicroVigene software (Vigene Tech, Version 3.0) to produce quantified data.

2.3. PKC δ and FOXO3A knockdown cells

FOXO3A was knocked down in OCI-AML3 cells by lentiviral transduction using a gene-specific shRNAmir transfer vector (Clone V3LHS_375386 mature target sequence CGGAACGTGATGCTTCGCA, which targets 1862–1880 on RefSeq NM_001455.3 Clone V3LHS_641765 mature target sequence CGGCACAACCTGTCACTGC, which targets 974–992 on RefSeq NM_001455.3). PKC δ was knocked down in THP-1 and HL60 cells by lentiviral transduction using a gene-specific shRNA transfer vector (Clone TRCN000010202 mature target sequence GCAAGACAACAGTGGGACCTA, which targets 1334–1354 on RefSeq NM_006254.3) (Open Biosystems, Huntsville, AL). Lentivirus was prepared by cotransfection of HEK293T cells (ATCC) with an equimolar mix of transfer vector and packaging plasmids (psPAX2 and pMD2.G from Addgene, Cambridge, MA) using JetPrime transfection reagent as directed by the manufacturer (Polyplus, Illkirch, France). Fresh lentiviral supernatants were passed through 0.45 μ m pore surfactant-free cellulose acetate membranes and then used at once to infect OCI-AML3, THP-1, and HL60 cells by incubation overnight at 37 °C under 5% CO₂. OCI-AML3 cells were the kind gift from Mark Minden (Ontario Cancer Institute; Toronto, Canada). THP-1 and HL60 cells were purchased from ATCC (Manassas, VA). Cells were subject to selection with puromycin (Invivogen, San Diego CA) starting at 1 μ g/mL. In parallel, all cells were transduced using lentivirus delivering a non-specific control (Open Biosystems). Knockdown was verified by Western blot analysis and by real time PCR.

2.4. Western blot analysis

Cells were treated with various doses of MK-2206 (Selleck Chemicals) for 6 h or 5 μ M GSK3 Inhibitor XVI (Calbiochem) for 6 h. Cells were sonicated in lysis buffer (62.5 mM Tris (pH 8.0), 2% SDS, 10% glycerol, 100 μ M AEBSF, 80 nM aprotinin, 5 μ M bestatin, 1.5 μ M E-64, 2 μ M leupeptin, 1 μ M pepstatin, 500 μ M sodium orthovanadate, 500 μ M glycerol phosphate, 500 μ M sodium pyrophosphate and 50 μ M DTT) and protein (5 \times 10⁵ cell equivalents) was subjected to electrophoresis using 10–14% acrylamide/0.1% SDS gels. Proteins were transferred to a nitrocellulose membrane and Western blotting analysis was performed with antibodies against phospho-PKC δ (Cell Signaling, Beverly, MA), PKC δ (Santa Cruz Biotechnology), phospho-GSK3 α/β (serine 9/21; Cell Signaling), GSK3 α/β (Cell Signaling), MYC (Cell Signaling), Foxo3A (Cell Signaling), and Tubulin (Sigma). Signals were

detected by using the Odyssey Infrared Imaging System and quantitated by Odyssey software version 3.0 (both LI-COR Biosciences, Lincoln, NE, USA). Tubulin was used as a loading control.

2.5. Statistical analyses

For RPPA, super curve algorithms were used to generate a single value from the five serial dilutions [20]. Loading control and topographical normalization procedures accounted for protein concentration and background staining variations [21]. Analysis using unbiased clustering perturbation bootstrap clustering, and principle component analysis was then done as fully described in a previous publication [7]. The cohort was divided into sixths based on the range of expression of all 511 samples. Based on a similarity of outcomes these were collapsed into two groups with expression in the upper third vs. the lower two thirds for phospho-GSK3 α/β . Comparison of the protein levels between paired samples was done by performing paired t test. Association between protein expression levels and categorical clinical variables were assessed in R using standard *t*-tests, linear regression, or mixed effects linear models. Association between continuous variable and protein levels were assessed by using the Pearson and Spearman correlation and linear regression. Bonferroni corrections were done to account for multiple statistical parameters for calculating statistical significance. The Kaplan–Meier method was used to generate the survival curves. Univariate and multivariate Cox proportional hazard modeling was done to investigate association with survival with protein levels as categorized variables using the Statistica version 12 software (StatSoft, Tulsa, OK). Although some molecular markers (NPM1, FLT3-ITD, and RAS mutations) are known for this dataset other more recently discovered prognostic markers (e.g., DNMT3A, CEBP α , Wilms Tumor 1) are not; therefore, the multivariate analysis did not contain all known AML prognostic markers. Overall survival was determined based on the outcome of 415 newly diagnosed AML patients treated at UTMACC and RD was based on the 237 patients that achieved remission.

3. Results

3.1. Phosphorylated GSK3 β levels are elevated in a subset of AML patient cell samples compared to normal CD34⁺ cells

We have used RPPA for the analysis of protein expression from clinical samples in many hematologic malignancies [7,8,19,22–24]. Analysis of proteins of interest is possible as long as a validated antibody is available. We used RPPA to study total and phosphorylated GSK3 α/β (at serine 21 and serine 9, respectively) in AML blast cells obtained from 511 newly diagnosed AML patients and CD34⁺ cells obtained from 11 normal bone marrow donors. We compared phosphorylation status of the kinase in the AML patient set with that in normal CD34⁺ cells. Total levels of GSK3 β protein were significantly ($p = 0.0059$) lower in the blast cells from the AML patients compared to normal CD34⁺ cells as shown in the histogram in Fig. 1A. Levels of phosphorylated GSK3 α/β protein were lower in the AML cells overall ($p < 0.0273$; Fig. 1B) with 17.4% of cases having levels above normal and 35.8% with levels below normal CD34⁺ cells. There is a set of patients that show high levels of phosphorylated GSK3 α/β on the histogram in Fig. 1B. Differences in phosphorylation status of the kinase among various AML sub-types determined by French–American–British (FAB) class and cytogenetic category were very pronounced ($p < 0.0001$ for both categories; Supplemental Fig. S1A and B). Corresponding plots for total GSK3 α/β are shown in Supplementary Fig. S1C and D. Levels of total and phosphorylated GSK3 α/β did not differ between diagnosis and relapse in 47 patients with paired samples ($p = 0.21$ for total protein and $p = 0.31$ for phosphorylated protein).

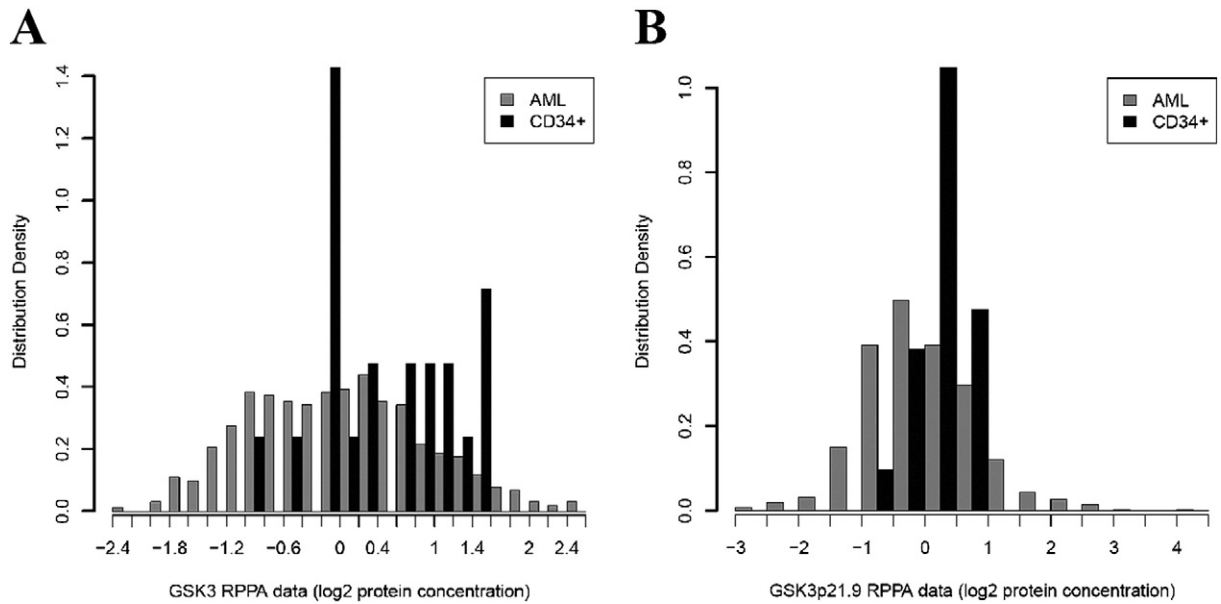


Fig. 1. GSK3 α/β total and serine 21/9 phosphorylated protein expression in AML blast cells compared to normal counterpart CD34+ cells. RPPA was performed as described in the “Materials and methods” section. Expression of total (A) and phosphorylated (B) GSK3 α/β was compared in blast cells from 511 AML patients versus CD34+ bone marrow cells from 11 normal donors.

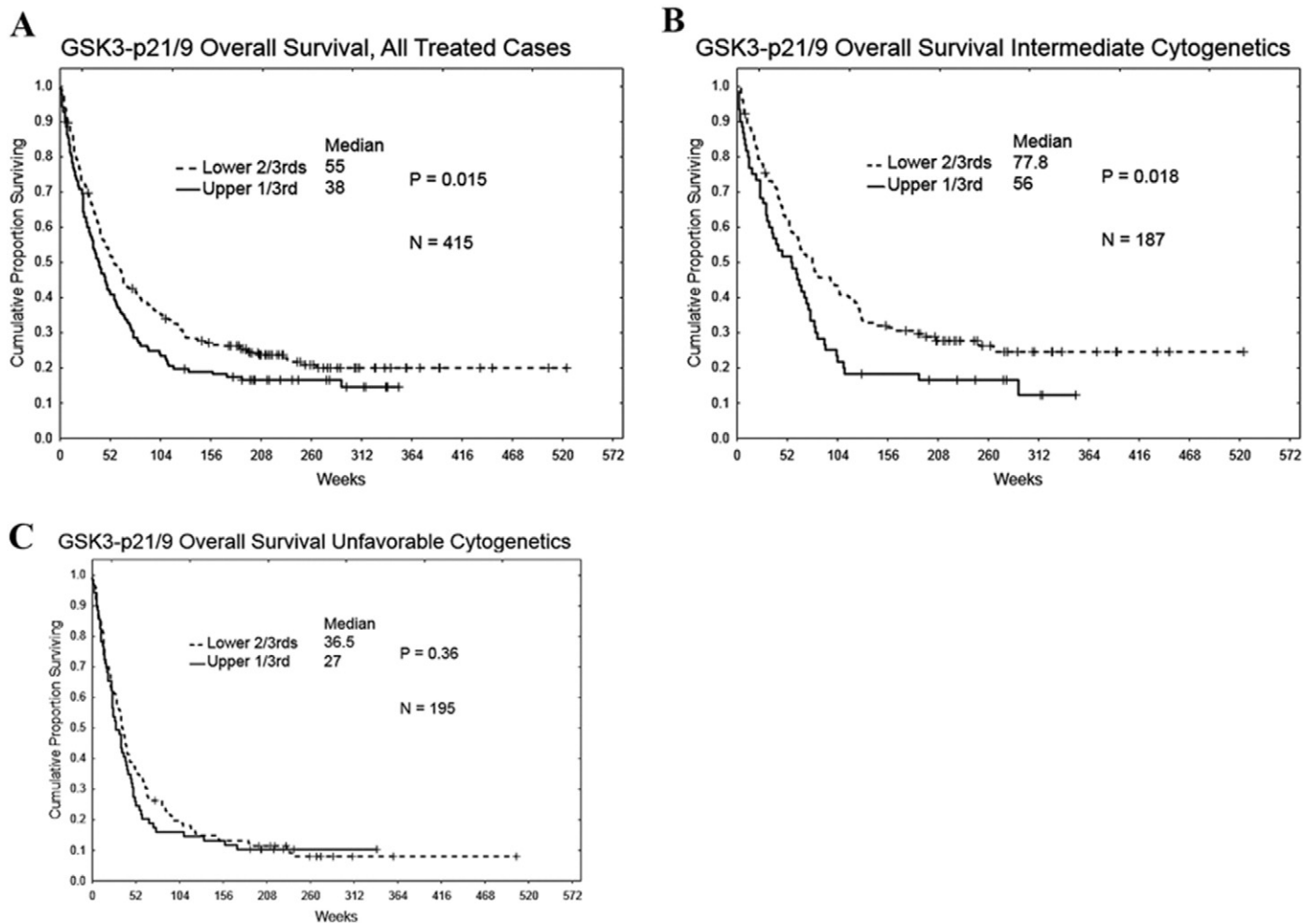


Fig. 2. Correlation of overall survival with serine 21/9 GSK3 α/β phosphorylation levels in AML patient cytogenetic subsets. The Kaplan-Meier curve for overall survival (OS) for the total AML patient set (A; N = 415), intermediate cytogenetic patient set (B; N = 187), and unfavorable cytogenetic patient set (C; N = 195) based on expression of phosphorylated GSK3 α/β .

3.2. Phosphorylated GSK3β levels are associated with poor survival outcome and shorter RD

The level of total GSK3α/β, divided at the median, was not predictive of remission attainment (55.5 vs. 54.8%, respectively) RD (46 vs. 46 weeks, respectively, $p = 0.68$), or overall survival (49 vs. 49 weeks, respectively, $p = .80$). For survival analysis, RPPA values for phospho-GSK3α/β were categorized into two groups (i.e., lower two-thirds and upper one-third) based on the range of expression in all 511 cases. However among all AML patients, those with higher levels of phosphorylated GSK3α/β exhibited shorter overall survival duration compared to those with low levels of the phosphorylated kinase (40 vs 54 weeks, respectively; $p = 0.015$; Fig. 2A). For patients with intermediate cytogenetics, phosphorylated GSK3α/β predicted poor overall survival as patients with elevated levels of phosphorylated kinase exhibited significantly shorter overall survival duration compared to patients with lower levels of phosphorylated GSK3α/β (Fig. 2B; 56 vs 77.8 weeks, respectively; $p = 0.018$). Phosphorylation status of GSK3α/β had no effect on overall survival duration in patients with unfavorable cytogenetics (Fig. 2C). Likewise no difference was seen among those with favorable cytogenetics though the patient sample size was small ($N = 32$; $p = 0.39$; data not shown).

The phosphorylation of GSK3 kinases also influenced RD. Among all categories of AML patients that achieved remission ($N = 237$), patients

with higher levels of phosphorylated GSK3α/β exhibited shorter RD compared to those with low levels of phosphorylated GSK3α/β (Fig. 3A; 43 weeks vs 67 weeks respectively; $p = 0.022$). Among intermediate cytogenetic patients ($N = 121$), the influence of phosphorylation of GSK3 kinase on RD was more pronounced as patients with higher levels of phosphorylated GSK3α/β displayed much shorter RD compared to patients with low levels (Fig. 3B; 45 weeks vs 95 weeks respectively; $p = 0.0075$). This correlation was observed even in the intermediate cytogenetic patients ($N = 35$) with FLT3 mutation (Fig. 3C; 24 weeks vs 50 weeks respectively; $p = 0.009$).

3.3. Phosphorylation of GSK3 kinases correlates with activation of AKT and other signaling molecules in blast cells from AML patients

We have previously used RPPA to study the survival signaling pathways in AML blast cells including those that involve AKT [7,8,19,22–24]. RPPA was used to examine correlations of total or phosphorylated GSK3α/β with 229 proteins in blast cells from 511 AML patients. Sixty four proteins are significantly correlated with total GSK3 expression in the AML cells ($p < 0.0001$; $R > 0.30$; Supplemental Fig. 2). As shown in Fig. 4, forty-four proteins showed statistically significant ($p < 0.0001$, $R > 0.20$) correlation with phosphorylated GSK3α/β. Twenty four proteins displayed a positive correlation while twenty proteins displayed a negative correlation with the phospho-kinase. The two proteins that

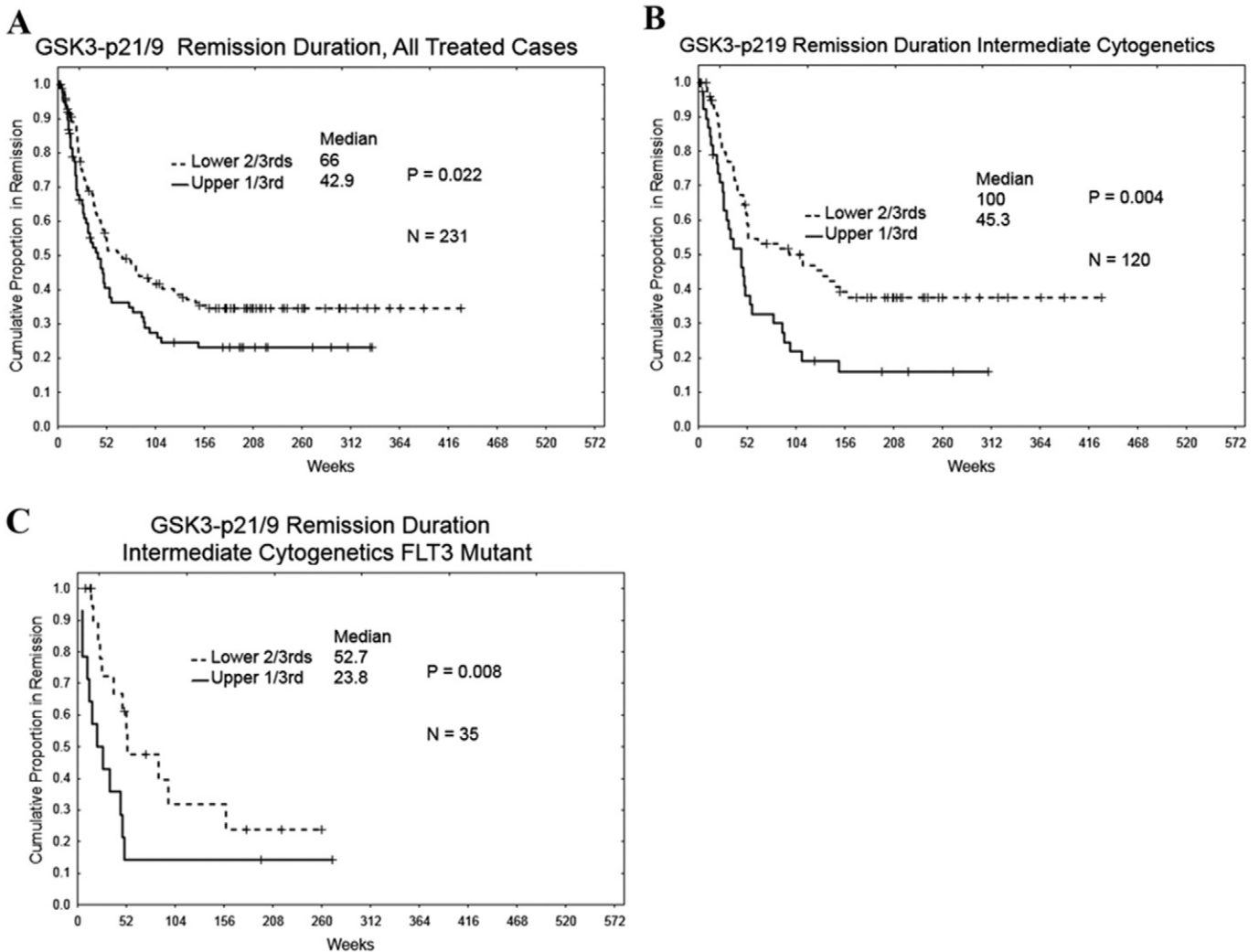


Fig. 3. Correlation of remission duration with serine 21/9 GSK 3α/β phosphorylation levels in AML patient cytogenetic subsets. The Kaplan–Meier curve for remission duration for the total treated AML patient set (A; $N = 231$), treated intermediate cytogenetic patient set (B; $N = 120$), and treated intermediate cytogenetic with the FLT3 mutant patient set (C; $N = 35$) based on expression of phosphorylated GSK3α/β.

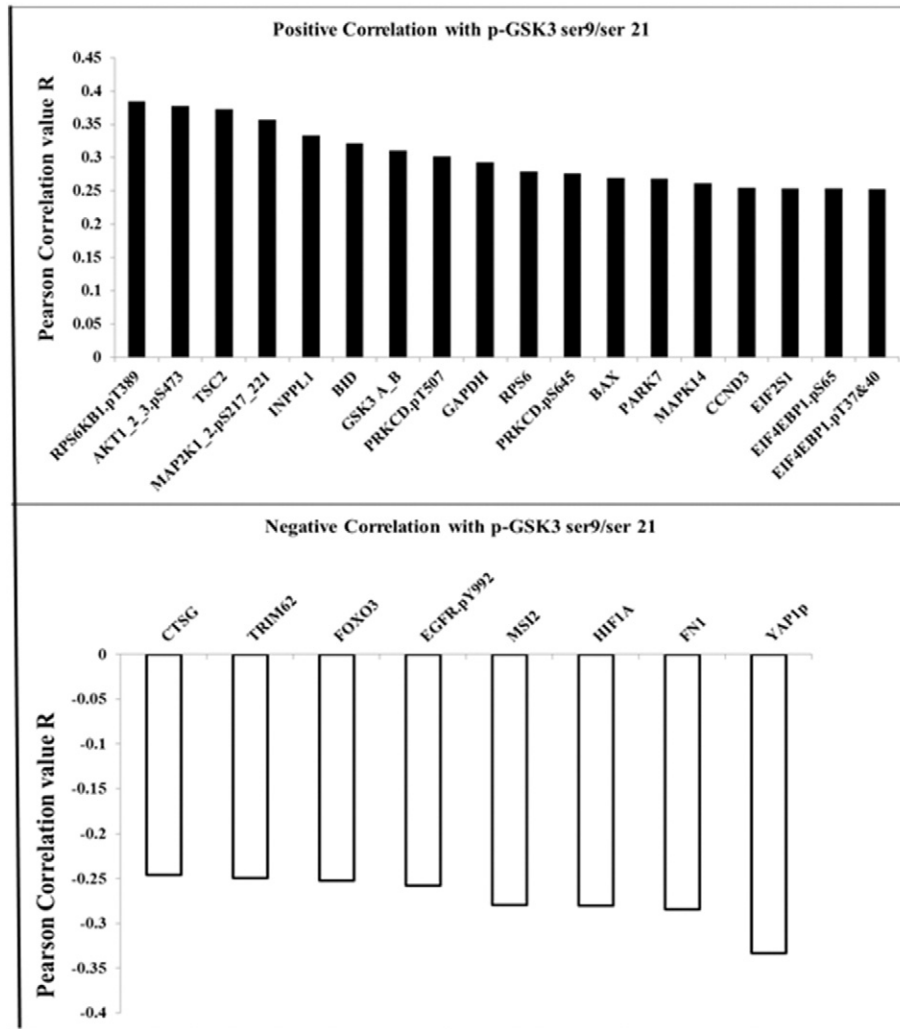


Fig. 4. Proteins correlated with phosphorylated GSK3 α/β expression in AML patients. Negative and positive correlation from a list of 229 proteins also assayed compared to phosphorylated GSK3 α/β expression using the same reverse phase proteomics analysis on the same samples; Pearson correlation coefficient $R > 0.25$, $p < 0.001$.

exhibited the greatest positive correlation with phosphorylated GSK3 α/β are phospho-S6K1 (T389) and phospho-AKT (S473). AKT phosphorylated at S473 reflects the active form of the kinase. That higher levels of phosphorylated GSK3 α/β are found when AKT is active is consistent with AKT's role as a GSK3 kinase [11,25]. S6K1 has also been shown to phosphorylate GSK3 kinases, though usually in the absence of TSC2 [26]. Interestingly, phosphorylated PKC δ (at both T507 and S645) was found to be positively correlated with phospho-GSK3 levels. PKC δ has often been viewed as a stress kinase but recent studies suggest that the enzyme can have pro-survival properties [17,18,27,28]. A recent study by Kinehara and colleagues suggest that PKC δ may phosphorylate GSK3 kinases in human pluripotent stem cells as part of a mechanism to regulate stem cell renewal [28].

RPPA revealed a negative correlation between FOXO3A and phospho-GSK3. Since FOXO3A is negatively regulated by AKT, the negative association between phospho-GSK3 and the forkhead protein is likely an indicator of AKT activity in the individual patients. Included among the proteins exhibiting negative correlation with phosphorylated GSK3 is the epidermal growth factor receptor (EGFR) phosphorylated at tyrosine 922 (Y922). EGFR is a membrane tyrosine kinase that is activated by factors such as EGF and transforming growth factor α (TGF α). EGFR can act as an upstream regulator of many kinase pathways including PI3K/AKT, so a negative correlation between phosphorylated (i.e., active) EGFR and phospho-GSK3 would be predicted. Implications of EGFR regulation of GSK3 activity, however, may be very

important as Du and colleagues have recently demonstrated that the stem cell phenotype of cancer stem cells may be dependent on suppression of GSK3 via an EGFR/AKT axis [29]. The protein showing the strongest negative correlation with phospho-GSK3 was serine 127 phosphorylated Yes Associated Protein 1 (YAP1). YAP1 is a transcriptional co-activator that is part of the Hippo regulatory pathways [30, 31]. When YAP1 is phosphorylated on serine 127, it is expelled from the nucleus and YAP1 driven gene expression decreases. While EGFR supports YAP1 phosphorylation via PI3K, the mechanism is independent of AKT [31]. There is no evidence that GSK3 is a YAP1 kinase. However, it is possible that GSK3 could be promoting YAP1 phosphorylation via suppression of a protein phosphatase. GSK3 suppresses PP2A function [32] and PP2A has been shown to regulate phosphorylation of YAP1 [30].

Considering that GSK3 serine 9/serine 21 phosphorylation levels were significant to survival characteristics in the intermediate cytogenetic AML patients, we examined protein correlations between the phospho-kinase and the 229 proteins and the AML patients in this subset. Most of the proteins that were represented in all AML patients were also found in the intermediate cytogenetic group. As shown in Fig. 5, PKC δ , phosphorylated on either threonine 507 or serine 645, serine 473 phosphorylated AKT and serine 217/221 phosphorylated MEK1 (MAP2K1) are still among the proteins with the strong positive correlation with phospho-GSK3. A few proteins such as ATG7 and EIF2S1 did not correlate with phosphorylated GSK in the intermediate

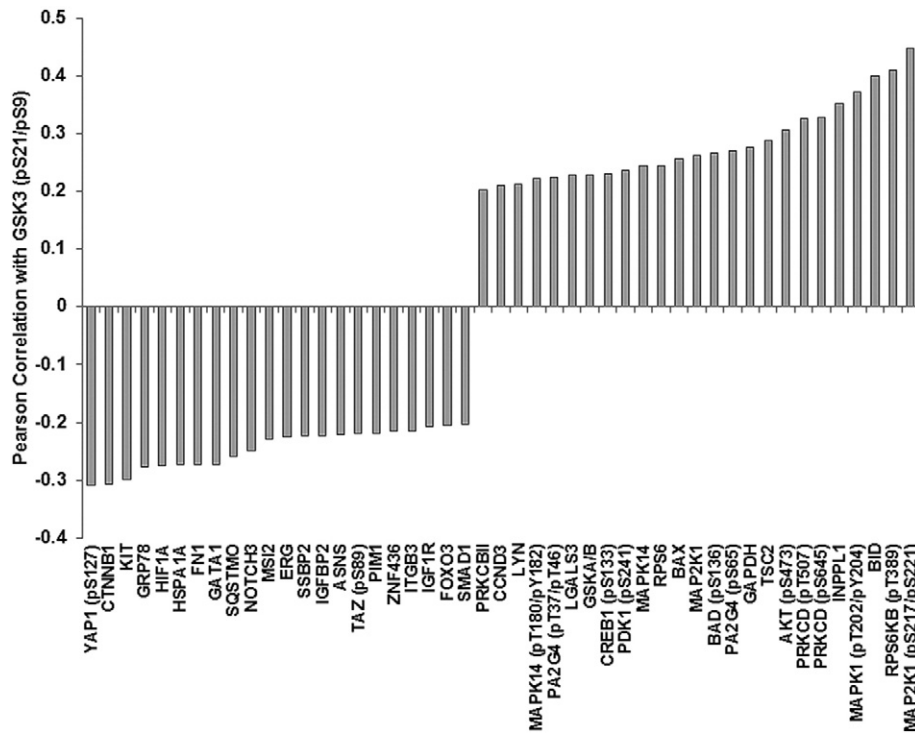


Fig. 5. Proteins correlated with phosphorylated GSK3 α/β expression in the intermediate cytogenetic group AML patients. Negative and positive correlation from a list of 229 proteins also assayed compared to phosphorylated GSK3 α/β expression using the same reverse phase proteomics analysis on the same samples; Pearson correlation coefficient $R > 0.25$, $p < 0.001$.

cytogenetic patients compared to all AML patients (Fig. 5). Also, Galectin 3 (LGALS3) was found to correlate in the Intermediate group though this protein was not in the group of total AML patients. Serine 127 phosphorylated YAP1, β -catenin (CTNNB1), KIT, and HIF1 α (HIF1A) were still among the proteins with strong correlation with phospho-GSK, and two proteins not found in the total AML set, PIM1 and Asparagine Synthase (ASNS), were found in the Intermediate subset of patients (Fig. 5).

3.4. AKT is a positive regulator of FOXO3A and PKC δ that is independent of GSK3

AKT is well known to regulate FOXO3A [4,5]. OCI-AML3 cells exhibit basal levels of FOXO3A phosphorylated at threonine 32 (T32; Fig. 6A). As shown in Fig. 6A, treatment of OCI-AML3 cells with the specific AKT inhibitor MK-2206 suppresses T32 phosphorylation of FOXO3A indicating that AKT is likely the FOXO3A kinase in these cells. While GSK3 is known to phosphorylate a number of transcription factors including MYC, MAX, and USF1 [33], the kinase is not known to target FOXO3A. To investigate the relationship between FOXO3A and GSK3, expression and phosphorylation status of GSK3 was compared in OCI-AML3 cells expressing a lentiviral control vector targeting a non-human gene (NH; target sequence for green fluorescent protein; GFP) with cells expressing lentiviral shRNA against FOXO3A. As shown in Fig. 6B, two transductants displayed roughly 40% reduction of FOXO3A. However, there was no difference in either total GSK3 α/β or phosphorylated kinase in either FOXO3A shRNA transductant suggesting that FOXO3A does not act directly on GSK3. The association between GSK3 phosphorylation status and FOXO3A levels in the AML patient samples likely reflects AKT activity in those samples (i.e. both GSK3 and FOXO3A are AKT substrates). The inhibition of FOXO3A phosphorylation by MK-2206 (Fig. 6A) supports this hypothesis.

The RPPA data suggests that there is a positive association between phospho-GSK3 and phospho-PKC δ (Figs. 4 and 5). Phosphorylated GSK3 indicates that the kinase is inactive while phosphorylation of PKC δ indicates that the kinase is active. A logical assumption would be

that PKC δ may act as a GSK3 kinase. Such a function for the PKC has been suggested by Kinehara and colleagues [28]. PKC δ expression was suppressed by lentiviral shRNA in two AML derived cell lines (i.e., THP-1 and HL60). As shown in Fig. 6B, there was roughly 80% reduction of the kinase in THP-1 cells and approximately 60% reduction of the PKC in HL60 cells (when compared to cells with control NH vector). There was no difference in either total GSK3 α/β or phosphorylated GSK3 α/β in cells with PKC δ shRNA in either cell line suggesting that PKC δ does not act directly on GSK3. The study by Kinehara and colleagues [28] involved human pluripotent stem cells so the action of the PKC on GSK3 may be different in leukemia cells compared to normal stem cells. A second possibility is that GSK3 acts on the PKC. OCI-AML3 cells were treated with 5 μ M dose of a GSK3 α/β inhibitor (Calbiochem Inhibitor XVI) for 6 h. As shown in Fig. 6C, the inhibitor blocked GSK3 phosphorylation though the molecule induced expression of total protein. However, inhibition of GSK3 had no effect on levels of phospho-PKC δ (Fig. 6C). MK-2206 is a specific inhibitor of AKT [35]. Treatment of OCI-AML3 cells with 1 μ M dose of MK-2206 blocked phosphorylation of both GSK3 and PKC δ (Fig. 6C). This novel finding suggests that AKT positively regulates PKC δ though it remains to be determined if the kinase acts directly on the PKC. These results suggest that the correlation between phospho-GSK3 levels and phospho-PKC δ levels in the AML patient samples likely represent AKT activity in the samples (phosphorylation of both GSK3 and PKC δ are positively regulated by AKT).

4. Discussion

The GSK3 kinases are involved in diverse cellular functions regulating cell growth, proliferation, survival, adhesion, and differentiation [9–11]. These enzymes are critical for maintaining cellular homeostasis by regulating metabolic processes (e.g., glycolysis), transcription (e.g., MYC and WNT pathways), translation (e.g., via mTOR), cell death processes including apoptosis (e.g., via MCL-1) and autophagy (e.g., via Bif-1) to name a few processes [9–14]. The GSK3 kinases are clearly important cellular rheostats. Since GSK3 phosphorylation of survival proteins such as MCL-1, β -catenin, and various cyclins results in the

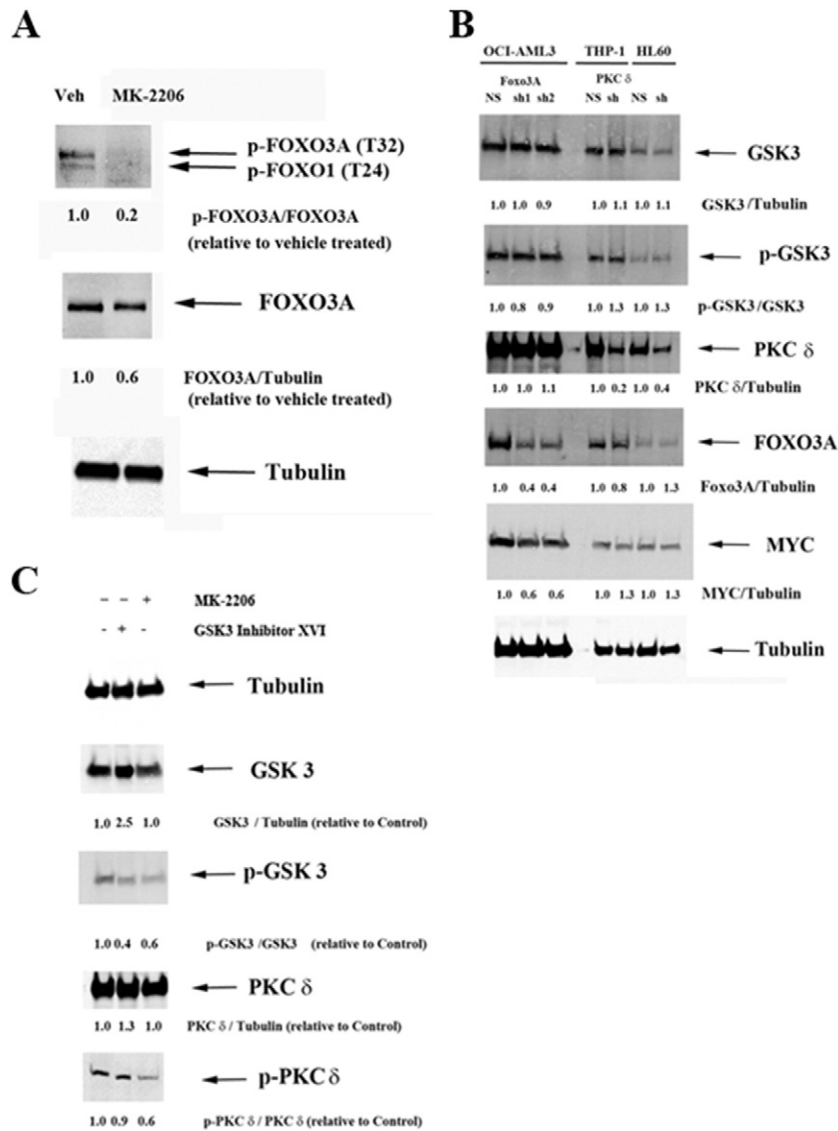


Fig. 6. Role of GSK3 α/β on AKT suppression of FOXO3A and PKC δ in AML cell lines. (A) Protein lysate (200,000 cell equivalents) from OCI-AML3 cells containing control (NS) lentiviral plasmid or cells containing FOXO3A shRNA using 2 different lentiviral plasmids, and THP-1 cells or HL60 cells containing control (NS) lentiviral plasmid or cells containing PKC δ shRNA lentiviral plasmid were subject to SDS/PAGE and Western analysis performed for GSK3 α/β , phosphorylated GSK3 α/β , PKC δ , FOXO3A, MYC, and Tubulin. Ratios were determined by densitometric analysis of the bands depicted in the figure and normalized to each NS control. (B) Protein lysate (200,000 cell equivalents) from OCI-AML3 cells treated with vehicle (-/-; 0.1% DMS) or Calbiochem GSK3 inhibitor XVI (5 μ M) or MK-2206 AKT inhibitor (1 μ M) for 6 h were subject to SDS/PAGE and Western analysis performed for GSK3 α/β , phosphorylated GSK3 α/β , PKC δ , phosphorylated PKC δ , and Tubulin. Ratios were determined by densitometric analysis of the bands depicted in the figure and normalized to vehicle treated control.

proteolytic destruction of these proteins, GSK3 is often viewed as a tumor suppressor [11,13]. Indeed as AKT is viewed as a pro-survival kinase that inactivates GSK3 in many cancers including AML, this would support strategies for AML therapy to suppress AKT and if possible, support GSK3 function. Consistent with this notion, in our RPPA analysis of phosphorylated GSK3 AML patients from the intermediate cytogenetic groups fare better in overall survival and in RD when they express lower levels of phosphorylated GSK3 (see Figs. 2 and 3).

GSK3 phosphorylation status had no impact on survival or RD in AML patients from the unfavorable cytogenetic category (see Figs. 2 and 3). The PI3K/AKT/GSK axis may not be as potent in this population of patients or the various enzymes have different roles in these AML cells compared to leukemia cells from the favorable/intermediate cytogenetic groups. Recent studies suggest there are instances where GSK3 can function as a tumor promoter [11]. Banerji and colleagues have found that GSK3 α may act as a negative regulator of differentiation in AML cells and thus targeting at least the α isoform of the kinase could have therapeutic benefits [34]. Scadden's group has evidence that AKT

may not be critical in leukemia stem cells and that actually FOXO3A may play a more prominent role maintaining these cells [36]. The possibility exists that GSK may have a role in supporting these leukemic stem cells [11,36]. Wang and colleagues have found that GSK3 supports association of CREB and MEIS1, which is critical for HOX-mediated transformation in leukemia [37]. It is likely that the role of GSK3 (or AKT for that matter) in AML is complex and likely varies from patient to patient.

Among the 229 other proteins examined by RPPA, many of the well-established GSK3 substrates including SMAD1, HIF-1 α , and β -catenin were negatively correlated with phosphorylated GSK3 (Fig. 4 and Fig. 4B) as expected. This finding is consistent with having low levels of these target proteins when active (i.e., unphosphorylated) GSK3 is present. Still, a number of other GSK3 targets either showed no correlation (e.g. p21, MCL1; data not shown) or positive correlation (SMAD3, Cyclin E; Fig. 4). This variability likely reflects the complexity of the regulatory pathways at work in AML and warrants further investigation. Some of the proteins, such as FOXO3A that displayed a negative

correlation with phospho-GSK3, likely do not directly interact with GSK3, but rather are substrates of AKT like GSK3. Suppression of FOXO3A by shRNA in the AML cell line OCI-AML3 had no effect on phosphorylation status of GSK3 (Fig. 6B). However, inhibition of AKT with a specific inhibitor (i.e., MK-2206) suppresses phosphorylation and reduces FOXO3A levels in the OCI-AML3 cells (Fig. 6A).

Consistent with conditions where AKT is active, RPPA revealed that phospho-GSK3 levels were positively correlated with AKT phosphorylated at serine 473, with BAD phosphorylated at serine 136, and with Ribosomal Protein S6 phosphorylated at serine 235 and 236 (Fig. 4). These events would suggest support of leukemia survival as phosphorylation of BAD at S136 inhibits its pro-apoptotic function while phosphorylation of Ribosomal Protein S6 supports protein translation [38]. Interestingly, there was also a strong correlation with phosphorylation (i.e., activation) of PKC δ at both threonine 507 and serine 645 (Fig. 4). Regulation of PKC δ by GSK3 or AKT is not clear. The reduction of phosphorylated PKC δ with the AKT inhibitor and not the GSK3 inhibitor suggests that AKT may act as a PKC δ kinase. The novel possibility suggests a new survival cascade that AKT may regulate since there is emerging evidence that the PKC δ can act as a survival kinase [17,18]. A model is depicted in Fig. 7 showing some of the possible survival pathways that may be critical for AML cell survival that depend on activation of AKT with inactive GSK3.

While it is not yet determined if inactivation of GSK3 by AKT is required for its pro-survival function, a recent report from the Grant lab suggests that inactivation of GSK3 is at least necessary if not sufficient for apoptotic pathways involving inhibition of AKT in leukemia cells [39]. In that study, U937 cells in which GSK3 is suppressed either by a pharmacologic inhibitor such as CHIR-98,014 or by shRNA become resistant to killing by the PI3K/mTOR inhibitor BEZ235 in combination with the BH3 mimetic ABT-737 [39]. A mechanism whereby GSK3 activation alters expression of targets such as MCL-1, FOXO3A, CTNNB1 (β -catenin), or MYC was suggested. While in the RPPA analysis in the

present study no significant correlation between phospho-GSK3 and MCL-1 was observed, we did see expression of FOXO3A and β -catenin negatively correlated with the phosphorylated (i.e., inactive) kinase (Fig. 4). Studies are ongoing to determine the mechanistic role of GSK3 activation for apoptosis in AML cells.

In summary, the findings presented here suggest that GSK phosphorylation is likely an important event in AML patients with intermediate cytogenetics. Phosphorylation (i.e. inactivation) of the GSK3 kinases is associated with poor survival outcome in these AML patients. The GSK3 targets that may be critical for leukemia cell survival remain to be determined. Still, strategies to promote GSK3 activation by inhibiting GSK3 kinases such as AKT or by inducing protein phosphatases such as PP2A could be beneficial to improving therapy for at least some AML patients.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbaci.2015.07.001>.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

This work was supported by the Leukemia Lymphoma Society (Translational Research Grant to SMK) and National Institutes of Health (PO1 grant CA-55164 to MA).

References

- [1] A.M. Martelli, C. Evangelisti, F. Chiarini, J.A. McCubrey, The phosphatidylinositol 3-kinase/Akt/mTOR signaling network as a therapeutic target in acute myelogenous leukemia patients, *Oncotarget* 1 (2010) 89–103.
- [2] A.T. Fathi, D.G. Grant, J.E. Karp, Exploiting cellular pathways to develop new treatment strategies for AML, *Cancer Treat. Rev.* 36 (2010) 142–150.
- [3] I. Samudio, M. Konopleva, B. Carter, M. Andreeff, Apoptosis in leukemias: regulation and therapeutic targeting, *Cancer Treat. Res.* 145 (2010) 197–217.
- [4] A.M. Martelli, C. Evangelisti, F. Chiarini, C. Grimaldi, L. Manzoli, J.A. McCubrey, Targeting the PI3K/AKT/mTOR signaling network in acute myelogenous leukemia, *Expert Opin. Investig. Drugs* 18 (2009) 1333–1349.
- [5] A.M. Martelli, M. Nyäkern, G. Tabellini, R. Bortul, P.L. Tazzari, C. Evangelisti, et al., Phosphoinositide 3-kinase/Akt signaling pathway and its therapeutic implications for human acute myeloid leukemia, *Leukemia* 20 (2006) 911–928.
- [6] C. Scholl, D.G. Gilliland, S. Fröhling, Deregulation of signaling pathways in acute myeloid leukemia, *Semin. Oncol.* 35 (2008) 336–345.
- [7] S.M. Kornblau, R. Tibes, Y.H. Qiu, W. Chen, H.M. Kantarjian, M. Andreeff, et al., Functional proteomic profiling of AML predicts response and survival, *Blood* 113 (2009) 154–164.
- [8] S.M. Kornblau, M. Womble, Y.H. Qiu, C.E. Jackson, W. Chen, M. Konopleva, et al., Simultaneous activation of multiple signal transduction pathways confers poor prognosis in acute myelogenous leukemia, *Blood* 108 (2006) 2358–2365.
- [9] G.V. Rayasam, V.K. Tulasi, R. Sodhi, J.A. Davis, A. Ray, Glycogen synthase kinase 3: more than a namesake, *Br. J. Pharmacol.* 156 (2009) 885–898.
- [10] F. Takahashi-Yanaga, Activator or inhibitor? GSK-3 as a new drug target, *Biochem. Pharmacol.* 86 (2013) 191–199.
- [11] J.A. McCubrey, L.S. Steelman, F.E. Bertrand, N.M. Davis, S.L. Abrams, G. Montalto, et al., Multifaceted roles of GSK-3 and Wnt/ β -catenin in hematopoiesis and leukemogenesis: opportunities for therapeutic intervention, *Leukemia* 28 (2014) 15–33.
- [12] R. Linding, L.J. Jensen, G.J. Ostheimer, M.A. van Vugt, C. Jørgensen, I.M. Miron, et al., Systematic discovery of in vivo phosphorylation networks, *Cell* 129 (2007) 1415–1426.
- [13] C. Xu, N.G. Kim, B.M. Gumbiner, Regulation of protein stability by GSK3 mediated phosphorylation, *Cell Cycle* 8 (2009) 4032–4039.
- [14] J. Yang, Y. Takahashi, E. Cheng, J. Liu, P.F. Terranova, B. Zhao, et al., GSK-3 β promotes cell survival by modulating Bif-1-dependent autophagy and cell death, *J. Cell Sci.* 123 (Pt 6) (2010 Mar 15) 861–870.
- [15] Z. Kazemi, H. Chang, S. Haserodt, C. McKen, N.E. Zachara, O-linked beta-N-acetylglucosamine (O-GlcNAc) regulates stress-induced heat shock protein expression in a GSK-3 β -dependent manner, *J. Biol. Chem.* 285 (50) (2010 Dec 10) 39096–39107.
- [16] T. Jin, I. George Fantus, J. Sun, Wnt and beyond Wnt: multiple mechanisms control the transcriptional property of β -catenin, *Cell. Signal.* 20 (2008) 1697–1704.
- [17] V.R. Ruvolo, K.B. Karanjeet, T.F. Schuster, R. Brown, Y. Deng, E. Hinchcliffe, et al., Role for PKC δ in fenretinide-mediated apoptosis in lymphoid leukemia cells, *J. Signal. Transduct.* 2010 (2010 Jan 1) 584657.
- [18] A. Basu, D. Pal, Two faces of protein kinase C δ : the contrasting roles of PKC δ in cell survival and cell death, *Sci. World J.* 10 (2010) 2272–2284.

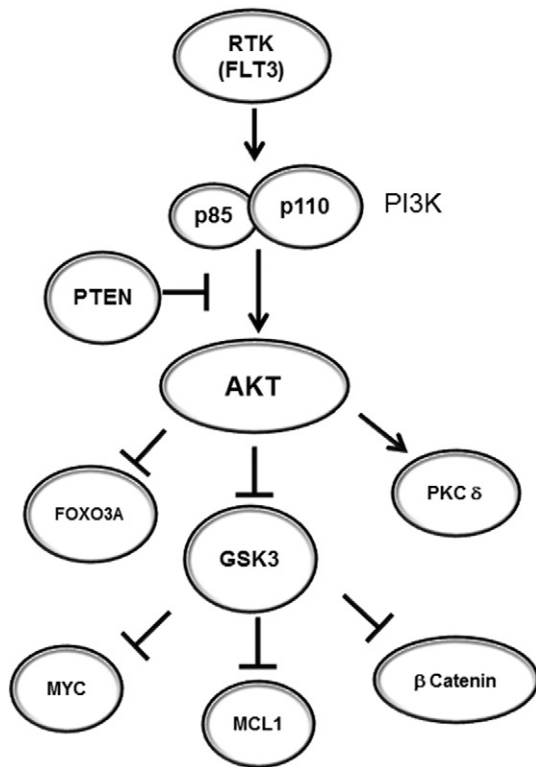


Fig. 7. Model of AKT and GSK3 α/β -mediated pathways in AML cells. Extracellular survival signals activate AKT which suppresses GSK3 α/β . When AKT is suppressed, FOXO3A and PKC δ are inhibited independent of GSK3 α/β . GSK3 α/β when active will suppress MYC, MCL-1, and β -catenin.

- [19] S.M. Kornblau, N. Singh, Y. Qiu, W. Chen, N. Zhang, K.R. Coombes, Highly phosphorylated FOXO3A is an adverse prognostic factor in acute myeloid leukemia, *Clin. Cancer Res.* 16 (2010) 1865–1874.
- [20] J. Hu, X. He, K.A. Baggerly, K.R. Coombes, B.T. Hennessy, G.B. Mills, Non-parametric quantification of protein lysate arrays, *Bioinformatics* 23 (2007) 1986–1994.
- [21] E.S. Neeley, S.M. Kornblau, K.R. Coombes, K.A. Baggerly, Variable slope normalization of reverse phase protein arrays, *Bioinformatics* 25 (2009) 1384–1389.
- [22] R. Tibes, Y. Qiu, Y. Lu, B. Hennessy, M. Andreeff, G.B. Mills, et al., Reverse phase protein array: validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells, *Mol. Cancer Ther.* 5 (2006) 2512–2521.
- [23] P.P. Ruvolo, Y.H. Qui, K.R. Coombes, N. Zhang, V.R. Ruvolo, G. Borthakur, et al., Low expression of PP2A regulatory subunit B55 α is associated with T308 phosphorylation of AKT and shorter complete remission duration in acute myeloid leukemia patients, *Leukemia* 25 (2011) 1711–1717.
- [24] S.M. Kornblau, A. Qutub, H. Yao, H. York, Y.H. Qiu, D. Graber, et al., Proteomic profiling identifies distinct protein patterns in acute myelogenous leukemia CD34⁺ CD38⁻ stem-like cells, *PLoS One* 8 (2013) e78453.
- [25] D.A. Cross, D.R. Alessi, P. Cohen, M. Andjelkovich, B.A. Hemmings, Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B, *Nature* 378 (1995) 785–789.
- [26] H.H. Zhang, A.I. Lipovsky, C.C. Dibble, M. Sahin, B.D. Manning, S6K1 regulates GSK3 under conditions of mTOR-dependent feedback inhibition of Akt, *Mol. Cell* 24 (2006) 185–197.
- [27] Y. Emoto, Y. Manome, G. Meinhardt, H. Kasaki, S. Kharbanda, M. Robertson, et al., Proteolytic activation of protein kinase C delta by an ICE-like protease in apoptotic cells, *EMBO J.* 14 (1995) 6148–6156.
- [28] M. Kinehara, S. Kawamura, D. Tateyama, M. Suga, H. Matsumura, S. Mimura, et al., Protein kinase C regulates human pluripotent stem cell self-renewal, *PLoS One* 8 (1) (2013) e54122.
- [29] W.W. Du, L. Fang, X. Yang, W. Sheng, B.L. Yang, A. Seth, et al., The role of versican in modulating breast cancer cell self-renewal, *Mol. Cancer Res.* 11 (2013) 443–455.
- [30] R. Johnson, G. Halder, The two faces of hippo: targeting the hippo pathway for regenerative medicine and cancer treatment, *Nat. Rev. Drug Discov.* 13 (2014) 63–79.
- [31] R. Fan, N.G. Kim, B.M. Gumbiner, Regulation of hippo pathway by mitogenic growth factors via phosphoinositide 3-kinase and phosphoinositide-dependent kinase-1, *Proc. Natl. Acad. Sci. U. S. A.* 110 (2013) 2569–2574.
- [32] X.Q. Yao, X.X. Zhang, Y.Y. Yin, B. Liu, D.J. Luo, D. Liu, et al., Glycogen synthase kinase-3 β regulates Tyr307 phosphorylation of protein phosphatase-2A via protein tyrosine phosphatase 1B but not Src, *Biochem. J.* 437 (2011) 335–344.
- [33] J. Terragni, G. Nayak, S. Banerjee, J.L. Medrano, J.R. Graham, J.F. Brennan, et al., The E-box binding factors max/Mnt, MITF, and USF1 act coordinately with FoxO to regulate expression of proapoptotic and cell cycle control genes by phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3 signaling, *J. Biol. Chem.* 286 (2011) 36215–36227.
- [34] V. Banerji, S.M. Frumm, K.N. Ross, L.S. Li, A.C. Schinzel, C.K. Hahn, et al., The intersection of genetic and chemical genomic screens identifies GSK-3 α as a target in human acute myeloid leukemia, *J. Clin. Invest.* 122 (2012) 935–947.
- [35] H. Hirai, H. Sootome, Y. Nakatsuru, K. Miyama, S. Taguchi, K. Tsujioka, MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo, *Mol. Cancer Ther.* 9 (2010) 1956–1967.
- [36] S.M. Sykes, S.W. Lane, L. Bullinger, D. Kalaizidis, R. Yusuf, B. Saez, et al., AKT/FOXO signaling enforces reversible differentiation blockade in myeloid leukemias, *Cell* 146 (2011) 697–708.
- [37] Z. Wang, M. Iwasaki, F. Ficara, C. Lin, C. Matheny, S.H. Wong, et al., GSK-3 promotes conditional association of CREB and its coactivators with MEIS1 to facilitate HOX-mediated transcription and oncogenesis, *Cancer Cell* 17 (2010) 597–608.
- [38] A.M. Martelli, C. Evangelisti, W. Chappell, S.L. Abrams, J. Bäsecke, F. Stivala, et al., Targeting the translational apparatus to improve leukemia therapy: roles of the PI3K/PTEN/Akt/mTOR pathway, *Leukemia* 25 (7) (2011 Jul) 1064–1079.
- [39] M. Rahmani, M.M. Aust, E. Attkisson, D.C. Williams Jr., A. Ferreira-Gonzalez, S. Grant, Dual inhibition of Bcl-2 and Bcl-xL strikingly enhances PI3K inhibition-induced apoptosis in human myeloid leukemia cells through a GSK3- and Bim-dependent mechanism, *Cancer Res.* 73 (2013) 1340–1351.