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Clinical Significance of *PTEN* Deletion, Mutation, and Loss of PTEN Expression in *De Novo* Diffuse Large B-Cell Lymphoma

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Abstract

PTEN loss has been associated with poorer prognosis in many solid tumors. However, such investigation in lymphomas is limited. In this study, PTEN cytoplasmic and nuclear expression, *PTEN* gene deletion, and *PTEN*

mutations were evaluated in two independent cohorts of diffuse large B-cell lymphoma (DLBCL). Cytoplasmic PTEN expression was found in approximately 67% of total 747 DLBCL cases, more frequently in the activated B-cell–like subtype. Nuclear PTEN expression was less frequent and at lower levels, which significantly correlated with higher *PTEN* mRNA expression. Remarkably, loss of PTEN protein expression was associated with poorer survival only in DLBCL with AKT hyperactivation. In contrast, high PTEN expression was associated with Myc expression and poorer survival in cases without abnormal AKT activation. Genetic and epigenetic mechanisms for loss of PTEN expression were investigated. *PTEN* deletions (mostly heterozygous) were detected in 11.3% of DLBCL, and showed opposite prognostic effects in patients with AKT hyperactivation and in *MYC* rearranged DLBCL patients. *PTEN* mutations, detected in 10.6% of patients, were associated with upregulation of genes involved in central nervous system function, metabolism, and AKT/mTOR signaling regulation. Loss of PTEN cytoplasmic expression was also associated with *TP53* mutations, higher *PTEN*-targeting microRNA expression, and lower PD-L1 expression. Remarkably, low *PTEN* mRNA expression was associated with down-regulation of a group of genes involved in immune responses and B-cell development/differentiation, and poorer survival in DLBCL independent of AKT activation. Collectively, multi-levels of PTEN abnormalities and dysregulation may play important roles in PTEN expression and loss, and that loss of PTEN tumor-suppressor function contributes to the poor survival of DLBCL patients with AKT hyperactivation.

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common and heterogeneous type of B-cell lymphoma. Gene expression profiling (GEP) has classified DLBCL into two molecularly distinctive subtypes: germinal center B-cell–like (GCB) and activated B-cell–like (ABC) types, with gene expression profiles resembling those of normal germinal center B cells and those of mitogenically activated blood B cells, respectively [1].

The current standard regimen of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) has clearly improved the outcome of DLBCL patients over the past decades [2], but because some patients with refractory disease or with early relapse still have worse outcomes [3], further clarification of disease subgroups with distinct pathology mechanisms is needed. Recent studies showed that the phosphatidylinositol-3 kinase (PI3K)-AKT pathway was constitutively activated in 25-52% of DLBCL [4,5], which prompted us to study the significance of PTEN (phosphatase and tensin homologue), a major negative regulator of the PI3K/AKT signaling, in the pathogenesis of DLBCL. PTEN antagonizes PI3K signaling through dephosphorylation of phosphoinositide-3-phosphate (PIP3). PTEN deficiency leads to PIP3 accumulation and thereby de-repression of the PI3K/AKT pathway, which in turn promotes cell growth, proliferation, angiogenesis, and other cellular processes [6].

The phosphatase activities of PTEN in the plasma membrane are finely regulated by complex mechanisms. Dynamic PTEN binding to the plasma membrane, as a critical step for PI3K signaling inhibition by PTEN, is determined by local PIP2 and PIP3 gradients [7,8] and PTEN conformation which is regulated by posttranslational modifications such as phosphorylation, ubiquitination, acetylation, and SUMOylation. Phosphorylation of the C-terminal tail prevents PTEN from membrane binding and keeps PTEN inactive in the cytoplasm [8,9].

PTEN localizes not only to the cytoplasm but also to the nucleus and other subcellular compartments [8]. PTEN localized in the nucleus has tumor-suppressive functions in maintaining chromosomal stability by up-regulation of RAD51 and interaction with p53 promoting p300-mediated p53 acetylation, independent of its enzymatic activities against the PI3K/AKT pathway [10]. Several regulatory mechanisms for PTEN nuclear localization have been proposed, including passive diffusion, active transport mediated by major vault protein, nuclear localization signal, interaction with GTPase Ran, and monoubiquitination of PTEN [8,11,12].

Loss of PTEN function is significantly related to advanced disease, chemotherapy resistance, and poor survival in patients with prostate, breast, melanoma, colorectral, esophageal, and head and neck cancers [13–20]. PTEN can be inactivated by genetic and epigenetic mechanisms. *PTEN* is one of the most frequently mutated genes, and *PTEN* gene alterations play critical roles in the pathogenesis of many human cancers [21–25]. In DLBCL, Lenz and colleagues found that *PTEN* gene deletion was associated with the GCB subtype [26]; Pfeifer et al demonstrated that absence of PTEN expression defines a PI3K/AKT-dependent GCB-DLBCL subtype in both cell lines and primary samples [27]. However, a few studies have suggested different prognostic effects of PTEN loss/expression in small DLBCL cohorts [28–31]. Large-scale studies are needed to establish the clinical significance of PTEN expression/loss and genetic abnormalities in DLBCL.

In this study, we analyzed cytoplasmic and nuclear expression of PTEN protein, *PTEN* deletions, and *PTEN* mutations and their prognostic significance in a large number of patients with *de novo* DLBCL treated with R-CHOP, and explored the potential regulatory mechanisms for PTEN deficiency in DLBCL.

Materials and Methods

Patients

Patients were organized as a part of the International DLBCL Rituximab-CHOP Consortium Program study, and were selected

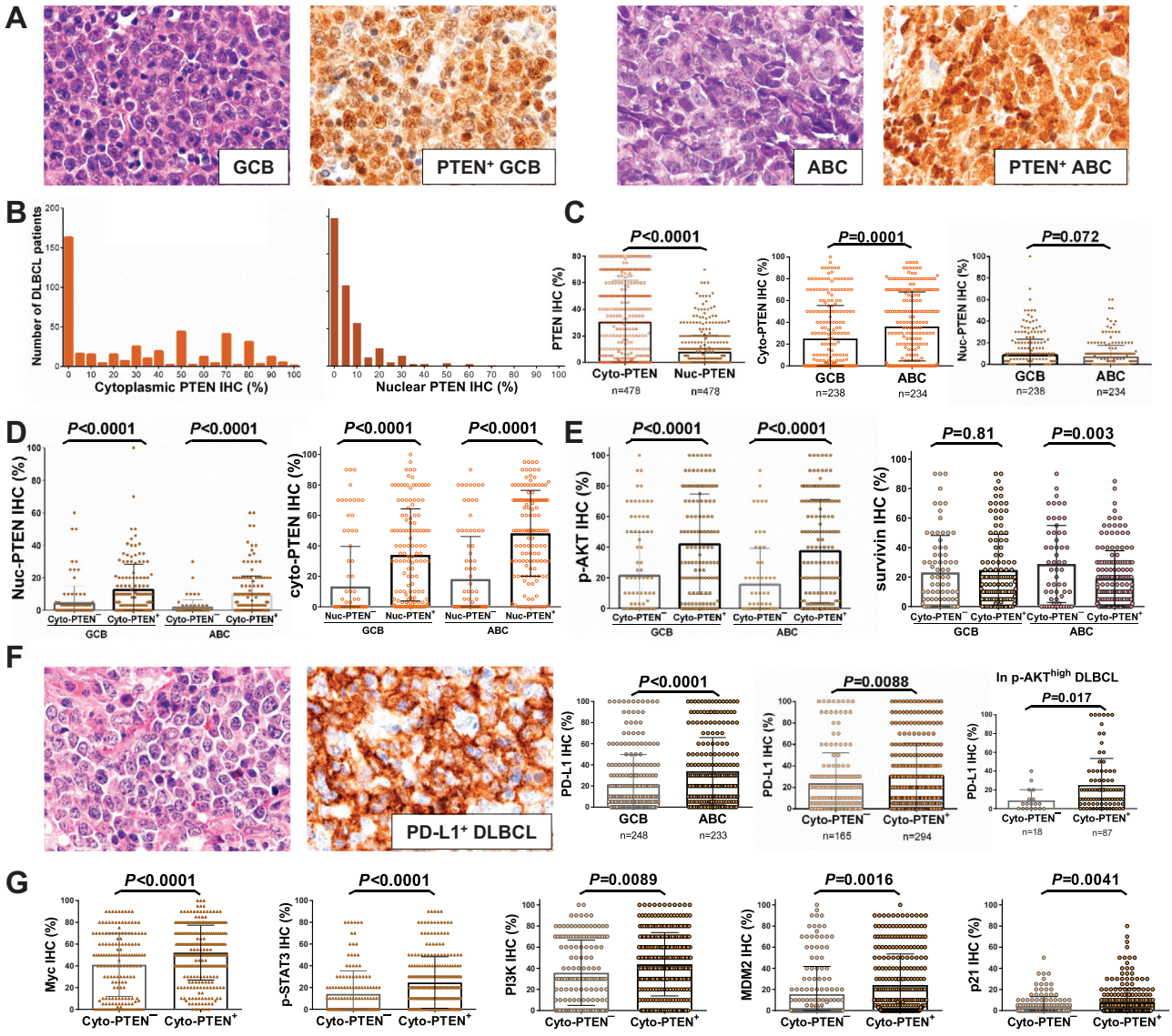


Figure 1. Analysis of PTEN expression by immunohistochemistry (IHC). (A) Representative hematoxylin and eosin and immunohistochemistry of PTEN expression in GCB-DLBCL and ABC-DLBCL. (B and C) Histograms and comparison of cytoplasmic (Cyto-) and nuclear (Nuc-) PTEN expression in DLBCL and between GCB/ABC subtypes (training cohort). (D) Cytoplasmic PTEN expression was associated with higher nuclear PTEN expression in both GCB-DLBCL and ABC-DLBCL. (E) Cytoplasmic PTEN expression was associated with higher p-AKT expression in GCB-DLBCL and ABC-DLBCL, and inversely associated with survivin expression in ABC-DLBCL. (F) Representative hematoxylin and eosin and immunohistochemistry of PD-L1 expression in DLBCL. The ABC compared with the GCB subtype had a significantly higher mean level of PD-L1 expression. Cytoplasmic PTEN expression was associated with a higher mean level of PD-L1 expression in overall DLBCL and in cases with high p-AKT expression. (G) Cytoplasmic PTEN expression was associated with higher mean levels of Myc, p-STAT3, PI3K, MDM2, and p21 expression in DLBCL. Significant *P* values are in bold.

according to the eligibility and exclusion criteria (fulfilling the DLBCL diagnostic criteria and treated with R-CHOP or R-CHOP–like therapy, and excluding patients with transformation from lower grade B-cell lymphoma, primary mediastinal large B-cell lymphoma, primary cutaneous DLBCL, primary central nervous system DLBCL, or acquired immunodeficiency) which have been described previously [32,33]. PTEN staining was achieved initially in 478 cases (training cohort) and additionally in 269 cases, a later assembled validation cohort. The institutional review boards of each participating center approved this study as being of minimal to no risk or as exempt. Nuclear expression of phospho-AKT-Ser^{473} (p-AKT, activated form of AKT) has been evaluated in the training cohort [34] and data were available in 461 cases. Cell-of-origin classification was according to GEP and/or immunohistochemistry (IHC) algorithms as described previously [32,35].

PTEN and PD-L1 Immunohistochemistry

Hematoxylin and eosin–stained slides from DLBCL cases were reviewed, and representative areas of the formalin-fixed and paraffin-embedded (FFPE) tissue sections with the highest percentages of tumor cells were selected for tissue microarray construction and subject for IHC staining. PTEN expression was evaluated by IHC using a PTEN antibody (138G6, Cell Signaling). PTEN expression was analyzed for positive versus negative (i.e., loss of) expression status, as well as high versus low expression. The cutoff used for high cytoplasmic PTEN expression was >40% and the cutoff for high

Table 1. Comparison of clinical and molecular features of patients with diffuse large B-cell lymphoma (DLBCL) with and without PTEN cytoplasmic expression in the training cohort

	in DLBCL			in GCB-DLBCL			in ABC-DLBCL			in p-AKT^{high} DLBCL		
	Cytoplasmic PTEN$^+$	Cytoplasmic PTEN$^-$		Cytoplasmic PTEN$^+$	Cytoplasmic PTEN$^-$		Cytoplasmic PTEN$^+$	Cytoplasmic PTEN$^-$		Cytoplasmic PTEN$^+$	Cytoplasmic PTEN$^-$	
	n=306	n=172	*P*	n=137	n=101	*P*	n=165	n=69	*P*	n=89	n=18	*P*
GCB/ABC Subtype												
GCB	137	101	**.004**							41	12	.13
ABC	165	69								48	6	
Age, years												
< 60	128	76	.63	70	52	1.0	55	22	.88	44	9	1.0
≥ 60	178	96		67	49		110	47		45	9	
Sex												
Male	190	92	.081	86	55	.23	102	37	.25	56	8	.19
Female	116	80		51	46		63	32		33	10	
Stage												
I - II	134	85	.21	72	56	.42	60	27	.88	39	5	.41
III - IV	164	80		63	39		99	41		47	11	
B-symptoms												
No	190	103	.92	95	61	.38	92	40	.88	57	9	.57
Yes	103	57		37	31		65	26		29	7	
LDH												
Normal	109	51	.12	52	31	.26	56	20	.29	30	4	.28
Elevated	169	110		73	61		93	47		48	14	
Extranodal sites												
0 - 1	227	126	.64	107	74	1.0	117	51	.74	64	8	.067
≥ 2	71	35		27	18		43	16		21	8	
ECOG score												
0 - 1	230	124	.89	105	71	1.0	121	51	.71	65	10	.47
≥ 2	46	26		18	12		28	14		15	4	
Tumor size												
< 5 cm	135	67	.73	62	40	1.0	71	26	.73	35	2	**.035**
≥ 5 cm	95	51		43	29		52	22		23	8	
IPI score												
0 - 2	182	101	.76	92	64	1.0	86	35	.89	51	6	.11
> 2	118	61		43	29		75	32		36	11	
Therapy response												
CR	237	120	.079	105	72	.37	128	46	.10	65	11	.39
PR	35	29		12	16		23	13		13	4	
SD	11	11		7	6		4	5		7	0	
PD	23	12		13	7		10	5		4	3	
Nuclear PTEN expression												
0%	69	129	**< .0001**	30	72	**< .0001**	39	55	**< .0001.**	25	13	**.0008**
> 0%	237	43		107	29		126	14		64	5	
***TP53* mutations**												
No	214	115	**.044**	90	67	.41	121	46	.065	62	13	.81
Yes	51	44		28	27		23	17		12	3	
MDM2 expression												
≤ 10%	169	119	**.001**	82	72	**.03**	86	47	**.022**	53	10	.75
> 10%	131	47		54	25		77	21		36	8	
BCL6 expression												
≤ 30%	62	47	**.05**	12	16	.07	50	31	**.025**	16	2	.58
> 30%	237	116		124	80		113	36		72	14	
BLIMP-1 expression												
< 5%	173	116	**.033**	94	77	.24	78	39	.32	44	15	**.017**
≥ 5%	118	51		37	21		80	30		43	3	
IgA IHC												
0%	302	163	**.011**	134	96	.24	164	65	**.012**	89	17	**.026**
100%	4	9		3	5		1	4		0	1	
IgG IHC												
0%	282	146	**.013**	126	88	.22	152	56	**.015**	82	17	.73
100%	24	26		11	13		13	13		7	1	
PD-L1 IHC												
< 5%	50	48	**.003**	33	43	**.0045**	17	5	.62	14	7	**.047**
≥ 5%	244	117		99	56		142	60		73	11	

Abbreviations: LDH, lactate dehydrogenase; ECOG, Eastern Cooperative Oncology Group; IPI, International Prognostic Index; CR, complete remission; PR, partial response; SD, stable disease; PD, progressive disease; GCB, germinal center B-cell–like; ABC, activated B-cell–like. Significant *P* values are highlighted in bold.

nuclear PTEN expression was >30% of tumor cells, which were determined by the X-tile software (Yale School of Medicine, New Haven, CT).

Expression of p-AKT, IL-6, PI3K [34], Myc [36], p-STAT3 [37], MDM2 [38], p21 [39], BLIMP-1 [40], IgA and IgG [41] had been assessed by previous studies; the cutoff for p-AKT^{high} expression (AKT hyperactivation) was ≥70% as described previously [34].

PD-L1 expression was assessed by IHC using a DAKO PD-L1 antibody. The IHC results were scored independently by three pathologists (J.K., Y.X, and K.H.Y), and final scores were based on consensus. The cutoff for PD-L1 positivity is ≥5% of tumor cells.

Fluorescence in situ Hybridization and Gene Sequencing

Fluorescence *in situ* hybridization (FISH) analysis was performed and data were available for 359 cases of the training cohort and 248 cases of the validation cohort. To evaluate *PTEN* gene (chromosome 17p13.1) deletions, a commercial *PTEN* probe was utilized (ZytoLight® SPEC PTEN/CEN 10 Dual Color Probe Z-2078-200; Zytovision, Bremerhaven, Germany). The ratio of *PTEN* signals (green) to CEP10 signals (red) was counted in 200 tumor cells. If this ratio was lower than 0.81, heterozygous *PTEN* deletion was considered to be present. Ratios lower than 0.46 were considered to be suggestive of homozygous deletions. The ratios were calculated as ratios below the mean plus three standard deviations of green to red signal ratios in reference cases (5 tonsils) and subtraction of tumor-infiltrating T cells, which accounted for 15% of undeleted alleles.

For *PTEN* sequencing, genomic DNA was extracted from FFPE tissues of 368 cases and then subjected to Sanger sequencing. The sequencing results were compared to the National Center for Biotechnology Information (NCBI) reference sequence NM_000314 (PTEN) to identify non-synonymous *PTEN* mutations. Single nucleotide polymorphisms documented by the NCBI dbSNP database (build 147) have been excluded.

Gene Expression Profiling and microRNA Profiling

Gene expression profiling was performed by using the Affymetrix GeneChip Human Genome HG-U133 Plus Version 2.0 Array as described previously (GSE31312) [32,42]. Microarray data were normalized for further supervised clustering analysis. Multiple *t*-tests were used to identify differentially expressed genes between groups with and without *PTEN* abnormalities, and the *P* values obtained were corrected for the false discovery rate (FDR) using the beta-uniform mixture method.

microRNA (miRNA) profiling was performed by HTG Molecular Diagnostics Inc. (Tucson, AZ) using FFPE tissue sections (unpublished preliminary data). miRNAs targeting PTEN are according to the literature review [43] and TargetScan: http://www.targetscan.org).

Statistical Analysis

The clinical and pathological features of DLBCL patients were compared using the Fisher's exact or chi-square test. The unpaired *t*-test (2-tailed) was used to compare mean expression levels of biomarkers between DLBCL groups. Overall survival (OS) was calculated from time of diagnosis to last follow-up or death due to any cause. Progression-free survival (PFS) was calculated from time of diagnosis to disease progression, relapse, or death from any cause. Patients who were alive and free of disease progression at last follow-up were censored. Survival analysis was performed using the Kaplan-Meier method with the Prism 5 program (GraphPad Software, San Diego, CA), and differences in survival were compared using the log-rank (Mantel-Cox) test. Multivariate survival analysis was performed using a Cox proportional hazards regression model with the SPSS software program (version 19.0; IBM Corporation, Armonk, NY). All differences with $P \leq .05$ were considered statistically significant.

Results

PTEN is Expressed in Both Cytoplasm and Nucleus and the Cytoplasmic Expression is More Frequently Lost in GCB-DLBCL

In view of PTEN's distinct functions in the cytoplasm and nucleus, we evaluated PTEN expression in the cytoplasm and nucleus compartments separately. Representative PTEN$^+$ IHC staining and the expression histogram for the training cohort are shown in Figure 1, *A* and *B*. We found cytoplasmic PTEN expression was significantly higher than that in the nuclei (Figure 1*C*). Expression of cytoplasmic PTEN (Cyto-PTEN$^+$) was observed in 306 (64%) of 478 DLBCL in the training cohort, and showed significant differences between GCB and ABC subtypes: 57.6% (137/238) of GCB-DLBCL versus 70.5% (165/234) of ABC-DLBCL ($P = .004$, Table 1). The mean level of Cyto-PTEN expression for GCB-DLBCL was also significantly lower than that for ABC-DLBCL (Figure 1*C*). On the other hand, nuclear expression of PTEN (Nuc-PTEN$^+$) was observed in 280 (58.6%) of 478 DLBCL, including 57.1% (136/238) of GCB-DLBCL and 59.8% (140/234) of ABC-DLBCL. In contrast with the higher cytoplasmic PTEN expression in ABC-DLBCL, there was a trend of higher nuclear PTEN expression in GCB than in ABC DLBCL ($P = .072$, Figure 1*C*), although nuclear PTEN expression significantly correlated with cytoplasmic PTEN expression (Table 1, Figure 1*D*). Regardless of the expression compartments, totally 129 (26.7%) of 478 DLBCL did not have any PTEN expression (Cyto-PTEN$^-$ and Nuc-PTEN$^-$).

To validate the results, we assembled an independent DLBCL cohort (n = 204). Compared with the training cohort, the validation cohort showed a similar pattern of PTEN expression, with a slightly lower frequency of Cyto-PTEN loss, whereas a higher frequency of Nuc-PTEN loss compared with the training cohort: 25% of DLBCLs were Cyto-PTEN$^-$, and 69% of DLBCLs were Nuc-PTEN$^-$; 11% of DLBCLs did not show either cytoplasmic or nuclear PTEN expression. Consistent with the results in the training cohort, in the validation cohort cytoplasmic expression is predominant and the cytoplasmic PTEN and nuclear PTEN expression are significantly correlated (Supplementary Figure S1*A*).

Surprisingly, PTEN expression (cytoplasmic and/or nuclear) was associated with a higher mean level of phospho-AKT-Ser^{473} protein (p-AKT) nuclear expression but not *AKT1* mRNA expression (Figure 1*E* and Supplementary Figure S1*A* for the training and validation cohort, respectively). However, Cyto-PTEN$^+$ (but not Nuc-PTEN$^+$) expression was associated with significantly decreased survivin expression (a downstream target of the PI3K/AKT pathway [44]) in ABC-DLBCL (Figure 1*E*) independent of *TP53* mutation status, which may suggest a correlation between PTEN expression and decreased AKT function.

Cyto-PTEN$^+$ expression, but not p-AKT^{high}, PI3K^{high}, or Nuc-PTEN$^+$ expression, showed significant association with PD-L1 expression, which is considered as a tumor immune evasion mechanism of DLBCL [45] (Table 1, Figure 1*F*). Conversely,

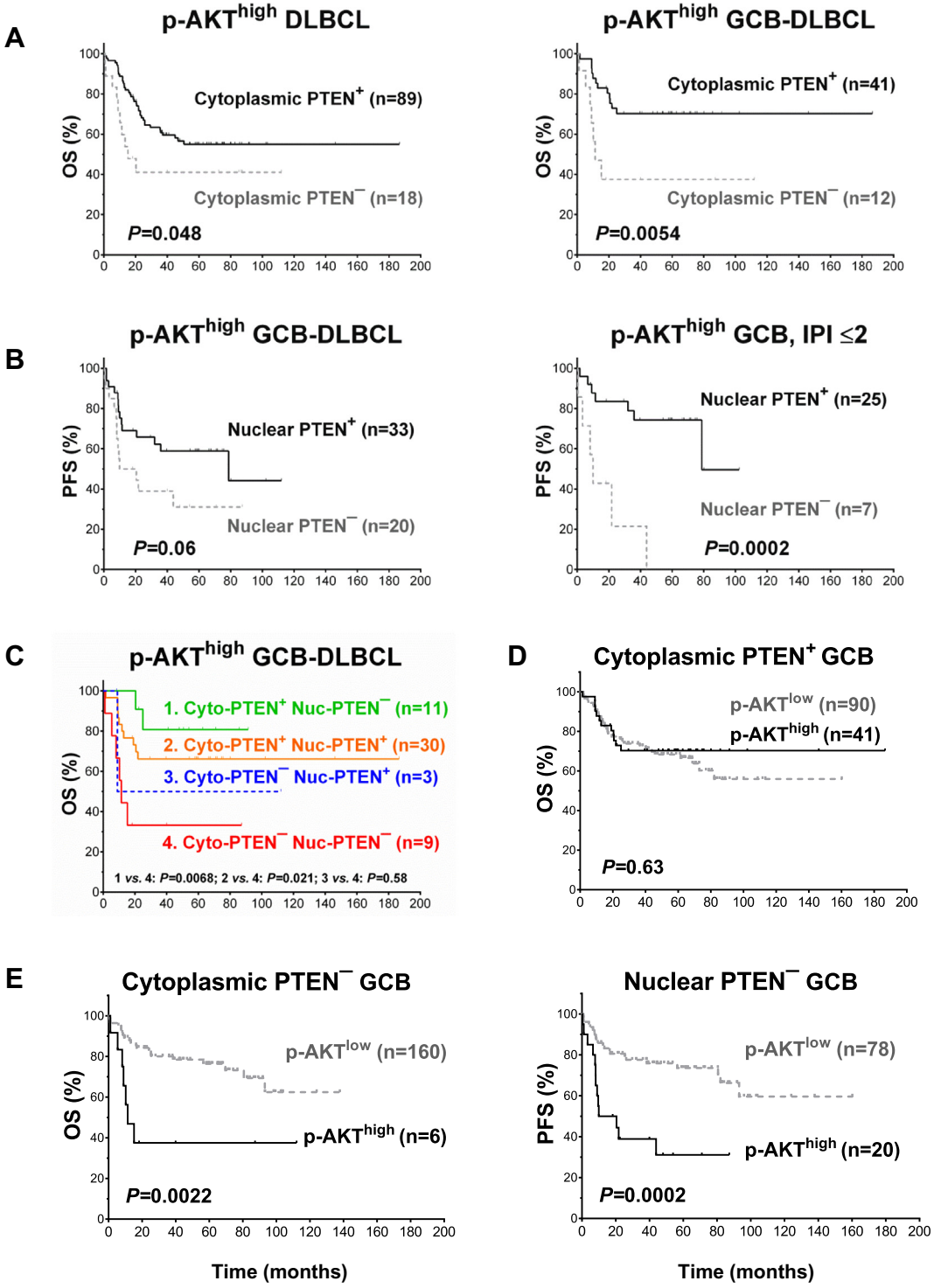


Figure 2. Survival analysis for PTEN expression/loss in DLBCL with high phosphorylated-AKT expression (p-AKT^{high}, cutoff: ≥70%). (A) Loss of PTEN cytoplasmic expression was associated with significantly poorer overall survival rate (OS) in patients with high p-AKT expression, especially in GCB-DLBCL. (B) Loss of PTEN nuclear expression was associated with decreased progression-free survival rate (PFS) in GCB-DLBCL patients with high p-AKT expression. This effect was only significant in the group with an International Prognostic Index (IPI) score ≤2. (C) Survival analysis in respect to both cytoplasmic and nuclear PTEN^{+} status in patients with p-AKT^{high} GCB-DLBCL. (D) In GCB-DLBCL cases with cytoplasmic PTEN expression, p-AKT expression level was not prognostic. (E) In GCB-DLBCL patients without cytoplasmic/nuclear PTEN expression, p-AKT^{high} expression was associated with significantly poorer survival.

PD-L1^{+} cases had a higher mean level of PTEN expression than PD-L1^{-} cases (P = .0015). Like Cyto-PTEN expression, PD-L1 expression was significantly higher in the ABC subtype (Figure 1*F*). Cyto-PTEN^{+} status was also associated with significantly higher mean levels of Myc, p-STAT3, PI3K, MDM2, and p21/CDKN1A expression (Figure 1*G*).

Table 2. Comparison of clinicopathologic features of patients with p-AKT overexpressing diffuse large B-cell lymphoma (DLBCL) respective to the status of cytoplasmic or nuclear PTEN expression, *PTEN* deletion, and *PTEN* mutation in the training cohort

	p-AKT^{high} GCB			p-AKT^{high} GCB			p-AKT^{high} DLBCL			p-AKT^{high} DLBCL		
	Cyto-PTEN$^+$	Cyto-PTEN$^-$		Nuc-PTEN$^+$	Nuc-PTEN$^-$		*PTEN* deletion	No *PTEN* deletion		*MUT-PTEN*	*WT-PTEN*	
	N (%)	N (%)	*P*	N (%)	N (%)	*P*	N (%)	N (%)	*P*	N (%)	N (%)	*P*
Variables	41 (100)	12 (100)		33 (100)	20 (100)		7 (100)	75 (100)		9 (100)	74 (100)	
Age, years												
< 60	24 (58.5)	8 (66.7)	.74	20 (60.6)	12 (60.0)	1.0	3 (42.9)	32 (42.7)	1.0	3 (33.3)	40 (54.1)	.3
≥ 60	17 (41.5)	4 (33.3)		13 (39.4)	8 (40.0)		4 (57.1)	43 (57.3)		6 (66.7)	34 (45.9)	
Sex												
Male	29 (70.7)	5 (41.7)	.09	24 (72.7)	10 (50.0)	.14	4 (57.1)	45 (60.0)	1.0	7 (77.8)	45 (60.8)	.47
Female	12 (29.3)	7 (58.3)		9 (27.3)	10 (50.0)		3 (42.9)	30 (40.0)		2 (22.2)	29 (39.2)	
Stage												
I-II	21 (52.5)	3 (30.0)	.29	17 (53.1)	7 (38.9)	.39	2 (28.6)	27 (39.1)	.7	2 (25.0)	26 (36.6)	.71
III-IV	19 (47.5)	7 (70.0)		15 (46.9)	11 (61.1)		5 (71.4)	42 (60.9)		6 (75.0)	45 (63.4)	
B symptoms												
No	33 (82.5)	7 (70.0)	.4	28 (84.8)	12 (70.6)	.28	4 (57.1)	44 (62.0)	1.0	5 (71.4)	46 (64.8)	1.0
Yes	7 (17.5)	3 (30.0)		5 (15.2)	5 (29.4)		3 (42.9)	27 (38.0)		2 (28.6)	25 (35.2)	
LDH												
Normal	14 (38.9)	2 (16.7)	.29	13 (46.4)	3 (15.0)	**.031**	3 (42.9)	31 (47.7)	1.0	2 (25.0)	27 (42.2)	.46
Elevated	22 (61.1)	10 (83.3)		15 (53.6)	17 (85.0)		4 (57.1)	34 (52.3)		6 (75.0)	37 (57.8)	
Extranodal sites												
0 - 1	33 (82.5)	5 (50.0)	**.046**	28 (87.5)	10 (55.6)	**.017**	5 (71.4)	45 (66.2)	1.0	4 (44.4)	48 (70.6)	.26
≥ 2	7 (17.5)	5 (50.0)		4 (12.5)	8 (44.4)		2 (28.6)	23 (33.8)		5 (55.6)	20 (29.4)	
ECOG score												
0 - 1	31 (83.8)	6 (75.0)	.62	25 (89.3)	12 (70.6)	.23	6 (85.7)	50 (82.0)	1.0	6 (85.7)	52 (78.8)	1.0
≥ 2	6 (16.2)	2 (25.0)		3 (10.7)	5 (29.4)		1 (14.3)	11 (18.0)		1 (14.3)	14 (21.2)	
Tumor size												
< 5 cm	14 (53.8)	1 (16.7)	.18	12 (52.2)	3 (33.3)	.44	3 (50.0)	35 (58.3)	.69	2 (66.7)	31 (60.8)	1.0
≥ 5 cm	12 (46.2)	5 (83.3)		11 (47.8)	6 (66.7)		3 (50.0)	25 (41.7)		1 (33.3)	20 (39.2)	
IPI score												
0 - 2	28 (68.3)	4 (36.4)	**.081**	25 (75.8)	7 (36.8)	**.008**	4 (57.1)	38 (52.8)	1.0	2 (25.0)	42 (58.3)	.13
3 - 5	13 (31.7)	7 (63.6)		8 (24.2)	12 (63.2)		3 (42.9)	34 (47.2)		6 (75.0)	30 (41.7)	
Therapy response												
CR	29 (70.7)	7 (58.3)	1.0	24 (72.7)	12 (60.0)	.38	7 (100)	54 (72.0)	.18	4 (44.4)	56 (75.7)	.11
PR	4	3		2	5		0	12		4	10	
SD	4	0		3	1		0	3		0	5	
PD	4	2		4	2		0	6		1	3	
***TP53* mutations**												
No	26 (78.8)	8 (72.7)	.69	23 (79.3)	11 (73.3)	.71	4 (57.1)	64 (88.9)	**.02**	7 (77.8)	59 (86.8)	.61
Yes	7 (21.2)	3 (27.3)		6 (20.7)	4 (26.7)		3 (42.9)	8 (11.1)		2 (22.2)	9 (13.2)	
PD-L1 IHC												
< 5%	9 (22.5)	7 (58.3)	**.031**	10 (31.3)	6 (30)	1.0	0 (0)	15 (22.7)	.33	13 (19.7)	2 (22.2)	1.0
≥ 5%	31 (77.5)	5 (41.7)		22 (68.8)	14 (70)		6 (100)	51 (77.3)		53 (80.3)	7 (77.8)	

Abbreviations: Cyto-PTEN, cytoplasmic PTEN expression; Nuc-PTEN, nuclear PTEN expression; LDH, lactate dehydrogenase; ECOG, Eastern Cooperative Oncology Group; IPI, International Prognostic Index; CR, complete remission; PR, partial response; SD, stable disease; PD, progressive disease; GCB, germinal-center B-cell–like; MUT, mutated, WT, wild-type.

Absence of PTEN Expression is Associated with Unfavorable Clinical Features and Outcomes Only in DLBCL with AKT Hyperactivation

Clinical features for PTEN$^+$ and PTEN$^-$ DLBCL groups are shown in Table 1 (cytoplasmic expression) and Supplementary Table S1 (nuclear expression). Cyto-PTEN$^-$ expression was not significantly associated with any clinical parameters (only trend of more female sex). Nuc-PTEN$^-$ status was associated with elevated serum lactate dehydrogenase (LDH) level ($P < .0001$). In the DLBCL subset with p-AKT hyperactivation (p-AKT^{high}) [34], Cyto-PTEN$^-$ status was associated with a larger tumor size ($P = .035$), and Nuc-PTEN$^-$ status was associated with elevated LDH, extranodal sites >1, ECOG performance status >1, tumor size ≥5cm, and International Prognostic Index (IPI) score >2.

Neither cytoplasmic nor nuclear PTEN$^+$ status showed significant prognostic impact in overall DLBCL. However, Cyto-PTEN$^-$ status was associated with a lower complete remission rate, with a trend of significance in the overall DLBCL cohort ($P = .079$), and significantly in the p-AKT^{high} ABC-DLBCL subset ($P = .0007$, Table 1). In p-AKT^{high} DLBCL, Cyto-PTEN$^-$ status was associated with lower mean levels of p-AKT ($P = .042$) and PD-L1 expression ($P = .042$, Figure 1*F*), but with higher frequency of survivin expression (26% vs. 8.9%, $P = .031$) and significantly poorer OS ($P = .048$), particularly in the GCB subtype ($P = .0054$) (Figure 2*A*). Moreover, in p-AKT^{high} GCB-DLBCL, loss of nuclear PTEN expression was associated with poorer PFS with borderline significance ($P = .06$, Figure 2*B*), although it was associated with significantly lower mean levels of antiapoptotic Bcl-2 ($P = .0068$) and MDM2 ($P = .0011$) expression.

Notably, patients with Nuc-PTEN$^-$ GCB-DLBCL more frequently had IPI >2, extranodal sites >1, and elevated LDH (P = .008, .017, and .031, respectively) (Table 2). To eliminate the confounding effects by these unfavorable clinical factors, we further compared survival of Nuc-PTEN$^+$ and Nuc-PTEN$^-$ patients with high and low IPI individually, and found that Nuc-PTEN$^-$ status was associated with markedly shorter PFS durations only for patients with an IPI ≤2 (P = .0002, Figure 2*B*).

Incorporating both cytoplasmic and nuclear PTEN$^+$ status in the survival analysis found Cyto-PTEN but not Nuc-PTEN expression had significant prognostic impact in p-AKT^{high} GCB-DLBCL patients (Figure 2*C*). However, the significance was lost in multivariate survival analysis adjusting clinical factors in p-AKT^{high} GCB-DLBCL. In contrast, in p-AKT^{high} ABC-DLBCL, Nuc-PTEN$^+$ expression was an independent prognostic factor for better OS (P = .003; hazard ratio [HR] 0.16; 95% confidence interval [CI] 0.049-0.53) and PFS (P = .008; HR 0.22; 95% CI 0.07-0.67) after adjusting clinical factors (Table 3). In the validation cohort, loss of Cyto-PTEN expression was also associated with signficantly shorter PFS in p-AKT^{high} DLBCL (P = .029, Supplementary Figure S1*B*) but not in the overall DLBCL cohort. However, in the multivariate survival analysis adjusting for clinical factors, Cyto-PTEN$^-$ status lost signficance as an independent prognostic factor in the validation p-AKT^{high} DLBCL cohort (data not shown).

Consistent with the role of PTEN in suppressing AKT activation and activity, the adverse prognostic significance of p-AKT^{high} expression in GCB-DLBCL that we have reported previously [34] was only significant in the Cyto-PTEN$^-$ GCB-DLBCL (P = .0022 for OS and P = .0029 for PFS, respectively) and Nuc-PTEN$^-$ GCB-DLBCL subsets (P = .12 for OS and P = .0002 for PFS, respectively), but not in the Cyto-PTEN$^+$ GCB-DLBCL (P = .63 for OS and P = .18 for PFS, respectively) or Nuc-PTEN$^+$ GCB-DLBCL subset (P = .89 for OS and P = .50 for PFS, respectively) (Figure 2, *D* and *E* and Supplementary Figure S2).

Table 3. Multivariate analysis for PTEN expression (positive or high), *PTEN* deletion and *PTEN* mutations in overall DLBCL, cases with ≥70% p-AKT expression (p-AKT^{high}), and cases with ≤30% p-AKT expression (p-AKT$^-$)

	OS			PFS		
Variables	HR	95% CI	*P*	HR	95% CI	*P*
In p-AKT^{high} ABC-DLBCL						
IPI >2	3.28	1.02-10.48	.046	3.84	1.20-12.33	.024
Female	.27	.096- .74	.011	.34	.13- .89	.028
Tumor size >5cm	1.91	.67-5.49	.23	1.94	.71-5.28	.19
B-symptoms	10.2	2.67-39.02	.001	6.37	1.88-21.56	.003
*Nuclear PTEN$^+$	**.16**	**.049- .53**	**.003**	**.22**	**.07- .67**	**.008**
In p-AKT^{high} ABC-DLBCL						
IPI >2	3.84	1.20-12.33	.024	2.35	.85-6.51	.10
Female	.19	.063- .60	.004	.28	.10- .79	.016
Tumor size >5cm	2.10	.73-6.09	.17	2.12	.79-5.71	.14
B-symptoms	7.27	1.88-28.17	.004	4.42	1.37-14.29	.013
*Cytoplasmic PTEN$^+$	**.47**	**.12-1.77**	**.26**	**.53**	**.14-1.95**	**.34**
In overall DLBCL						
IPI >2	2.31	1.63-3.28	< .001	2.28	1.64-3.18	< .001
Female	.75	.52-1.07	.12	.72	.51-1.02	.064
Tumor size >5cm	1.15	.80-1.64	.45	1.12	.79-1.57	.53
B-symptoms	1.62	1.12-2.33	.01	1.59	1.12-2.26	.009
*Nuclear PTEN^{high}	**.39**	**.16- .97**	**.043**	**.34**	**.14- .84**	**.02**
Myc^{high}	2.15	1.49-3.09	< .001	2.10	1.48-2.97	< .001
In overall DLBCL						
IPI >2	2.38	1.68-3.38	< .001	2.34	1.67-3.26	< .001
Female	.86	.60-1.23	.40	.84	.60-1.19	.33
Tumor size >5cm	1.36	.96-1.92	.084	1.31	.94-1.82	.11
B-symptoms	1.41	.98-2.03	.063	1.39	.98-1.98	.061
*Cytoplasmic PTEN^{high}	**1.19**	**.84-1.69**	**.33**	**1.42**	**1.02-1.98**	**0.036**
In p-AKT$^-$ DLBCL						
IPI >2	2.59	1.65-4.05	< .001	2.80	1.81-4.32	< .001
Female	.95	.60-1.49	.82	.85	.54-1.32	.47
Tumor size >5cm	1.07	.68-1.69	.77	1.03	.67-1.59	.89
B-symptoms	.97	.61-1.56	.90	1.00	.64-1.57	.99
*Cytoplasmic PTEN^{high}	**1.33**	**.84-2.09**	**.22**	**1.62**	**1.05-2.50**	**.03**
Myc^{high}	1.71	1.05-2.79	.03	1.63	1.02-2.61	.041
In p-AKT^{high} DLBCL						
IPI >2	4.80	1.78-12.98	.002	3.47	1.51-7.96	.003
Female	.48	.21-1.07	.072	.34	.15- .78	.011
Tumor size >5cm	1.38	.63-3.02	.43	1.72	.80-3.71	.17
B-symptoms	2.59	1.12-5.98	.026	3.07	1.36-6.94	.007
**PTEN* deletion	**4.53**	**.98-20.89**	**.052**	**5.30**	**.99-28.33**	**.051**
**PTEN* mutation	**4.53**	**.97-21.12**	**.054**	**3.78**	**1.02-13.97**	**.046**

Abbreviations: OS, overall survival; PFS, progression-free survival; HR, hazard ratio; CI, confidence interval; GCB, germinal center B-cell–like; ABC, activated B-cell–like; IPI, International Prognostic Index.

* Data for PTEN factors are highlighted in bold. Cutoffs for Nuclear PTEN^{high} and Cytoplasmic PTEN^{high}: >30% and >40%, respectively.

High Cytoplasmic PTEN Expression is Associated with Poorer Survival Only in DLBCL Patients with Low AKT Activation

In contrast to the results above indicating that loss of PTEN expression was associated with unfavorable clinical outcomes only in DLBCL with AKT hyperactivation, in the p-AKT-deficient training subcohort (p-AKT$^-$, cutoff: ≤30% which was approximate to the mean p-AKT expression level, 33%), high Cyto-PTEN expression (Cyto-PTEN^{high}, cutoff: >40%; frequency: 36%) was associated with inferior OS (P = .014) and PFS (P = .012), which was only significant in the GCB subtype (Figure 3*A*). In contrast, high Nuc-PTEN expression (Nuc-PTEN^{high}, cutoff: >30%%; frequency: 5.2%) was associated with better OS and PFS in p-AKT$^-$ DLBCL cases (Figure 3*B*), overall GCB-DLBCL cases, and the p-AKT$^-$ GCB-DLBCL subset.

Notably, Cyto-PTEN^{high} expression was associated with higher mean levels of p-AKT (in both GCB and ABC), PI3K (P = .039), Myc (in GCB only), p21 (P = .0011), MDM2 (in both GCB and ABC), and p-STAT3 (in ABC only) expression at the protein level (Figure 4*A*) but not at the mRNA level, and associated with both Bcl-2 protein (P = .0021) and *BCL2* mRNA (P = .0003) expression. Restricting the analysis in the p-AKT$^-$ DLBCL subset in which PTEN^{high} expression showed prognostic effect, Cyto-PTEN^{high} expression remained to be associated with high Myc (an unfavorable prognostic factor [36]) and p-AKT expression, significantly only in the GCB subtype (Figure 4*A*). Nuc-PTEN^{high} expression was associated with higher mean levels of p-AKT and PI3K but not Myc expression, and the association with p-AKT expression was significant only in the ABC subtype (Figure 4*B*).

In multivariate survival analysis adjusting for clinical parameters, Cyto-PTEN^{high} remained as an unfavorable factor for PFS in overall DLBCL and the p-AKT$^-$ DLBCL subcohort (P = .009; HR 1.77; 95% CI 1.15-2.72), whereas Nuc-PTEN^{high} was a favorable factor for PFS independent of clinical factors only in the overall cohort (P = .032; HR 0.37; 95% CI 0.15-0.92). After adding the factor of Myc^{high} in the Cox regression models, Cyto-PTEN^{high} remained as an independent factor for unfavorable PFS only in p-AKT$^-$ DLBCL cases but not in the overall cohort, whereas Nuc-PTEN^{high} was a favorable factor for both OS (P = .043; HR 0.39; 95% CI 0.16-0.97) and PFS in the overall cohort but not in the p-AKT$^-$ DLBCL subcohort (Table 3).

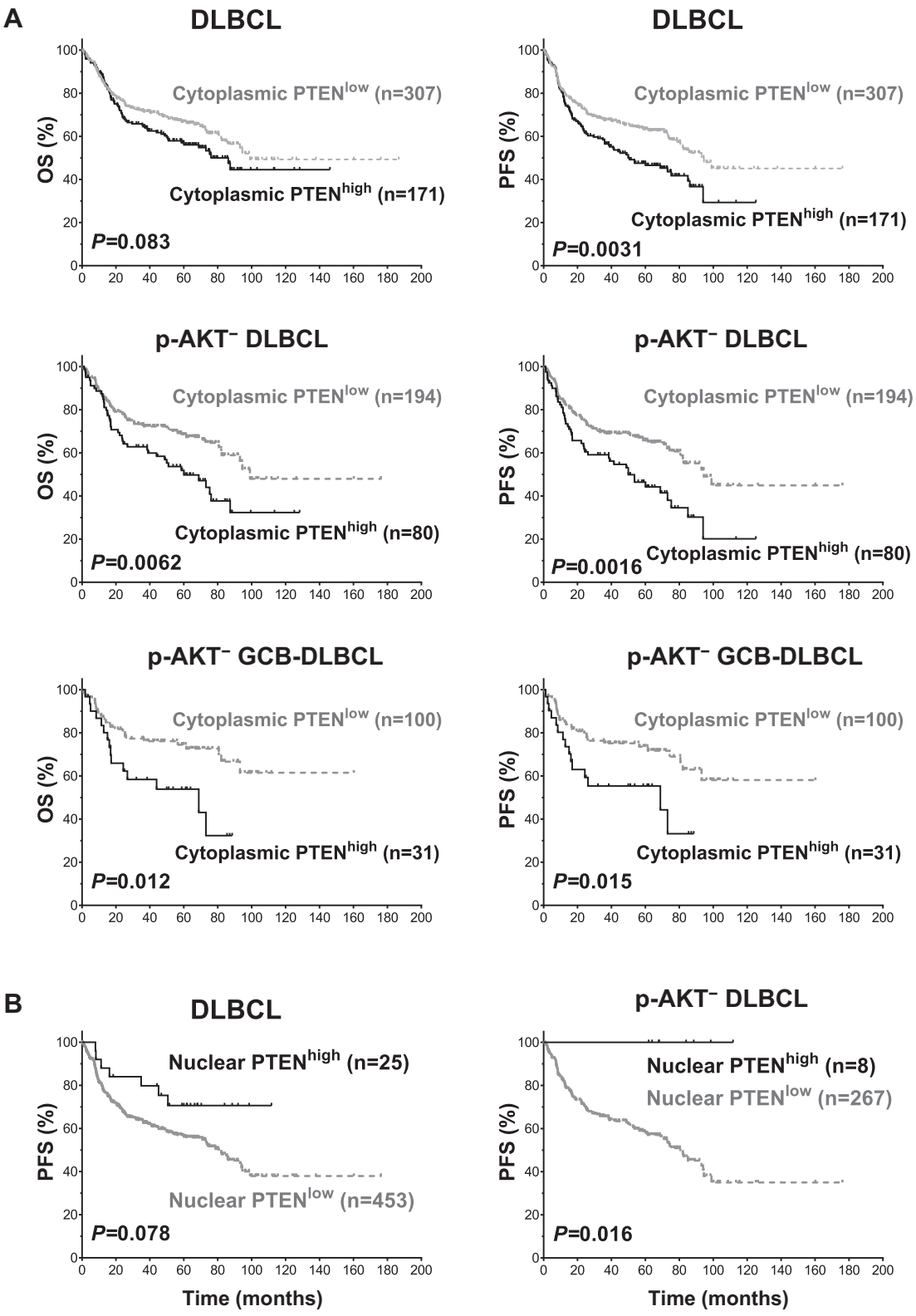


Figure 3. Survival analysis for high levels of PTEN expression in DLBCL. (A) DLBCL patients with high cytoplasmic PTEN^{+} expression (cutoff: >40%) had a significant poorer progression-free survival rate (PFS) compared with patients with low PTEN expression. The adverse prognostic effect was only significant in DLBCL with no or low p-AKT expression (p-AKT^{-}, cutoff: ≤30%), and GCB-DLBCL with low p-AKT expression. (B) High nuclear PTEN^{+} expression (cutoff: >30%) was associated with trend of better PFS in DLBCL with no or low p-AKT expression. The favorable prognostic effect was only significant in patients with no or low p-AKT expression.

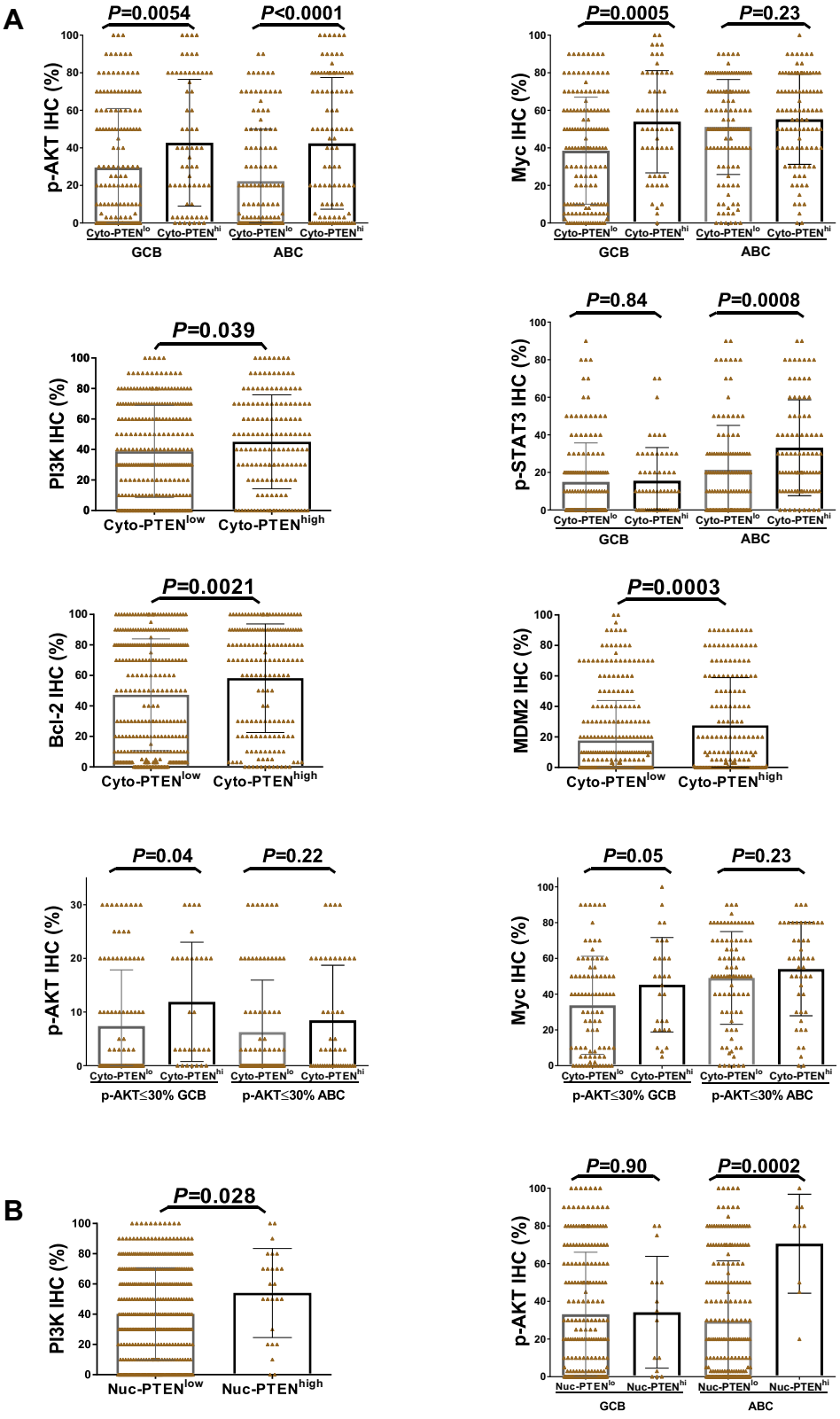


Figure 4. Biomarker expression analysis for high PTEN expression. (A) High cytoplasmic PTEN expression (>40%) was associated with higher mean levels of p-AKT, Myc (in GCB only), PI3K, p-STAT3 (in ABC only), Bcl-2, and MDM2 expression. Only in DLBCL with no or low p-AKT expression, high cytoplasmic PTEN expression was associated with higher mean levels of p-AKT and Myc expression. (B) High nuclear PTEN expression (>30%) was associated with higher mean levels of p-AKT (in ABC only) and PI3K expression. Significant *P* values are in bold.

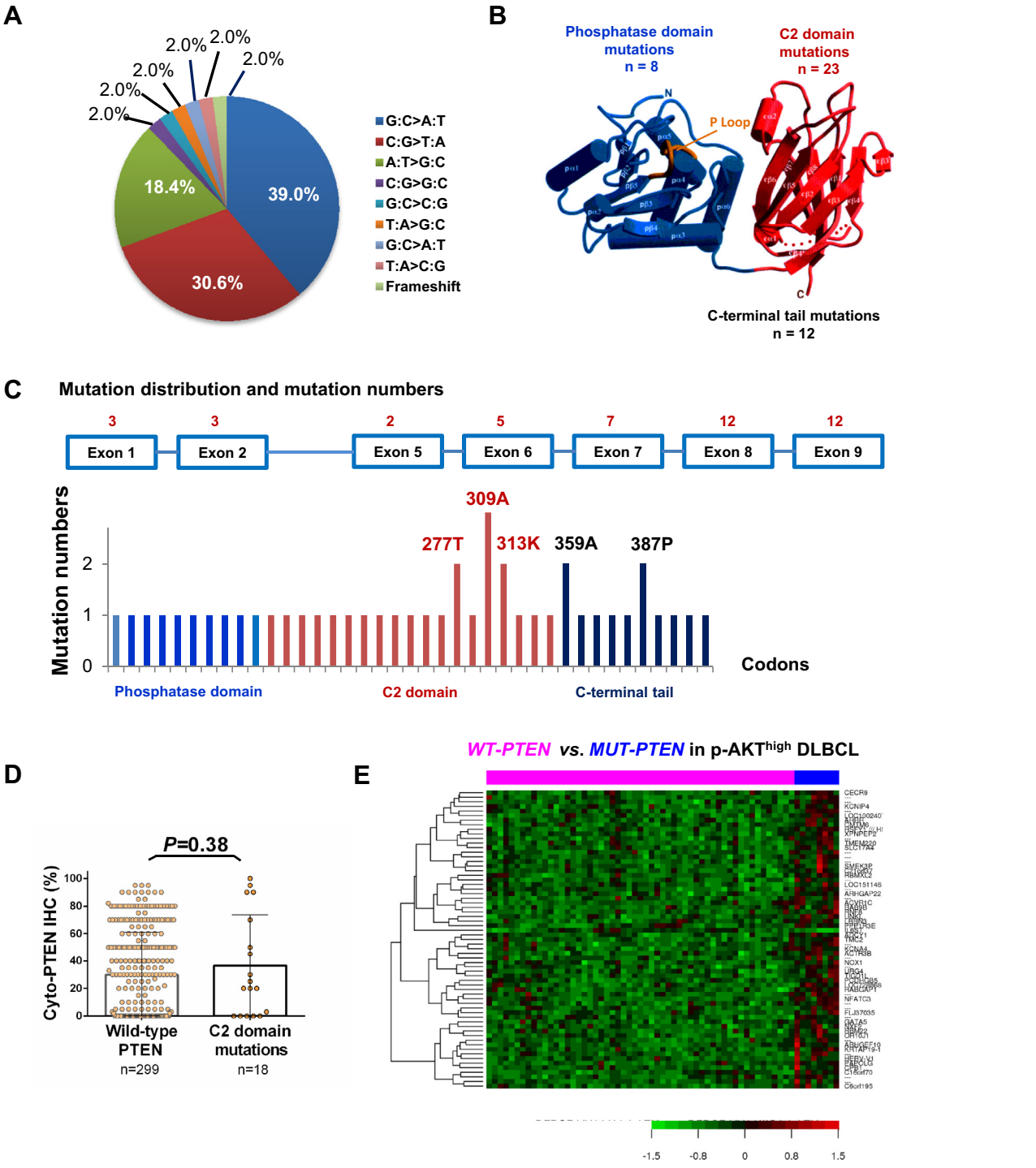


Figure 5. *PTEN* mutation analysis in the DLBCL training cohort. (A) Proportions of classified point mutations. (B) PTEN mutation numbers in the phosphatase domain (blue), C2 domain (red) and C-terminal tail. PTEN crystal figure is edited from Lee et al 1999; reference [41]. (C) Distribution of mutation numbers according to *PTEN* exons and codons. (D) *PTEN* mutations in the C2 domain were associated with a trend of higher mean cytoplasmic PTEN level but it was not significant. (E) Genes significantly differently expressed between wild-type *PTEN* and mutated-*PTEN* groups in DLBCL with AKT hyperactivation.

Compared with the training cohort, the validation cohort had a higher frequency of Cyto-PTEN^{high} expression (52%) and lower frequency of Nuc-PTEN^{high} expression (1.5%). As in the training cohort, in the validation cohort Cyto-PTEN^{high} expression was associated with higher mean levels of p-AKT and Myc expression (Supplementary Figure S2*A*). In p-AKT^{-} cases (≤30% p-AKT expression), Cyto-PTEN^{high} expression was associated with trend of poorer survival, whereas Nuc-PTEN^{high} was associated with trend of better survival. In contrast, in p-AKT^{+} cases (>30% p-AKT expression), Cyto-PTEN^{high}

Table 4. Gene expression profiling analysis

	PTEN^{low} *vs. PTEN*$^{not\ low}$ in GCB-DLBCL (FDR<0.01, fold >2)		*PTEN*^{low} *vs. PTEN*$^{not\ low}$ in ABC-DLBCL (FDR<0.01, fold >1.74)	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Signaling, receptors, B-cell development and differentiation		*PTEN*, *PTEN/PTENP1*, *STAP1*, *BLNK*, *FCRL1*, *KLHL6*, *LPAR5*, *RGS1*, *RGS13*, *FCRL5*, *BANK1*		*PTEN*, *PKN2*, *RANBP9*, *MAP3K13*, *RGS13*
Transcriptional regulation, mRNA processing and regulation		*INTS7*, *PABPC1*, *CBFB*, *EZH2*, *ZNF117*, *IGF2BP3*, *HNRNPD*, *MYBL1*		*RFX7*, *ZEB1*, *HIF1A*, *TBL1XR1*, *OVOS/OVOS2*, *SMCHD1*, *MBD4*, *TCF4*, *PRDM2*
Cell cycle		*NIPBL*, *CASC5*		*GPSM2*, *DPY30/MEMO1*, *SMC1A*, *PTP4A1*, *C7orf11*, *ZYG11B*, *MARK4*, *SFI1*
Immune response, inflammation		*HLA-DMA/HLA-DMB*, *HLA-DPA1*, *HLA-DOA*, *SERPINB9*, *HLA-DQB1*, *LYZ*		*POLR3E*
Metabolism, ribosomes		*AMD1*, *RPL15*, *PGK1*, *SAMM50*, *CIRH1A*		*C11orf54*, *AMD1*, *FUT8*, *RPL15*, *PDE7A*, *DERA*, *PNPLA8*, *C21orf57*, *SLC16A1*
Posttranslational modification, protein degradation, transport		*IDE*, *LRMP*, *CSE1L*, *UBE2G1*, *FBXO6*		*CCDC91*, *C18orf55*, *OSBPL8*, *USP1*
Actin, cytoskeleton, cell adhesion, extracellular matrix, motility		*ANXA7*, *RABEP2*, *SYNE2*, *TMEM163*, *FGD6*, *ENPP2*, *POSTN*		*ANXA7*, *RABEP2*, *KIAA1217*, *DMD*
Unknown function	*ZDHHC11*	*FAM82B*		*FAM82B*, *C12orf66*, *RP6-213H19.1*, *BAGE2/BAGE4*

	MUT-PTEN vs. WT-PTEN in DLBCL (FDR< 0.05)		*MUT-PTEN vs. WT-PTEN* in p-AKT^{high} DLBCL (FDR< 0.25)	
	Upregulated	Downregulated	Upregulated	Downregulated
Signaling, receptors	*BOC*, *GPC4*, *GLRA3*, *PTPRF*, *C7orf16*, *ACVR1C*, *UNC5C*	*DENND4C*	*ACVR1C*, *ARHGAP22*, *OR10J1*, *CMTM6*	*IL6ST*
Transcriptional regulation	*SOX10*, *DPPA4*, *TFAP2A*		*NFATC3*, *ARHGEF10*, *GATA5*, *HSFY1/HSFY2*	
Cell growth and differentiation, development			*ADCY1*, *URG4*, *RABGAP1*, *AHRR*	
Immune response, inflammation	*PDCD6*	*MYD88*	*XPNPEP2*	
Metabolism	*FAM19A5*	*PGS1*	*NOX1*, *CPB1*, *PPP1R3E*	
mRNA processing and regulation, protein folding, posttranslational modification, degradation	*C2orf30*, *AFF2*, *CUL7*, *RNF7*, *USP46*, *PSMG4*, *ELAVL4*, *HERC6*	*ICMT*	*UNKL*, *PAPOLG*, *RBM22*, *NXF2*, *RNF8*, *RBMXL2*	
Transport, actin, cytoskeleton, motility	*MYO1C*, *DYNLRB1*, *STARD13*, *TTLL2*, *RHO*	*KIF5B*	*RAB9B*, *SLC17A4*, *ACTR3B*	
Cell adhesion, extracellular matrix, ion channels	*PCDHGB5*, *NLGN3*, *GJA3*, *ATP4B*		*KCNA4*, *TMC2*, *PCDHGB5*, *KRTAP19-1*, *KCNIP4*	
IncRNA, pseudogene, unknown function	*ADCK2*, *PCA3*, *LOC283140*, *HYDIN/HYDIN2*, *PRAMEF12*, *LOC100129175*, *C20orf12*, *TMEM174*, *HEATR4*, *HSPC072/LINC00652*, *C7orf45*, *LOC219731*, *HMCN2*, *LOC404266*	*LRPPRC*, *DCTN6*	*C16orf70*, *LOC728868*, *LRRN3*, *LOC151146*, *C6orf195/LINC01600*, *HERV-V1*, *FLJ37035*, *CECR9*, *TIGD1L*, *LOC100240734*, *SMEK3P*, *C21orf37/LINC01549*, *TMEM220*	

Abbreviations: *MUT-PTEN*, *PTEN* mutated genotype; *WT-PTEN*, *PTEN* wild-type genotype; FDR, false discovery rate.

expression was associated with significantly better PFS, whereas Nuc-PTEN^{high} (only two cases) was associated with poorer PFS (Supplementary Figure S2*B*). In the multivariate analysis, only Cyto-PTEN^{high} expression in the p-AKT^{+} subcohort showed trend toward being an independent factor for better PFS (P = .085; HR 0.30; 95% CI 0.076-1.18).

PTEN Gene Deletions and Mutations are Infrequent in DLBCL but are Independent Unfavorable Prognostic Factors in p-AKT^{high} DLBCL

To understand the mechanisms for PTEN deficiency in DLBCL, *PTEN* gene deletion status was assessed in a total of 607 cases of DLBCL (359 plus 248 from the training and validation cohorts, respectively), and *PTEN* mutation status was assessed in 368 cases from the training cohort.

Totally 44 *PTEN* mutations were detected in 39 (10.6%) of 368 patients, including 23 (12.2%) patients with GCB-DLBCL and 16 (9.0%) patients with ABC-DLBCL. Of these, 8 (18%) mutations were in the regions encoding the phosphatase domain (corresponding to aa15-aa185, [46]) of the PTEN protein, 23 (52%) in the C2 domain (aa185-aa351), and 12 (27%) in the C-terminal tail (aa351-aa403) (Figure 5, *A*–*C*). No correlation between *PTEN* mutation and *PTEN* deletion or PTEN protein expression was observed, although the expression levels of PTEN with C2 domain mutations were slightly increased (P = .38, Figure 5*D*).

A distinct GEP signature was identified for *PTEN* mutations (FDR < 0.25, Figure 5*E*). Seven genes were down-regulated and 43 were up-regulated in the mutated-*PTEN* DLBCL subgroup compared with the wild-type *PTEN* DLBCL subgroup (FDR < 0.05, Table 4). Notably, *PDCD6* which encodes programmed cell death 6, a calcium-binding protein required for T-cell receptor-, Fas-, and glucocorticoid-induced cell death and having inhibitory function towards PI3K/AKT/mTOR signaling [47], was up-regulated in the mutated-*PTEN* DLBCL group. Many genes related to neural function (such as *BOC*, *GLRA3*, *UNC5C*, *GPC4*, *GLRA3*, and *NLGN3*) and protein degradation (such as *CUL7*, *RNF7*, *USP46*, and *HERC6*) were also up-regulated in the mutated-*PTEN* DLBCL group, whereas *MYD88*, which was recurrently mutated in primary central nervous sytem (CNS) lymphoma [48], was downregulated. We further compared mutated-*PTEN* DLBCL group with the wild-type *PTEN* DLBCL group in the p-AKT^{high} DLBCL subset. In the mutated *PTEN* subgroup, 43 genes were up-regulated whereas only

IL6ST (interleukin 6 signal transducer, involved in STAT3 activation) was downregulated (Table 4). Up-regulated genes included CNS-related genes (*ADCY1* and *ARHGEF10*), genes invovled in protein degradation, and *PPP1R3E* invovled in glycogen metabolism.

Heterozygous or homozygous *PTEN* deletion (Figure 6*A*) was only present in 44 (12.3%) of 359 patients in the training cohort, including 27 (61.4%) patients with GCB-DLBCL and 17 (38.6%) patients with ABC-DLBCL. Only two of these cases had homozygous deletion. Figure 6*B* depicted the case distribution of *PTEN* mutation and *PTEN* deletion in the training cohort. *PTEN* deletion cases overlapped with approximately 17.7% of Cyto-PTEN$^-$ DLBCL cases, and 14.6% of Nuc-PTEN$^-$ DLBCL cases. Similar frequency of *PTEN* deletion (heterozygous or homozygous) was observed in the validation cohort (10.1%, 25 of 248 patients, including 4 patients [1.6%] who had homozygous deletion), which overlapped with approximately 13.6% of the Cyto-PTEN$^-$ cases, and 15.9% of the Nuc-PTEN$^-$ cases (Supplementary Figure S1*C*).

PTEN deletion was associated with lower mean levels of Cyto-PTEN expression in both the training and validation cohorts (P = .015 and P = .013, respectively; Figure 6*C*, and Supplementary Figure S1*D*). Only a trend of decrease in Nuc-PTEN expression was associated with *PTEN* deletion (P = .24) likely due to the low nuclear PTEN expression and small number of positive cases. Among cases with *PTEN* deletion, Cyto-PTEN$^+$ expression status was associated with trend of better OS in the training cohort (P = .065, Figure 6*C*), and significant better OS in the combined training and validation cohort (P = .031). Conversely, among Cyto-PTEN$^+$ cases, *PTEN* deletion was associated significantly better OS (P = .02 in the training cohort and P = .006 in the combined cohort), despite the association with decreased Cyto-PTEN expression (P = .008 in the combined cohort).

The clinical features of patients with and without *PTEN* deletion/mutation in the training cohort are shown in Supplementary Tables S2 and S3. *PTEN* deletion was associated with age <60 years (P = .024) in ABC-DLBCL. *PTEN* mutation tended to be associated with age ≥60 years (P = .078) in GCB-DLBCL and elevated LDH levels (P = .051) in ABC-DLBCL. Different from PTEN$^-$ expression status, *PTEN* deletion/mutation was not associated with decreased p-AKT, PI3K, survivin, Myc, p-STAT3, p21, or PD-L1 expression. However, *PTEN* deletion was associated with lower mean levels of MDM2 and BLIMP-1 expression (P = .01 and .027, respectively). We have shown previously that BLIMP-1 expression was associated with the ABC subtype [40].

Similarly with the prognostic effects associated with PTEN protein loss, *PTEN* deletion and *PTEN* mutation were associated with trends towards poorer survival in p-AKT^{high} DLBCL despite the small case numbers (Figure 6*D*), although not in overall DLBCL (data not shown). The effect of *PTEN* deletion was stronger in the GCB compared with the ABC subtype. Moreover, multivariate analysis adjusting for clinical factors showed that both *PTEN* mutation and deletion were independent prognostic factors for poorer OS and PFS in p-AKT^{high} DLBCL (Table 3).

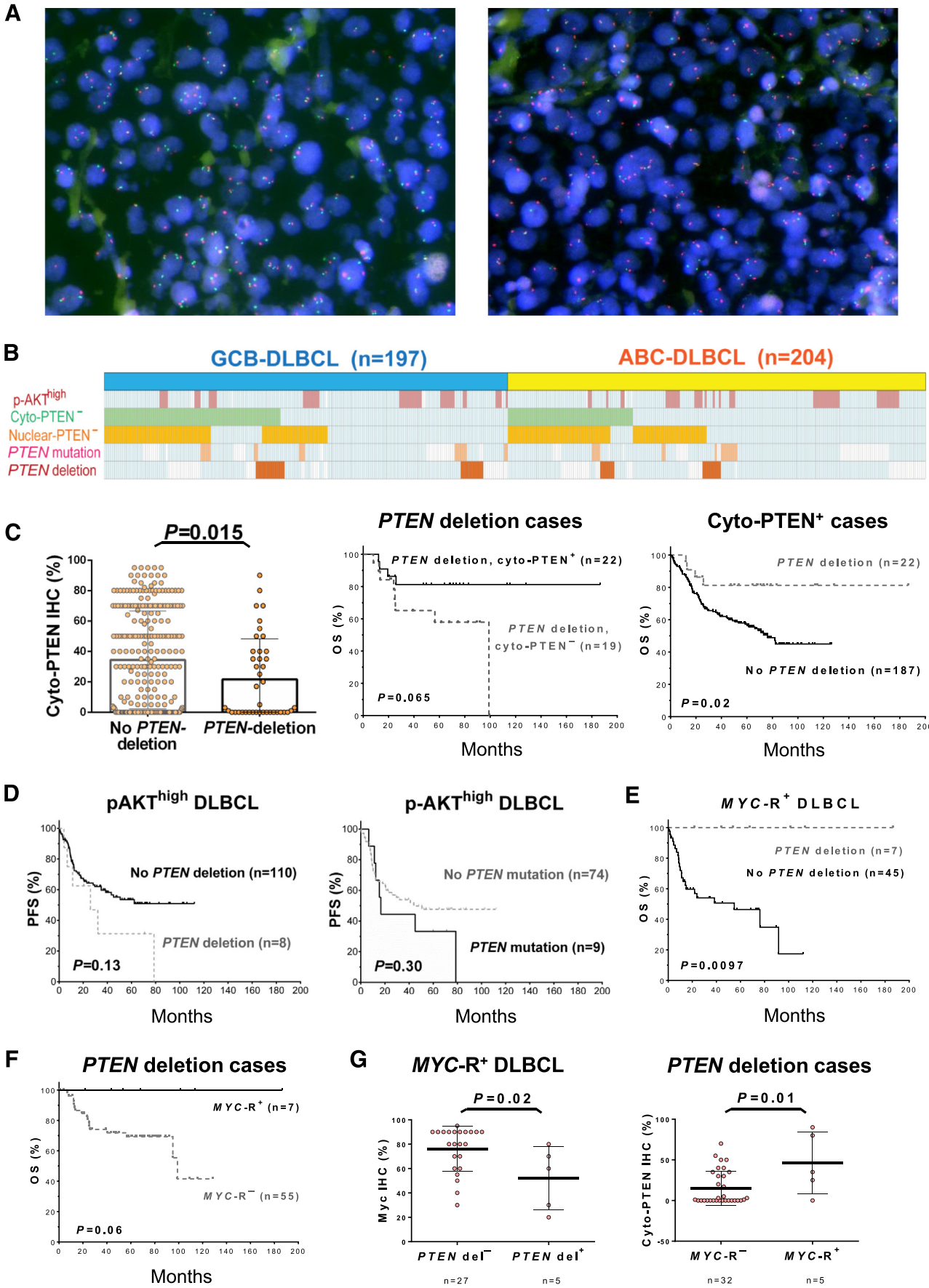
Opposite to this observation in p-AKT^{high} DLBCL, in *MYC*-rearranged DLBCL cases, seven cases had *PTEN* deletion (heterozygous or homozygous) and significantly better survival (in the training cohort, P = .033 for OS and P = .064 for PFS; in the combined traning and validation cohorts, P = .0097 for OS and P = .025 for PFS; Figure 6*E*). Similar favorable effect of *PTEN* deletion was also shown in GCB-DLBCL with *BCL2* rearrangement (P = .08 for OS in the training cohort and P = .048 for OS in the combined cohorts). Conversely, among DLBCL cases harboring *PTEN* deletion, *MYC* rearrangement was associated with trend of better OS (P = .096 in the training cohort, P = .06 in the combined training and validation cohort; Figure 6*F*). However, multivariate analysis indicated that *PTEN* deletion was not a prognostic factor independent of clinical factors in *MYC*-rearranged patients. The better survival may be attributable to the decreased Myc protein expression in these *MYC*-rearranged cases harboring *PTEN* deletion (P = .02 in the training cohort); we have shown previously that *MYC*-rearranged DLBCL cases without Myc overexpression had superior survival [36]. In addition, PTEN expression was positive in five of seven *MYC*-rearranged cases with *PTEN* deletion. Among cases with *PTEN* deletion, *MYC* rearrangement was associated with increased cytoplasmic PTEN expression (P = .01, Figure 6*G*), which is similar to the association of Myc overexpression with cytoplasmic PTEN expression (P = .0022, figure not shown).

Both Transcriptional and Post-transcriptional Mechanisms are Involved in Nuclear and Cytoplasmic PTEN Expression Regulation

The above data showed that *PTEN* genetic lesions only contributed to a small proportion of DLBCL with PTEN deficiency. We further correlated PTEN expression to biologic data from our previous studies [32,33,38] and found that loss of PTEN expression was associated with *TP53* mutation and IgA/IgG positive immunophenotypes (Table 1, Supplementary Table S1). Notably, previous studies have shown that wild-type but not mutated p53 transactivates *PTEN* [49,50].

At the transcription level, we found that Nuc-PTEN negativity was associated with significantly lower *PTEN* mRNA expression (P = .0054), more signficant in GCB-DLBCL (P = 0.0092) than in ABC-DLBCL (P = 0.081). Comparably, the association between *PTEN* downregulation and Cyto-PTEN$^-$ status was not significant (P = 0.065), with a stronger trend in ABC-DLBCL (P = 0.087) than in GCB-DLBCL (P = 0.36).

The lack of significant association of Cyto-PTEN expression with *PTEN* mRNA expression may suggest the important role of posttranscriptional regulation in Cyto-PTEN expression. We further extracted PTEN-targeting miRNA from miRNA profiling data and found that Cyto-PTEN$^-$ status was associated with significantly higher expression of several PTEN-targeting miRNAs, including miR-106b-3p, miR-200c-5p, miR-486-5p, miR-141-5p, and miR-130b-5p (Figure 7, *A* and *B*). When further analyzed in GCB/ABC subtypes, Cyto-PTEN negativity was associated with higher miR-486, miR-130b, and miR-106b expression in GCB-DLBCL, and with higher miR-200c and miR-222 in ABC-DLBCL. In comparison, loss of Nuc-PTEN expression did not show correlations with expression of most PTEN-targeting miRNAs except for higher miR-106b-3p expression (P = .042, figure not shown). Interestingly, absence of PD-L1 expression was also associated with significantly higher levels of miR-106b-3p (P = .0088, Figure 7*C*) and miR-130b-5p (P = .036, figure not shown) expression. These data may suggest that posttranscriptional regulation including miRNA-mediated epigenetic mechanism played a significant role in regulating cytoplasmic PTEN expression, whereas nuclear PTEN expression was mainly regulated at the transcription level in GCB-DLBCL.


A
B
GCB-DLBCL (n=197)
ABC-DLBCL (n=204)
p-AKT high
Cyto-PTEN −
Nuclear-PTEN −
PTEN mutation
PTEN deletion
C
P=0.015
Cyto-PTEN IHC (%)
No PTEN-deletion
PTEN-deletion
PTEN deletion cases
PTEN deletion, cyto-PTEN+ (n=22)
PTEN deletion, cyto-PTEN− (n=19)
P=0.065
OS (%)
Months
Cyto-PTEN+ cases
PTEN deletion (n=22)
No PTEN deletion (n=187)
P=0.02
D
pAKT high DLBCL
No PTEN deletion (n=110)
PTEN deletion (n=8)
P=0.13
PFS (%)
p-AKT high DLBCL
No PTEN mutation (n=74)
PTEN mutation (n=9)
P=0.30
E
MYC-R+ DLBCL
PTEN deletion (n=7)
No PTEN deletion (n=45)
P=0.0097
F
PTEN deletion cases
MYC-R+ (n=7)
MYC-R− (n=55)
P=0.06
G
MYC-R+ DLBCL
P=0.02
Myc IHC (%)
PTEN del−
PTEN del+
n=27
n=5
PTEN deletion cases
P=0.01
Cyto-PTEN IHC (%)
MYC-R−
MYC-R+
n=32
n=5

Striking Prognostic Effect and Gene Expression Signatures Associated with Low PTEN mRNA Expression

PTEN mRNA expression showed much greater prognostic effect than PTEN protein expression. Low *PTEN* mRNA levels (*PTEN*-mRNA^{low}) was associated with significantly poorer OS and PFS in overall DLBCL and GCB-DLBCL, ABC-DLBCL, p-AKT^{low}, and p-AKT^{high} subsets with multiple cutoffs (Figure 7*D*, with a cutoff at 21st percentile).

Moreover, distinct GEP signatures were identified for low *PTEN* mRNA expression, but not for Cyto- or Nuc-PTEN protein negativity. In GCB-DLBCL, up to 11,556 transcripts were up- or down-regulated in *PTEN*-mRNA^{low} patients compared with *PTEN*-mRNA$^{not\ low}$ patients with a FDR threshold of 0.01. In ABC-DLBCL, 2,358 transcripts were differentially expressed between *PTEN*-mRNA^{low} and *PTEN*-mRNA$^{not\ low}$ groups (FDR < 0.01). When use another cutoff at 50th percentile (median) for *PTEN*-mRNA^{high}, a greater number of significant transcripts were differentially expressed between *PTEN*-mRNA^{high} ABC-DLBCL and other ABC-DLBCL patients (n = 10,361, FDR<0.01, data not shown). The spectrum of *PTEN*-mRNA^{low} genes in ABC-DLBCL was similar with that in GCB-DLBCL, and both showed downregulation of genes involved in immune responses, B-cell receptor (BCR) signaling, gene expression, and metabolism, such as downregulation of *HLA-DRB4*, *CD58*, *MS4A1*/*CD20*, *FCRL3*, *CSE1L*, *RPL15*, and *HNRNPA1*. Notably, GEP analysis for AKT hyperactivation also demonstrated downregulation of many genes involved in immune responses, microenvironment, and metabolism in p-AKT^{high} GCB-DLBCL patients [34]. Two genes regulating mRNA turnover (*PABPC1* and *IGF2BP3*) were downregulated in both GCB and ABC subtypes of *PTEN*-mRNA^{low} DLBCL, including *IGF2BP3* which protects mRNAs against miRNA-mediated degradation [51]. *PTEN*-mRNA^{low} gene signatures in GCB-DLBCL and in ABC-DLBCL with >2-fold and >1.74-fold differences, respectively, are shown in Figure 7*E* and Table 4.

Discussion

In two large cohorts of DLBCL, PTEN expression was observed mainly in the cytoplasmic compartments of the tumor cells (64–75% of cases); PTEN expression in the nucleus was less frequent and at lower levels. PTEN cytoplasmic expression was more frequent and higher (by mean level) in the ABC compared with GCB subtype. The frequency of loss of cytoplasmic PTEN expression observed in this study (25–36%) is comparable to those by other studies in DLBCL (31-37%) [28,30]. Complete loss of both cytoplasmic and nuclear PTEN expression was observed in 27% of the training cohort and 11% of the validation cohort, which was comparable to the frequency of complete loss of PTEN expression reported in melanoma (25%) [25], and lower than those in some solid tumors, such as hepatocellular (57%), prostate (52%), colorectal (48%) [25], glioblastoma (53%) [52], and triple-negative breast cancer (48%) [19]. Loss of cytoplasmic and/or nuclear PTEN expression was associated with poorer clinical outcomes only in DLBCL with high p-AKT (Ser^{473}) nuclear expression, which were mainly manifested in the GCB subtype by univariate survival analysis but were retained only in the ABC subtype by multivariate analysis adjusting for clinical parameters. In contrast, in patients without abnormal AKT activities, high cytoplasmic PTEN expression was associated with poorer survival, which is also only significant in the GCB subtype.

These findings may explain the inconsistent prognostic results in DLBCL by previous studies, and strongly suggest that the tumor-suppressor function of PTEN is limited to the negative regulation of the AKT signaling pathway. Supportingly, recent studies demonstrated that the dependence of GCB-DLBCL on surface BCR density and signaling is only in the presence of PTEN [53], and that most AKT inhibitor-sensitive DLBCL models did not express PTEN and were of GCB subtype; in contrast, PI3K inhibitor is selectively effective in ABC-DLBCL through NF-κB inhibition [54]. These findings are consistent with that PTEN inhibits BCR-induced AKT activation in DLBCL [55,56], and intracellular PTEN levels determine whether BCR signaling promotes cell death or cell survival via differential regulation of PI3K/AKT and NF-κB pathways [57]; loss of the *PTEN* gene was preferentially detected in GCB-DLBCL, and loss of PTEN expression defined a PI3K/AKT-dependent GCB-DLBCL [26,27,54]. On the other hand, studies also showed that besides the well-known inhibition of PI3K/AKT via lipid phosphatase activity, PTEN has many other functions including those in the nucleus [8,58–60], negative regulation of central B-cell tolerance checkpoints [61], and roles in B-cell homeostasis in the immune system [62]. Paradoxically, PTEN is required for both initiation and maintenance of pre-B acute lymphoblastic leukemia cells, and loss of PTEN causes rapid cell death of transformed pre-B leukemia cells [61]. Such multi-directional functions of PTEN may explain the opposite prognostic effects of PTEN expression in AKT-hyperactive DLBCL and p-AKT^{-} DLBCL cases, the lack of synergy between *PTEN* deletion and *MYC* rearrangement, and lack of distinct GEP signatures for PTEN expression and *PTEN* deletion.

As we have discussed in the previous review [43], loss/deficiency of PTEN expression can be attributed to genetic alterations and

Figure 6. *PTEN* deletion and *PTEN* mutation analysis in the DLBCL training cohort. (A) Representative FISH results for normal (left) and *PTEN* deletion (right). Red signals: centromere 10; green signals: *PTEN* gene. (B) Distribution of *PTEN* deletions and mutations in GCB-DLBCL and ABC-DLBCL cases, and their correlations with PTEN expression deficiency and p-AKT overexpression. Each column represents one patient; cases with *PTEN* deletion, mutation, PTEN loss, and p-AKT overexpression are highlighted in corresponding colors; cases without indicated abnormalities are shown in light blue or white color (for negative or unknown status, respectively). (C) The mean level of cytoplasmic PTEN expression was significantly lower in patients with *PTEN* gene deletion than that in patients without *PTEN* gene deletion. Among patients with heterozygous or homozygous *PTEN* deletion, patients with cytoplasmic PTEN expression had trend of better overall survival rate (OS) in the training cohort. Among patients with positive *PTEN* cytoplasmic expression, PTEN deletion was associated with significantly better OS. (D) *PTEN* deletion/mutation showed trends towards decreased progression-free survival (PFS) rates in DLBCL cases with p-AKT overexpression. (E) In combined training and validation cohort, *PTEN* deletion was associated with significantly better OS in DLBCL cases with *MYC* gene rearrangement. (F) In DLBCL cases with *PTEN* deletion, *MYC* gene rearrangement was associated with better OS with borderline significance. (G) In *MYC* rearranged DLBCL cases (training cohort), *PTEN* deletion was associated with a significantly lower mean level of Myc expression. In DLBCL cases with *PTEN* deletion, *MYC* rearrangement was associated with a significantly higher mean level of PTEN cytoplasmic expression.

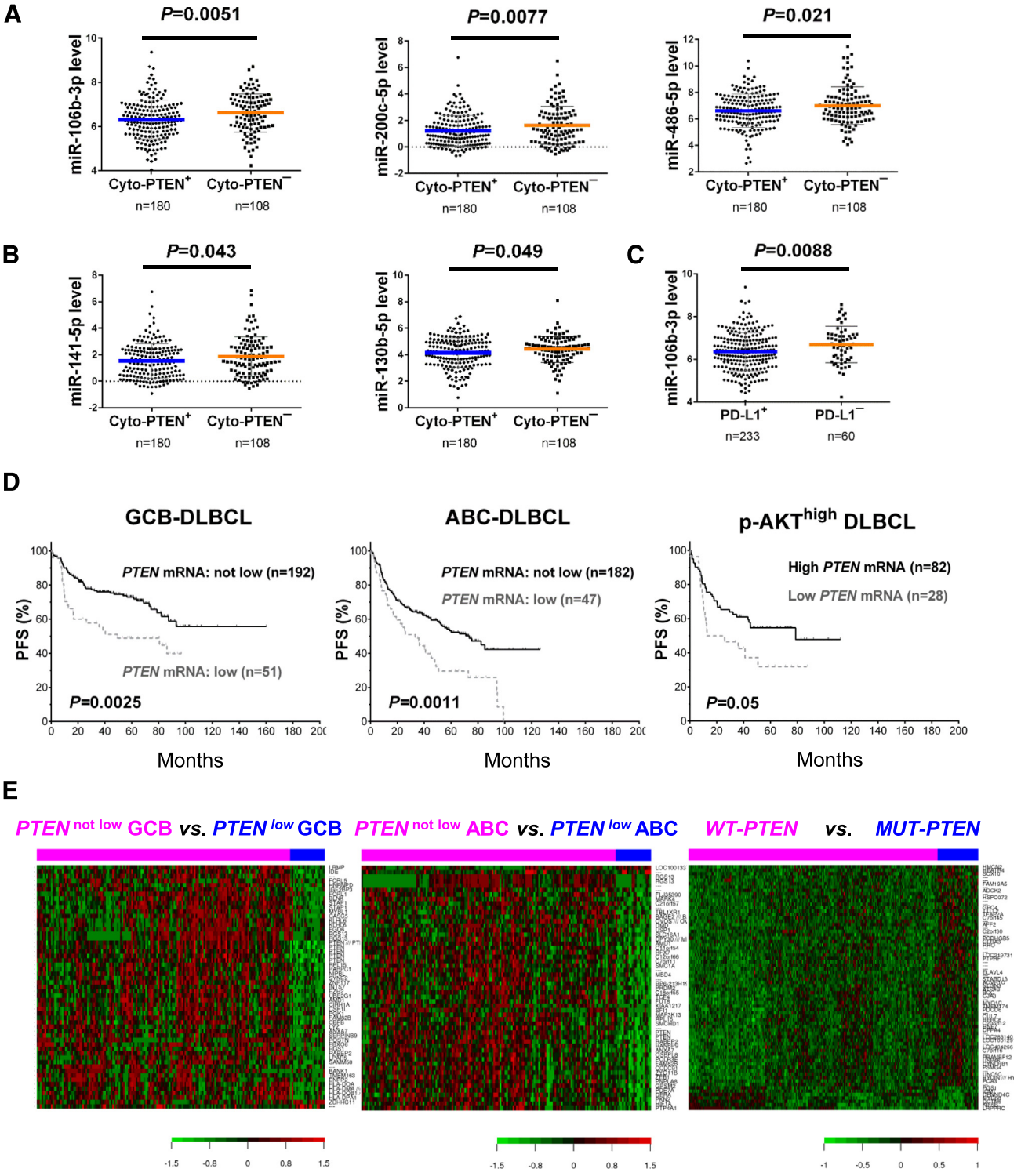


Figure 7. miRNA profiling and gene expression profiling analysis in the training cohort. **(A-B)** Loss of cytoplasmic PTEN expression was associated with significantly higher levels of miR-106b-3p, miR-200c-5p, miR-486-5p, miR-141-5p, and miR-130b-5p expression in DLBCL. **(C)** Absence of PD-L1 expression was associated with significantly higher miR-106b-3p expression. **(D)** Low *PTEN* mRNA expression was associated with significantly worse progression-free survival (PFS) in GCB-DLBCL, ABC-DLBCL, and the p-AKT^{high} DLBCL subset. **(E)** Genes significantly differently expressed between DLBCL groups with low *PTEN* mRNA expression and other cases (designated as *PTEN*^{low} and *PTEN*$^{not\ low}$, respectively), and between DLBCL patients with wild-type *PTEN* (*WT-PTEN*) and mutated *PTEN* (*MUT-PTEN*).

transcriptional, translational, and post-translational dysregulations. *PTEN* deletion (mostly heterozygous) and mutation as genetic mechanisms for PTEN deficiency and inactivation were observed in only approximately 11.3% and 10.6% of DLBCLs, respectively. These frequencies of *PTEN* gene alterations are much lower than those in some solid tumors (homozygous deletion, up to 42.5%; mutation, up to 44%) [20,21,63], which is consistent with previous studies in DLBCL and high-grade non-Hodgkin lymphoma [31,64]. Distinct GEP signatures were identified for low *PTEN* mRNA expression and *PTEN* mutations, which suggest that BCR signaling

and B cell differentiation regulate PTEN expression at the transcription level, and that PTEN is involved in CNS and immune response regulation in addition to its function in AKT/mTOR signaling. It has been reported that PTEN loss was associated with brain metastasis in melanoma patients [16].

We further explored the biological correlations and regulation mechanisms of PTEN expression. We surprisingly found that p-AKT (Ser^{473}) nuclear expression and PTEN cytoplasmic expression were positively correlated in both training and validation cohorts, although no correlations were found between PTEN protein expression and *AKT1* mRNA, nor between p-AKT protein expression and *PTEN* mRNA. As this was opposite to what one would expect (PTEN loss should correlate with increased p-AKT expression), we stained a separate set of FFPE tissue samples for the entire DLBCL training and validation cohorts using another PTEN monoclonal antibody from DAKO (clone 6H2.1). However, again we found that PTEN positivity and high expression were associated with p-AKT expression in DLBCL samples (data not shown). Such surprising positive (instead of negative) correlation between AKT and PTEN expression was also found in breast cancer, melanomas, and urinary bladder cancer by other studies [65–67]. Although paradoxical at the first glance, these results may reflect the complex regulation network of PI3K/AKT/PTEN with divergent activating and inactivating [68] mechanisms as demonstrated by previous studies [54]. It is known that phosphorylation at the Ser^{473} residue of AKT is mainly regulated by mTORC2 [69,70]; AKT activation in GCB-DLBCL is the principal consequence of tonic BCR signaling but AKT activation must not depend soly on the BCR signaling [53]. Notably, our results [34] showed that p-AKT (Ser^{473}) expression was primarily associated with Myc and Bcl-2 expression (targets of mTORC2 and BCR signaling) in GCB-DLBCL ($P < 0.0001$), and with IL-6 expression in ABC-DLBCL ($P = 0.0005$), whereas the association with PI3K was rather weak ($P = 0.019$ in the overall DLBCL cohort only). It is possible that the inhibitory effect of PTEN on AKT activation did not dominate the divergent mechanisms activating p-AKT (Ser^{473}) expression among DLBCL cases; these divergent mechanisms may also indirectly up-regulate PTEN expression, since *PTEN* mRNA expression showed correlation with BCR signaling gene signatures, and Cyto-PTEN expression was associated with the ABC subtype, whereas loss of PTEN was associated with IgA/IgG expression. However, as cytoplasmic PTEN expression was associated with significantly decreased survivin expression (an indicator of AKT function in antiapoptosis) in ABC-DLBCL (Figure 1*E*), and we did not examine the p-AKT (Thr^{308}) expression, PTEN may still have a significant role in repressing AKT function in DLBCL.

Moreover, the complexity between PTEN stability and function by posttranslational modifications may also contributes to the positive correlation between p-AKT and PTEN IHC results. Earlier studies indicated that phosphorylation of the PTEN's C-terminal tail causes a conformation change that stabilizes PTEN but at the same time inhibits its phosphatase activity and binding to the plasma membrane [9]; PD-1 inhibits this stabilizing/inactivating phosphorylation [71]. Since the antibody we used detected total PTEN, it is possible that the observed PTEN positivity also included stabilized phosphorylated PTEN (which has no tumor suppressor function), and PTEN expression levels were not linearly correlated with PTEN function; common mechanisms for the phosphorylation modification of PTEN and AKT could exists. Notably, although *PTEN* mRNA expression showed significantly favorable prognostic effect and striking GEP signatures, such effect and distinct GEP signatures were lacking for PTEN protein expression in overall DLBCL. Therefore, the effect and interpretation based on *PTEN* mRNA expression in DLBCL by previous studies may deserve precaution.

In our DLBCL cohort, p-AKT expression was significantly associated with both PTEN and Myc expression; accordingly, Myc expression also showed positive association with Cyto-PTEN expression. *MYC* rearrangement and *PTEN* deletion showed antagonistic rather than synergistic prognostic effect, but the case number was small. Whether the antagonistic effect resulted from *MYC*/*PTEN* gene structures is unknown; comparably lower Myc expression and increased PTEN expression in these cases with concurrent *MYC*/*PTEN* abnormalities (Figure 6*G*) could be relevant. Notably, earlier functional studies demonstrated that Myc transcriptionally activates *PTEN* expression [72]; on the other hand, Myc negatively regulates PTEN expression posttranscriptionally through miRNAs [73,74]. Conversely, PTEN represses Myc expression by inhibiting PI3K/AKT signaling and transcriptional modulation [27,75]; scenario that *PTEN* deletion did not cooperate with Myc activation in tumorigenesis was also reported [76]. Again, these findings suggested the complexity of PTEN-involved molecular network.

In this study, loss of cytoplasmic PTEN expression was also associated with *TP53* mutations and increased miRNA expression. Among the PTEN-targeting miRNAs showing negative correlations with Cyto-PTEN expression, miR-106b, miR-222, miR-200c, and miR-130b have been associated with poor prognosis [77–80]. Targeting these overexpressing miRNAs could be a feasible strategy to increase PTEN expression in DLBCL.

Interestingly, we found that loss of Cyto-PTEN expression was associated with a lower mean level of PD-L1 expression in DLBCL, whereas *PTEN* deletion/mutation and expression of p-AKT, PI3K, or Nuc-PTEN had no association with PD-L1 expression. There was no correlation between *PTEN* and *PD-L1* (*CD274*) mRNA expression. These data did not support the finding *in vitro* that loss of PTEN function was associated with increased PD-L1 expression [81]. However, *in vivo* studies found that PTEN loss through *PTEN* deletion and mutation did not increase PD-L1 expression in several mouse models. Because our data showed that both PD-L1 and Cyto-PTEN expression were associated with the ABC subtype and decreased miR-106b-3p and miR-130b-5p expression, which have been shown to target PD-L1 expression in cancer cells [82,83], and PTEN-targeting miR-200c [73] is a key regulators of PD-L1 expression in acute myeloid leukemia [84], we speculated that common regulators of PD-L1 surface expression and PTEN cytoplasmic expression possibly underlie the positive correlation between Cyto-PTEN and membrane PD-L1 expression in this DLBCL cohort. Furthermore, because PD-L1 expression was often associated with greater likelihood of response to PD-1 blockade [85], our results may suggest that Cyto-$PTEN^-$ DLBCL cases with lower PD-L1 expression would be less likely to respond to PD-1 blockade, which is consistent with the observation that PTEN loss was associated with inferior outcomes in patients with metastatic melanoma who received PD-1 inhibitor therapy [86].

Conclusions

In summary, the prognostic significance of PTEN loss and high expression in *de novo* DLBCL treated with R-CHOP

depends on AKT activities. *PTEN* deletion and mutation may have limited significance for poorer clinical outcome in DLBCL. PTEN protein and *PTEN* mRNA expression showed totally different prognostic effects and gene signatures in DLBCL. Our data suggest that the PI3K/PTEN/AKT and Myc signaling pathways are divergent rather than linear. Epigenetic and posttranslational mechanisms may play important roles in PTEN and PD-L1 expression.

Conflict of Interest Disclosure

The authors declare no conflict of interest.

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Author Contributions

X.W., X.C., R.S., Z.Y.X-M., A.T., Y. L., and K.H.Y designed the study, conducted the research, and performed the statistical analysis. X.W., X.C., R.S., C.T., A.T., J. Z., G.C.M., M. X., Y.M., K.J., X.T., Y.P., C.V., Y.X., K.D., A.C., A.O., Y.Z., G.B., K.L.R., E.D.H., W. W.L.C., J.H.K., J.H., M.P., A.J.M.F., M.B.M., B.M.P., J.N.W., M. A.P., S.L., R.N.M., L.J.M., Y.L., Z.Y.X-M., and K.H.Y. contributed vital new reagents, resources, technology, and analytical tools. X.W., A.T., C.V., W.C., K.D., A.C., A.O., Y.Z., G.B., K.L.R., E.D.H., W. W.L.C., J.H.K., J.H., M.P., A.J.M.F., M.B.M., B.M.P., J.N.W., M. A.P., Y.L., Z.Y.X-M., and K.H.Y. collected clinical and follow-up data under approval by the institutional review boards and the material transfer agreement. X.W., X.C., R.S., Z.Y.X-M., L.J.M., and K.H.Y. edited the manuscript. All authors contributed vital strategies, participated in discussions, and provided scientific input.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2018.03.002.

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