

Targeting Positive Cofactor 4 Induces Autophagic Cell Death In MYC-Expressing Diffuse Large B Cell Lymphoma

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Research Article

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Abstract

Background: The MYC-expressing diffuse large B-cell lymphoma (DLBCL) is one of the refractory lymphomas. The pathogenesis of MYC-expressing DLBCL is still unclear, and there is a lack of effective therapy. In this study, we have explored the clinical significance and the molecular mechanisms of transcription co-activator 4 (PC4) in MYC-expressing DLBCL.

Methods: We investigated PC4 expression in 54 cases of DLBCL patients' tissues and matched normal specimens, and studied the molecular mechanisms of PC4 in MYC-expressing DLBCL both in vitro and in vivo.

Results: We reported for the first time that targeting c-Myc could induce autophagic cell death in MYC-expressing DLBCL cell lines. We next characterized that PC4 was an upstream regulator of c-Myc, and PC4 was overexpressed in DLBCL and was closely related to clinical staging, prognosis and c-Myc expression. Further, our in vivo and in vitro studies revealed that PC4 knockdown could induce autophagic cell death of MYC-expressing DLBCL. And inhibition of c-Myc mediated aerobic glycolysis and activation of AMPK / mTOR signaling pathway were responsible for the autophagic cell death induced by PC4 knockdown in MYC-expressing DLBCL. Through the DLRTM and EMSA assay, we also found that PC4 exerted its oncogenic functions by directly binding to c-Myc promoters.

Conclusions: PC4 exerts its oncogenic functions by directly binding to c-Myc promoters. Inhibition of PC4 can induce autophagic cell death of MYC-expressing DLBCL. Our study provides novel insights into the functions and mechanisms of PC4 in MYC-expressing DLBCL, and suggests that PC4 might be a promising therapeutic target for MYC-expressing DLBCL.

Background

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of adult non-Hodgkin lymphoma with tremendous heterogeneity in terms of clinical manifestations, histological morphology and prognosis^{1,2}. Rituximab in the combination with CHOP chemotherapy (R-CHOP) is the standard therapeutic regimen for DLBCL, but nearly 40% of patients have a poor prognosis due to recurrence and drug resistance, while only 10% of patients can be completely cured². According to previous studies, most patients show chromosomal abnormalities, including the translocation and amplification of c-Myc, Bcl-2 and Bcl-6 genes³⁻⁵. Among them, MYC-expressing DLBCL is one of the refractory lymphoma with unclear pathogenesis, rapid progression, chemo-resistance and low cure rate, which is an urgent problem to be solved⁶⁻⁷. However, c-Myc is a natural disordered protein and lacks drug recognition sites, presenting a significant challenge for drugs targeting c-Myc and thus showed side effects. Therefore, the development of anti-tumor drugs targeting c-Myc is challenging and exhibits various hurdles⁸.

The c-Myc is an oncogene, which not only regulates tumor growth and differentiation, but also is a key regulator in energy metabolism, indicating a crucial therapeutic target for DLBCL⁹. In recent years, a

promising phenomenon has been found in the study of tumor energy metabolism, the rapid reduction of the energy charge below a critical limit can trigger autophagy cell death rather than an adaptive autophagic response¹⁰. Different from apoptosis (Type I programmed cell death), autophagic cell death (Type II programmed cell death) occurs in various types of cancer¹¹. In previous studies, inhibition of MYC could induce autophagic cell death in Burkitt lymphoma cell lines¹², but the specific mechanism was unclear. The combination of the antidepressants maputiline and fluoxetine induce autophagic cell death in drug-resistant Burkitt's lymphoma¹³. In multiple myeloma, metformin can induce autophagic cell death through the AMPK/mTOR pathway¹⁴⁻¹⁵. This type of cell death can have a contribution to anticancer efficacy or drug resistance, respectively^{16,17}. Therefore, targeting autophagy may provide a new potential therapeutic strategy to overcome drug resistance¹⁸.

In this study, we firstly revealed that targeting PC4 would induce autophagic cell death in MYC-expressing DLBCL cell lines. Through bioinformatics analysis, we found that PC4 was abnormally high expressed in DLBCL and was positively correlated with MYC expression. Considering the key role of c-Myc in DLBCL, it is crucial to elucidate the potential role and underlying molecular mechanisms of PC4 in DLBCL. Then, we first reported that PC4 was highly expressed in DLBCL, and positively correlated with c-Myc expression and poor prognosis of patients. Next, our findings revealed that PC4 knockdown induced autophagic cell death in MYC-expressing DLBCL, which is a new type of cell death. Surprisingly, knockdown of PC4 had no significant influence on the c-Myc low expression cell lines and non-cancerous lymphocytic cell lines. We also found that inhibition of c-Myc mediated aerobic glycolysis and excessive activation of the AMPK / mTOR signaling pathway were responsible for the autophagic cell death induced by PC4 knockdown in MYC-expressing DLBCL. Furthermore, PC4 regulated the transcription of c-Myc to exert its oncogenic functions. Our study provides novel insights into the functions and mechanisms of PC4 in MYC-expressing DLBCL, and suggested that PC4 might be a promising therapeutic target for MYC-expressing DLBCL.

Materials And Methods

Cell lines and clinical samples

The human lymphocytic cell lines (CCRF-SD) and diffuse large B-cell lymphoma cell lines (DOHH2, OCL-LY10, HBL-1 and TMD8) were purchased from the Cell Bank of the Chinese (Shanghai, China). All cells were cultured in the RPMI-1640 medium (Hyclone, USA), supplemented with 1% streptomycin/penicillin (Beyotime, Shanghai, China) and 10% FBS (Gibco, USA), and incubated at 37°C with 5% CO₂. A number of 24 DLBCL patients' RNA samples and 24 normal lymph gland RNA samples and a total of 30 paraffin-embedded DLBCL patients' tissues with paired adjacent tissues were obtained at Department of Hematology of Southwest Hospital of Third Military Medical University. All DLBCL patients were diagnosed by Lymph node biopsy and confirmed diagnosis by at least two experienced pathologists. The study was approved by the Ethics Committee of Third Military Medical University.

Immunohistochemical Staining

The paraffin-embedded patients' tissues were dewaxed and rehydrated and incubated with human PC4 antibody (1: 200; Sigma, St. Louis, Missouri, USA) at 4°C overnight. Then, the slides were sequentially incubated with secondary antibody at 37°C for 1 hour, and used DAB to visualize positive staining. PC4 expression in DLBCL patients' tissues was evaluated by percentage of positive-staining cells. Intensity was graded as follows: 0, no signal; 1, weak (light yellow); 2, moderate (brown); and 3, strong staining. The percentage of positive cells was evaluated quantitatively and scored as: 0 (< 5% positive tumor cells), 1 (5–25% positive tumor cells), 2 (26–50% positive tumor cells), 3 (51–75% positive tumor cells) and 4 (> 75% positive tumor cells). The final quantification of each staining was obtained by multiplying these 2 scores. A total staining score of 0–12 was calculated and graded as negative (–, score 0–1), weak (+, score 2–4), moderate (++, score 5–8), or strong (+++, score 9–12). All the use rights of patient specimens were approved by the Ethics Committee of the First Affiliated Hospital of The Army Medical University and obtained the informed consent of the patients

Quantitative RT-PCR

Total RNA was collected from DLBCL cells using Trizol (Cwbiotech, China). 1 µg RNA was reverse transcribed into cDNA according to the recommended protocol by the RevertAid First Strand cDNA Synthesis kit. (#K1622, Thermo Fisher Scientific, Inc.) Quantitative RT-PCR was performed according to the recommended protocol by SYBR Green qPCR master mix (Takara). When the reactions were completed, the relative gene expression was calculated by the comparative threshold cycle (Ct) method. GAPDH expression was used as control. Human-specific primers sequences are shown in Additional file 1: Table S1.

RNA interference

The shRNA lentivirus vector targeting human PC4 (shPC4#1: 5'-GACAGGUGAGACUUCGAGATT -3'; 5'-UCUCGAAGUCUCACCUGUCTT -3'. shPC4#2: 5'-ACAGAGCAGCAGCAGCAGATT-3'; 5'-UCUGCUGCUGCUGCUCUGUTT-3') ; and c-Myc (sh-c-Myc#1: 5'-ATGTCAAGAGGCGAACACA-3'; 5'-TGTGTTTCGCCTCTTGACAT-3'. sh-c-Myc#2: 5'-ACGATTCTTCTAACAGAAAT-3'; 5'-ATTTCTGTTAGAAGGAATCGT-3'.) negative control shRNA (5'-UUCUCCGAACGUGUCACGUTT-3'; 5'-ACGUGACACGUUCGGAGAATT-3') were purchased and constructed by GenePharma (Shanghai, China). The human PC4 plasmid and control were purchased and constructed from GeneChem (Shanghai, China). TMD8 and HBL-1 cells were transfected with shRNA or plasmid according to the recommended instructions.

Cell proliferation assay

Cells were cultured in 96-well plates with a density of 3000 cells each well and 100 µl RPMI-1640 medium. Cellular proliferation was measured with the Cell Counting Kit-8 (Dojindo, Japan) at the wavelength of 450 nm. Data were read by a microplate reader (Multiskan Go Multimode Reader; Thermo Scientific).

Cell apoptosis analysis by flow cytometry

Cells were treated with AnnexinV- 7-AAD (BD Biosciences) for 30 min at 37°C in the dark for apoptosis analysis, then analyzed by flow cytometry.

Western blotting analysis

The cell lines were harvested, washed, and lysed with RIPA buffer (Beyotime, China) which contain protease inhibitor cocktail (Roche) for 30min on ice. Total protein was collected and quantitated by a BCA kit (Beyotime, China) according to the recommended instruction. The protein samples were separated by electrophoresis in gel, and then transferred onto PVDF membranes (Millipore). Blotted membranes were incubated with primary antibodies overnight at 4°C. The membranes were washed 5min for 3 times with TBST, and then incubated with HRP-linked secondary antibody (Cell Signaling Technology, USA) 1h at room temperature. The band intensities were detected and visualized by an enhanced chemiluminescence detection system (Bio-Rad Laboratories). Primary antibodies against c-Myc, LC3, SQSTM1, ATG7, PARP, CASPASE3, Bcl-2, BAX, PI3K, S6K1, AKT, mTOR, 4EBP1, AMPK, P38, P53, HIF-1 α , GLUT1, PKM2, HK2, LDHA and β -actin were obtained from Cell Signaling Technology. Primary antibodies against PC4 were obtained from Sigma.

Immunofluorescence staining

Cells were fixed in 4.0% formaldehyde for 10 min and permeabilized with ice-cold methanol or 0.5% Triton X-100 for 5 min. The following primary antibodies were used, and subsequently using secondary antibodies to detect Primary antibodies. Conjugating to AlexaFluor 488 or 555 (Invitrogen). Cells were counterstained with DAPI and mounted in Vectashield (Vector Laboratories).

In vivo tumor growth model

For in vivo tumor growth model, 100 μ l PBS containing 1×10^7 PC4 stable knockdown TMD8 cells or negative control cells or controls were injected subcutaneously at one dorsal site of athymic male nude mice. Tumor growth was measured every 2 days, which was calculated by the following formula: volume (mm^3) = (width² * length)/2. At the endpoint, the mice were sacrificed; Carbon dioxide (CO₂) administration was used to euthanize mice. Put the mice into the euthanasia box (Clean, see-through airtight box), do not pour CO₂ into the euthanasia box first. Attach CO₂ and exhaust pipes to the box and make sure other areas are sealed. 100%CO₂ was poured into the box at the rate of 20% replacement of box contents per minute for 10 minutes to ensure that the mice did not move and breathe (The mice gradually lost consciousness when CO₂ was injected into the box for 2-3 minutes). CO₂ was turned off, and the animals were observed for 2 minutes. After that, the animals were taken out and confirmed to be completely dead by respiratory arrest, cardiac arrest and dilated pupils. And then xenografts were dissected, weighed and fixed in 4% paraformaldehyde. Each group of n = 5. The animal carcasses were packed in opaque plastic bags for infectious substances and delivered to the Experimental Animal Center of Army Military Medical University for unified treatment.

Glucose uptake, lactate and ATP assays

Lactate production (Lactate Assay Kit) was measured according to the manufacturer (BioVision). For glucose uptake analysis, cells were cultured with a fluorescent D-glucose derivative, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG; APEX-BIO) for 30 min at 37°C. The fluorescence intensity of 2-NBDG was measured through flow cytometry (BD FACSCanto II™). ATP production (Enhanced ATP Assay Kit) was measured according to the recommended manufacturer's protocol (Beyotime).

Transmission electron microscopy

Cells were harvested and immediately fixed in 3% glutaraldehyde overnight at 4°C and postfixed with 2% osmium tetroxide for 1 hour at 37°C. And then, cells were embedded and stained using uranylacetate/lead citrate. The samples were imaged using a TEM (JEM-1400PLUS, Japan).

RNA-seq assay

Total RNA was extracted using Trizol reagent (Invitrogen, CA, USA) following the manufacturer's procedure. The total RNA quantity and purity were analyzed by Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA, USA) with RIN number >7.0. Approximately 10 µg of total RNA representing a specific adipose type was subjected to isolate Poly (A) mRNA with poly-T oligo attached magnetic beads (Invitrogen). Following purification, the mRNA is fragmented into small pieces using divalent cations under elevated temperature. Then the cleaved RNA fragments were reverse-transcribed to create the final cDNA library in accordance with the protocol for the mRNA-seq sample preparation kit (Illumina, San Diego, USA), the average insert size for the paired-end libraries was 300 bp (±50 bp). And then we performed the paired-end sequencing on an Illumina sequence platform.

Luciferase reporter assay

TMD8 cells were cultured at a density of 5×10^4 cells/well in 96-well culture plates and transfected with 0.2 µg of dual-luciferase reporter construct SUB1, or co-transfected with 0.2 µg of the luciferase reporter construct c-Myc and the internal control vector pRL-TK, pRL-SV40, or pRL-CMV (Promega, Madison, WI) at a ratio of 20:1 (reporter construct: control vector) using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to instruction of the recommended manufacturer.

EMSA (electrophoretic mobility shift assay)

The DNA binding assays were performed using purified GST-PC4 protein and biotin-labelled fragments of the promoters containing the W-boxes, using GST protein as a negative control, and non-labeled fragments were used as competitors. The bands at the upper and lower part of membranes indicate shift (protein-probe complex) and unbound free probes, respectively.

Statistical analysis

Statistical analysis was carried out using SPSS 22.0 software (SPSS Inc., Chicago, USA), and all data were presented as means ± SD. Comparisons between two groups were performed using the Student's t-test. Comparisons among three or more groups were performed using a one-way analysis of variance

(ANOVA). The survival data was carried out using the Kaplan-Meier method. Correlation between PC4 expression and clinical parameters was determined using the Pearson's χ^2 method. $P < 0.05$ indicated a statistically significant difference.

Results

PC4 is highly expressed in DLBCL and positively correlated with c-Myc expression and poor prognosis of patients.

We began our studies by exploring the potential clinical significance of PC4, we firstly analyzed PC4 mRNA expression level in tumor tissues compared with normal tissues (Figure 1A), the mRNA expression of PC4 in tumor was apparently higher than that in normal tissues. We also analyzed GEPIA database and revealed that PC4 was positively correlated with MYC expression ($P < 0.001, R = 0.46$) in human whole blood (Supplemental Figure 1A). Moreover, GEPIA database showed that PC4 mRNA expression level was significantly higher in DLBCL patients tissues ($n = 47$) than in normal tissues ($n = 337$) (Supplemental Figure 1B). Then, we analyzed PC4 mRNA expression level and found that c-Myc(+) tissues had higher PC4 mRNA expression compared to c-Myc(-) tissues (Figure 1B). In addition, we analyzed PC4 expression in DLBCL specimens with or without positive expression of c-Myc protein, and found that average staining score of PC4 in c-Myc(+) tissues were significantly increased compared to c-Myc(-) tissues (Figure 1C). The data above suggested a possible positive correlation between PC4 expression levels and c-Myc expression level in DLBCL. Meanwhile, through analyzing 159 and 414 cases of DLBCL from public cancer databases (GSE4475 and GSE10846, respectively) we found that the higher PC4 expression group had poorer overall survival compared with lower PC4 expression group (Figure 1D and 1E). Although c-Myc expression demonstrated no significant change on the basis of gender, age, subtype, Ki-67 expression, it had poorly differentiated in DLBCL with a higher Ann Arbor stage and poorly event-free survival (Table1). Finally, we detected the protein level of PC4 and c-Myc in non-cancerous lymphocytic cell lines (CCRF-SD) and DLBCL cell Lines (DOHH2, OCL-LY10, HBL-1 and TMD8), our result showed that PC4 protein expression were much higher in TMD8 and HBL-1 cells compared to other cell lines (Figure 1F). The qPCR (Figure 1G) and Immunofluorescent staining (Supplemental Figure 2A) assays confirmed that the mRNA and protein level of PC4 was up-regulated in DLBCL cell lines (DOHH2, OCL-LY10, TMD8 and HBL-1 cells) compared with non-cancerous lymphocytic cell lines (CCRF-SD cells). Collectively, these results suggest that PC4 is a potential oncogene and positively correlated with c-Myc in DLBCL.

Table 1

Correlations between c-Myc expression level and clinic characteristics of patients with DLBCL.

Clinicopathological parameters	patients	c-Myc		P
	Number (%)	Negative (%)	Positive (%)	Value
All cases	54(100)	27(50)	27(50)	P=1.000
Gender				
Female	27(50)	12(22.2)	15(27.8)	P=0.587
Male	27(50)	15(27.8)	12(22.2)	
Age				
< 60	37(31.5)	19(35.2)	18(33.3)	P=1.000
≥ 60	17(68.5)	8(14.8)	9(16.7)	
Subtype				
GCB type	29(53.7)	17(31.5)	12(22.2)	P=0.243
Non-GCB type	22(40.7)	8(14.8)	14(25.9)	
Unclassified DLBCL	3(5.6)	2(3.7)	1(1.9)	
Ki-67 expression				
≥ 70%	22(40.7)	8(14.8)	14(25.9)	P=0.097
< 70	32(59.3)	19(35.2)	13(24.1)	
Ann Arbor stage				
I-II	17(31.5)	16(29.6)	1(1.9)	P=0.000
III-IV	37(68.5)	11(20.4)	26(48.1)	
Time from enrollment		Event-free survival (standard error)		
12 months	66.7(6.7)	92.6(2.1)	77.8(4.4)	P=0.000
24 months	63.0(7.3)	92.6(2.1)	74.1(5.1)	P=0.000
Corrected with continuity correction of Pearson's χ^2 test. DLBCL, Diffuse large B-cell lymphoma;				

Knockdown of PC4 induces cell apoptosis in MYC-expressing DLBCL in vitro and in vivo.

To investigate the functional significance of increased PC4 expression in DLBCL, TMD8 and HBL-1 cells were used for the subsequent loss-of-function study. The stable cell lines with PC4 knockdown were established by specific shRNA (shPC4#1 and shPC4#2) (Figure 2A). The CCK-8 assays demonstrated that PC4 knockdown inhibited the proliferation (Figure 2B). Then, the expression of apoptosis protein

markers, including poly ADP-ribose polymerase (PARP), cleaved caspase 3, B-cell lymphoma-2 (BCL-2) and B-cell lymphoma-2-Associated X (BAX) were detected by western blotting in the constructed cells (Figure 2C). As shown in Figure 2D, knockdown of PC4 increased apoptosis in stable PC4 Knockdown cell lines than controls, Gene Set Enrichment Analysis (GSEA) showed that the gene sets of apoptosis enrichment in PC4 low (shPC4) in TMD8 cell lines (Figure 2E), which means that PC4 silencing can induce cell apoptosis. We observe cell vacuoles and cell debris in the constructed cells through a microscope (Supplemental Figure 3A). Moreover, TEM images showed autophagic vacuole (AV) formation in the constructed cells (Figure 2F). These finding suggested that knockdown of PC4 was associated with autophagy. In addition, we established a subcutaneous xenograft model to study the biological function of PC4 in vivo. TMD8 cells with or without stable PC4-knockdown were inoculated into athymic male nude mice. During the in vivo experiments, xenograft growth in the sh-PC4#1 group was dramatically inhibited compared to the sh-NC group and control group (Figure 2H). The average tumor weight and tumors size at the experimental endpoint was reduced by PC4 knockdown (Figure 2G and 2I). Besides, knockdown of PC4 had no significant impact on mice body weight (Figure 2J). Taken together, the above data suggests that PC4 promotes DLBCL cell proliferation, and knockdown of PC4 can induce apoptosis and autophagy.

Knockdown of PC4 induces apoptosis in MYC-expressing DLBCL by inducing excessive autophagy.

Non-selective autophagy occurred due to lack of energy¹¹. As envisioned, Silencing of PC4 could induce the expression of the LC3II and down-regulated the SQSTM1 protein in DLBCL cells (Figure 3A). The associated autophagy proteins (beclin1, ULK1) also up-regulated after silencing of PC4 (Figure 3B). In addition, TEM images showed AV formation in PC4 knockdown cells, which could be reversed by 3-methyladenine (3MA; an autophagy inhibitor) (Figure 3C). Above-mentioned data suggested that autophagy was induced by PC4-knockdown. PC4 knockdown significantly suppressed the proliferation of TMD8 and HBL-1 cells, which was partly reversed by 3MA (Figure 3D). Similarly, the cell apoptosis was also reversed by 3MA (Figure 3E). Therefore, our results confirmed that PC4 knockdown could induce autophagic cell death in TMD8 and HBL-1 cells. To assess the effect of knockdown of PC4 in c-Myc low expression cell lines (DOHH2 and OCL-LY10) and CCRF-SD were established with PC4 knockdown by specific shRNA (Supplemental Figure 4A). Interestingly, we found no effect on the level of LC3II and cleaved PARP and cell proliferation (Supplemental Figure 4B and 4C). Collectively, these results suggested that PC4 inhibition can induce autophagic cell death only in the c-Myc high expressing DLBCL cell lines. Galluzzi reported that a rapid reduction in energy charge below a critical limit is likely to trigger autophagic cell death¹¹. This is well established that c-Myc is a key regulator in energy metabolism⁹, suggesting that PC4 may directly regulate c-Myc or metabolism. This indicated that PC4 is a tumor-specific oncogene and may be a novel therapeutic target for MYC-expressing DLBCL.

Knockdown of PC4 induces excessive autophagy through AMPK/mTOR signaling pathway in MYC-expressing DLBCL.

We conducted Genome-wide analysis to compare the gene expression profiles in TMD8 with or without stable PC4-knockdown to explore the potential mechanism of PC4 on cell proliferation inhibition and apoptosis in DLBCL. The heat map showed that 4 genes, including c-Myc, reduced their expression, while the expression of 36 genes were increased after PC4 knockdown (Figure 4A). PC4 affects the expression of 12669 genes in TMD8 stable PC4 knockdown cells, with 12172 genes that overlap between TMD8 stable PC4 knockdown cells and control cells (Figure 4B). Then, we used KEGG enrichment analysis in TMD8 stable PC4 knockdown cells and controls (Figure 4C), which revealed that after PC4-knockdown AMPK signaling pathway was most increased and PI3K-Akt signaling pathway was most inhibited. Moreover, we established Gene ontology analysis to understand the PC4 full function, the results demonstrated that PC4 could positive regulate the metabolic process and cellular process in DLBCL (Figure 4D). Therefore, PC4 promotes proliferation in DLBCL through regulating metabolism. According to the data from Genome-wide analysis, we established mTOR signaling pathway and downstream protein (4EBP1 and S6K1) by western blot, our result showed that the level of phosphorylation was decreased after PC4 knockdown (Figure 4E). Then we detected mTOR upstream signaling pathway including AMPK, P53, P38 and AKT, which revealed that the level of AMPK phosphorylation was comparatively more increased than the other proteins (Figure 4F). GSEA showed that the gene sets of mTORC1 were enriched in PC4^{high} (Control) compared with PC4^{low} (shPC4) in TMD8 cell lines (Figure 4G). A previous study showed that alteration of the above pathways was associated with energy metabolism declension¹⁹.

PC4 regulates c-Myc transcription to perform it's oncogene function.

To further illustrate the underlying mechanisms of PC4 in DLBCL, we conducted metabolism-related experiments. We found that the 2-NBDG uptake and the production of Lactate and ATP were significantly inhibited after PC4-knockdown (Figure 5A, 5B and 5C). Furthermore, Silencing of PC4 inhibited the key enzymes of glycolysis including GLUT1, PKM2, HK2 and LDHA (Figure 5D). Previous studies showed that glycolysis metabolism was regulated by c-Myc²⁰ and HIF-1 α ²¹. Then we performed western blotting and q-PCR on c-Myc and HIF-1 α and found that the protein and mRNA level of c-Myc was dramatically decreased in stable PC4 knockdown cell lines (Figure 5E and 5F). GSEA showed that the gene sets of MYC were enriched in PC4^{high} (Control) compared with PC4^{low} (shPC4) in TMD8 cell lines (Figure 5G). To further demonstrate the relationship between PC4 and c-Myc, we subsequently examined the relationship between PC4 and c-Myc. Schematic presentation of PC4 and c-Myc binding sites on the c-Myc locus are shown in Figure 5H, BS: binding site, BS1: CCAACAAATGCAATGGGAGT and BS2: CAGGAGGGGCGGTATCTG. We conducted luciferase reporter assays, which revealed that PC4 regulated c-Myc transcription through two PC4 binding sites in c-Myc promoters (Figure 5I). However, EMSA confirmed PC4 as a DNA binding protein, which is associated with the c-Myc binding sequence in BS1, not in BS2 (Figure 5J). To further verify the BS1 function, we conducted BS1 mutation in TMD8 and HBL-1 cell lines, BS mutation sequence: TTGGTGGGCATGGCAAAGAC. Our result confirmed that PC4 directly activate c-Myc transcription through BS1 by EMSA (Figure 5K). In addition, the protein and mRNA level of PC4 has no significant influence after c-Myc stable knockdown (Supplemental Figure 5A and 5B). In addition, we conducted c-Myc overexpression experiment in PC4 knockdown cell lines by the specific

plasmid (Supplemental Figure 6A) to prove that c-Myc was responsible for the oncogenic functions of PC4. As expected, c-Myc overexpression rescued the apoptosis (Supplemental Figure 6B, 6D and 6E) and cell proliferation inhibition (Supplemental Figure 6C). The results of the above experiments indicated that PC4 regulated c-Myc transcription to perform its oncogenic function. The potential mechanism of PC4 in diffuse large B-cell lymphoma was shown in Figure 6

Discussion

Owing to the increased recurrence and chemotherapy resistance, current strategies for the treatment of c-Myc (+) DLBCL are still facing with many difficulties and challenges²². And c-Myc is a natural disordered protein and lacks drug recognition sites that can be utilized⁸. Therefore, it is critical to identify the c-Myc related targets for precise individualized therapy and better prognosis. Here, we find a novel oncogene, PC4, which is overexpressed in DLBCL and positively correlated with c-Myc expression. Moreover, targeting PC4 can induce therapeutic autophagy by inhibiting c-Myc mediated aerobic glycolysis and activating AMPK / mTOR pathway in c-Myc (+) DLBCL. Interestingly, there is no significant effects on the c-Myc low expressing cells and non-cancerous lymphocytic cells after PC4 stable knockdown. These findings indicate that PC4 is a tumor-specific oncogene in c-Myc (+) DLBCL.

PC4 is a nuclear protein which also known as SUB1. As a multifunctional nuclear protein, PC4 is initially isolated, purified and identified from the upstream stimulatory activity (USA) in mammalian cell nuclear extracts²³⁻²⁵. Apart from its transcriptional co-activation function that facilitates RNA polymerase II-driven gene transcription²⁶⁻³², PC4 also plays an important role in various cellular process including DNA replication, DNA repair and chromatin organization³³⁻⁴². PC4 also participated in the regulation of autophagy⁴³⁻⁴⁵. During the malignant transformation of normal dermal multipotent fibroblasts, we reported that PC4 is up-regulated and positively correlated with K-Ras and MAPK pathway, implying the potential role of PC4 in tumorigenesis for the first time⁴⁶. Our study and previous studies confirmed that PC4 is highly expressed in lung cancer⁴⁷, breast cancer⁴⁸, prostate cancer⁴⁹, astrocytoma⁵⁰ and esophageal squamous cell carcinoma⁵¹, and regulates cancer growth, lymphatic metastasis, and chemoradiosensitivity. However, the PC4 role in hematological cancers, including DLBCL are still unknown. Here, we specifically characterized the expression and biological function of PC4 in DLBCL. Through bioinformatics and clinical samples analysis, PC4 was found to be upregulated in DLBCL, positively correlated with c-Myc expression and showed poor prognosis of patients. These results encourage us to further explore the functional significance of increased PC4 expression in DLBCL, especially in c-Myc(+) DLBCL. Then, in vivo and in vitro studies showed that targeting PC4 could induce autophagic cell death through blocking c-Myc mediated aerobic glycolysis in c-Myc(+) DLBCL.

Autophagic cell death is different from apoptosis, known as type II programmed cell death, which is mainly characterized by the appearance of abundant vacuole enveloping cytoplasm and organelles, and the degradation of various components inside the vacuole via lysosome^{15,18}. In response to metabolic stress, including hunger and energy deficiency, autophagy is mainly regulated by mTOR kinase⁵². In

recent years, autophagic cell death has gained enormous attention. Suzanne found that combination of the antidepressants maputiline and fluoxetine can induce autophagic cell death in drug-resistant Burkitt's lymphoma¹³. In multiple myeloma, metformin can induce autophagic cell death through the AMPK/mTOR pathway¹⁴. Furthermore, mTOR inhibitors have been widely used for the treatment of various cancers, and autophagic cell death is one of the main pathways. However, the knowledge of its regulatory mechanism is still insufficient. Galluzzi reported that a rapid reduction in energy charge below a critical limit is likely to trigger the cell death rather than an adaptive autophagic response¹¹. As it is well established, that aerobic glycolysis is the main source of energy in cancers⁵³. The evaluating index of the DLBCL patients' condition and prognosis, such as lactate dehydrogenase (LDH) activity⁵⁴ and 18F-FDG⁵⁵ PET/CT, reflect the dependence of DLBCL cells on aerobic glycolysis. Owing to the critical role of c-Myc in aerobic glycolysis, targeting PC4 could block c-Myc mediated aerobic glycolysis and induce autophagic cell death in c-Myc(+) DLBCL. The autophagic cell death induced by PC4 knockdown is a promising therapeutic target for c-Myc (+) DLBCL as a new type of cell death. PC4 could emerge as a drug target site in cancer for c-Myc through upstream regulation.

Conclusion

In the current study, we firstly reported the expression pattern, diagnostic and prognostic value of PC4 in DLBCL. Furthermore, targeting PC4 can induce autophagic cell death through blocking c-Myc mediated aerobic glycolysis and energy supply, and activate AMPK / mTOR signaling in c-Myc(+) DLBCL. Therefore, our study provides novel insights into the functions and mechanisms of PC4 in c-Myc(+) DLBCL, and suggest that PC4 may be a novel therapeutic target for c-Myc(+) DLBCL.

Abbreviations

DLBCL

Diffuse large B cell lymphoma

PC4

Positive Cofactor 4

Declarations

Acknowledgements

Not applicable.

Authorship Contributions

LM, QG, PL, JC, and CS conceived and designed the project. LM performed the mostly experiments, analyzed the data, drafted the manuscript; QG and MX contributed to the collection of human tissues; PL took part in animal experiments; PL, and JF took part in cellular and molecular biology experiments, QG

and PL, edited the manuscript; CS designed the study, supervised the experiments and revised the manuscript. All authors have read and approved the final manuscript.

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Data availability

The datasets generated and/or analysed during the current study are available in the NCIB repository, BioProject ID: PRJNA769815. <http://www.ncbi.nlm.nih.gov/bioproject/769815>

Ethics approval and consent to participate

4-5 weeks old male athymic nude mice were obtained from the Center and followed the care and use of Guidelines from Laboratory Animals of the Army Medical University. All animals experimental procedures were approved by the Army Medical University Animal Care and Use Committee (AMUWEC20201334). The studies involving human data and samples were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Army Military Medical University in accordance with the Ethical review of biomedical research involving people (2016,China) and National Health Commission (Standard for Quality Control of Drug Clinical Trials,2020,China) and WMA Helsinki Declaration, CIOMS International Ethical Guidelines for Human Biomedical Research. The patients/participants provided their written informed consent to participate in this study. All the participants had the opportunity to discuss any questions or issues. All the animal experiments in the study was carried out in compliance with the ARRIVE guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest and any commercial affiliations.

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Supplementary Material

Supplementary Figures and Tables are not available with this version

Figures

Figure 1

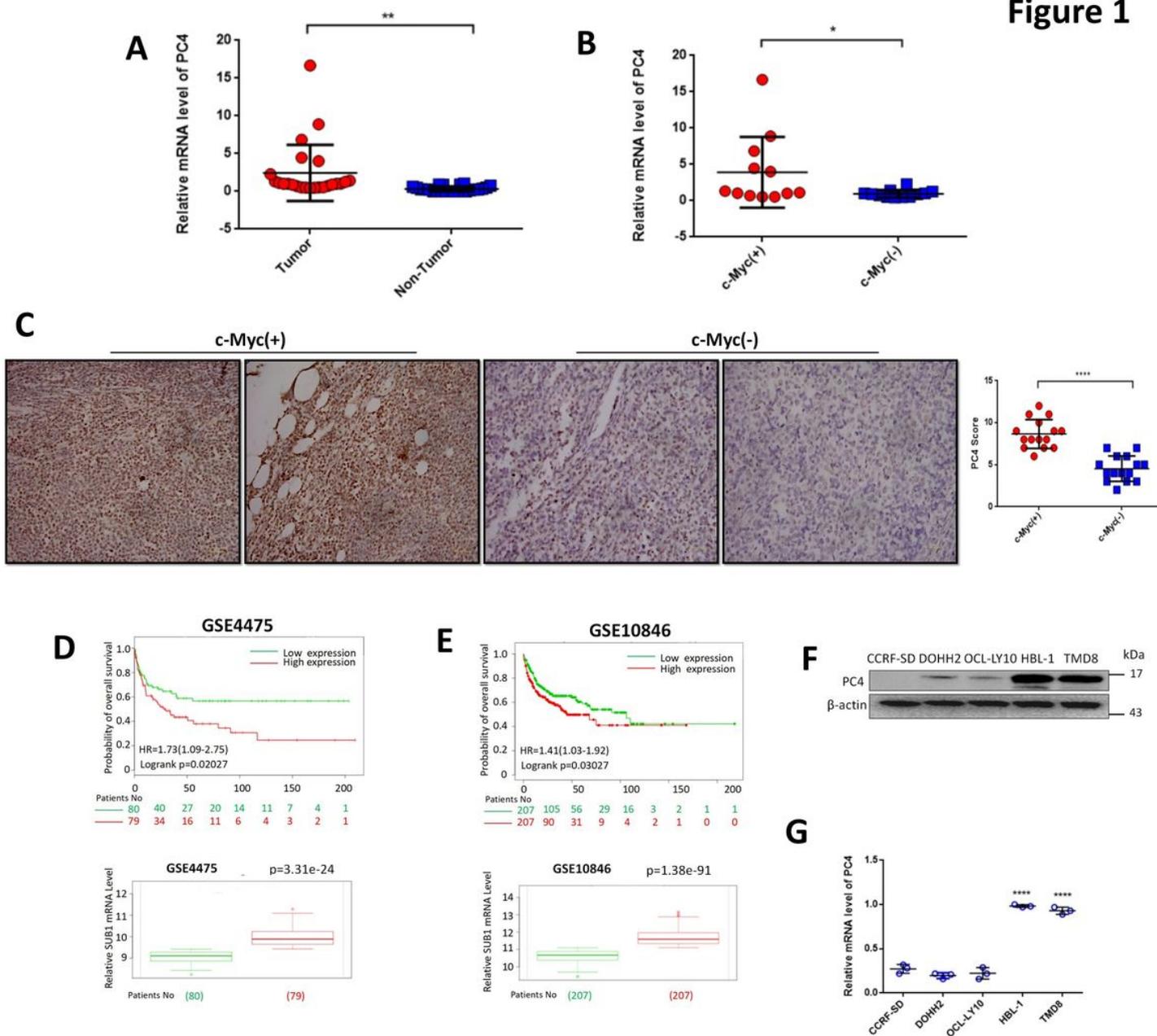


Figure 1

PC4 is significantly up-regulated in diffuse large B-cell lymphoma with c-Myc expression patients and cell lines, and is correlated with poor prognosis of patients.

(A) The mRNA level of PC4 in DLBCL patients' tissues (n=24) and normal tissues (n=24). (B) The mRNA level of PC4 in c-Myc(+) (n=12) and c-Myc(-) (n=12) DLBCL patients' tissues. (C) Immunohistochemical staining of PC4 in c-Myc(+) (n=15) and c-Myc(-) (n=15) of DLBCL tissues. Scale bar represents 50um. (D) Kaplan-Meier analysis for the association of PC4 expression levels with overall survival time in DLBCL (n=159). (E) Kaplan-Meier analysis for the association of PC4 expression levels with overall survival time in DLBCL (n=417). (F) The protein level of PC4 in CCRF-SD cells and DLBCL cell Lines. (G) The mRNA

level of PC4 in CCRF-SD cells and DLBCL cell Lines. All data are represented as mean \pm SD. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

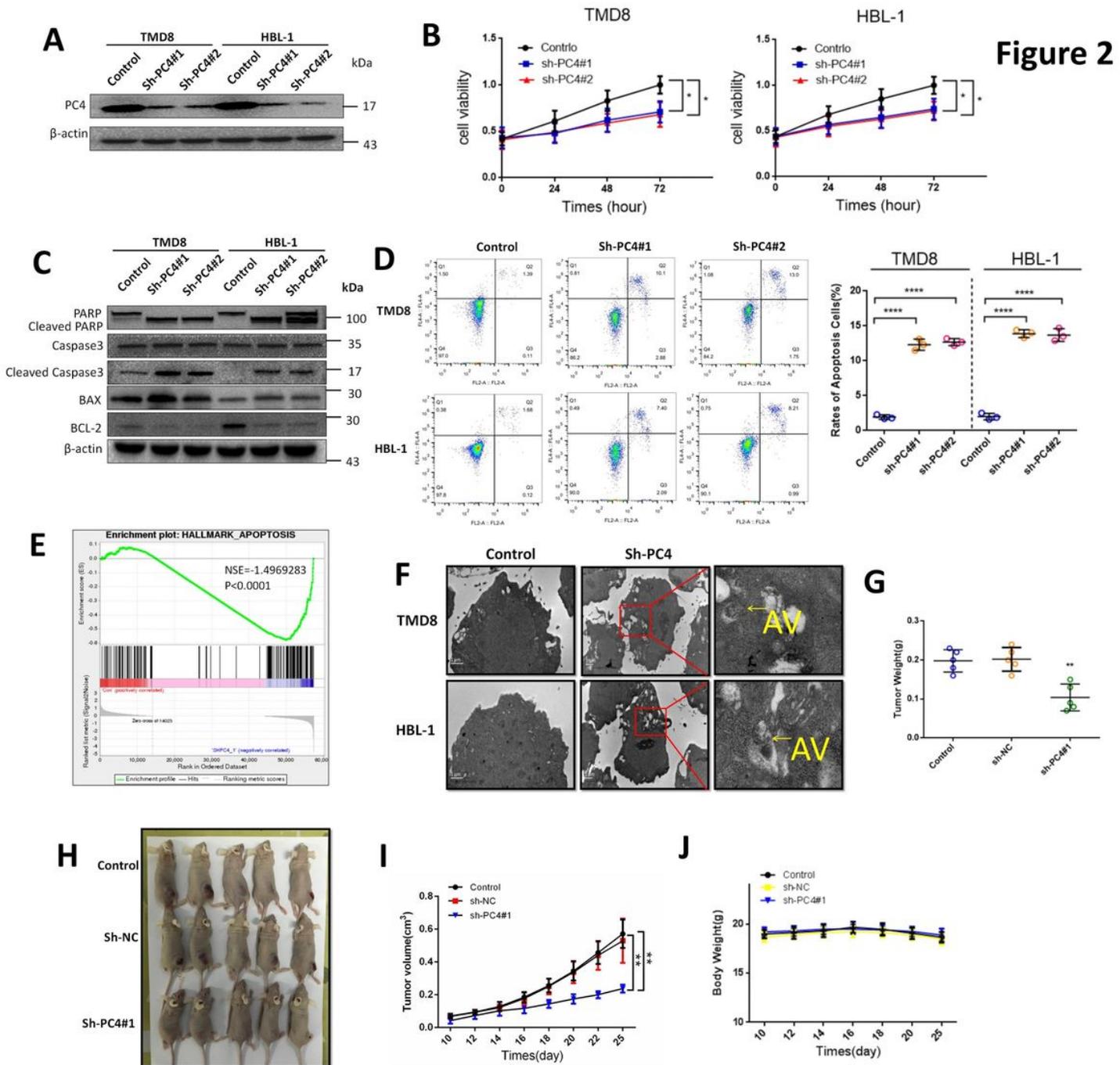


Figure 2

Knockdown of PC4 induces cell apoptosis in MYC-expressing DLBCL in vitro and in vivo.

(A) Two shRNAs (shPC4#1 and shPC4#2) were used to establish the stable PC4 knockdown cell lines. The PC4 knockout efficiency was examined by western blot. (B) Cell viability in the constructed cells was determined by CCK-8 assay. (C) The expression of apoptosis markers (c-caspase 3, PARP, Bax and Bcl-2) was detected by western blotting in the constructed cells (samples derive from the same experiment and that gels/blots were processed in parallel). (D) Apoptosis rate in the constructed cells was determined by flow cytometry. Experiments were triplicated independently. (E) GSEA comparing the gene sets of APOPTOSIS in the constructed TMD8 cells. (F) The constructed cells were observed by TEM. Scale bar represents 1 μ m. (I) The TMD8 cell with stable PC4-knockdown was inoculated into athymic male nude mice (Each group of n=5). At the endpoint, the xenografts were photographed (H) dissected and weighed (G). (J) The body weight of the mice was measured. All data indicate the mean \pm SD. * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001.

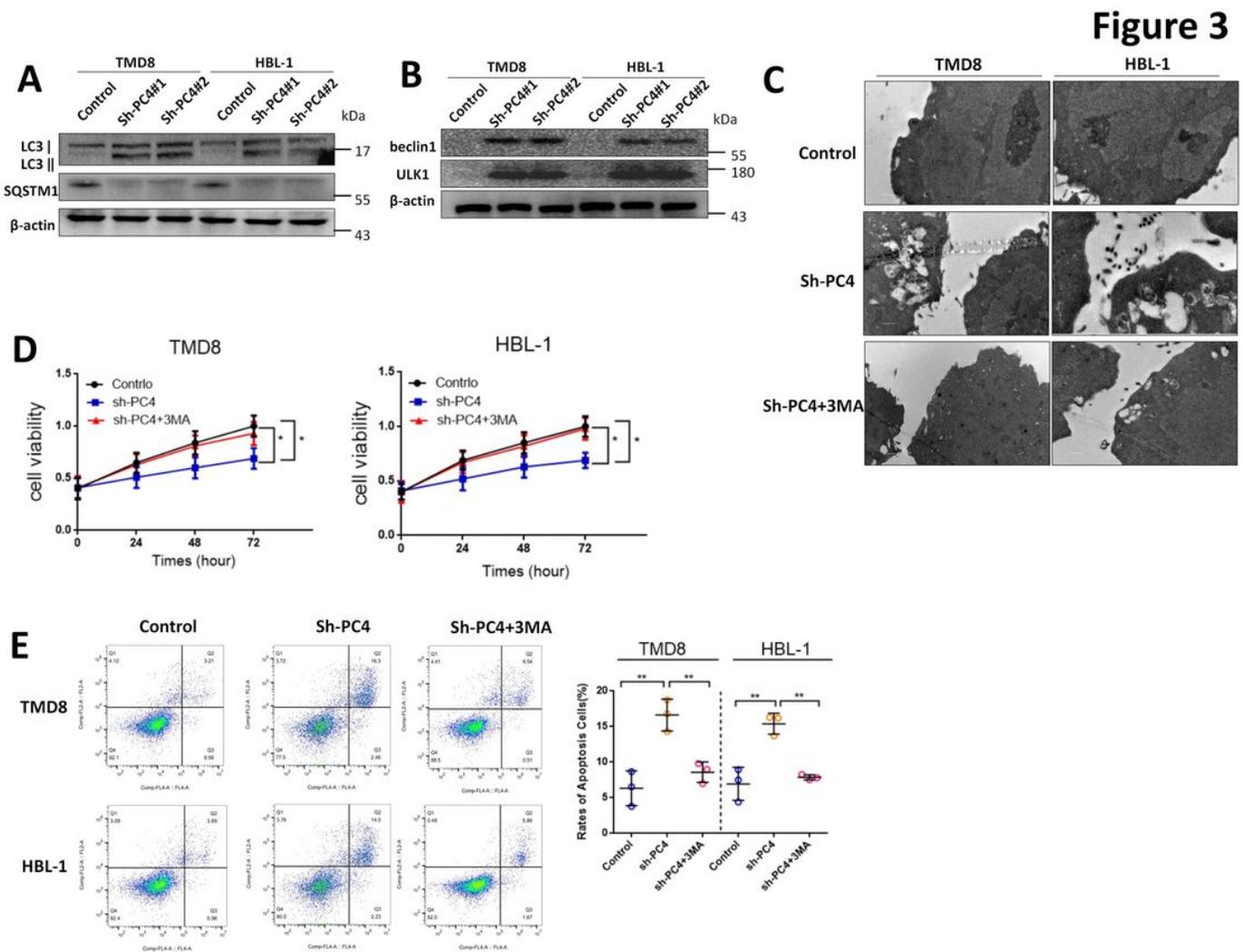


Figure 3

Inhibition of PC4 induces apoptosis in MYC-expressing DLBCL by inducing excessive autophagy.

(A) The expression of LC3 and SQSTM1 was detected by western blotting in the constructed cells. (B) The protein level of beclin1, ULK1 was detected by western blotting in the constructed cells. (C) The constructed cells with or without 3MA (4mM) were observed for autophagy using TEM. Scale bar represents 1um. (D) Cell viability in the constructed cells was determined by CCK-8 assay. (E) Apoptosis rate in the constructed cells was determined by flow cytometry. Experiments were triplicated independently. All data are represented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

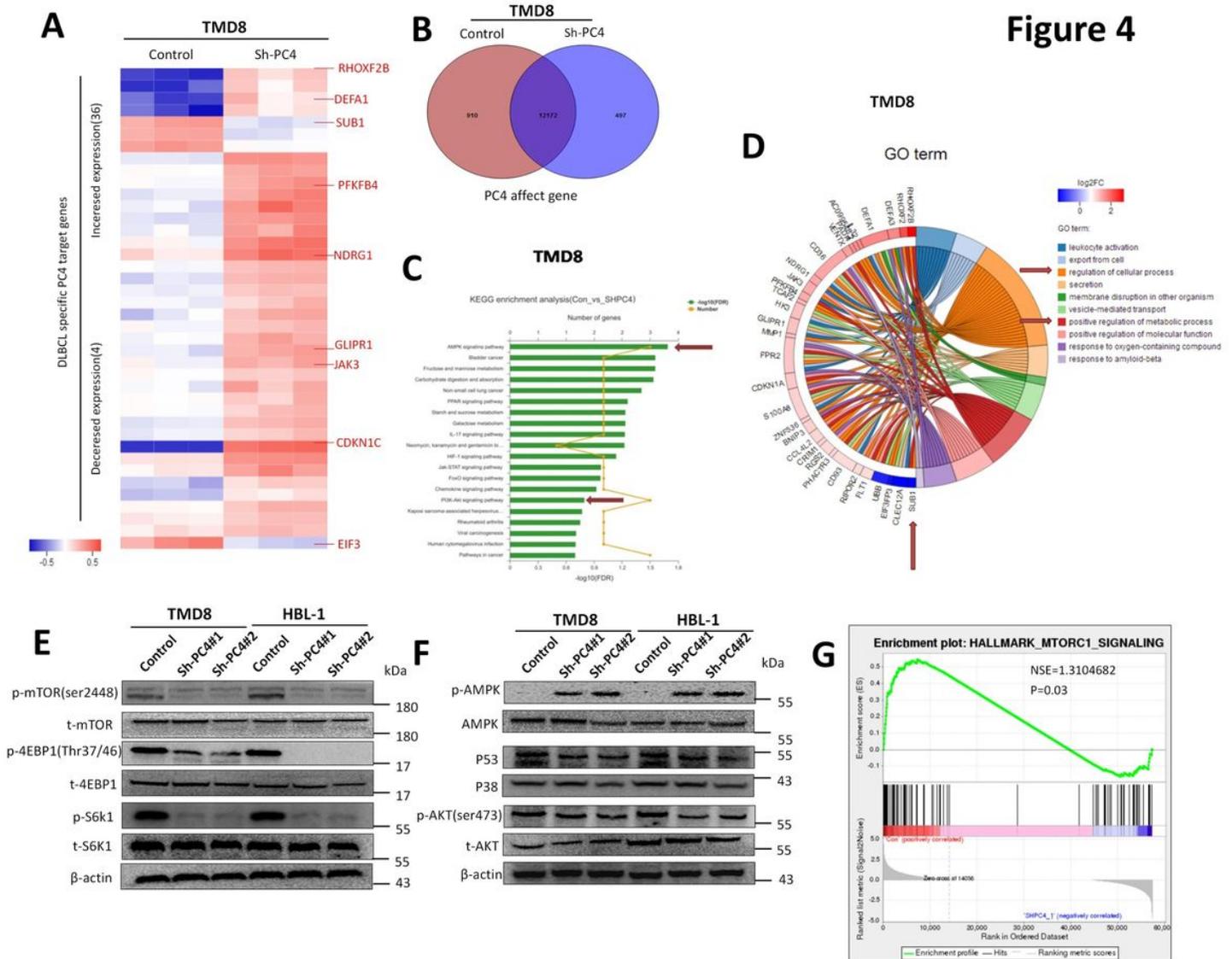


Figure 4

Knockdown of PC4 induces apoptosis in MYC-expressing DLBCL by inducing excessive autophagy.

(A) Heat maps show mRNA levels of target genes in the constructed cells. (B) Venn diagram in the constructed cells. (C) KEGG enrichment analysis in the constructed cells.(D) GO analysis in the constructed cells. (E) The mTOR and AMPK (F) and their related protein was detected by western blotting

in the constructed cells (the Phosphorylated and non-phosphorylated samples derive from the same experiment and that gels/blots were processed in parallel). (G) GSEA comparing the gene sets of MTORC1 in the constructed TMD8 cells. All data are represented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

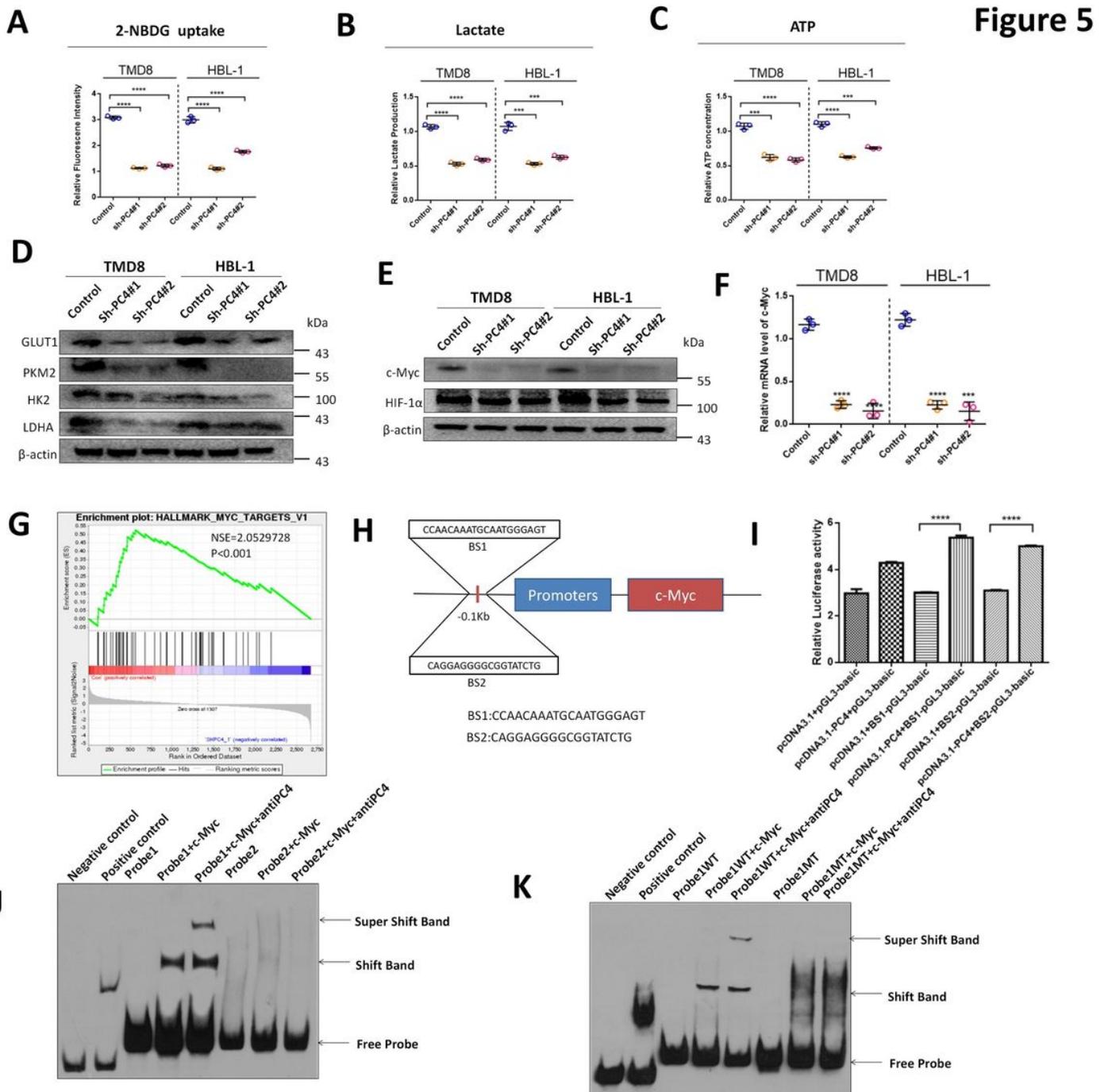


Figure 5

PC4 directly regulates c-Myc transcription by binding to c-Myc's promoters.

(A) 2-NBDG uptake, (B) Lactate and (C) ATP were determined in the constructed cells. (D) The expression of GLUT1, PKM2, HK2 and LDHA were detected by western blotting in the constructed cells (samples derive from the same experiment and that gels/blots were processed in parallel). (E) The Protein levels of c-Myc and HIF-1 α were detected by western blotting. (F) The mRNA levels of c-Myc were detected by qPCR. (G) GSEA comparing the gene sets of MYC in the constructed TMD8 cells. (H) Schematic presentation of PC4 and c-Myc binding sites on the c-Myc locus. (I) Luciferase reporter activities were assessed in c-Myc promoter along with or without co-expressing PC4. (J) EMSA was conducted to detect the binding of PC4 to c-Myc's promoters. (K) EMSA were conducted to detect the BS1 binding sites of PC4 to c-Myc's promoters. All data are represented as mean \pm SD. ***p<0.001, ****p<0.0001.

Figure 6

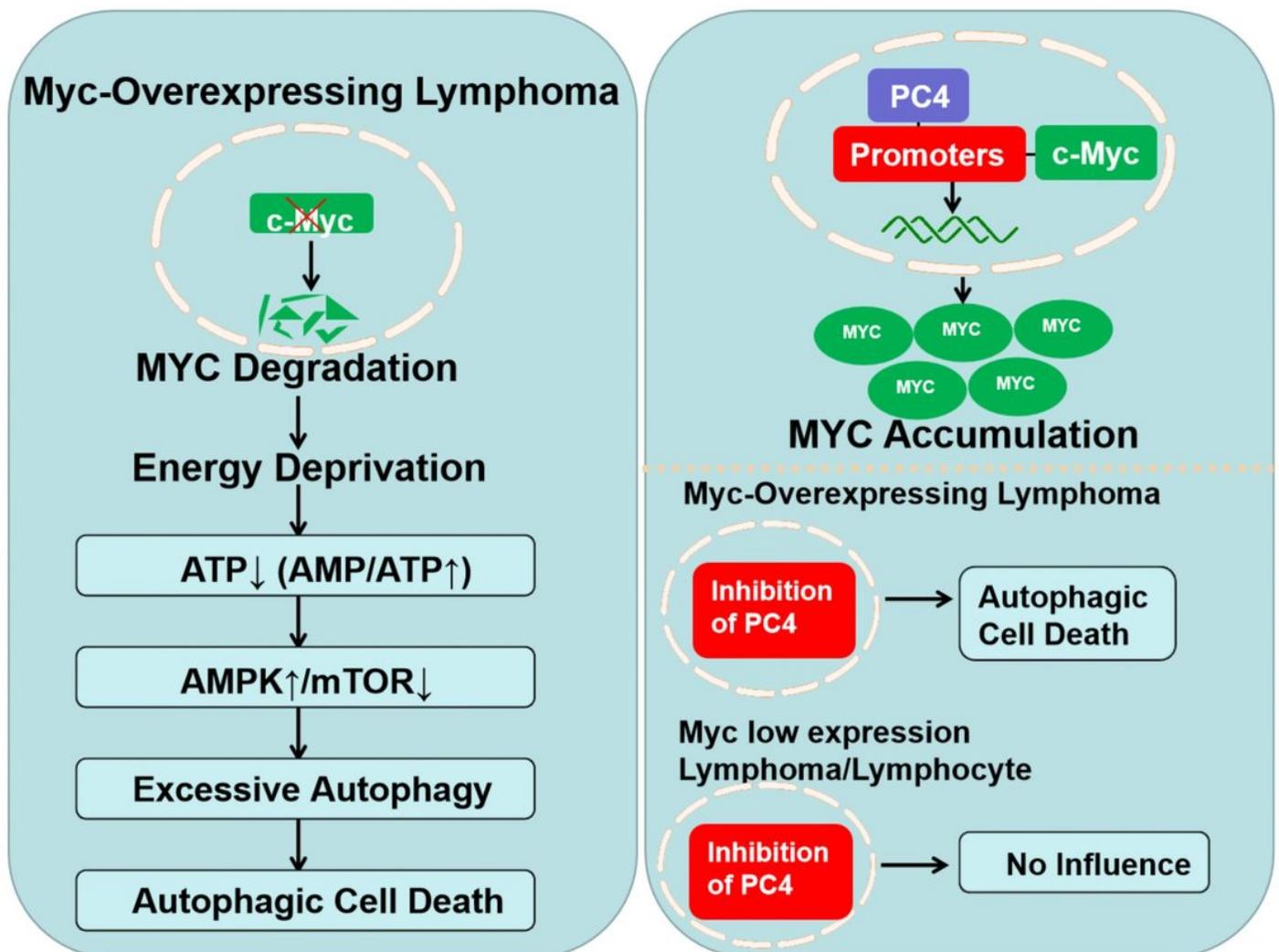


Figure 6

Schematic illustration for the potential mechanisms of PC4 in diffuse large B-cell lymphoma progression.

In c-Myc(+) DLBCL, PC4 regulated the c-Myc transcription by binding to c-Myc's promoters, targeting PC4 induced autophagic cell death through Inhibited their glycometabolism.