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Original contribution

Aberrant differential expression of EZH2 and H3K27me3 in extranodal NK/T-cell lymphoma, nasal type, is associated with disease progression and prognosis☆,☆☆

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Summary Enhancer of zeste homolog 2 (EZH2), an H3K27-specific histone methyltransferase, has been shown to be frequently overexpressed in various human cancers including lymphoma. Here we investigate the expression and functionality of EZH2 and H3K27me3 in extranodal NK/T-cell lymphoma, nasal type (ENKTL). Results of NanoString analysis revealed that EZH2 and related histone H3 families were up-regulated genes in ENKTL tissues. Results of immunohistochemistry demonstrated that EZH2 and trimethylation of Lys-27 in histone (H3K27me3) were highly expressed in 55.2% and 78.0% of patients with ENKTL, respectively. EZH2 overexpression was significantly associated with higher tumor cell proliferation ($r = 0.582$, $P = .000$), advanced stage ($P = .012$), and predicted poorer overall survival ($P = .016$) in ENKTL. H3K27me3-positive expression was correlated with lower tumor cell proliferation ($r = -0.623$, $P = .036$), earlier stage ($P = .043$), and predicted better overall survival ($P = .020$). In addition, EZH2 and H3K27me3 showed inverse correlations ($r = -0.652$, $P = .002$) in clinical samples by immunohistochemistry. Furthermore, inhibition of EZH2 by 3-deazaneplanocin A significantly suppressed tumor cell growth. Interestingly, pharmacologic suppression of the JAK3/STAT3 pathway effectively reduced EZH2 and enhanced H3K27me3 in NK/T tumor cell lines. Our data suggest that EZH2 and H3K27me3 are important prognostic markers and potential therapeutic targets in ENKTL.

1. Introduction

Extranodal NK/T-cell lymphoma, nasal type (ENKTL), is an aggressive malignancy with a poor prognosis [1,2]. It is particularly prevalent in Asian countries and parts of Latin America. Although chemotherapy and radiotherapy help improve the disease outcome, the prognosis of NKTCL remains poor [2]. There is an urgent need for

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effective targeted therapy. Recently, the combination of genomic and functional analysis has identified some candidate tumor suppressor genes in ENKTL, such as *PRDM1*, *HACE1*, *AIM*, and *FOXO3* [3], but none of them are an independent prognostic factor for this disease [4]. Gene expression profiling studies also have identified a number of oncogenes and signaling pathways, with differential expression and activation in ENKTL [1,5]. However, few aberrant molecules contribute to ENKTL pathogenesis and can potentially serve as therapeutic targets. Thus, a novel diagnostic molecular predictor of tumor behavior is currently required to help guide therapeutic decisions.

Enhancer of zeste homolog 2 (EZH2) is an H3K27-specific histone methyltransferase, which plays a key role in the epigenetic maintenance of repressive chromatin mark. EZH2 protein must partner with other noncatalytic proteins, such as EED and SUZ12, to form the polycomb repressive complex 2 (PRC2) to carry out its histone methyltransferase activity [6]. When the PRC2 complex is recruited to chromatin, EZH2 induces H3K27 dimethylation and trimethylation (H3K27me2/3). H3K27me3 is frequently associated with gene repression, and it is a critical epigenetic event during tissue development [7]. EZH2 has been shown to be frequently overexpressed in various human cancers including lymphoma [8], and its overexpression is associated with invasive growth and poor clinical outcomes in solid tumors such as prostate, breast, gastric, and endometrial cancers [9-11]. Very few studies have suggested that EZH2 is overexpressed in NK/T-cell lymphoma [12]. However, the relationship between EZH2 and H3K27me3 remains unclear, and their effect on prognosis has not been reported. In our study, we demonstrated that EZH2 and H3K27me3 were aberrantly overexpressed in most ENKTL, and both had important clinicopathological significance. We discovered that EZH2 overexpression was inversely associated with H3K27me3, and there is an opposite effect in clinicopathological significance and prognosis. In addition, we found that JAK3 inhibitor may reduce the growth of ENKTL cells by decreasing EZH2 expression and increasing H3K27me3 expression. Our findings may provide new ideas for the prognosis and treatment of ENKTL.

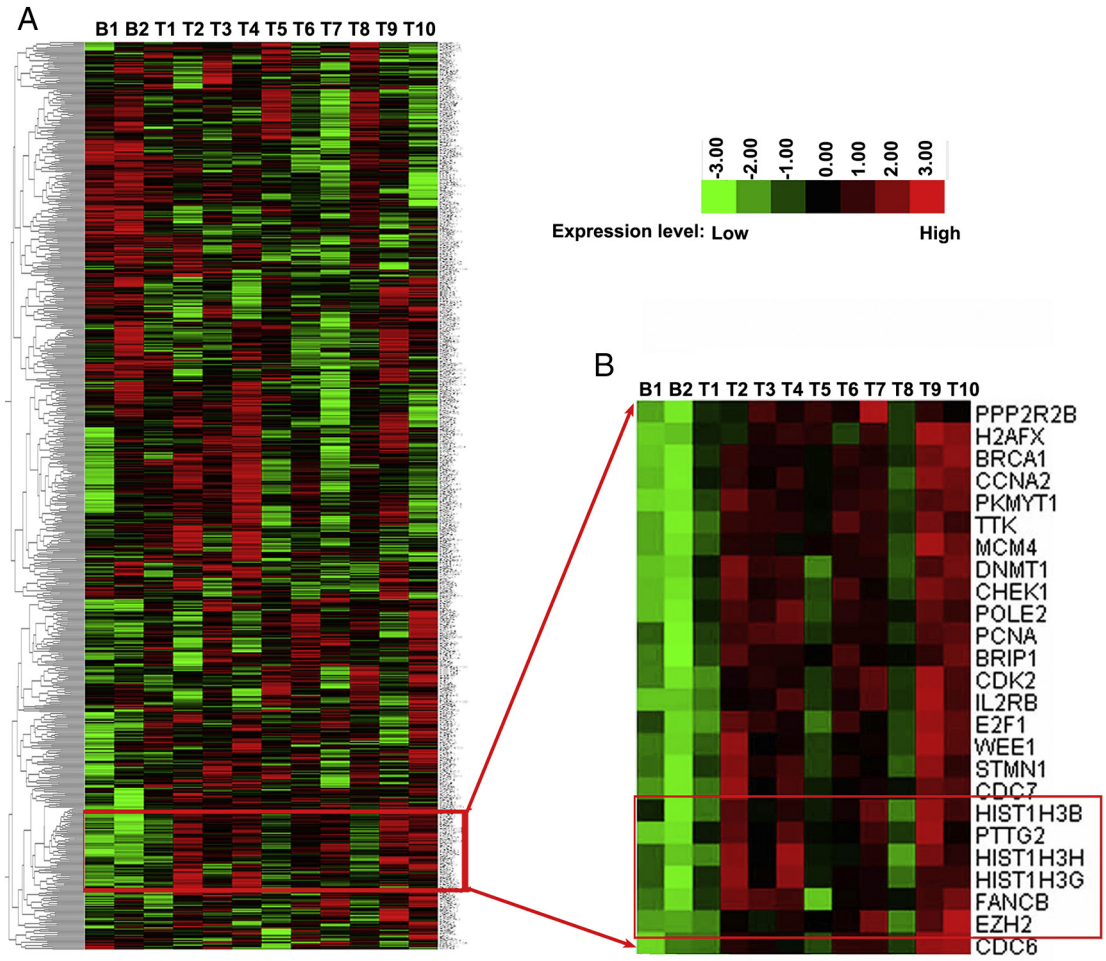


Fig. 1 NanoString technology detected the expression 770 genes of 13 pathways oncogenes in 12 cases. A, The heat map lists the expression of 770 genes in 12 cases. B, This panel is a partial enlargement of panel A. Green represents low expression, and red represents high expression. The genes in the red box are histone-related genes, which exhibited high expression in ENKTL tumor samples.

2. Materials and methods

2.1. Patients and samples

We collected archival formalin-fixed, paraffin-embedded tumor blocks of 38 Chinese patients who were diagnosed as having ENKTL from the Department of Pathology, Peking University First Hospital. We confirmed the diagnosis of ENKTL according to World Health Organization classification [13]. Follow-up data were available for 38 patients. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Medical Ethics Committee of the Peking University First Hospital (No. 2013[571]) and with the Declaration of Helsinki and its later amendments or comparable ethical standards.

2.2. Gene expression profiling by Nanostring nCounter assay

Total RNA was extracted from formalin-fixed, paraffin-embedded tissues (10 tumor samples and 2 normal nasal mucosa) using RNeasy Mini Kit (Qiagen, Hilton, German) in accordance with the manufacturer's instructions. RNA quality was evaluated with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). For Nanostring nCounter assay, we used a PanCancer Pathways Panel that included 770 essential genes representing 13 canonical pathways: Notch, Wnt, Hedgehog,

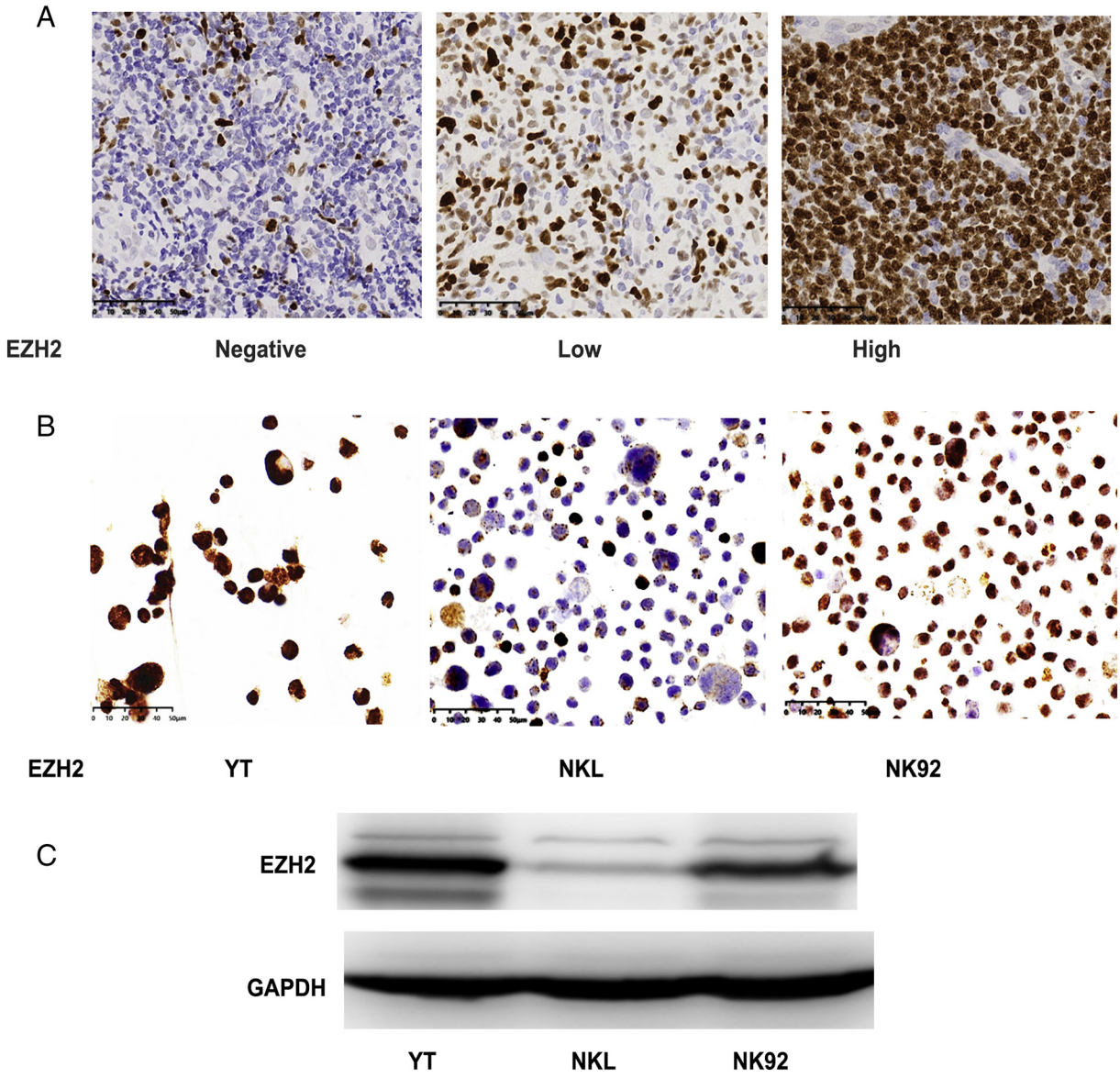


Fig. 2 IHC, ICC, and Western blot analysis of EZH2 expression in ENKTL cases and cell lines. A, EZH2 immunostaining in the nuclei of ENKTL primary tumor cells. Representative images of negative, low, and high expression. EZH2-positive staining was detected in YT and NKL92 but not in NKL by ICC (B) and Western blot analysis (C). A and B, Original magnification ×400.

TGF-β, MAPK, STAT, P13K, RAS, chromatin modification, transcriptional regulation, DNA damage control, cell cycle, and apoptosis. We performed the NanoString nCounter assay according to NanoString's standard protocol. Raw counts obtained for each sample were normalized using nSolver software version 3.0 (NanoString Technologies, Seattle, Washington, USA). All procedures related to RNA quantification including sample preparation, hybridization, detection, and scanning were carried out as recommended by NanoString Technologies.

2.3. Cell culture, treatment, and viability

We used 3 nasal NK/T-cell lymphoma cell lines in the current study: NKL, NK92, and YT. Cell culture methods are described in our previous studies [14]. Cells were seeded at 2 × 10^5 cells/mL per well in 24-well plates and treated with 3-deazaneplanocin A (DZNep; Sigma-Aldrich, St Louis, MO) at indicated concentrations for 48 hours with dimethyl sulfoxide (DMSO) as a control before being subjected to CellTiter 96

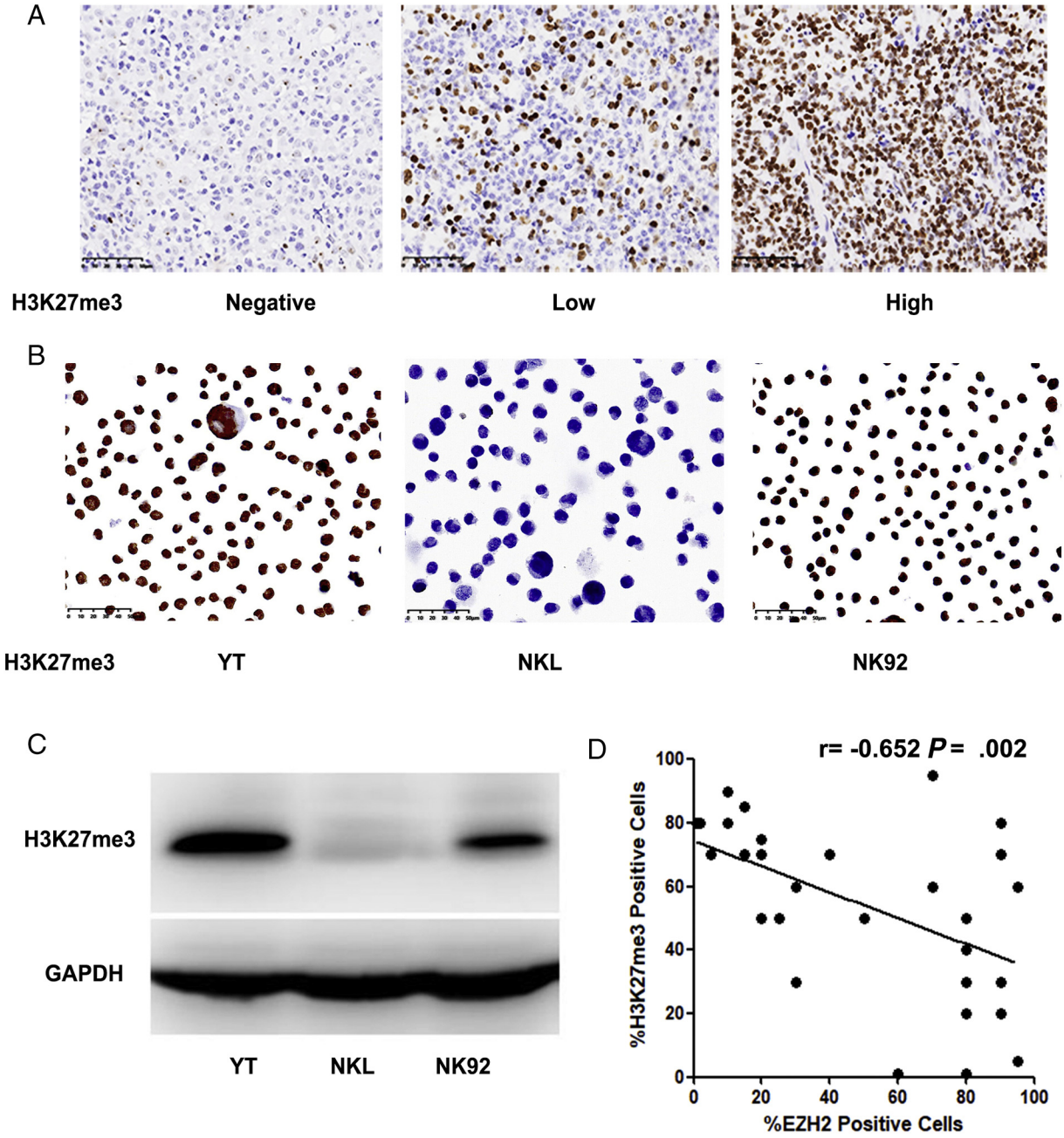
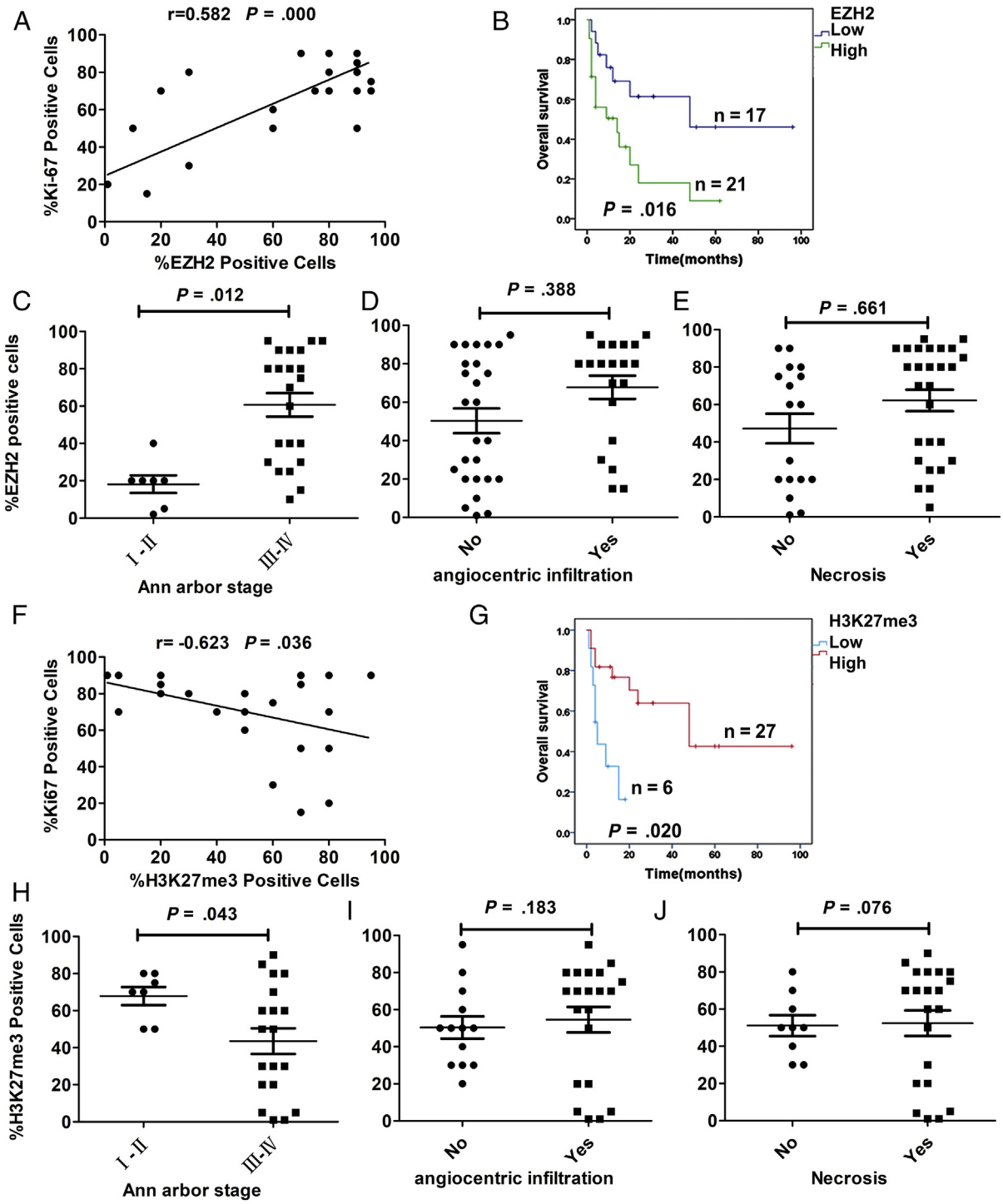


Fig. 3 IHC, ICC, and Western blot analysis of H3K27me3 expression in ENKTL cases and cell lines. A, H3K27me3 immunostaining in the nuclei of ENKTL primary tumor cells. Representative images of negative, low, and high expression. H3K27me3-positive staining was detected in YT and NKL92, but not in NKL by ICC (B) and Western blot analysis (C). D, Scatterplot showing the correlation between H3K27me3 and EZH2 expression by IHC. Spearman correlation coefficient: $r = -0.652$, $P = .002$. A and B, Original magnification ×400.

(Corning, NY, USA) AQueous One Solution Cell Proliferation Assay (MTS). The MTS assay was performed as previously described [15].

2.4. Immunohistochemistry and immunocytochemistry

Immunohistochemistry (IHC) and immunocytochemistry (ICC) were performed as previously described [15,16]. IHC staining was performed using the Dako Envision detection kit (Dako, Gilstrap, Denmark). The tissue sections were subjected to heat-induced antigen retrieval in EDTA buffer (pH 9.0). We used primary antibodies against EZH2 (no. 5246; Cell Signaling) and H3K27me3 (no. 9733; Cell Signaling Technology, MA, USA). Positive nuclear staining pattern was interpreted as representing EZH2 and H3K27me3 immunoreactivity. We defined a high expression of EZH2/H3K27me3 as moderate/strong nuclear staining in 30% or more of the tumor cell population and a low



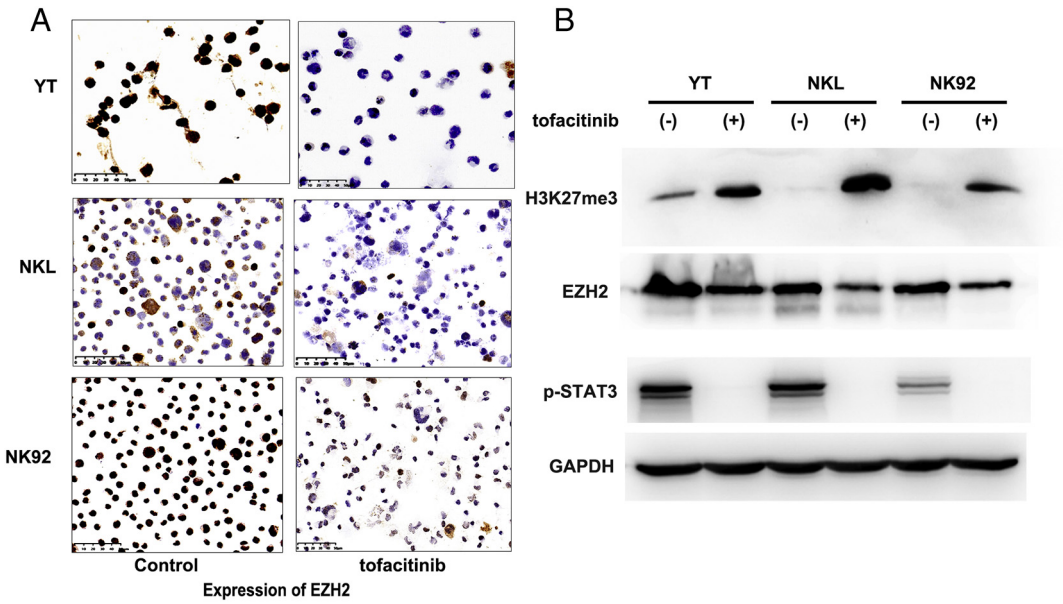


Fig. 5 Regulation of EZH2 and H3K27me3 expression by tofacitinib. A, ICC showed the different expression of EZH2 protein in YT, NKL, and NK92 after treated with or without tofacitinib. B, Western blot analysis of phosphorylated STAT3, EZH2, and H3K27me3 levels in YT, NKL, and NK92 cells treated with or without tofacitinib. A, Original magnification ×400.

expression of EZH2/H3K27me3 as less than 30% nuclear staining. The mean percentage of positive tumor cells was determined by evaluating at least 5 areas under a high-power field microscopy [17]. Breast carcinoma known to be positive for EZH2 expression was used as a positive control. B-cell lymphoma was used as a positive control for H3K27me3 staining. For the negative control reactions, phosphate-buffered saline was used instead of the primary antibody.

2.5. Western blot

Western blot analysis was performed as described previously [18]. Primary antibodies against EZH2 and H3K27me3 were purchased from Cell Signaling Technology, and GAPDH (sc-47724; 1:1,000) was from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Horseradish peroxidase–conjugated secondary antibodies included antirabbit (1:5000; ZSGB-BIO, Beijing, China) and antimouse (1: 5000; ZSGB-BIO). We quantified protein expression by densitometry and normalized it to GAPDH.

2.6. Flow cytometry

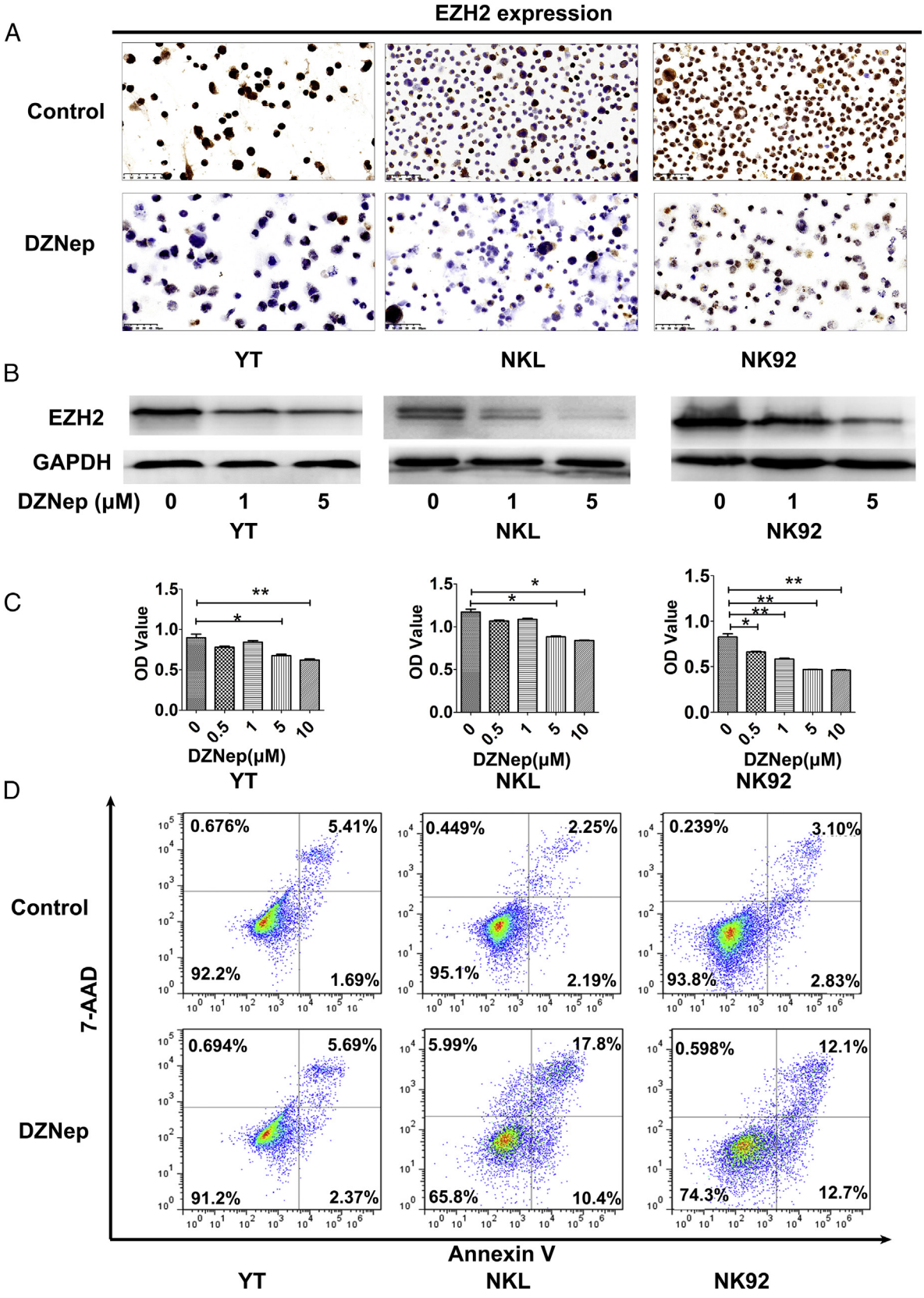
After treatment with DZNep for 48 hours, all cells were collected and analyzed with PE–Annexin V Apoptosis Detection Kit I (BD Pharmingen, Franklin, New Jersey, USA) following the manu-facturer's instructions. Flow cytometry was performed using FACS Aria II instruments (BD Biosciences, San Jose, CA). The same time points of DMSO-treated cells were examined as controls. The experiments were repeated 3 individual times.

2.7. Statistical analysis

We used Student *t* test to analyze cell proliferation and the Fisher exact test to analyze the association between EZH2 or H3K27me3 expression and clinical parameters of the subjects.

Fig. 4 Correlation of EZH2 or H3K27me3 with clinicopathological features of ENKTL. A, Scatterplot representation of the correlation between the percentage of Ki67-positive cells and the percentage of EZH2-positive cells. Spearman correlation coefficient: $r = 0.582$, $P = .000$. B, Kaplan-Meier curves and log-rank test survival analysis showed that OS time was longer in the EZH2 low-expression group than in the high-expression group ($P = .016$). Blue indicates low expression, and green indicates high expression of EZH2. C, The percentage of EZH2 expression is significantly higher in Ann Arbor III-IV than in Ann Arbor I-II ($P = .012$). D, The percentage of EZH2-positive cells was similar between the 2 groups with or without vascular infiltration ($P = .388$). E, The percentage of EZH2-positive cells was similar between the 2 groups with or without necrosis, ($P = .661$). F, The expression of H3K27me3 was negatively correlated with Ki67 expression. Spearman correlation coefficient: $r = -0.623$, $P = .036$. G, Kaplan-Meier curves and log-rank test survival analysis showed that the OS time of patients with H3K27me3 in the high-expression group was longer than that in the low-expression group ($P = .020$). Red lines represent the high-expression group, and blue lines represent the low-expression group. H, The percentage of H3K27me3-positive cells was significantly higher in Ann Arbor stage I-II than in Ann Arbor III-IV stage ($P = .043$). I, The percentage of H3K27me3-positive cells was similar between the 2 groups with or without vascular infiltration ($P = .183$). J, The percentage of H3K27me3-positive cells was similar between the 2 groups with or without necrosis ($P = .076$).

The Spearman rank-correlation coefficient test was used to correlate the expression of EZH2 and H3K27me3. We calculated estimated overall survival (OS) using the Kaplan-Meier method and compared this with log-rank tests. Differences were considered statistically significant when the 2-sided *P* value was less than .05. All analyses were performed using



SPSS (Statistical Package for the Social Sciences) 19.0 software (Chicago, IL).

3. Results

3.1. Identification of key aberrant oncogenic genes in ENKTL

To identify potential key oncogenic genes in ENKTL, we chose a panel of 770 genes in classical oncogenic pathways to perform gene expression analysis using the NanoString nCounter system. We analyzed the expression of 770 genes in 10 cases of ENKTL and 2 control cases using unsupervised hierarchical clustering (Fig. 1A). Genes associated with the histone modification (eg, *EZH2, HIST1H3B, PTTG2, HIST1H3H,* and *HIST1H3HG*) and the cell cycle (eg, *CDC7, CDC6, CCNA2, CDK2*, *PCNA*, *E2F1, WEE1*) were more highly expressed in neoplastic samples compared with normal controls (Fig. 1B).

3.2. EZH2 and H3K27me3 were overexpressed in ENKTL

Previous studies indicated that aberrant hypermethylation of promoters is an important mechanism of tumor suppressor gene silencing in NK/T-cell lymphoma [15,19,20]. In addition, high expression of *EZH2* and histone H3-related genes were most prominent in our initial screening. We focused our investigation on the expression of EZH2 and one of its key substrates H3K27me3 in more ENKTL samples (38 cases). High expression of EZH2 was detected in 55.2% (21/38) of ENKTL specimens. Fig. 2A shows representative IHC results of negative, low, and high EZH2 expression in ENKTL samples. In addition, high expression of EZH2 was detected in YT and NK92, but not in NKL cell lines (Fig. 2B and C). H3K27me3 was detected to be highly expressed in 78% (27/33) of ENKTL specimens. Fig. 3A show representative results of negative, low, and high H3K27me3 expression in ENKTL samples by IHC, respectively. Results of ICC and Western blot analysis also showed that H3K27me3 was highly expressed in YT and NK92 cells, but was little expressed in NKL cells (Fig. 3B and C). EZH2 showed a negative association with H3K27me3 ($r = -0.652$, $P = .002$) in ENKTL cases (Fig. 3D).

3.3. Clinicopathological significance of EZH2 and H3K27me3

Because EZH2 and H3K27me3 were overexpressed in most ENKTL samples, but EZH2 and H3K27me3 showed inverse correlation in ENKTL unexpectedly, we further analyzed the clinicopathological significance of EZH2 and H3K27me3. EZH2 expression showed a considerable correlation with Ki67 labeling index, indicating that EZH2 plays a role in cell proliferation in ENKTL (Fig. 4A). Kaplan-Meier analysis revealed that patients with EZH2 overexpression exhibited a worse prognosis on OS ($P = .016$; Fig. 4B). In addition, EZH2 expression was significantly correlated with advanced stage of ENKTL (Fig. 4C) but was not correlated with angiocentric infiltration (Fig. 4D) and tissue necrosis (Fig. 4E). Then, we analyzed the relationships between H3K27me3 expression and clinicopathological features. The expression of H3K27me3 was negatively correlated with that of Ki67 in ENKTL (Fig. 4F). Univariate analysis revealed that patients with H3K27me3 overexpression exhibited longer survival time ($P = .020$; Fig. 4G). In addition, higher H3K27me3 expression was associated with earlier stage of ENKTL (Fig. 4H) but was not correlated with angiocentric infiltration (Fig. 4I) and tissue necrosis (Fig. 4J). These data suggested that aberrant expression of EZH2 and H3K27me3 is associated with ENKTL progression and prognosis. However, EZH2 may not function as an H3K27-specific histone methyltransferase through the canonical pathway but may be exerted in other ways.

3.4. Regulation of EZH2 and H3K27me3 expression by JAK3 inhibitor (tofacitinib)

Several studies have provided evidence supporting the role JAK/STAT pathway in ENKTL lymphomagenesis [5,21,22]. It has also been reported that phosphorylation of EZH2 by JAK3-mediated resulted in EZH2 oncogenic function independent of its enzymatic activity in ENKTL [12,23]. Thus, we tested the effect of the JAK3/STAT3 pathway on EZH2 and H3K27me3 expression by using JAK3 inhibitor (tofacitinib). ICC results showed that tofacitinib could markedly decreased EZH2 expression in YT, NKL, and NK92 cells (Fig. 5A). Consistently, results of Western blot analysis showed that tofacitinib reduced EZH2 expression. On the contrary, H3K27me3 expression was evidently elevated after treatment by tofacitinib in YT, NKL, and NK92 cells

Fig. 6 Effects of DZNep on cell growth and apoptosis in ENKTL. A, YT, NKL, and NK92 cells were treated with DMSO or DZNep for 48 hours, and expression of EZH2 was detected by ICC. B, YT, NKL, and NK92 cells were treated with DMSO or DZNep (1 μM, 5 μM) for 48 hours, and cell lysates were immunoblotted for EZH2. C, YT, NKL, and NK92 cells were treated with DZNep for indicated concentration for 48 hours, respectively, and proliferation was evaluated by optical density value using the MTS assay. *P<.005, **P<.001. Error bars indicate the SEM from 3 independent experiments. D, YT, NKL, and NK92 cells were treated with DMSO or DZNep for 48 hours, and apoptosis was evaluated by annexin V−7AAD staining using flow cytometry. The experiment shown is representative of 3 independent experiments. A, Original magnification ×400.

(Fig. 5B). Therefore, EZH2 may be regulated by the JAK/STAT3 pathway and plays a role as oncogene in ENKTL.

3.5. Inhibition of EZH2-induced ENKTL cell growth inhibition and apoptosis

The role oncogene plays and its positive correlation with poor prognosis suggested that EZH2 may be a therapeutic target in ENKTL. Thus, we explored the function of EZH2 inhibition by using DZNep in ENKTL cell lines. Consistent with previous reports [24,25], DZNep effectively reduced the expression of EZH2 in YT, NK92, and NKL cells in a dose-dependent manner (Fig. 6A and B). Furthermore, we evaluated the effects of DZNep on cell growth in ENKTL lines. Results of MTS assays demonstrated that YT, NK92, and NKL cell lines responded to DZNep treatment (Fig. 6C). Last, we evaluated whether DZNep induces apoptosis. Apoptosis that occurred in tofacitinib-treated cells was analyzed by flow cytometry after annexin V staining. The results revealed that DZNep induced an increase in apoptotic cells in NKL and NK92 cell lines, as shown by an increase in the annexin V–positive and 7-aminoactinomycin D–negative fraction, when compared with DMSO-treated cells. However, no significant increase in the number of apoptotic cells was observed in YT cell lines tested (Fig. 6D).

4. Discussion

EZH2 is a subunit of the polycomb repressive complex, which trimethylates lysine 27 on histone 3, a repressive marker for gene expression. EZH2 is important for cancer cell proliferation, migration, and invasion, all of which are associated with cancer initiation, progression, and metastasis. More importantly, EZH2 is closely related to cancer stem cell properties and tumor-initiating cell function [26,27]. In the present study, we identified that EZH2 and H3K27me3 were aberrantly overexpressed in ENKTL. Moreover, strong EZH2 expression was associated with increased tumor cell proliferation and showed significant prognostic effect in ENKTL. In agreement with previous reports, EZH2 overexpression is associated with cell proliferation, inhibition of apoptosis, and poor prognosis in a number of cancer types, including breast, ovarian, melanoma, and prostate, and hematopoietic malignancies [9,12,17,28,29]. H3K27me3 also was overexpressed in ENKTL cases and cell lines, but the clinicopathological significance is contrary to our expectations. H3K27me3 overexpression was correlated with lower proliferation rates and predicted a better prognosis in accordance with the fact that H3K27me3 expression is a superior prognostic indicator for clinical outcome in patients with breast, ovarian, and pancreatic cancers [30].

Previously, it was considered that the function of EZH2 is gene silencing through the methylation of H3K27. Several previous studies have shown a positive correlation between EZH2 and H3K27me3 activation, and both are inferior predictors in various cancers. Contrary to those previous data, our clinical samples show that expression of EZH2 and H3K27me3 is inversely correlated with each other and show a contrary effect on prognosis. These results highlight a noncanonical EZH2 function in ENKTL. Previous studies have also reported that EZH2 expression lacked an association with abundance of H3K27me3 in breast tumor subtypes [31], renal cell carcinoma [32], ovarian cancer, and pancreatic cancers [30]. In addition, it was reported that the expression of EZH2 was correlated with lower level of H3K27 methylation with enhanced PCNA expression, and high expression of H3K27me3 predicts better prognosis in non–small cell lung cancer [33]. In glioblastoma, AKT signaling activation leads to phosphorylation of EZH2 and inhibits its H3K27me3 enzymatic activity [34]. In glioblastoma multiforme and in a prostate cancer model, EZH2 has also been implicated in the methylation of nonhistone substrates and via binding and methylating STAT3, which promotes tumorigenesis of glioblastoma stem–like cells [34]. In ENKTL, EZH2 behaves unconventionally in that its promotion of growth is independent of its methyltransferase activity [12]. Phosphorylation of EZH2 by JAK3 mediates this switch from histone methyltransferase to transcriptional coactivator, leading to the upregulation of a series of genes that are involved in DNA replication, cell cycle, biosynthesis, and invasiveness [23]. Thus, pro-proliferative function of EZH2 in ENKTL is not completely dependent of its methyltransferase activity.

STAT3 signaling pathway is hyperactive in various cancer types including ENKTL [21]. Gene expression profiling has revealed that members of the STAT3 pathway are differentially expressed in ENKTL tumor cells compared with normal NK cells [22,35]. STAT3 activation often results from constitutive JAK3 phosphorylation at Tyr980. A recent study reported that EZH2 binds to and methylases STAT3, enhancing STAT3 activity in glioblastoma stem–like cells [34]. An analysis of the EZH2 promoter in the National Center for Biotechnology Information database identified that EZH2 contained 3 conserved STAT3-binding sites. Chromatin immunoprecipitation–polymerase chain reaction analysis also revealed that STAT3 signaling enhances EZH2 promoter activity in gastric cancer cells [17]. Furthermore, Yan et al [23] demonstrated that JAK3 phosphorylates EZH2, altering EZH2 activity and promoting the survival and proliferation of NK/T-cell lymphoma cells. All of the above studies are suggestive of potential interaction between EZH2 and STAT3. Tofacitinib, a JAK3 inhibitor, was reported to be able to induce G1 cell-cycle arrest and inhibited cell growth in Epstein-Barr virus–positive T- and NK-cell lines [36]. According to our data, tofacitinib not only decreased the expression of p-STAT3 but also decreased EZH2 expression and increased H3K27me3 expression. Thus, the JAK/STAT3 pathway may be an upstream signaling pathway controlling EZH2 and H3K27me3 expression in malignant NK/T cells [37].

DZNep is a PRC2 inhibitor that inhibits *S*-adenosylhomocysteine hydrolase, resulting in cellular accumulation of *S*-

adenosylhomocysteine. *S*-adenosylhomocysteine is a competitive inhibitor of methyl donor for methyltransferases [38]. DZNep targets EZH2 by reduction in the level of the enzyme H3K27me3 and by induction of apoptosis in various tumor cells [39,40]. We found that DZNep significantly reduced EZH2, inhibited growth of NK tumor cells, and induced apoptosis of tumor cells. Currently, EZH2 inhibitors are being investigated in clinical trials for B-cell lymphomas [41], Therefore, targeting EZH2 may have potential therapeutic value in clinical strategies of this lymphoma.

In conclusion, our study identified aberrant differential expression of EZH2 and H3K27me3 in ENKTL, which is associated with disease progression and prognosis, and suggested that targeting EZH2 may have therapeutic usefulness in management of this lymphoma.

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