OncoTargets and Therapy

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

PIK3CA expression in diffuse large B cell lymphoma tissue and the effect of its knockdown in vitro

This article was published in the following Dove Press journal:
OncoTargets and Therapy
20 April 2017
Number of times this article has been viewed

Wenli Cui[1–4,*]
Shutao Zheng[5,6,*]
Zebing Liu[1–3]
Weige Wang[1–3]
Ying Cai[1–3]
Rui Bi[1–3]
Bing Cao[1–3]
Xiaoyan Zhou[1–3]

[1]Department of Pathology, Shanghai Cancer Center, Fudan University, [2]Department of Oncology, Shanghai Medical College, Fudan University, [3]Institute of Pathology, Fudan University, Shanghai, [4]Department of Pathology, The First Affiliated Hospital of Xinjiang Medical University, [5]Clinical Medical Research Institute, First Affiliated Hospital of Xinjiang Medical University, [6]State Key Lab Incubation Base of Xinjiang Major Diseases Research, First Affiliated Hospital of Xinjiang Medical University, Xinjiang Uygur Autonomous Region, Urumqi, People's Republic of China

*These authors contributed equally to this work

Abstract: PIK3CA has been extensively investigated from its molecular mechanism perspective and epidemiological association with its mutations in different types of cancers. However, little has been reported regarding the clinicopathological significance of PIK3CA expression in diffuse large B cell lymphoma (DLBCL). In the present study, we investigated the clinicopathological significance of PIK3CA in DLBCL by performing immunohistochemical evaluation of PIK3CA in tissue microarrays consisting of 199 cases of DLBCL. Kaplan–Meier survival analysis was performed to analyze the association between PIK3CA expression and overall prognosis. To further investigate the role of PIK3CA mediated in the proliferation, cell cycle and apoptosis of DLBCL cells, Cell Counting Kit-8 (CCK-8) and flow cytometry assays were carried out in DLBCL cell lines after successful, stable knockdown of PIK3CA using lentiviral short hairpin RNA inference. Our results indicated that although PIK3CA was shown to be extensively expressed in DLBCL, no significant association was observed between PIK3CA expression and clinical outcome or between PIK3CA expression and other clinicopathological parameters, except between performance state (PS) and phosphorylated AKT (p-AKT) expression. In vitro studies revealed that in DLBCL cell lines OCI-LY8 and OCI-LY1, knockdown of PIK3CA could significantly reduce proliferation and promote apoptosis in a G1-phase arrested manner. Additionally, p27 was shown to be markedly upregulated, whereas p-AKT and cyclin D1 were found to be pronouncedly downregulated after stable knockdown of PIK3CA. Together, our results support the oncogenic property of PIK3CA in DLBCL.

Keywords: diffuse large B cell lymphoma, PIK3CA, proliferation, prognosis

Introduction

Diffuse large B cell lymphoma (DLBCL) is one of the most common types of aggressive B cell non-Hodgkin lymphomas. However, only 40%–60% of the patients with DLBCL respond to the current standard therapy, and recurrence after the initial remission is quite common. Therefore, there is an urgent need to develop novel therapies to improve survival of patients with DLBCL.

Gene amplification has been considered as one of the mechanisms of pathogenesis[1,2] in non-Hodgkin lymphomas. In our previous work, we have found that copy number variations (CNVs) frequently occurred in the PIK3CA subunit at the DNA level, and ensuing investigation showed that CNVs of PIK3CA were significantly important in predicting inferior prognosis in patients with DLBCL.[3] As far as PIK3CA is concerned, numerous investigations have been carried out on mechanistic aspects, mainly in the cell culture system and animal models, in the setting of malignancies, including B cell lymphomas. Nevertheless, limited data are available regarding PIK3CA in DLBCL,

Correspondence: Xiaoyan Zhou
Department of Pathology, Fudan University Shanghai Cancer Center, Dong'an Road 270, Shanghai 200032, People's Republic of China
Tel +86 21 6417 5590
Email xyzhou100@163.com

submit your manuscript | www.dovepress.com
Dovepress
http://dx.doi.org/10.2147/OTT.S129970

especially in terms of its clinicopathological significance and its impact on outcomes. Herein, we present our results of our characterization efforts of PIK3CA in DLBCL and its mechanistic and clinical relevance.

Materials and methods

Clinical tissues

The current study was approved by the Medical Ethics Committee of Fudan University Shanghai Cancer Center (Shanghai, China), and written informed consent was obtained from each patient implicated prior to undergoing biopsy. A total of 199 cases of clinical DLBCL blocks undergoing formalin-fixed and paraffin-embedded (FFPE) process and 20 cases of reactive hyperplasia (RH) lymph nodes that were chosen as normal control of DLBCL were enrolled. All cases enrolled in the study were retrieved from our biobank and the Department of Pathology in the Shanghai Cancer Center from 2005 to 2012. Diagnoses were pathologically confirmed and reviewed by two separate clinical pathologists (Wenli Cui and Zebing Liu) on the basis of the 2010 World Health Organization (WHO) classification. Clinicopathological variables, including gender, age, body mass, tumor location, subtypes (germinal center B [GCB] or non-GCB cells), international prognostic index (IPI), performance system, B symptoms, clinical stage, extra-axillary lymph node (EXLN), lactate dehydrogenase (LDH) concentration, therapy effects, overall prognosis, cure with bleomycin, doxorubicin, vincristine, prednisone (CHOP) and R-CHOP rituximab, bleomycin, doxorubicin, vincristine, prednisone (R-CHOP) were electronically retrieved from the Hospital Information System (HIS) of Fudan University Shanghai Cancer Center.

Human lymphoma cell lines

Four kinds of DLBCL cell lines (DOHH2, OCI-Ly1, OCI-Ly8 and SUDHL-4) and two kinds of Burkitt's lymphoma cell lines (Raji and Namalwa) were available in our laboratory. Of note here, OCI-Ly1 and OCI-Ly8 were cultured in Iscove's Modified Dulbecco's Medium (IMDM; HyClone/Thermo Fisher Scientific, South Logan, UT, USA) supplemented with 10% bovine serum. The remainder four cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% calf serum (HyClone/Thermo Fisher Scientific), unless otherwise stated.

Immunohistochemistry (IHC)

Briefly, serial sections from the FFPE samples were collected onto poly-L-lysine-coated slides and processed with a standard manual streptavidin–peroxidase technique using a biotin-free detection system (Dako, CO, Carpinteria, CA, USA) after a heat-induced antigen retrieval procedure with ethylenediaminetetraacetic acid (EDTA) for phosphorylated AKT (p-AKT) and citric acid for PIK3CA for 3 min. Primary antibodies were incubated with sections overnight. Omission of the primary antibody and its replacement with an anti-rabbit antibody IgG was used as the negative control. A biotin-free detection kit (EnVisionTM; Dako) was utilized following the instructions of the manufacturer. The source and dilution of the primary antibodies involved are described in Table 1. Immunostaining of PIK3CA was evaluated blindly and independently by two separate pathologists (Wenli Cui and Zebing Liu). The immunoscoring was from 0 to 3: 0, no recognizable staining in tumor cells (referred to as negative); 1, slight staining in tumor cells (weak positive); 2, moderate staining in tumor cells (moderate positive) and 3, distinct staining in tumor cells (strong positive). Cases were considered to be positive if ≥30% of the tumor cells were stained with the primary antibody. In our setting, a high expression of PIK3CA was defined as cases with immunostaining of PIK3CA being strong positive (+++), whereas a low expression of PIK3CA was defined as cases with negative (−), weak positive (+) and moderate positive staining (++) of PIK3CA.

Table 1 Primary antibodies used in Western blot and IHC experiments

Antibody	No, clone	Western blot dilution	IHC dilution	Production
p110α	4249, clone C73F8	1:2,000	1:400	Cell Signaling Technology
p-AKT (Ser473)	4060, clone D9E	1:2,000		Cell Signaling Technology
p-AKT (Ser473)	2118-1, clone EP2109Y		1:200	Epitomic
p27	SX53G5	1:1,000		Cell Signaling Technology
AKT	40D4	1:1,000		Cell Signaling Technology
PARP	46D11	1:1,000		Cell Signaling Technology
Bcl-2	50E3	1:1,000		Cell Signaling Technology
Cyclin D1	DCS6	1:1,000		Cell Signaling Technology
GAPDH	60004-1-Ig	1:100,000		Proteintech Group

Abbreviations: IHC, immunohistochemistry; p-AKT, phosphorylated AKT.

Western blot

Protein samples (30 μg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), followed by blocking in Tris-buffered saline (TBS) containing 5% bovine serum albumin (BSA) and 0.1% Tween 20 at room temperature for 1 h and then incubation with diluted primary antibody overnight at 4°C with gentle shaking. After washing for three times with Tris Buffered Saline with Tween (TBST), with each time for 5 minutes, the protein samples were incubated with secondary antibody for 1 h at room temperature. The working dilutions of primary antibodies are described in Table 1. The secondary antibodies were either anti-rabbit IgG or anti-mouse IgG peroxidase conjugates (Proteintech Group, Wuhan, People's Republic of China). Signals were developed with enhanced chemiluminescence substrates (Pierce Biotechnology, Rockford, lL, USA) following the protocols provided by the manufacturer and visualized by Image Quantity One (Bio-Rad, La Jolla, CA, USA).

Transfection and selection of stable OCI-LY1 and OCI-LY8 cell lines

Briefly, 1.0×10^5 cells/well were plated in 24 plates. Next, 300 μL complete culture medium containing recombinant lentivirus and control lentivirus (all containing 6 μg/mL polybrene; GeneChem, Shanghai, China) was added into the plates. Two days later, the virus-containing medium was replaced with fresh complete medium. The expression of GFP was observed under a microscope after 3 days. The medium containing puromycin (6 μg/mL; Sigma-Aldrich) was added every 3 or 4 days to screen the stable infected cell lines (OCI-LY8/PIK3CA or OCI-LY8/GFP) until the uninfected cells were almost completely removed.

Cell proliferation assay

Cell viability was measured after transfection using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the protocol provided by the manufacturer. A total of 10 μL of CCK-8 was added into each well of a 96-well plate, and the cells were incubated for 4 h at 37°C. Then, measurement was performed at a wavelength of 450 nm in a microplate reader (BioTek Instruments, Winooski, VT, USA). Each experiment was performed in triplicate.

Apoptosis and cell-cycle analysis

After transfection with lentivirus for 72 h, OCI-LY8 cells were collected after washing twice with phosphate-buffered saline (PBS). For cell cycle, cells were fixed in 70% cold ethanol overnight, followed by incubation with 5 μg/mL 7-aminoactinomycin D (7-AAD; Sigma-Aldrich, St Louis, MO, USA) and staining with PBS buffer and 10 μg/mL RNase A in the dark for 15 min at room temperature, and then subjected to analysis by flow cytometry (BD FACSCalibur; BD Biosciences, San Jose, CA, USA). A total of 3×10^4 cells were counted for each sample. For apoptotic analysis, cells were stained using the Annexin V-FITC kit (Invitrogen™, Life Technologies Inc., Carlsbad, CA, USA); viable cells were not stained with Annexin V. The necrotic cells were Annexin V and 7-AAD double positive, whereas apoptosis cells were Annexin V positive and 7-AAD negative.

Statistical analysis

SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) software was used for statistical analysis. Data were expressed as mean ± standard error of mean (SEM) of three independent experiments. Two-tailed Student's *t*-test was used to analyze the difference between two groups of measurement data that showed normal distribution, whereas Mann–Whitney *U* test was used for those that showed abnormal distribution. The chi-square test and Fisher's extract test were used to statistically analyze the difference between categorical data. Survival curves were plotted using the Kaplan–Meier method and compared by the log-rank test. Spearman's correlation tests were used to evaluate the association between PIK3CA expression and clinicopathological variables. A *P*-value <0.05 was considered to be statistically significant.

Results

PIK3CA expression in DLBCL tissue

To investigate the expression status of PIK3CA in DLBCL tissue obtained from patients and reactive hyperplasic lymph nodes, which served as control, IHC was carried out with a DLBCL tissue microarray (Figure 1). Histopathologically, the positive immunostaining of PIK3CA was mainly sublocalized on both membrane and cytoplasmic compartments of cells. In terms of immunostaining intensity, PIK3CA was observed to be highly heterogeneous in DLBCL with its expression being rarely negative but overwhelmingly showed weak positive, moderate positive and strong positive results. In reactive hyperplasic lymph nodes, PIK3CA was shown to be uniformly negative in germinal center cells, whereas moderately or strongly positive in non-germinal center cells. On the whole, PIK3CA expression was shown to be extensively expressed in DLBCL tissues compared to that in reactive hyperplasic lymph nodes, despite those cases being limited.

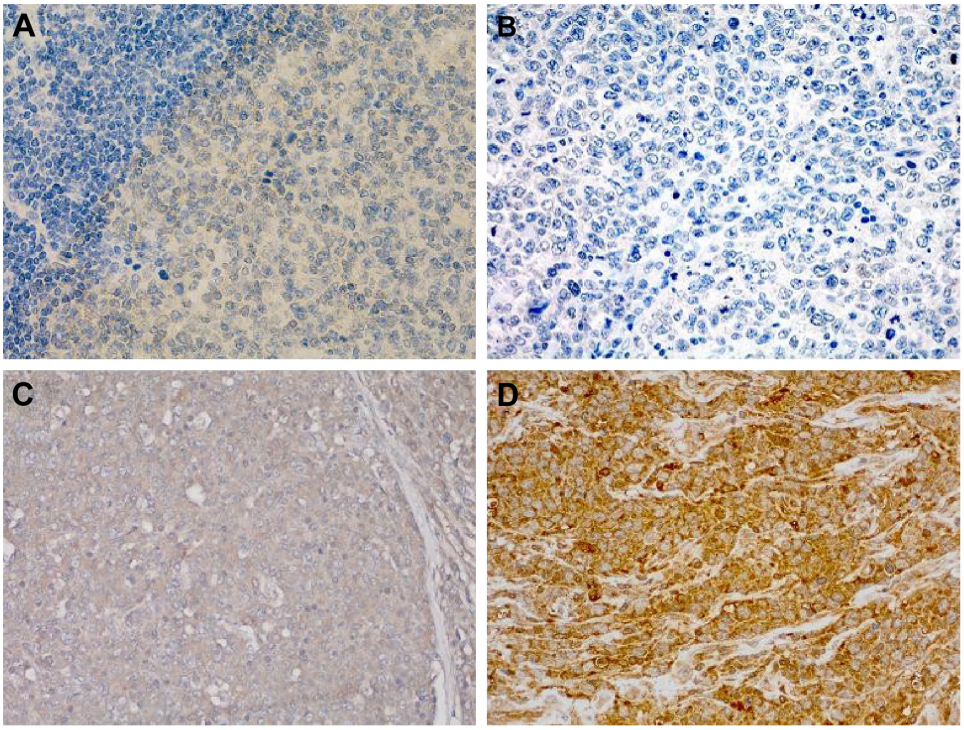

Figure 1 Expression of PIK3CA was evaluated using IHC in both DLBCL and RH tissues.
Notes: (**A**) Weak positive immunostaining of PIK3CA in cells dispersed in and out of the germinal center of RH lymph node. The sublocalization of positive immunostaining of PIK3CA was both cytoplasmic and membranous. (**B**–**D**) Negative, weak positive and strong positive immunostaining of PIK3CA in DLBCL tissues (envision method; magnification fold: ×400).
Abbreviations: IHC, immunohistochemistry; DLBCL, diffuse large B cell lymphoma; RH, reactive hyperplasia.

Association between PIK3CA expression and overall prognosis of patients with DLBCL

Having found that PIK3CA expression was extensively expressed in DLBCL tissues compared to that in hyperplasic lymph nodes, we next sought to analyze the clinicopathological significance of PIK3CA expression. Based on the expression status of PIK3CA, we categorized both the negative, weak positive and moderate positive cases into a group, defined as low expression, while the strong positive cases were grouped together as high expression. The correlation between expression status of PIK3CA and clinicopathological characteristics was analyzed using Pearson chi-square statistics (Table 2). Two statistically significant differences were observed, one between the expression of PIK3CA and performance state (PS) and the other between PIK3CA expression and p-AKT expression. No statistically significant difference was observed between the expression of PIK3CA and other clinicopathological variables, including gender, age, tumor size, tumor location, histopathological subtypes, IPI, B-system, clinical stage, number of EXLNs, concentration of LDH, therapeutic effects and treatment with CHOP or R-CHOP (Table 2).

Of the 199 cases of DLBCL we enrolled, clinicopathological information was unavailable for 98 cases; therefore, the Kaplan–Meier survival analysis was performed on 101 cases of DLBCL (Figure 2). Patients with DLBCL expressing high levels of PIK3CA (n=23) had a decreased overall survival compared to that of patients with DLBCL expressing low levels of PIK3CA (n=88); this was not statistically significant (P=0.212).

In vitro expression of PIK3CA

Similar to DLBCL tissues, detection of PIK3CA expression was extended in vitro in six different kinds of lymphoma cell lines (Figure 3A). p-AKT was also detected in the meantime, and the expression for both was endogenous. The endogenous PIK3CA expression in an OCI-LY8 cell line was shown to be downregulated using lentiviral-based short hairpin RNA interference (shRNA) vector directed against PIK3CA (Figure 3B, inset). On the basis of successful knockdown of PIK3CA in OCI-LY8 cells, proliferation was evaluated using CCK-8 methods (Figure 3B). It can be seen that proliferation of OCI-LY8 cells was significantly suppressed in comparison with control cells, suggesting that knockdown of PIK3CA could suppress the proliferation of DLBCL cells.

Table 2 Clinicopathological significance of PIK3CD expression in DLBCL

Variables	Total cases	PIK3CA expression		χ^2	*P*-value
		Low (–, +, ++)	High (+++)		
Gender					0.716
Male	113	92	21	1.356	
Female	75	62	13		
Age (years)					0.630
<60	115	94	21	1.729	
≥60	72	59	13		
Size (cm)					0.431
<10	153	129	24	2.752	
≥10	21	15	6		
Location					0.366
Extranodular	129	105	24	3.168	
Intranodular	59	49	10		
Subtype					0.303
GCB	99	85	14	3.643	
Non-GCB	89	69	20		
IPI					0.793
0	100	81	19	5.456	
1	40	35	5		
2	20	16	4		
3	15	13	2		
PS					0.044
ECOG 0–1	157	131	26	8.121	
ECOG 2–4	27	22	5		
B-system					0.793
Yes	92	76	16	1.034	
No	92	77	15		
Stage					0.842
I–II	105	86	19	0.832	
III–IV	70	59	11		
EXLN					0.764
≤1	147	123	24	1.156	
>1	28	23	5		
LDH (U/L)					0.278
<240	98	81	17	3.853	
≥240	75	62	13		
Effects					0.397
PR + CR	84	68	16	2.966	
PD + SD	17	16	1		
CHOP					0.794
CHOP	88	74	14	1.032	
R-CHOP	75	63	12		
p-AKT					0.036
Negative	57	50	7	13.519	
Weak	51	45	6		
Moderate	50	39	11		
Strong	30	20	10		

Notes: Of 199 cases with clinicopathological variables, in 10 cases, gender was unavailable; in 13 cases, age was unavailable; in 25 cases, tumor size was unavailable; in 11 cases, location, subtype and p-AKT status were unavailable; in 24 cases, IPI, stage and EXLN were unavailable; 15 cases were without PS and B-system; in 26 cases, LDH concentration was unavailable; 88 cases were without therapeutic effects and in 36 cases, therapeutic scheme, that is, using CHOP or R-CHOP, was unclear. Effects mean therapeutic effects.

Abbreviations: DLBCL, diffuse large B cell lymphoma; GCB, germinal center B; IPI, International Prognostic Index; PS, performance state; ECOG, Eastern Cooperative Oncology Group; EXLN, extra-axillary lymph node; LDH, lactate dehydrogenase; PR, partial response; CR, complete response; PD, progressive disease; SD, stable disease; p-AKT, phosphorylated AKT.

To determine how downregulation of PIK3CA could inhibit cellular proliferation, apoptosis was analyzed by flow cytometry in the shRNA-PIK3CA cell line. The flow cytometry analysis showed that knockdown of PIK3CA significantly increased the percentage of apoptosis compared to the control (Figure 3C and D). Analysis of the cell cycle indicated that the cell cycle was arrested at the G1 phase (Figure 3E and F). These results suggest that knockdown of PIK3CA was capable of promoting proliferation and preventing apoptosis.

To investigate the underlying mechanism by which PIK3CA promotes proliferation and prevent apoptosis, several important biomarkers involved in apoptosis and cell-cycle regulation were analyzed by Western blot in both of the knockdown cell lines, OCI-LY1 and OCI-LY8 (Figure 4). Following the knockdown of PIK3CA, p-AKT and cyclin D1 were significantly downregulated, whereas p27 and PARP were markedly upregulated, indicating that knockdown of PIK3CA could upregulate p-AKT and cyclin D1 while downregulating p27.

Discussion

In our present study, we have investigated the clinicopathological significance of PIK3CA expression in DLBCL tissues, finding that there was no significant association between PIK3CA expression and overall prognosis of patients with DLBCL in our setting. However, a significant association was observed between the expression of PIK3CA and Performance State (PS) and the expression of PIK3CA and expression of p-AKT. In contrast, no significant association was observed between PIK3CA expression and other clinicopathological variables, except between PS and p-AKT expression. In vitro, knockdown of PIK3CA in DLBCL cell lines was found to be capable of promoting cell apoptosis and preventing proliferation through cell-cycle arrest at the G1 phase, suggesting that PIK3CA plays an oncogenic role in DLBCL.

DLBCL is a heterogeneous diagnostic category consisting of molecularly distinct subtypes that differ in gene expression, oncogenic aberrations and clinical outcome[4] and whose histopathological subtypes include activated B cell (ABC)-like subtypes and GCB cell-like subtypes.[5] It has been extensively reported[6–10] that DLBCL relies on constitutive PI3K signaling to block apoptosis in the setting of B cell lymphoma or leukemia. This suggests the important and translational significance of the whole PI3K signal pathway in the clinical therapeutic intervention of DLBCL.[11] In contrast, there are limited publications available studying

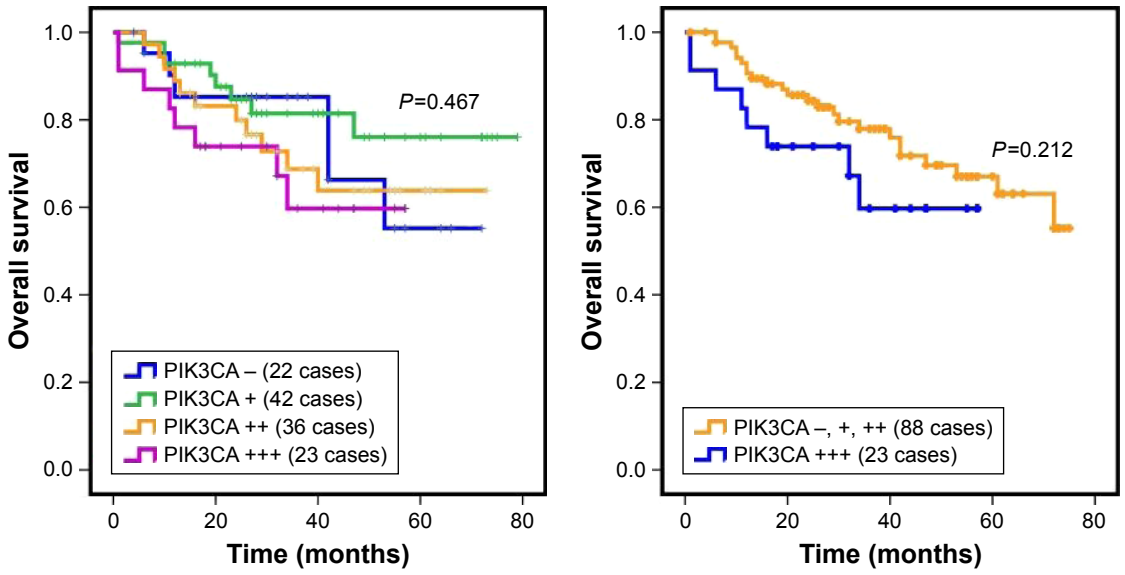

Figure 2 Overall prognostic significance of PIK3CA expression, which was analyzed using the Kaplan–Meier survival curve in DLBCL.
Notes: Of all the patients diagnosed with DLBCL, totaling 205, there were only 101 cases retrieved with the overall prognosis information. Based on the expression status of PIK3CA, 101 cases with prognosis were categorized into two subcohorts, those with low expression of PIK3CA and those with high expression of PIK3CA, with the number of cases being 88 and 23, respectively. The statistical significance was analyzed using the log-rank test.
Abbreviation: DLBCL, diffuse large B cell lymphoma.

the individual catalytic subunit of PI3K, separated from the whole PI3K signaling pathway. Furthermore, in comparison with the majority of mechanistic studies on cell culture and animal models, clinicopathological studies with regard to the individual catalytic subunit of PI3K have been particularly scarce. Consequently, we have focused more on the clinicopathological significance of PIK3CA expression in this study rather than the mechanistic aspect of PIK3CA in DLBCL, which numerous studies have investigated previously.

The original report regarding PIK3CA[12] in the setting of DLBCL came from mutation research on both common and hot mutational spots in PIK3CA in Saudi Arabian patients with DLBCL. In contrast, a previous study from our group discovered that PIK3CA is rarely mutated in Shanghai patients diagnosed with DLBCL.[13] Other studies have also found that PIK3CA mutations were rare in malignancies of the hematopoietic system and chronic lymphocytic leukemia (CLL).[14,15] The discrepancy of the separate studies may be attributable to the different clinical tissues involved and detection methods used.

The majority of investigations involving PIK3CA are either mutational[12,13] or mechanistic,[16–18] but there are rare data in regard to the clinicopathological significance of PIK3CA in B cell lymphoma.[19] In addition, a study that examined the copy number of PIK3CA found that it was significantly amplified in DLBCL compared with RH,[3] which acted as a control. In our current study, we have investigated the expression of PIK3CA on the protein level, finding that PIK3CA was markedly upregulated in DLBCL, which is congruent with its amplified copy number at the DNA level[3] and is suggestive of the oncogenic role that it plays in the pathogenesis of DLBCL. Moreover, our clinicopathological analysis showed that there was no significant association between the expression of PIK3CA and poor overall prognosis in DLBCL. The association between PIK3CA expression and prognosis of patients with cancer has been controversial and inconsistent.[3] Our results showed that there was no significant correlation between PIK3CA expression and other clinicopathological parameters, including age, gender, location, B-system, tumor size, stage and grade and overall prognosis of patients diagnosed with DLBCL.

Two recent mechanistic studies from in vitro cell culture system in mantle cell lymphoma[16,18] have reported that blockage of PIK3CA using its specific chemically synthesized inhibitors can prominently induce cell-cycle arrest followed by induction of apoptosis through complete abolishment of p-AKT and its downstream targets. The effects exerted over cell growth could be synergized in combination with dual inhibition of PIK3CD, another important catalytic subunit of PI3K. These findings support the use of dual PIK3CA/PIK3CD inhibitors in the therapy of mantle cell lymphoma and the potential therapeutic role of PI3KCA. In our study, we discovered that a stable knockdown of PIK3CA using a lentiviral shRNA technique was able to prevent proliferation and promote apoptosis through cell-cycle arrest at

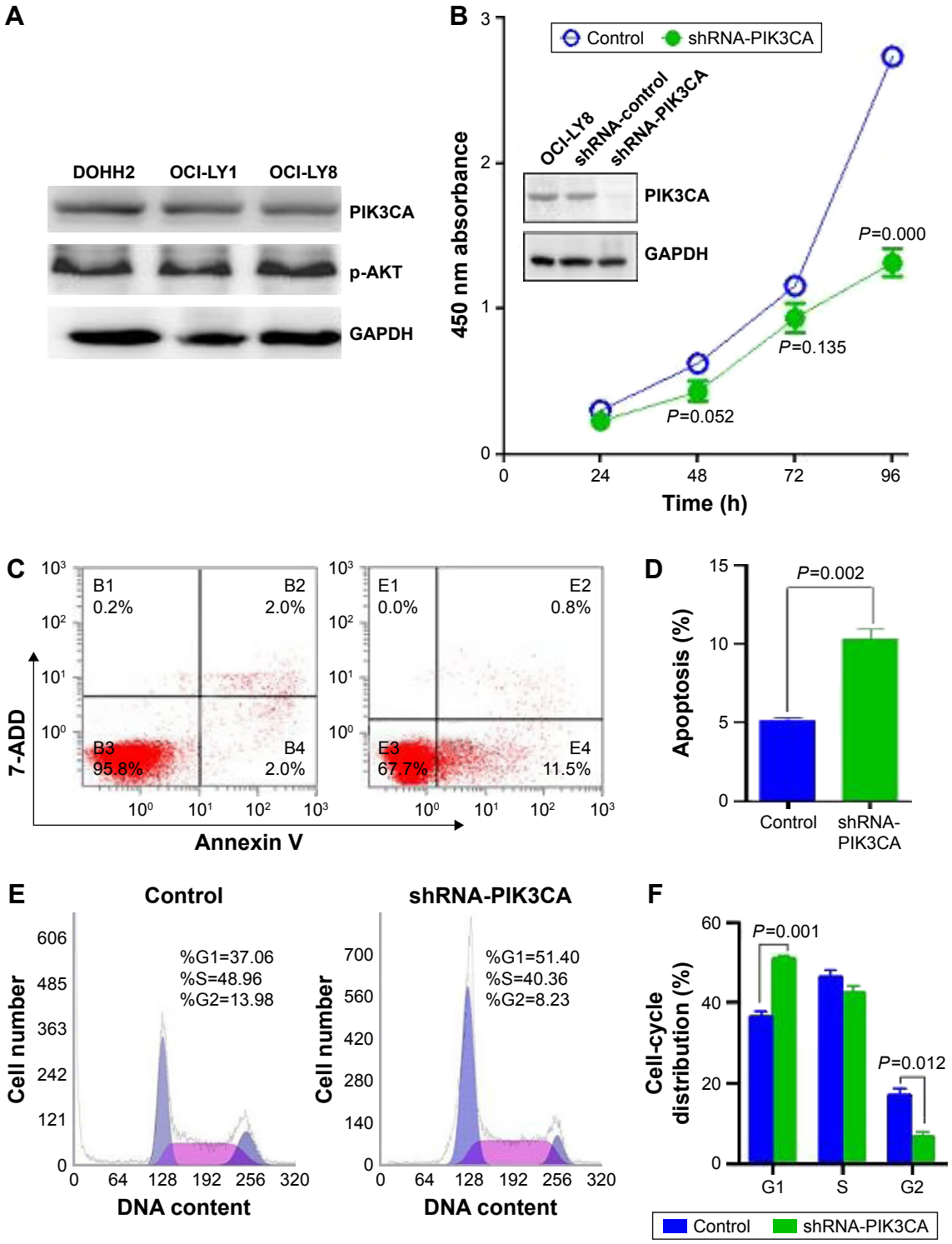

Figure 3 Knockdown of PIK3CA was pronouncedly capable of preventing proliferation and promoting apoptosis, rendering the cell-cycle arrest at the G1 phase in OCI-LY8 cells.

Notes: (**A**) The basal expression of PIK3CA and p-AKT was detected in six different kinds of DLBCL cell lines that were involved in the study. The molecular weights of PIK3CA, p-AKT and GAPDH were 110, 60 and 36 kDa, as indicated by the manufacturer's instruction. (**B**) Based on the successfully stable knockdown of PIK3CA (inserted immunoblotting of PIK3CA), proliferative variation was monitored using the CCK-8 approach. The proliferation of OCI-LY8 cells whose PIK3CA was stably knocked down was remarkably suppressed compared with OCI-LY8 transfected with control vector. (**C**) Cell apoptosis was assayed using flow cytometry before and after stable knockdown of PIK3CA in OCI-LY8. (**D**) Quantitative assay of cell late apoptosis. (**E**) Cell-cycle variation was evaluated using flow cytometry before and after stable knockdown of PIK3CA in OCI-LY8. (**F**) Quantitative assay of cell-cycle analysis. All the experiments were performed independently in triplicate, and representative figures are shown here. All the data were expressed as mean ± SEM. Two-tailed independent sample *t*-test was used to analyze the statistical difference. *P*-value was considered as statistically significant when <0.05 in comparison with the control group.

Abbreviations: p-AKT, phosphorylated AKT; DLBCL, diffuse large B cell lymphoma; SEM, standard error of mean; shRNA, short hairpin RNA interference; 7-AAD, 7-aminoactinomycin D.

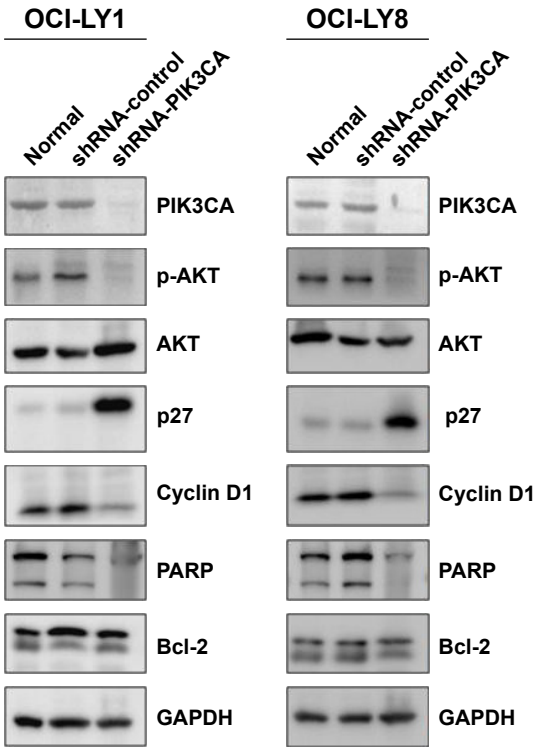

Figure 4 Expression variation of cell cycle- and apoptosis-relevant proteins was detected before and after stable knockdown of PIK3CA in OCI-LY1 and OCI-LY8 cells.

Notes: GAPDH was used as an internal loading control; all the blots were probed and re-probed with primary antibodies on the same membrane. On the basis of successful stable knockdown of PIK3CA, p-AKT, downstream target of PI3K signal pathway, as well as p27, PARP, Bcl-2 and cyclin D1 expression variations were detected using rabbit IgG mono-antibodies. The experiments were done independently in triplicate, and representative figures are presented.

Abbreviations: p-AKT, phosphorylated AKT; shRNA, short hairpin RNA interference.

the G1 phase in two different DLBCL cell lines OCI-LY1 and OCI-LY8, which is in agreement with the observations made in mantle cell lymphoma,[16,18] fully supporting the important roles PIK3CA played in the mediation of cell cycle and apoptosis in the setting of B cell lymphoma. Moreover, mechanistically, stable knockdown of PIK3CA can significantly reduce the expression of p-AKT, abolishing the activation of the constitutive PI3K signal pathway, which agrees with the findings reported by Iyengar et al[16] that cyclin D1 was also markedly downregulated, whereas p27 was significantly upregulated after knockdown of PIK3CA, further supporting the regulatory role of PIK3CA in the cell cycle of DLBCL cells. Cleaved PARP, a well-established apoptotic substrate, was apparently happened to give birth to cleaved PARP in comparison with control, biochemically confirming that PIK3CA prevents cell apoptosis observed by flow cytometry. Knockdown of PIK3CA was presented to have little effect on Bcl-2 expression, suggesting that there could be no direct crosstalk between PIK3CA and Bcl-2 in the present study. Our study supports in vitro that the oncogenic property of PIK3CA in DLBCL cell lines is mediated through the cell cycle and apoptosis.

Our study does have several technical limitations. First of all, both the limited clinical sample size[20] and number of cell lines used could potentially lead to an insufficient or even biased conclusion. Second, the specificity of the primary antibodies[21] used and the selection of cutoff value[22] in the scoring of IHC could also influence or even bias the final results. Third, the tissue microarray used[23] may not be able to reflect the real heterogeneous expression of PIK3CA in DLBCL in our study. Finally, a more in-depth mechanistic analysis is required to understand both the role and how PIK3CA functions in DLBCL.

Conclusion

In this study, PIK3CA was shown to be extensively expressed in DLBCL; however, there was no significant correlation with the majority of clinicopathological parameters, including poor prognosis. In vitro, PIK3CA was observed to be able to promote proliferation and prevent apoptosis in a DLBCL cell line OCI-LY8, suggesting that PIK3CA could be used as a potential therapeutic target in the curing of DLBCL.

Acknowledgments

The present study was supported by the National Natural Science Foundation of China (NSFC 81560035, 81470353, 30973391 and 81272630) and Science and Technology Talents Training Project of Xinjiang Uyghur Autonomous Region (qn2015bs011), Post-doctor Project of Xinjiang Medical University, Shanghai Hospital Development Center Emerging Advanced Technology Joint Research Project (HDC12014105), Shanghai Key Developing Disciplines (2015ZB0201) and Natural Science Foundation of Xinjiang Uygur Autonomous Region (2014211C032), Science and Technology Commission of Shanghai Municipality (15495810300), Shanghai Hospital Development Center Emerging Advanced Technology Joint Research Project (SHDC12014105), Shanghai Municipal Commission of Health and Family Planning 2015ZB0204. Furthermore, we are greatly appreciative of Professor Michael Liew of the Institute for Clinical and Experimental Pathology, ARUP Laboratories, Salt Lake City, UT, USA, and Doctor Farrukh T. Awan of the Ohio State University Comprehensive Cancer Center, Columbus, OH, USA, for their careful looking over and proofreading and giving critic comments in the preparation of the manuscript. Without their help, the

manuscript could get hardly published. We appreciate them with sincerity.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Conde L, Riby J, Zhang J, Bracci PM, Skibola CF. Copy number variation analysis on a non-Hodgkin lymphoma case-control study identifies an 11q25 duplication associated with diffuse large B-cell lymphoma. *PLoS One*. 2014;9(8):e105382.
2. Kwiecinska A, Ichimura K, Berglund M, et al. Amplification of 2p as a genomic marker for transformation in lymphoma. *Genes Chromosomes Cancer*. 2014;53(9):750–768.
3. Cui W, Cai Y, Wang W, et al. Frequent copy number variations of PI3K/AKT pathway and aberrant protein expressions of PI3K subunits are associated with inferior survival in diffuse large B cell lymphoma. *J Transl Med*. 2014;12:10.
4. Mondello P, Younes A. Emerging drugs for diffuse large B-cell lymphoma. *Expert Rev Anticancer Ther*. 2015;15(4):439–451.
5. Jardin F. Next generation sequencing and the management of diffuse large B-cell lymphoma: from whole exome analysis to targeted therapy. *Discov Med*. 2014;18(97):51–65.
6. Xu ZZ, Xia ZG, Wang AH, et al. Activation of the PI3K/AKT/mTOR pathway in diffuse large B cell lymphoma: clinical significance and inhibitory effect of rituximab. *Ann Hematol*. 2013;92(10):1351–1358.
7. Pfeifer M, Lenz G. PI3K/AKT addiction in subsets of diffuse large B-cell lymphoma. *Cell Cycle*. 2013;12(21):3347–3348.
8. Rahmani M, Aust MM, Benson EC, Wallace L, Friedberg J, Grant S. PI3K/mTOR inhibition markedly potentiates HDAC inhibitor activity in NHL cells through BIM- and MCL-1-dependent mechanisms in vitro and *in vivo*. *Clin Cancer Res*. 2014;20(18):4849–4860.
9. Waibel M, Gregory G, Shortt J, Johnstone RW. Rational combination therapies targeting survival signaling in aggressive B-cell leukemia/lymphoma. *Curr Opin Hematol*. 2014;21(4):297–308.
10. Zang C, Eucker J, Liu H, et al. Inhibition of pan-class I phosphatidylinositol-3-kinase by NVP-BKM120 effectively blocks proliferation and induces cell death in diffuse large B-cell lymphoma. *Leuk Lymphoma*. 2014;55(2):425–434.
11. Uddin S, Hussain AR, Siraj AK, et al. Role of phosphatidylinositol 3′-kinase/AKT pathway in diffuse large B-cell lymphoma survival. *Blood*. 2006;108(13):4178–4186.
12. Abubaker J, Bavi PP, Al-Harbi S, et al. PIK3CA mutations are mutually exclusive with PTEN loss in diffuse large B-cell lymphoma. *Leukemia*. 2007;21(11):2368–2370.
13. Baohua Y, Xiaoyan Z, Tiecheng Z, Tao Q, Daren S. Mutations of the PIK3CA gene in diffuse large B cell lymphoma. *Diagn Mol Pathol*. 2008;17(3):159–165.
14. Muller CI, Miller CW, Hofmann WK, et al. Rare mutations of the PIK3CA gene in malignancies of the hematopoietic system as well as endometrium, ovary, prostate and osteosarcomas, and discovery of a PIK3CA pseudogene. *Leuk Res*. 2007;31(1):27–32.
15. Marincevic M, Tobin G, Rosenquist R. Infrequent occurrence of PIK3CA mutations in chronic lymphocytic leukemia. *Leuk Lymphoma*. 2009;50(5):829–830.
16. Iyengar S, Clear A, Bodor C, et al. P110alpha-mediated constitutive PI3K signaling limits the efficacy of p110delta-selective inhibition in mantle cell lymphoma, particularly with multiple relapse. *Blood*. 2013;121(12):2274–2284.
17. Adams JR, Xu K, Liu JC, et al. Cooperation between Pik3ca and p53 mutations in mouse mammary tumor formation. *Cancer Res*. 2011;71(7):2706–2717.
18. Tabe Y, Jin L, Konopleva M, et al. Class IA PI3K inhibition inhibits cell growth and proliferation in mantle cell lymphoma. *Acta Haematol*. 2014;131(1):59–69.
19. Zhai L, Zhao Y, Ye S, et al. Expression of PIK3CA and FOXP1 in gastric and intestinal non-Hodgkin's lymphoma of mucosa-associated lymphoid tissue type. *Tumour Biol*. 2011;32(5):913–920.
20. Kaplan RM, Chambers DA, Glasgow RE. Big data and large sample size: a cautionary note on the potential for bias. *Clin Transl Sci*. 2014;7(4):342–346.
21. Baker M. Reproducibility crisis: blame it on the antibodies. *Nature*. 2015;521(7552):274–276.
22. Oh S, Koo DH, Suh C, et al. Prognostic value of immunohistochemical biomarkers at different cut-off values in patients with diffuse large B-cell lymphoma treated with CHOP chemotherapy. *J Korean Med Sci*. 2011;26(12):1556–1562.
23. Linderoth J, Ehinger M, Akerman M, et al. Tissue microarray is inappropriate for analysis of BCL6 expression in diffuse large B-cell lymphoma. *Eur J Haematol*. 2007;79(2):146–149.