INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common category of non-Hodgkin lymphoma (NHL), accounting for approximately 30% of NHL cases worldwide.\(^1\)\(^-\)\(^3\) DLBCL is more prevalent in elderly patients and tumor mass growing one or more lymph nodes and extranodal sites.\(^2\)\(^,\)\(^4\)\(^,\)\(^5\) The high incidence rate and heterogeneity of DLBCL have attracted extensive attention.\(^6\)\(^-\)\(^8\) Hence, it is important to reveal the underlying molecular mechanism of the origins and development of DLBCL for medical diagnosis and treatment.

USP21, a member of the ubiquitin-specific protease (USP) family, plays particularly crucial roles in regulating cellular signaling and disease development.\(^9\)\(^-\)\(^12\) USP21 is not only localized at microtubule to regulate microtubule dynamics but also posit on centriole to regulate primary cilium formation.\(^13\)\(^-\)\(^17\) Moreover, USP21 plays a vital role in stem cell differentiation by regulating the polyubiquitination of Nanog.\(^18\)\(^,\)\(^19\) USP21 can modulate cell cycle progression by deubiquitinating Forkhead box M1 (FOXM1) in basal-like breast cancer.\(^20\) However, the role of USP21 in DLBCL development has not been clarified.

In our study, we find that the USP21 mRNA level is enriched in DLBCL patients, which suggests that USP21 was associated with DLBCL growth. USP21 knockdown or overexpression in the DLBCL cell line shows that USP21 promotes cell proliferation. Furthermore, we then identify USP21 modulates the protein level of EZH2, a key regulatory gene of DLBCL growth.
MATERIALS AND METHODS

2.1 | Cell culture

The DLBCL cell line SU-DHL-4 and A20 were acquired from the American Tissue Culture Collection (ATCC). The cells were grown in RPMI 1640 medium (BI) supplemented with 10% fetal bovine serum (BI) and 100 U/ml Penicillin/Streptomycin (Sigma-Aldrich) at 37°C in a 5% CO₂ humidified atmosphere.

2.2 | Plasmid and cell transfection

Full-length USP21 was amplified by PCR from the cDNA library and cloned into pcDNA3.1 or pEGFP-N1 vectors. The USP21 siRNAs (#1: 5′-GCUAGAAGAACCUGAGUUA-3′; #2: 5′-GAGCUGUCUUCCAGAAAUA-3′) were synthesized by RiboBio. Cell transfection was performed as previously described.

2.3 | CCK-8 assay

Cell counting kit-8 (CCK-8) assay was used to measure cell viability and was performed as previously described. Cells were inoculated into a 96-well plate, with 1 × 10⁴ cells in each well, after transfection for 48 h. 10 μl CCK-8 was added into the well, and then, cells were incubated for 2 h at 37°C. Then, OD values at 450 nm and 630 nm of the cells were detected by a microplate reader.

2.4 | Cell death detection

Generally, PI can enter into the dead cells, but cannot enter into the viable cells. After 48 h of cell transfection, the cells were inoculated into 96 well plates. PI (10 μg/ml) was then added into the wells, and cells were incubated for 10 min at 37°C. All images were captured by Leica Inverted Microscope.

2.5 | Real-time PCR

Total RNA was harvested and isolated from DLBCL patient tissue using TRIzol reagent (Invitrogen). Reverse transcription was performed as previously described. All patients were informed that their tissue sample would be used for research before this study was carried out. RNA was reverse transcribed to synthesize cDNA using a reverse transcription kit (Takara). The relative expression of USP21 mRNA was normalized against GAPDH using SYBR Premix Ex Taq enzyme (Thermo Fisher Scientific) and LightCycler® 480 Instrument II (Roche Life Science). The primer sequences were listed as follows: USP21 forward primer: 5′-TCGTCCGCTGGATCTGA-3′; GAPDH reverse primer: 5′-CACCACCTTCTTGAATGTCATCATA-3′.

2.6 | Western blot

Western blot was performed as previously described. Total protein was harvested and extracted from cells using cell lysis buffer (1 mM EDTA, 150 mM NaCl, 50 mM Tris-HCL, 1% NP-40, 3% glycerol). The protein was separated by sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Millipore). After blocking for 1 h with 5% skim milk in TBST buffer, the membranes were incubated with indicated primary antibodies at 4°C overnight or 1 h at room temperature and then probed with HRP-conjugated secondary antibodies. Chemiluminescence detection was performed with ECL reagents (Millipore).

2.7 | Statistical analysis

All statistical analyses were performed with the GraphPad Prism 8 statistical software, and the statistical significance was analyzed by Student’s t test. The data were represented as the mean ± SEM, and the difference was considered significant at *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

RESULTS

3.1 | USP21 is enriched in DLBCL tissues and cell lines

To explore the effect of USP21 on DLBCL, we firstly investigated the Gene Expression Profiling Interactive Analysis (GEPIA) database and found that USP21 is highly expressed in tumor samples compared with paired normal tissues (http://gepia.cancer-pku.cn/detail.php?gene=USP21) (Figure 1A). Furthermore, the survival curve of the patients with high expression of USP21 or low expression of USP21 was examined, which shows high expression of USP21 correlated well with the low survival probability of the patients (Figure 1B). Then, we examined the mRNA expression level of USP21 in DLBCL tumor tissues, which was identified higher than adjacent normal tissues (Figure 1C). Consistently, the expression level of USP21 protein in DLBCL was higher than other normal cells (Figure 1D). Thus, these results illustrated that USP21 is enriched in DLBCL.

3.2 | USP21 knockdown restrains cell proliferation in DLBCL cells

Then, we induced depletion of USP21 using siRNA to explore the role of USP21 in tumor progression of DLBCL (Figure 2A). USP21
knockdown dramatically decreased cell proliferation rate examined by CCK8 assay (Figure 2B). To further illustrate whether USP21 affects cell death, we examined the percentage of dead cells (PI-positive) (Figure 2C). USP21 depletion did not interfere with cell death compared to control siRNA (Figure 2D). Therefore, USP21 stimulates cell proliferation but cannot cause cell death.
FIGURE 2. USP21 knockdown restraints cell proliferation. (A) Western blots show USP21 protein expression in SU-DHL-4 cells after transfection with control or USP21 siRNAs for 48 h. (B) Cell proliferation of SU-DHL-4 cells transfected with USP21 siRNA were measured by CCK8 assay. The siRNA was scaled down to siUSP21#1 only. (C) Representative images of Propidium Iodide (PI)-positive cells (Red) representing dead cells and DIC representing total cells. Scale bar, 100 μm. The siRNA was scaled down to siUSP21#1 only. (D) Quantification the number of dead cells. n.s., not significant; **p < 0.01; ***p < 0.001. Error bars indicate ±SEM.

FIGURE 3. USP21 promotes cell proliferation via Cys-221. (A) Overexpression of GFP-Vector, GFP-USP21, and GFP-USP21-C221A in SU-DHL-4 cells. (B) Cell proliferation of SU-DHL-4 cells transfected with GFP-Vector, GFP-USP21, and GFP-USP21-C221A were measured by CCK8 assay. (C) Representative images of Propidium Iodide (PI)-positive cells (Red) representing dead cells and DIC representing total cells. Scale bar, 100 μm. (D) Quantification of dead cells after transfection with GFP-Vector, GFP-USP21, or GFP-USP21-C221A. **p < 0.001, ****p < 0.0001. Error bars indicate ±SEM.
3.3 | The deubiquitinating activity of USP21 is required for DLBCL cell proliferation

USP21 acts as an efficient deubiquitinating enzyme. To examine the deubiquitinating activity of USP21 on DLBCL growth, we overexpressed USP21 wild-type or catalytically inactive mutant C221A (CA) in SU-DHL-4 cells (Figure 3A). We found that overexpression of USP21 dramatically increased cell proliferation, while USP21-CA could not (Figure 3B). In accordance with the USP21 knockdown results, there is no difference between USP21 expression and USP21-CA expression in inducing cell death (Figure 3C,D). These data demonstrate that USP21 promotes cell proliferation via its deubiquitinating active site.

3.4 | USP21 regulates the DLBCL cells proliferation by maintaining EZH2

Previous studies illustrated that EZH2 was necessary for the DLBCL development.28,29 To correlate USP21 with EZH2 in DLBCL, we overexpressed USP21 in SU-DHL-4 cells and found that the protein level of EZH2 was increased upon USP21 overexpression (Figure 4A,B). In contrast, the EZH2 protein level was decreased in USP21-depleted cells (Figure 4C,D). Therefore, these results demonstrated that USP21 can maintain the protein level of EZH2 in DLBCL cells.

4 | DISCUSSION

DLBCL is one of the common categories of NHL, which is a hazard for human health.30 According to relevant literature, there are about 25,000 new cases of DLBCL diagnosed in China each year, which accounts for 11–13% of diagnosed malignancies.31,32 Therefore, novel therapeutic targets are urgently needed for DLBCL patients in China. Here, we find that the USP21 mRNA level is enriched in DLBCL patient lymphoid tissue, in accordance with the data in the GEPIA database. Besides, the USP21 protein expression level in DLBCL cell lines is identified to be higher than that in normal cell lines, which verifies the mRNA result in the patient.

Ubiquitination acts as one of the post translational modifications of protein, which eliminates misfolded proteins and regulates different signaling pathways and cell functions.33–35 The ubiquitination level of protein relies on the synergistical effects of its ubiquitinases and deubiquitinases.36,37 In recent years, an increasing number of studies have reported that the deubiquitinating enzymes (DUBs) play important roles in cancer development and therapeutic resistance.38–41 For example, USP15 upregulates the TGF-β pathway to promote cell proliferation in glioblastoma pathogenesis.42 Kruppel-like factor 5 (KLF5) is stabilized by the DUB BRCA1-associated Protein 1 (BAP1), promoting breast cancer cell proliferation.43 The proteasomal cysteine deubiquitinase inhibitor b-AP15 inhibits proteasome DUB activities and induces cell apoptosis in DLBCL.30

However, the role of specific DUB in DLBCL development is rarely reported. We identify that knockdown of USP21 inhibits cell proliferation, and overexpression of USP21 promotes cell growth. Moreover, USP21-induced oncogenesis is dependent on its deubiquitinating activity.

EZH2, the key regulatory protein in DLBCL, modulates cell proliferation and other cell functions.28,44 GSK126, the specific inhibitor of EZH2, can silence transcription through trimethylation of histone H3 lysine 27 (H3K27me3) to depress cancer cells proliferation.45 USP21 could suppress EZH2 ubiquitination and thus promotes cell proliferation and metastasis in bladder carcinoma.11 In our study, we identified that USP21 can maintain the EZH2 protein level in DLBCL cells, which may due to deubiquitination and protein stabilization of EZH2 by USP21. Therefore, USP21 may be supposed to modulate DLBCL oncogenesis through this underlying mechanism.

FIGURE 4 USP21 maintains EZH2 protein levels in SU-DHL-4 cells. (A) Western blots show EZH2 expression level in SU-DHL-4 cells after transfection with GFP-Vector or GFP-USP21 for 48 h. (B) Quantification of the EZH2 protein level upon overexpression of GFP-Vector or GFP-USP21. (C) Western blots show EZH2 expression level in SU-DHL-4 cells after transfection with control or siUSP21 for 48 h. (D) Quantification of the EZH2 protein level upon transfection with control or siUSP21. **p < 0.01; ***p < 0.001. Error bars indicate ±SEM.
In conclusion, USP21 is enriched in DLBCL and is beneficial for tumor cell proliferation. Mechanistically, USP21 promotes cell growth by maintaining EZH2 protein level. Thus, we propose the model that USP21 and its downstream target EZH2 should be novel chemotherapeutic targets for DLBCL treatment (Figure 5).

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CONFLICT OF INTEREST
The authors declare no competing financial interests.

AUTHOR CONTRIBUTION
M.H., L.X., Z.P., and H.N. performed the experiments; M.H., L.X., Z.P., H.N., L.M., and X.W. analyzed the data; M.H., J.Z., L.M., and X.W. conceived and designed the experiments; and M.H. and X.W. wrote the article.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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