MYC overexpression in natural killer cell lymphoma: prognostic and therapeutic implications

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Abstract

The current clinical management of extranodal natural killer (NK)/T-cell lymphoma (ENKTL) primarily depends on conventional chemotherapy and radiotherapy, underscoring the need for innovative therapeutic strategies. This study explores the clinical significance and therapeutic implication of c-MYC (MYC) in ENKTL. Initially, we identified MYC protein overexpression in approximately 75% of cases within a large cohort of 111 patients. MYC overexpression was strongly correlated with lymphoma cell proliferation and poor clinical outcomes. Intriguingly, integrating MYC expression into the prognostic index of NK cells lymphoma with Epstein-Barr virus (PINK-E) prognostic model significantly enhanced its predictive power. Subsequently, we implemented MYC knockdown in NK malignancy cell lines with MYC overexpression, resulting in significant viability reduction. RNA sequencing used to determine MYC function revealed a high overlap with canonical MYC-regulated genes and enrichment in metabolism and cell cycle regulation. Integrative analysis of the RNA-sequencing data upon MYC knockdown with gene expression profiles of primary ENKTL cases identified a subset of genes closely associated with MYC overexpression. Among these, CDK4 emerged as a potential therapeutic target, and its inhibition not only abrogated MYC function but also decreased MYC expression in NK malignancy cells. Furthermore, the clinical-grade CDK4/6 inhibitor palbociclib exhibited a potent anti-tumor effect in xenograft mouse models, especially when combined with gemcitabine. In summary, our study firmly establishes MYC as an oncogene with prognostic significance in ENKTL and highlights CDK4 inhibition as a promising therapeutic strategy for treating ENKTL with MYC overexpression.

Introduction

Extranodal natural killer (NK)/T-cell lymphoma (ENKTL) is a distinct form of non-Hodgkin lymphoma (NHL) associated with Epstein-Barr virus (EBV) infection. The clinical outcome of patients with ENKTL is largely dependent on the clinical stage, and the median survival was only 7-20 months for those with advanced-stage disease.[1-3] Although significant advances have been made in our understanding of the pathogenesis and driver oncogenes of this disease,[4-10] treatment strategies have not led to a substantial improvement in survival. Clinical management, to a large extent, still relies on conventional chemotherapy and radiotherapy, and there is an unmet need to identify novel therapeutic approaches.

MYC is a transcription factor that promotes oncogenesis by activating and repressing downstream target genes controlling cell growth, metabolism, and survival.[11] In hematological malignancies, MYC is recognized as the essential driver in Burkitt lymphoma (BL) and high-grade B-cell

lymphoma, whereas its prognostic significance in ENKTL has not been well addressed. Despite only rare genetic alterations in ENKTL, MYC may still play an important role in tumor development because of its interactions with other disease drivers. Specifically, alterations in transcription factors, including activating mutations of *STAT3* and *STAT5*, and inactivating mutations/deletion of *PRDM1* and *TP53* were found to be important driving mechanisms in the oncogenesis of ENKTL.[4,7,9,10,12] These drivers have been shown to directly regulate MYC expression, which is of considerable significance in cancer development.[10,13-17] In particular, our recent study demonstrated that MYC expression was remarkably increased upon *PRDM1* deletion in primary NK cells.[10] Notably, a recent genomic study identified a genetic subtype of ENKTL named MB based on *MGA* mutation and 1p22.1/*BRDT* loss of heterozygosity.[9] This subtype is characterized by MYC overexpression and is associated with a poor clinical outcome. Based on these findings, we hypothesize that MYC is inclined to be transcriptionally activated by oncogenic drivers of ENKTL and that enhanced MYC expression significantly contributes to tumor biology and clinical outcome. In fact, previous studies have reported a correlation between MYC overexpression and an inferior clinical outcome in ENKTL.[18,19] Nevertheless, the biological significance and implications in clinical practice warrant further exploration.

In this study, we enrolled a substantial ENKTL patient cohort, scrutinizing MYC expression to comprehend its implications on clinical prognosis and risk assessment. Concurrently, we profiled MYC target genes, aiming to elucidate MYC function and pinpoint potential therapeutic targets pertinent to ENKTL cases with MYC overexpression.

Methods

Patients, samples, and clinicopathological data

A cohort of 111 patients with ENKTL diagnosed between 2009 and 2019 at Sun Yat-sen University Cancer Center was retrospectively investigated. All tissue specimens were formalin-fixed and paraffin-embedded (FFPE). The histology and immunophenotype were retrieved and reviewed by two experienced hematopathologists using the World Health Organization Classification.[20] Inclusion criteria required that cases have: i) relevant clinical and follow-up data; ii) sufficient pathology materials for review and further analysis; and iii) no history of immunodeficiency. All patients provided written informed consent for the tissue specimen collection and publication of their medical information during the first visit to the hospital. The registry was approved by the Institutional Review Board (SL-B2022-581-01).

Gene knockdown and RNA sequencing

For *MYC* and *RB* (RB transcriptional corepressor 1) KD, Dicer-Substrate Short Interfering RNA (DsiRNA) or negative control (NC1) (IDT, Coralville, IA, USA) were transfected into NK malignancy cell lines by electroporation using the Amaxa 4d nucleofector (Program CM-150). The siRNA sequences are listed in *Online Supplementary Table S1*. For RNA sequencing, total RNA was isolated and purified 48 hours (h) after siRNA transfection using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA). Before library preparation, RNA integrity number (RIN) was assessed using the Agilent Bioanalyzer 2100, and only RNA samples with RIN >7 were used for subsequent library preparation. Pooled libraries were sequenced by the Illumina NextSeq 500 system with PE150 reads.

In vivo experiments

All mouse experiments were approved by the Institutional Animal Care and Use Committee at the University of Nebraska Medical Center. In order to establish cell line-derived xenograft (CDX) and patient-derived xenograft (PDX) tumors, approximately $2x10^6$ tumor cells were subcutaneously implanted in 10-week-old NSG mice (The Jackson Laboratory, Bar Harbor, ME, USA). The PDX model was obtained from the Public Repository of Xenografts (ProXe, #DFTL-85005) with the fifth passage being used for the treatment assay. For IMC-1 CDX and PDX models, treatment was initiated 21 days post-xenograft implantation. Palbociclib was given by gavage of saline-dissolved isethionate daily, and gemcitabine was administered by interperitoneal injection of saline-dissolved hydrochloride weekly. Treatments of either drugs or saline control were continuously administered for 21 days, and the survival was monitored with the endpoints including death, body weight loss >20%, and severe morbidity. For the YT CDX model, treatments were initiated when the tumor volume reached approximately ~100 mm^3 and were continuously administered for 14 days. Immunohistological examination was performed after treatment or at the endpoints of the experiment, with the primary antibody information detailed in *Online Supplementary Table S2*.

Additional information can be found in the *Online Supplementary Appendix*.

Results

MYC overexpression in extranodal natural killer/T-cell lymphoma is associated with elevated cell proliferation

We retrospectively analyzed a cohort of 111 ENKTL cases, finding that most clinical characteristics aligned with prior reports[21-23] despite a slightly higher percentage of regional lymph node involvement (Table 1). MYC expression in the diagnostic samples was examined using immunohistochemistry (IHC), which showed a wide variation in the percentage of MYC-positive cells among these cases. Receiver operating characteristic (ROC) curve analysis identified 20% as the optimal cut-off value for predicting clinical outcomes,

Table 1. The baseline characteristics and therapeutic approaches for this cohort of patients (N=111).

Variables	N (%)
Age ≤60 years	103 (92.8)
Male sex	74 (66.7)
ECOG performance status	
<2	99 (89.2)
≥2	12 (10.8)
Ann Arbor staging	
I-II	85 (76.6)
III-IV	26 (23.4)
B symptoms	
No	57 (51.3)
Yes	54 (48.6)
Primary site	
UAT	91 (82.0)
Non-UAT*	20 (18.0)
PTI	
No	64 (57.7)
Yes	47 (42.3)
Regional LN involvement	
Absent	62 (55.9)
Present	49 (44.1)
Distant organ metastasis	
No	92 (82.9)
Yes	19 (17.1)
Elevated LDH	28 (25.2)
Detectable EBV DNA	84 (75.7)
PINK-E score	
<2	85 (76.6)
≥2	26 (23.4)
Primary treatment	
Chemotherapy	42 (37.8)
CMT#	69 (62.2)
Chemotherapy regimen	
Peg/Asp -based	86 (77.5)
Anthracyclines-based	21 (18.9)
Others	4 (3.6)

#Combinational modality treatment (CMT) indicates the combination of radiotherapy and chemotherapy. *Non-UAT sites: 9 in skin and soft tissues; 4 in the testis/suprarenal gland; 3 in the intestine; 4 in other sites. Asp: asparaginase; CMT: combinational modality treatment; ECOG: Eastern Cooperative Oncology Group; LDH: lactate dehydrogenase; LN: lymph node; PTI: primary tumor invasion; Peg: pegaspargase; PINK-E: prognostic index for natural killer cell lymphoma-Epstein-Barr virus; EBV: Epstein-Barr virus; UAT: upper aerodigestive tract.

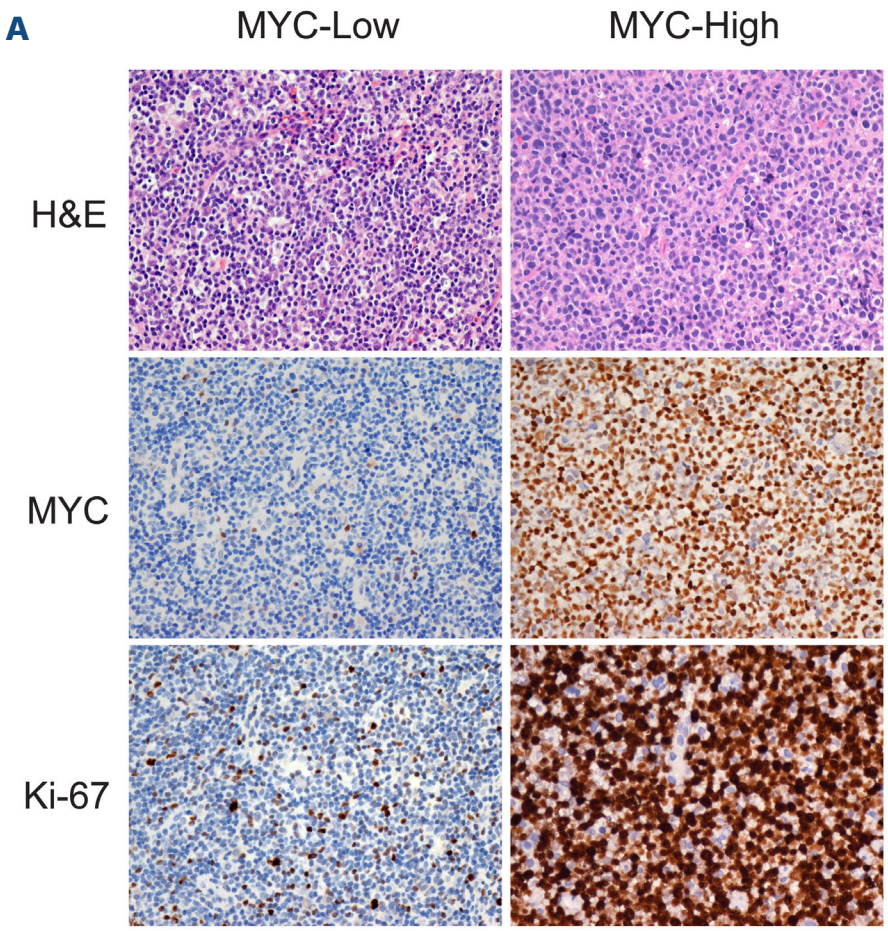
leading to the classification of 83 cases (74.9%) as exhibiting high MYC expression (Figure 1A, B). However, there was no discernible correlation between MYC expression and key clinical features (*Online Supplementary Table S3*). We also probed *MYC* rearrangement and copy number variation by fluorescence *in situ* hybridization (FISH) in 60 cases. This revealed *MYC* gene locus gain in three cases (5%), but no evidence of MYC gene rearrangement or amplification (*Online Supplementary Figure S1*). Considering that elevated MYC typically correlates with a higher proliferative rate, we evaluated the Ki-67 index in these cases. Defining a value of ≥60% as a high expression, based on the median value of this cohort, the Ki-67 index was found to be high in 63 of the 83 MYC-high cases (75.9%), compared to only one of 28 MYC-low cases (3.6%; Figure 1A, B). A strong correlation was demonstrated between the two markers (R=0.7989; Figure 1C), suggesting that MYC overexpression is closely related to cell proliferation in ENKTL. Moreover, in the analysis of 18 cases with both diagnostic and relapse biopsy samples, we found that 13 cases (72.2%) displayed a higher percentage of MYC expression in the relapse sample (Figure 1D, E). Taken together, this data indicates that MYC protein is frequently overexpressed in ENKTL, which is associated with increased proliferation of the lymphoma cells.

MYC overexpression is a marker of inferior clinical outcome in extranodal natural killer/T-cell lymphoma

The median progression-free survival (PFS) and overall survival (OS) for this cohort of patients were 28.1 and 45.4 months respectively. Notably, patients with high MYC expression exhibited significantly worse outcomes for both PFS and OS compared to those with low MYC expression (Figure 2A, B). In the subgroup analysis of patients treated with pegaspargase/asparaginase-based regimens, high MYC expression also correlated with inferior outcomes (*Online Supplementary Figure S2*). However, multivariate analysis did not identify MYC overexpression as a significant prognostic factor for either OS or PFS, suggesting potential overlap with other characteristics in this disease (*Online Supplementary Tables S4, S5*). In order to further explore the significance of MYC expression in clinical risk stratification, we assessed the commonly used prognostic index of NK cells lymphoma with Epstein-Barr virus (PINK-E) model[22] in this cohort of cases and found that in general, this model was able to stratify cases with different clinical outcomes. However, a notable limitation emerged as it classified more than 76% of cases into the low-risk group, where the 3-year PFS and OS were observed to be 59.0% and 71.0%, respectively (Figure 2C, D). Interestingly, when adding MYC expression to this model, we obtained a new stratification by using the score of 0-1 for low-risk, 2-3 for intermediate-risk, and ≥4 for high-risk, which exhibited improved efficacy, especially for distinguishing the low-risk group (Figure 2E, F). Similarly, we examined the integration of Ki-67 and also obtained an improved stratification than PINK-E (Figure 2G, H) when using the score of 0-1 for low-risk, 2 for intermediate-risk, and ≥3 for high-risk. We designated these two indexes as PINK-EM and PINK-EK, respectively, which have the potential to serve as useful tools in the clinical management of ENKTL.

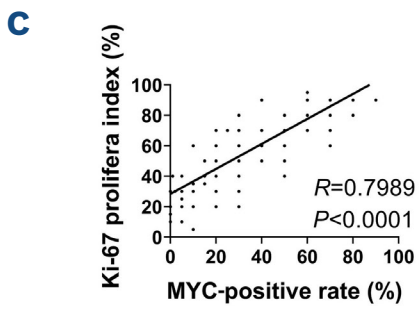
MYC overexpression mediates proliferation and survival in natural killer malignancy cells

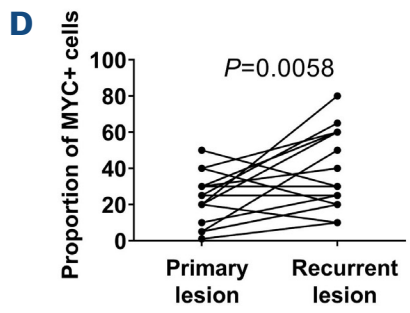
In order to deepen our understanding of MYC overexpression in ENTKL, we conducted *in vitro* functional analyses using a spectrum of NK malignancy cell lines, encompassing both NK lymphoma and leukemia, given the substantial overlaps



B

	MYC-Low	MYC-High
Ki-67-Low	27 (57.4%)	20 (42.6%)
Ki-67-High	1 (1.6%)	63 (98.4%)
Total	28 (25.2%)	83 (74.8%)





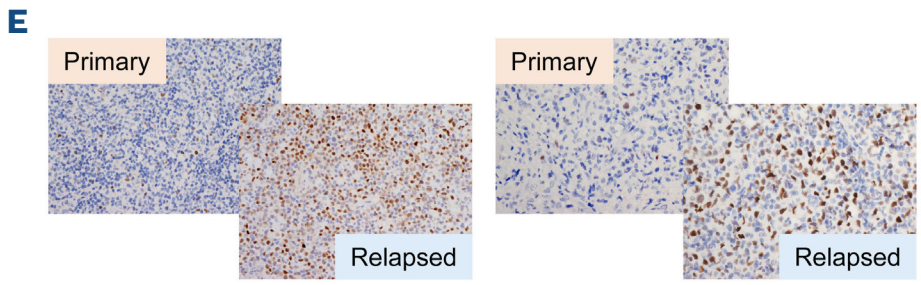


Figure 1. Overexpression of MYC in extranodal natural killer/T-cell lymphoma cases. (A) Hematoxilin and eosin and immunohistochemical staining for MYC and Ki-67 are shown in a representative case of MYC-Low and MYC-High extranodal natural killer/T-cell lymphoma (magnification 400X). (B) Summary of MYC and Ki-67 positivity in this cohort of cases; N (%). (C) The correlation between MYC and Ki-67 in this cohort of cases. (D) Comparison of MYC-positive percentages between diagnostic and relapsed samples in 18 paired cases. (E) Two representative cases showing a remarkable upregulation of MYC expression in the relapsed samples (magnification 400X). H&E: hematoxilin and eosin.

in morphological and genetic characteristics between these two entities, alongside normal NK cells. We observed that normal NK cells consistently showed low MYC mRNA and protein levels, in contrast to the notable variation seen in NK malignancy cells. Specifically, YT and NK-YS cells had MYC expression comparable to normal cells, while KHYG-1, NK-92, and IMC-1 cells exhibited significantly higher levels (Figure 3A, B). In pursuit of unraveling the functional role of MYC, we selected the cell lines with pronounced overexpression for MYC depletion. Given the oncogenic nature of MYC and the inherent challenges of transfecting blood cancer cells, we opted for the siRNA approach and used a blend of two siRNA to mitigate potential off-target effects. We evaluated three siRNA mixtures in NK-92 and IMC-1 cells, which displayed the highest MYC expression levels. The three mixtures exhibited varying KD efficacy with the first one (S1) being the highest and thus being chosen for subsequent experiments (Figure 3C). Notably, all tested siRNA mixtures significantly reduced cell viability, with the extent of this reduction closely mirroring the level of MYC depletion (Figure 3C, D). Specifically, after 72 hours of transfection with the S1 mixture, cell viability in treated cells decreased to approximately one-third of that in control cells. In addition, we noted a significant increase in cell apoptosis post MYC KD, by approximately 22% and 34% in NK-92 and IMC-1 cells, respectively (Figure 3E). This indicates that MYC overexpression contributes to both the proliferation and survival of NK malignancy cells. Moreover, MYC depletion in cell lines with low to intermediate MYC expression also led to reduced cell viability, albeit less pronounced compared to cells with high MYC expression (*Online Supplementary Figure S3*), which aligns with the established role of MYC as an oncogene.

Next, we performed RNA sequencing in NK-92 and IMC-1 cells following MYC KD, revealing substantial gene expression changes in both cell lines. Specifically, 24 hours after KD, we identified 3,995 significantly altered genes in NK-92 cells, with 2,474 showing decreased expression and 1,521 showing an increase. In IMC-1 cells, we observed significant alterations in 4,856 genes, including a decrease in expression for 2,931 genes and an increase for 1,925 genes (*Online Supplementary Figure S4A*). Gene set enrichment analysis (GSEA) showed that the differentially expressed genes (DEG) were highly enriched in canonical MYC target genes in both cell lines (*Online Supplementary Figure S4B*), suggesting that MYC exerts similar oncogenic functions in ENKTL as it does in other types of cancers. Comparative examination identified 1,746 downregulated and 742 upregulated genes commonly shared between the two cell lines. Pathway analysis showed that the downregulated genes were primarily involved in metabolic processes and cell cycle regulation, reinforcing the pro-proliferative func-

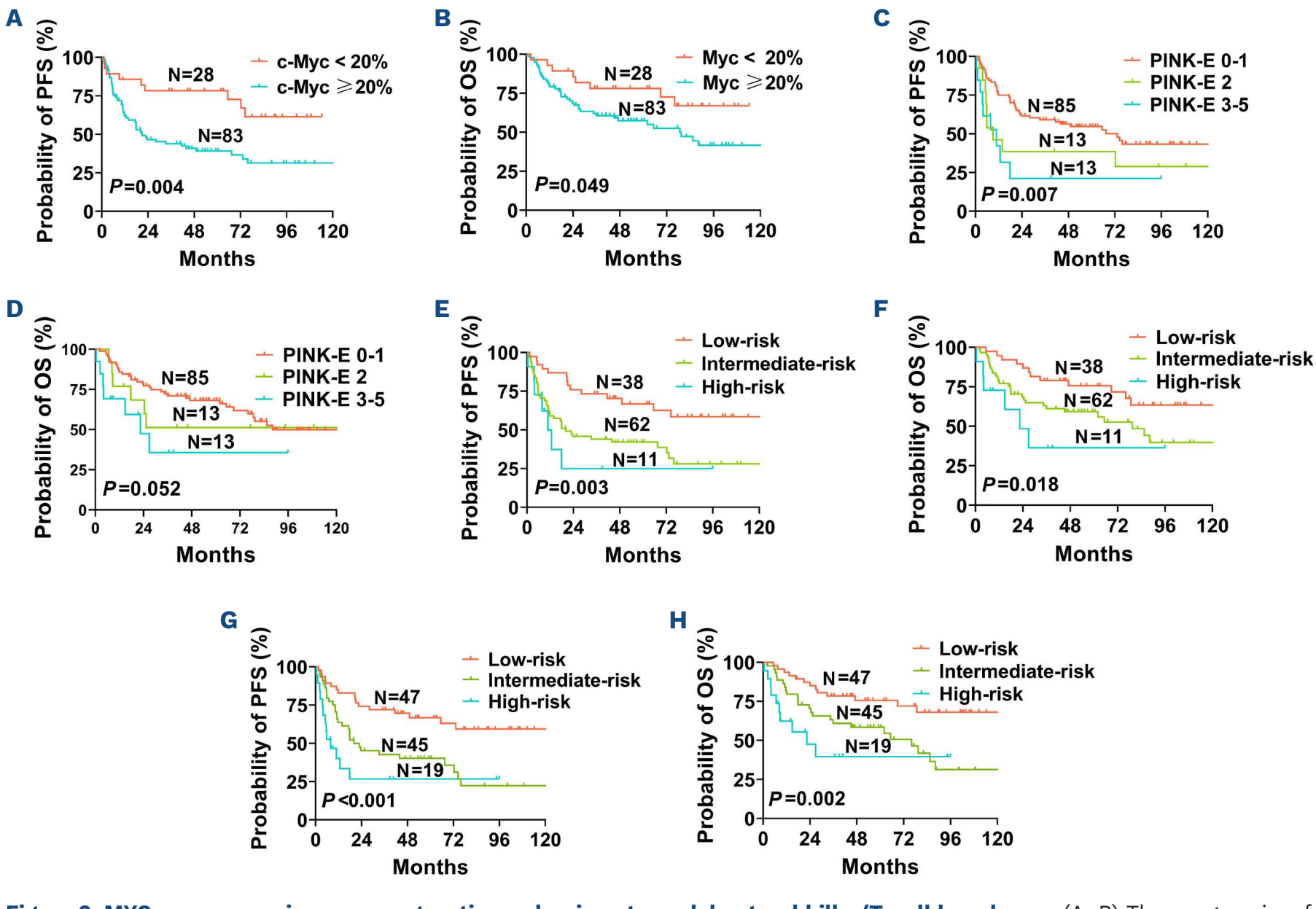


Figure 2. MYC overexpression as a prognostic marker in extranodal natural killer/T-cell lymphoma. (A, B) The progression-free survival (PFS) (A) and overall survival (OS) (B) curves for the cohort, categorized by MYC expression. PFS: median 50.4 months (95% confidence interval [CI]: 39.1-60.7) *versus* median 78.3 months (95% CI: 50.1-86.1); OS: median 60.6 months (95% CI: 48.9-70.5) *versus* median 72.5 months (95% CI: 57.8-89.6). (C, D) The cases were stratified by prognostic index of natural killer cells lymphoma with Epstein-Barr virus (PINK-E) model, and the PFS (C) and OS (D) curves are shown for each group. (E, F) The cases were stratified by the PINK-EM model which integrates PINK-E and MYC overexpression, and the PFS (E) and OS (F) curves are shown for each group. (G, H) The cases were stratified by the PINK-EK model which integrates PINK-E and Ki-67 index, and the PFS (G) and OS (H) curves are shown for each group.

tion of MYC (*Online Supplementary Figure 4C*). Conversely, upregulated genes were highly enriched in TNF-NF-κB and JAK-STAT signaling pathways, including both pathway activators/effectors and inhibitors, likely reflecting a feedback mechanism of oncogenic signaling (*Online Supplementary Figure S4D*). In addition, we also profiled the DEG 48 h after MYC KD and obtained a similar result for functional characterization (*Online Supplementary Figure S5*).

Identification of CDK4 as a potential therapeutic target in extranodal natural killer/T-cell lymphomaL with MYC overexpression

In order to confirm the identified MYC target genes, we analyzed gene expression profiling (GEP) data from 44 previously studied ENKTL cases.[5] We divided cases into three equal-sized groups according to MYC expression levels and then examined the DEG between the 15-case subsets of low- and high-MYC expression groups. On average, the MYC-high group exhibited approximately six-times the MYC level of the MYC-low group, with DEG analysis revealing 176 upregulated and 58 downregulated genes in the MYC-high group (Figure 4A, B). Then, we compared the DEG between the primary cases and the cell line data, and identified a list of 68 commonly shared genes, including 66 downregulated and two upregulated (Figure 4C, D; *Online Supplementary Table S6*). These genes likely represent *bona fide* target genes associated with MYC overexpression in ENKTL and have the potential to serve as therapeutic targets to impair MYC function given that direct MYC inhibition is impractical in current clinical practice. Theoretically, an ideal target needs to meet two essential criteria: it should be intimately relevant to MYC function and be pharmacologically targetable. By a holistic evaluation of the MYC functions demonstrated in the cell experiments, we set our sights on two well-defined MYC targets, HK2 and CDK4.[24,25] Notably, upon MYC KD, we observed a marked reduction in protein

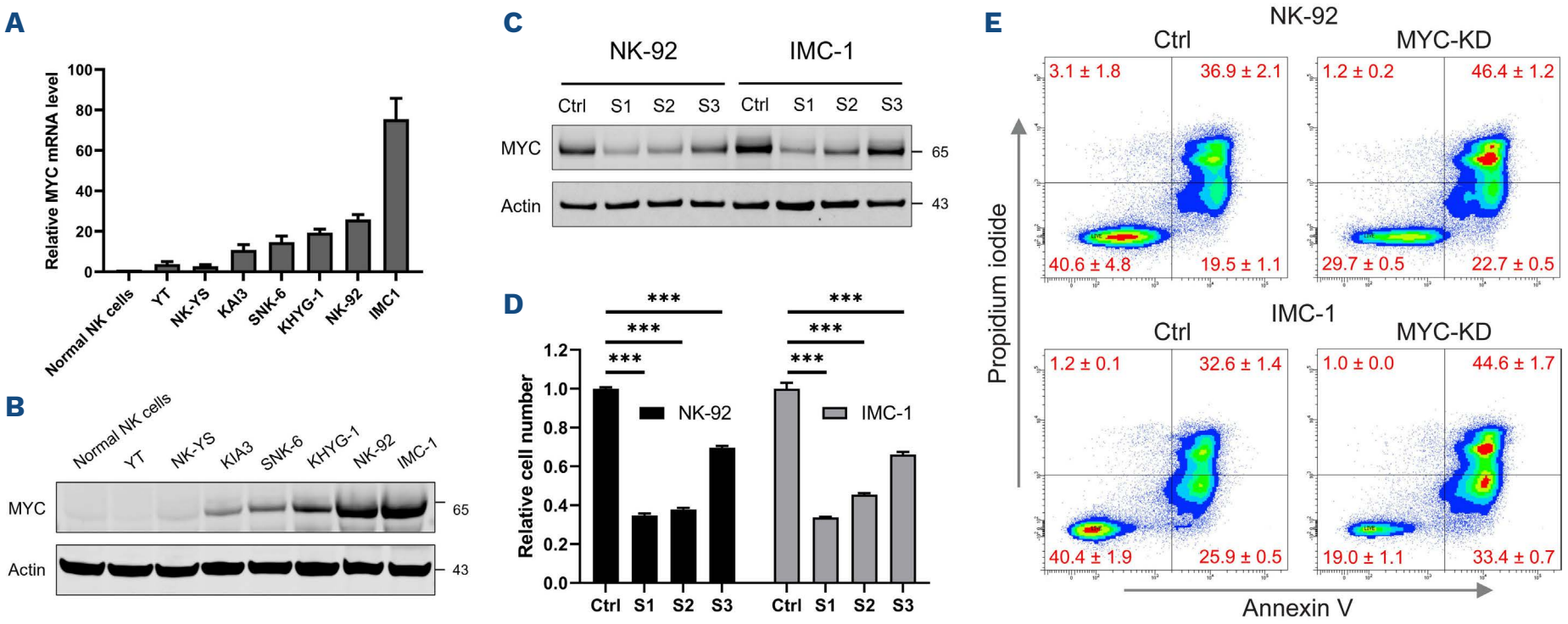


Figure 3. MYC knockdown decreased the viability of natural killer malignancy cells. (A, B) The mRNA and protein levels of MYC in 7 natural killer (NK) malignancy cell lines as measured by quantitative real-time polymerase chain reaction (A) and western blotting (B). (C) MYC knockdown (KD) in NK-92 and IMC-1 cells with the protein levels measured by western blotting. Three small interfering RNA mixtures, annotated S1, S2, and S3 were tested. (D) The cell viability was measured 72 hours after MYC KD in NK-92 and IMC-1 cells. The assay was performed in triplicate and the average values (AV) with standard deviation (SD) are shown. (E) Cell apoptosis was determined by Annexin V and propidium iodide staining followed by flow cytometry assay after 48 hours of MYC KD. The experiments were performed in duplicates and representative density plots with AV and SD were shown. Ctrl: control.

levels for both genes in the MYC-high NK lines (Figure 4E). For comparison, we also analyzed MYC-low NK lines and BL cell lines Raji and Namalwa, which harbor MYC/IgH rearrangements, revealing a reduction trend closely linked to the extent of MYC depletion (*Online Supplementary Figure S6A*). In terms of pharmacological intervention, benserazide, a drug to treat Parkinson's disease, was shown to be a selective HK2 inhibitor,[26] whereas several inhibitors targeting CDK4, such as palbociclib, have been approved for the treatment of breast cancer. Therefore, both targets were subjected to further inhibition testing.

We treated the seven NK malignancy cell lines with escalating doses of benserazide and palbociclib and observed a cell sensitivity profile strongly correlated with MYC expression level. Specifically, cells with MYC overexpression were more susceptible to the inhibition (Figure 5A; *Online Supplementary Figure S6B*). However, the effective inhibition of benserazide required doses (>10 μM) that would be prohibitive for potential *in vivo* application. In contrast, palbociclib demonstrated superior potency with effective doses in the nanomolar range and displayed better differentiation between MYC-high and MYC-low cells. Therefore, it was subjected to further investigation, which showed that the treatment induced both cytostatic and cytotoxic effects (Figure 5B). Because CDK4 promotes cell cycle progression through phosphorylating the tumor suppressor protein Rb, thereby releasing E2F transcription factors, we examined this signaling pathway with palbociclib treatment and observed time-dependent dephosphorylation of Rb at multiple sites (Figure 5C). Interestingly, we found that the MYC expression level was significantly decreased on both protein and mRNA levels, especially after 48 h of treatment (Figure 5C; *Online Supplementary Figure S6C*), suggesting that MYC repression likely resulted from transcription reprogramming due to Rb activation. This is supported by the significant rescue of MYC depletion following Rb KD (Figure 5D). Moreover, we applied palbociclib treatment in Raji and Namalwa cells and found that the MYC level was barely affected (*Online Supplementary Figure S6D*). In addition, to determine whether MYC repression was a simple consequence of cell cycle arrest, we performed a double thymidine block assay but did not observe the depletion of MYC as in the palbociclib treatment (*Online Supplementary Figure S6E*). Collectively, our data indicate that active cell cycle progression mediated by the E2F transcription program is essential for MYC overexpression in NK malignancy cells, whereby a regulatory feedback loop between MYC and CDK4 is thus formed (Figure 5E).

Palbociclib suppressed tumor growth in xenograft mouse models

For *in vivo* testing, we first established the IMC-1 CDX model, in which the tumor cells mainly resided in the viscera, especially the liver (*Online Supplementary Figure S7*). Compared to the vehicle control, palbociclib treatment at 50 mg/kg significantly prolonged the survival of animals, with a 50% increase in median survival (60 days in the treatment group *vs.* 40 days in the control group) (Figure

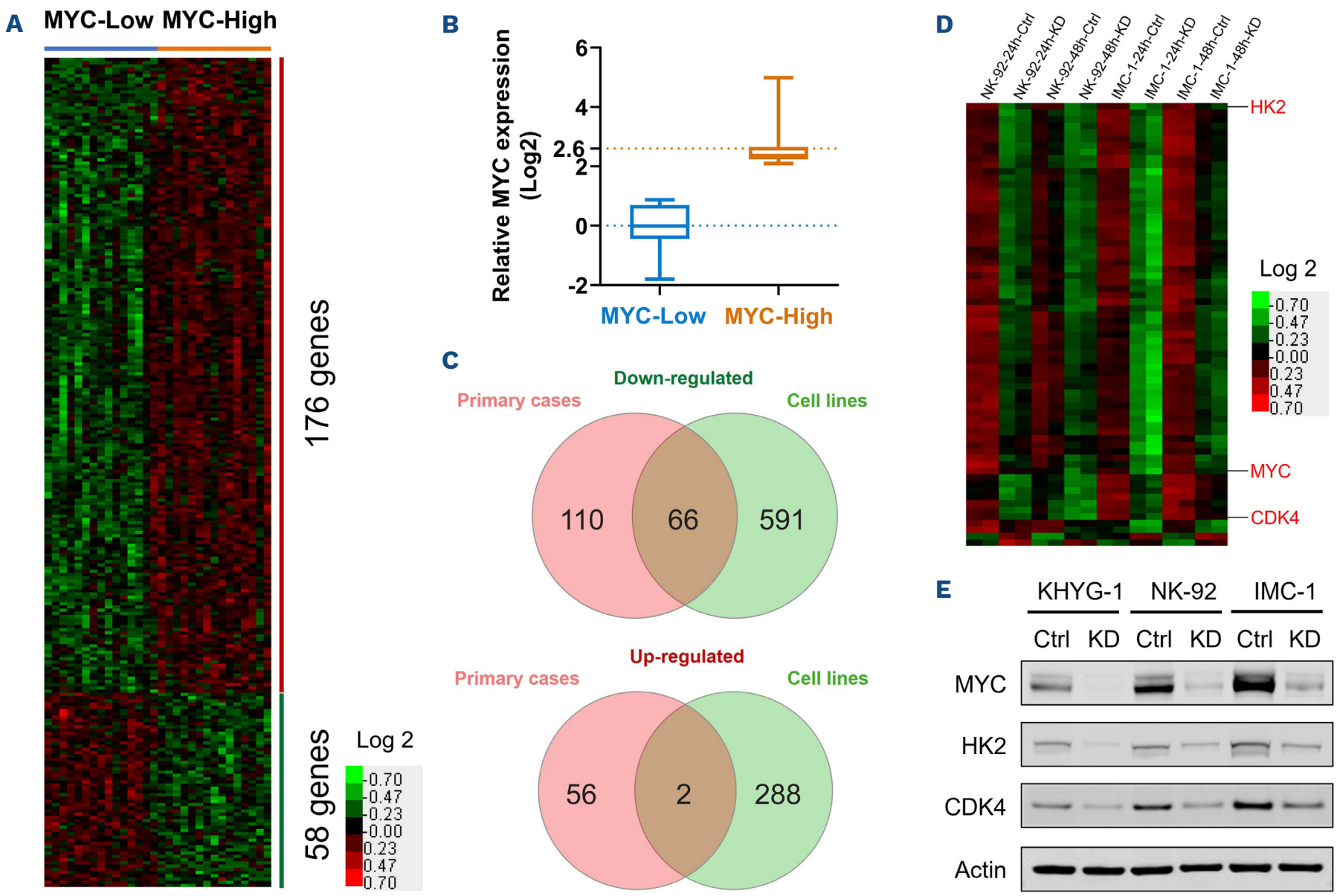


Figure 4. Identification of highly confident MYC targets in extranodal natural killer/T-cell lymphoma. (A) The Heatmap of differentially expressed genes (DEG) between the MYC-High and MYC-Low extranodal natural killer/T-cell lymphoma (ENKTL) cases (N=15/group). (B) Relative *MYC* mRNA level between MYC-High and MYC-Low groups. (C, D) The DEG from the analysis of the primary cases were cross-compared with the commonly altered genes in NK-92 and IMC-1 cells with MYC knockdown (KD). (C) The Venn diagrams show the relationship between the 2 sets of genes and (D) the heatmap shows the expression level of the overlapped genes in the MYC KD experiment. (E) KHYG-1, NK-92, and IMC-1 cells were knocked down with MYC for 48 hours and examined with MYC, HK2, and CDK4 by western blotting. The experiments were performed in triplicates and exhibited by a representative one. Ctrl: control.

6A). In order to assess the effectiveness under low MYC expression conditions, we conducted the treatment in the xenograft model of YT cells, characterized by minimal MYC expression. Consistent with *in vitro* data, the YT cell xenograft, which readily forms subcutaneous tumors, showed no response to the treatment (*Online Supplementary Figure S8*). In order to further evaluate this therapeutic effect, we employed a PDX model of ENKTL with MYC overexpression. Because in the IMC-1 CDX experiment we observed that male mice generally had longer survival, likely due to higher body weight in males at the comparable age, we performed the PDX studies separately for female and male mice. The growth pattern of the PDX model was similar to that of the IMC-1 CDX model, with the viscera organs, especially the liver predominantly involved. Beyond palbociclib single treatment, we also explored the potential enhancement of therapeutic efficacy with a combination of palbociclib and gemcitabine, a key chemotherapeutic agent in ENKTL treatment, particularly since palbociclib primarily induces a cytostatic effect. By preliminary testing, we established a well-tolerated treatment schedule in which gemcitabine was administered as 100 mg/kg on day 1, followed by palbociclib 100 mg/kg on days 4-6, continuously for 3 weeks. We found that either palbociclib or gemcitabine monotherapy, moderately prolonged the mouse survival, (median survival increases: 31-50% in female mice and 21-44% in male mice), whereas the combined treatment substantially improved the outcomes (median survival increases: 93% in female mice, P=0.0021; and 67% in male mice, P=0.0018) (Figure 6B). Besides the survival assessment, we also employed a cohort of mice (3 per treatment group) to examine the tumor growth in major organs at the end of treatment. Compared to control groups, palbociclib treatment at 100 mg/kg as a single agent, significantly decreased tumor burden in visceral organs, especially in the liver, along with a marked reduction in MYC expression. However, residual

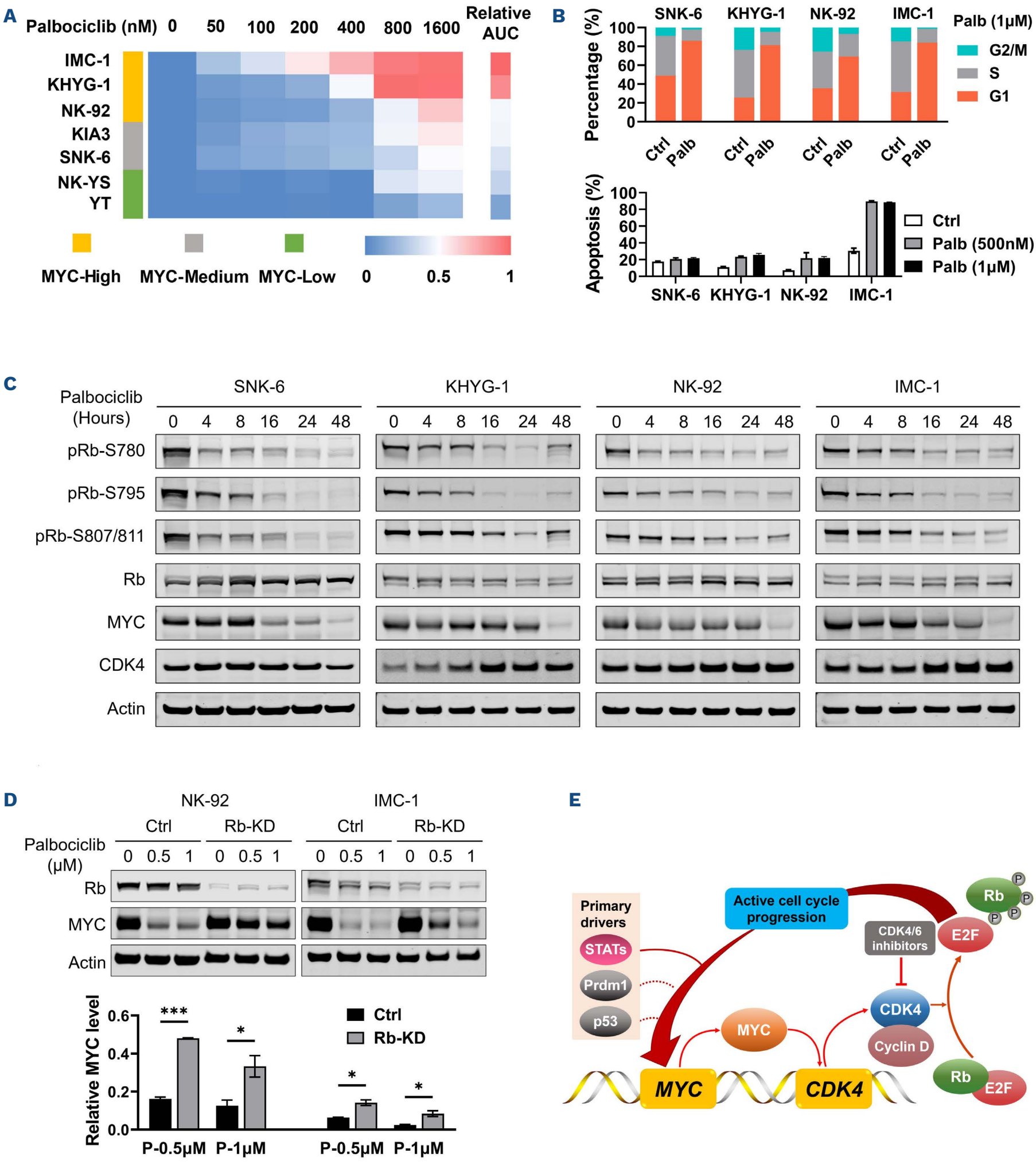
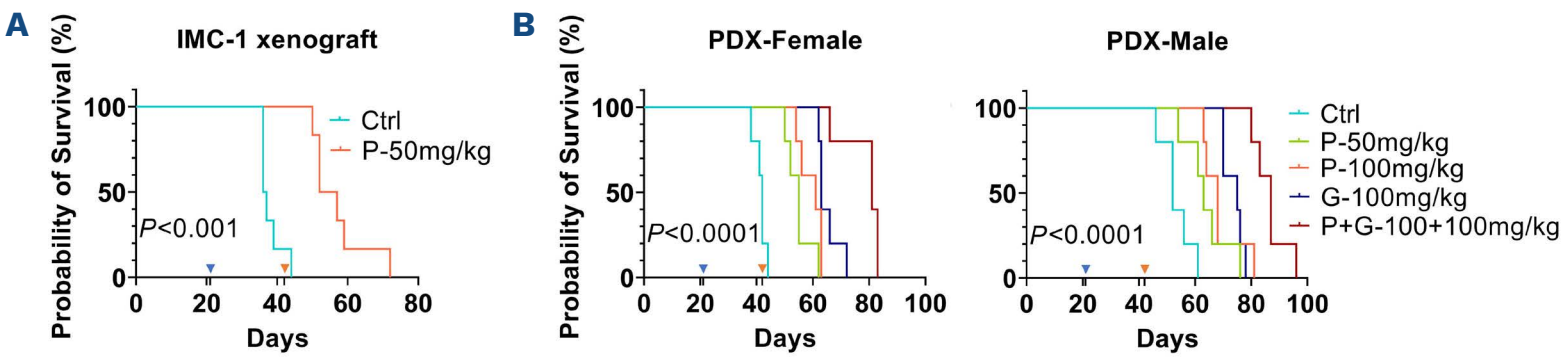


Figure 5. Palbociclib treatment selectively inhibited natural killer malignancy cells with MYC overexpression. (A) Natural killer (NK) malignancy cell lines were treated with increasing doses of palbociclib (Palb) for 72 hours (h) and examined with cell viability by prestoblue assay. The cell lines were ranked by the area under curve (AUC) of the inhibition plot. (B) SNK6, KHYG-1, NK-92, and IMC-1 cells were treated with Palb and measured with cell cycle after 24 h of treatment (upper) and apoptosis after 48 h of treatment (lower). (C) SNK-6, KHYG-1, NK-92, and IMC-1 cells were treated with Palb in a time-dependent manner and the CDK4 signaling and MYC levels were examined by western blotting. (D) Rb was knocked down by small interfering RNA in NK-92 and IMC-1 cells for 24 h, and then treated with Palb (P) for 48 h. Rb and MYC protein levels were measured by western blotting. The quantitation was carried out by analyzing the signal pixel-intensity of MYC with actin normalization. (E) Sketch illustration of the MYC-CDK4 regulation loop in NK cell malignancy. Ctrl: control, KD: knockdown.

tumor cells remain conspicuously present, especially along blood vessels. While gemcitabine monotherapy induced less significant tumor reduction compared to palbociclib, the combination of both marked improved therapeutic efficacy, leaving minimal residual tumor cells in various visceral organs (Figure 6C).

Discussion

Recent advances in treating ENKTL, including L-asparaginase integration, remain largely confined to conventional chemotherapy and radiotherapy. Advanced-stage ENKTL patients often face poor outcomes, with limited options



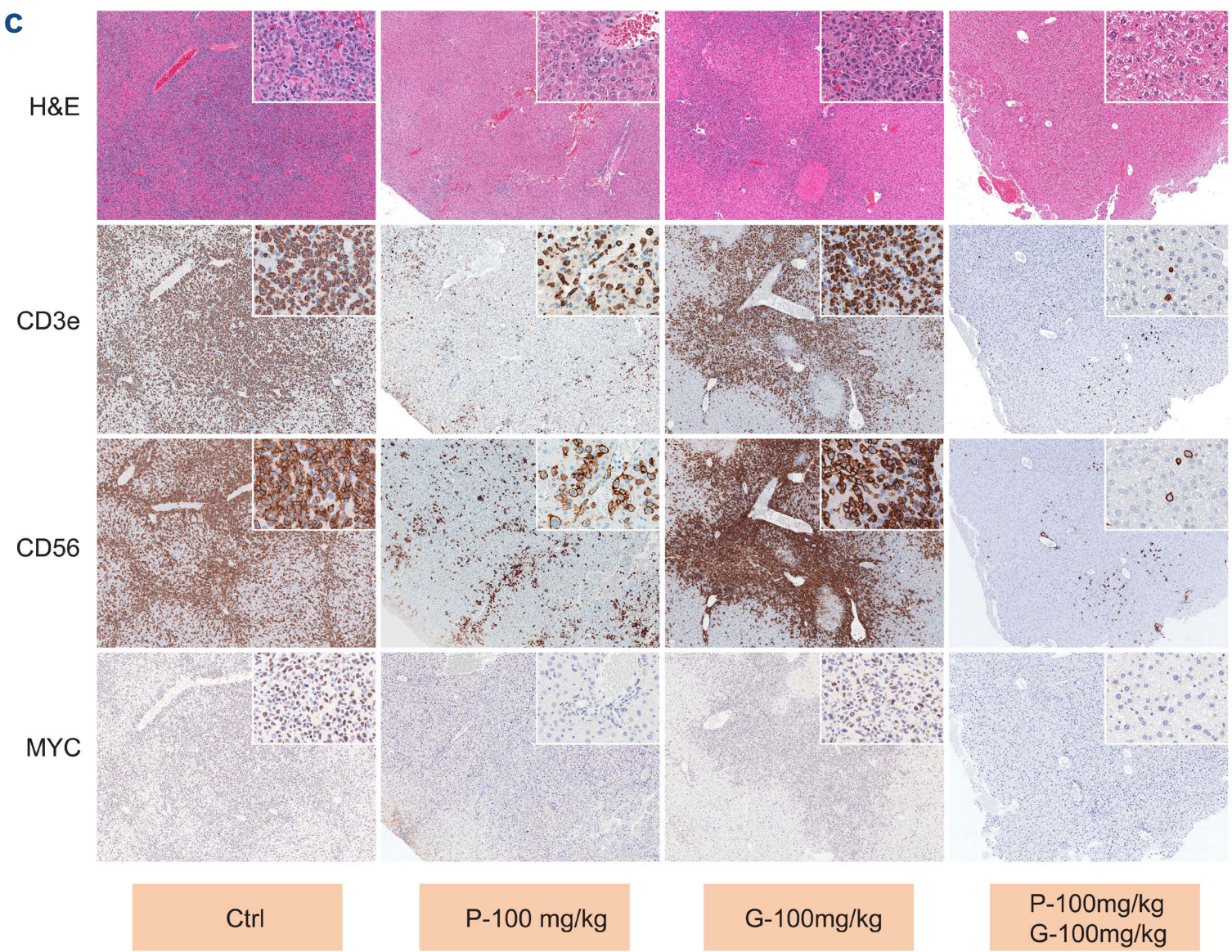


Figure 6. Palbociclib treatment repressed tumor growth in xenograft models of natural killer cell malignancy with MYC overexpression. IMC-1-CDX and an extranodal natural killer/T-cell lymphoma patient-derived xenograft (ENKTL-PDX) model were established in NSG mice. (A) For the IMC-1 CDX, the mice were treated with saline control (Ctrl, N=6) or palbociclib isethionate (P, N=6) daily, and monitored with survival time. (B, C) For the ENKTL-PDX, the treatment effect was investigated in female and male cohorts separately, and in each cohort, a saline control (Ctrl, N=8) and 4-drug treatment groups (N=8/group), including palbociclib isethionate (P) and gemcitabine hydrochloride (G), single and combined treatment, were examined. (B) The survival curve for the 5 treatment groups (N=5/each group) of the PDX model in the female and male cohorts, respectively. (C) Three mice from each group in every cohort were euthanized immediately after the treatment concluded (on day 21 of treatment) and examined with the key organs by pathological staining. The figure shows the tumor involvement in the liver upon different treatments (magnification 40X and 400X). For each representative case, the same area is shown for different staining. (A) and (B) blue triangles indicate treatment start and orange triangles indicate treatment end. H&E: hematoxilin and eosin.

for refractory or relapsed disease. While genomic studies have pinpointed key alterations in ENKTL, such as loss of *PRDM1*, *TP53*, and *FOXO3*, and gain-of-function mutations in the JAK/STAT pathway,[4,7,9,27,28] direct targeting of these drivers remains challenging in the clinical management of this disease. This study shifts focus to MYC, a critical oncogene in hematologic malignancies, investigating its therapeutic implications. Surprisingly, approximately three-quarters of the investigated ENKTL cases showed MYC overexpression, with few genetic alterations, aligning with the fact that MYC is downstream of multiple drivers in ENKTL.[7,9] Notably, MYC overexpression was more pronounced in most of the relapsed ENKTL cases. Coupled with findings from our *in vivo* study showing that gemcitabine treatment alone had little impact on the MYC expression, it raises a question of whether MYC also participated in the treatment resistance of ENKTL, especially given that some relevant mechanisms for this have been identified in pancreatic cancer.[29,30]

Cancer prognostic models are valuable tools to improve the management of patients by providing risk stratification. For ENKTL, the recently developed PINK prognostic model consists of four independent risk factors: age >60 years, stage III/IV disease, distant lymph node metastasis, and non-nasal-type disease. PINK-E, which further integrates detectable plalsma EBV DNA, is now commonly used in clinical practice. Nevertheless, since most patients present with localized or early-stage disease at diagnosis, they are likely to be stratified as low-risk by the PINK/PINK-E indexes. However, in some of the low-risk patients, the clinical outcomes were not as good as expected with standard treatment. This issue has been noticed repeatedly in previous studies,[31,32] and also was found in our study. In particular, we observed two notable declines in the survival curve for the low-risk group stratified by PINK-E, one within the first 2 years and the other 5 years after the diagnosis. This finding suggests that PINK/PINK-E stratification may fail to identify a subset of patients at higher risk of refractory or relapse. We speculated one major reason for this deficiency is the lack of biological indicators. Indeed, when either MYC expression or Ki-67 index was incorporated into the prognostic model, it significantly improved the discrimination with a substantial proportion of the cases being removed from the low-risk group. These two new models have their own advantages. The PINK-EK exhibited better discrimination for the low-risk group, whereas the PINK-EM outperformed in identifying high-risk cases. Further validations on a larger scale are required to evaluate their efficacy and determine which one would be more useful in clinical practice.

Given the important role of MYC in cancer development, considerable efforts have been dedicated to targeting it in cancer cells. However, to date, no direct targeting strategies have received clinical approval for application. In this study, we adopted an alternative strategy to target MYC downstream genes that are essential for its function in ENKTL cells. We considered CDK4 and HK2 as potential targets, not only because they are the key regulators of their respective pathways which are crucial for MYC function, but also because both are kinases that could be targeted by small molecule inhibitors. Our focus turned to CDK4, primarily because CDK4/6 inhibitors have been extensively tested in clinical trials for various types of cancers and have been approved for the treatment of breast cancer. However, as a canonical MYC target with an emerging role in cancer treatment,[33] HK2 holds considerable promise in the treatment of MYC-associated malignancies, especially given the recent advances in identifying potent and selective HK2 inhibitors.[34] Since MYC aberration is frequently observed in many types of cancers, it raises the possibility that CDK4 inhibition may also be efficacious in other cancers where MYC plays a significant role. An affirmative answer might be supported by the fact that CDK4 is a classic MYC target and there is a significant overlap between MYC and E2F bound genes.[35,36] Nevertheless, given that transcriptional regulation is a highly dynamic mechanism orchestrated by a large number of molecules, the impact of MYC on a gene is greatly dependent on the specific context of the cell. Besides, the contribution of the target gene to tumor biology and compensatory mechanisms upon inhibition are also important factors determining the therapeutic efficacy. Therefore, CDK4 inhibition as a treatment for MYC-associated malignancies needs to be specifically investigated in different cancers. Surprisingly, palbociclib treatment induced substantial MYC depletion in the NK malignancy models, both *in vitro* and *in vivo*, which might be an important underlying mechanism contributing to the effectiveness of the treatment. We speculate that this is mainly through transcription reprogramming upon RB activation, which also implies that the active proliferative status mediated by E2F is a prerequisite for MYC overexpression in NK malignancy cells (Figure 5E). Notably, genetic aberrations of the RB gene were barely identified in this disease, further highlighting the potential of this treatment strategy. One notable limitation is the predominant use of IMC-1 and NK-92 cells in the functional study. While these cells exhibit exceptionally high MYC expression, they are not derived from typical ENKTL patients and may not fully capture the diverse cellular characteristics of ENKTL. In the xenograft experiments, although the treatment did not eradicate the tumor and all mice eventually died from the outgrowth of tumor cells after treatment was discontinued, palbociclib exhibited a potent anti-tumor effect, especially when combined with gemcitabine. Given the challenges of tracking tumor growth in visceral organs, our treatments were limited to a 3-week period, followed by a survival assessment. We speculate that additional cycles of treatment and a more strategically optimized drug combination may further improve therapeutic efficacy.

In conclusion, our findings underscored that MYC, despite not being a primary driver, is an oncogene with prognos-

tic significance in ENKTL, and can serve as a biomarker to evaluate the aggressiveness of the tumor. Further, we demonstrated that the clinical-grade CDK4/6 inhibitor palbociclib is promising in the treatment of ENKTL with MYC overexpression. Clinical trials are desired to further determine the safety and efficacy of this treatment approach in patients with ENKTL.

Disclosures

No conflicts of interest to disclose.

Contributions

CB and KF designed the experiments. CB, YH, and RA performed the experiments and data analysis. FW, XY and LX assisted in performing experiments. AB assisted in data analysis. CB wrote the manuscript. XH, ML, WCC, JI, DDW, and JMV provided advice for the project design and writing of the manuscript.

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Data-sharing statement

Data on individual patients will not be shared. Other original data and protocols are available to other investigators upon request.

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