Clinical significance and functional validation of inorganic pyrophosphatase in diffuse large B cell lymphoma in humans

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Abstract Diffuse large B-cell lymphoma (DLBCL), the most common type of non-Hodgkin's lymphoma, displays marked heterogeneity. Although it is usually curable, 30–40% of patients die within 1–2 years due to refractory treatment or cancer relapse. In different types of cancer in humans, inorganic pyrophosphatase (PPA1) is deregulated, thereby contributing to tumorigenesis by supplying the tumor with an enormous energy source. However, the role of PPA1 in DLBCL is still unclear. Here, we analyzed PPA1 in 65 patients with DLBCL and 20 patients with reactive hyperplasia of the lymph nodes (control). The PPA1 level was significantly higher in patients with DLBCL than in control subjects ($p < 0.05$), and it is closely associated with B symptoms (i.e., fever, night sweats, and weight loss) and the IPI score ($p < 0.05$). Furthermore, PPA1 mRNA and protein levels were higher in most DLBCL cell lines than in the control HMy2.CIR cell line. Lastly, we investigated the effects of PPA1 knock-down on the proliferation and survival of the DLBCL

cell line. We found that p53 and p21 expression decreased in PPA1-silenced DLBCL cells. In addition, cell proliferation decreased and cell apoptosis increased. In conclusion, PPA1 is a novel molecule that may be useful in the development and prognosis of DLBCL in the future.

Keywords Diffuse large B-cell lymphoma · Inorganic pyrophosphatase · Prognosis · Proliferation

Introduction

Diffuse large B-cell lymphoma (DLBCL), the most common type of non-Hodgkin's lymphoma which accounts for 40% of its total cases (Coiffier 2005; Smith et al. 2010), displays a marked heterogeneity with respect to different clinical outcomes, as well as various histological, immunophenotypic, and molecular features (Harris et al. 1994; De Paepe and De Wolf-Peters 2007). Since the introduction of rituximab into the chemotherapeutic cocktail of cyclophosphamid, doxorubicin, vincristine and prednisolone (R-CHOP), the overall survival rates of these patients has drastically improved (Sehn et al. 2005; Perry et al. 2014; Sehn and Gascoyne 2015), although 30–40% of patients still die of the disease within 1–2 years (Akkaya et al. 2016). Furthermore, patients with a high-risk International Prognostic Index (IPI) have a 5-year progression-free-survival rate of only 40% (Micallef et al. 2006; Tay et al. 2011). Although gene expression profiling studies have provided new

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insights on the two main types of DLBCL, namely, germinal center B-cell (GCB) and non-germinal center B-cell (no-GCB), which has helped to predict patient response to therapy (Alizadeh et al. 2000; Hans et al. 2004; Berglund et al. 2005), its diagnosis and prognosis remain a challenge. Thus, identifying new molecular biomarkers may lead to the development of new therapies.

The altered metabolism of cells is a hallmark of cancer cells, because metabolic alterations are needed for their high proliferation rate (Cerella et al. 2013; Mishra et al. 2015). Inorganic pyrophosphatase (PPA1), a member of the inorganic pyrophosphatase (PPase) family, is an enzyme which catalyzes the hydrolysis of inorganic pyrophosphate (PPi) into two inorganic phosphates (Pi). Thus, it has critical roles in various metabolic reactions due to its highly exergonic catalytic functions (Baykov et al. 1999; Yi et al. 2012), such as in the synthesis of nucleic acids, proteins and carbohydrates, lipid metabolism and neurite growth (Fairchild and Patejunas 1999; Curbo et al. 2006; Polewski et al. 2010; Tezuka et al. 2013). The dysregulation of PPi metabolism has been associated with disease. For example, abnormal PPA1 expression has been described in many types of solid cancer in humans (Jeong et al. 2012; Yi et al. 2012; Bodnar et al. 2016; Luo et al. 2016). However, the significance of PPA1 expression in patients with DLBCL, as well as its correlation with the clinical characteristics and development of the disease, is still unclear.

In this study, we investigated the expression of PPA1 in DLBCL patients samples and cell lines, and we found that PPA1 was highly expressed in tissues of patients with DLBCL and several representative DLBCL cell lines. Moreover, PPA1 expression was associated with B symptoms, the IPI score. In addition, PPA1 knockdown by RNA interference decreased DLBCL cell proliferation and promoted apoptosis, which were regulated by the p53 and p21 signaling pathways. Our results may provide novel targets to DLBCL pathogenesis, diagnosis and treatment.

Materials and methods

Patients and tissue samples

A cohort of 85 patients, including 65 patients with DLBCL and 20 patients with reactive hyperplasia of

the lymph nodes, were recruited from the Department of Pathology at the Inner Mongolia People's Hospital between 2013 and 2015. Informed written consent was obtained from all patients, and the experimental protocol was approved by the Institutional Ethics Committee of the Inner Mongolia People's Hospital. All patients were diagnosed with DLBCL according to 2008 World Health Organization criteria. DLBCL was further sub-classified as either GCB or non-GCB on the basis of an immunohistochemical panel (Hans et al. 2004).

Immunohistochemistry

Immunohistochemistry was performed as previously described (Luo et al. 2016). Cross-sections of paraffin-embedded tissues were stained with an anti-PPA1 monoclonal mouse antibody (1:200 dilution; Lot:EB061-3B2, Sigma, St. Louis, MO, USA). Secondary staining was performed with HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:200). Images were acquired with a BX51 research microscope (Olympus, Co., Tokyo, Japan) using 20× and 40× objectives. The staining was evaluated by scoring positively-stained cells as 1 (no staining), 2 (light staining), 3 (moderate staining) or 4 (dark staining). Thereafter, the mean percentage of positive cells was scored as $0 \leq 25\%$, $1 = 26\text{--}50\%$, $2 = 51\text{--}75\%$, $3 = 76\text{--}100\%$. The staining results were calculated by multiplying the two scores and classified as weak (1–5) or strong (≥ 6).

Cell lines and cell culture

The B-cell line HMy2.CIR was obtained from the Cell Resources Center for the Biological Sciences at the Chinese Academy of Sciences (Shanghai, China) and cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA) and 100 U/ml penicillin–streptomycin (100× solution, Invitrogen, Carlsbad, CA, USA; No. 15070-63). The DLBCL cell lines SU-DHL-2 and SU-DHL-8 (ABC type) were purchased from American Type Culture Collection (Manassas, VA, USA). The other three DLBCL cell lines OCI-Ly19, SU-DHL-4 and VAL (GCB type) were kindly provided by Dr. Jun Cheng (Tianjin Medical University Cancer Institute and Hospital,

Tianjin, China). The DLBCL cell lines were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) Medium supplemented with 10 or 20% FBS (OCI-Ly19) (GIBCO) and 100 U/ml penicillin–streptomycin.

RNA isolation, RT-PCR and real-time PCR

Total RNA was isolated from HMy2.CIR and DLBCL cell lines using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. Real-time PCR was performed in triplicate using Platinum 1 SYBR 1 Green qPCR SuperMix-UDG (Invitrogen). The reaction consisted of 40–45 cycles at 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 10 s. The primers used for RT-PCR and real-time PCR were synthesized as follows:

Control 5'-GGCATCCACGAACTACCTT-3'
(sense primer) and 5'-
CTCGTCATACTCCTGCTTGC-3'
(antisense primer)
PPA1 5'-CGCTATGTTGCGAATTTGTTC-3'
(sense primer) and 5'-
CCAGTATGTTTATCATTGTGCC-3'
(antisense primer)

Protein extraction and western blotting analysis

HMy2.CIR and DLBCL cells were lysed in ice-cold radioimmunoprecipitation assay buffer in the presence of a protease inhibitor cocktail. The protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Western blotting analysis was carried out as previously described (Zhang et al. 2016), and anti-Ki67, anti-caspase3, anti-p53, anti-p21, anti-β-actin (Santa Cruz Biotechnology Inc.), and anti-PPA1 (Sigma) primary antibodies were used. Goat anti-mouse or anti-rabbit secondary antibodies were conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Immunoreactive proteins were detected with chemiluminescent reagents (Millipore, Billerica, MA, USA). The results were visualized using a chemiluminescent HRP substrate kit (Millipore) and analyzed using Image J software (National Institutes of Health, Baltimore, MD, USA). The densitometry results were first normalized with that of β-actin and then compared with the control to obtain relative fold changes. The mean value for each blot was averaged from three

independent experiments and indicated at the bottom of the blot.

RNA interference

Human DLBCL cells were transiently transfected with PPA1 siRNA, a scrambled control and NControl-CY3. Approximately 1×10^6 DLBCL cells were plated in a 12-well plate and cultured in antibiotic-free RPMI-1640 Medium supplemented with 2% FBS and then transfected with LipofectamineTM Transfection Reagent (Invitrogen) according to the manufacturer's instructions. The cells were lysed at 48 h after transfection, and protein levels were determined by western blotting analysis at 48 h after transfection. The efficiency of PPA1 knockdown was assessed by RT-PCR and real-time PCR. Proliferation and apoptosis were assessed in PPA1-silenced cells at 24 and 48 h after transfection, respectively. The siRNA sequences are as follows:

PPA1-siRNA-1 GCACAATGATAAACATACT
PPA1-siRNA-2 GGAAAGTCATTGCCATTAA

Cell proliferation assay

The CCK-8 Cell Proliferation Assay was carried out according to the manufacturer's instructions (CCK-8, Dojindo Laboratories, Shanghai, China). Approximately 2×10^3 cells were seeded in a 96-well plate, cultured in antibiotic-free RPMI-1640 Medium supplemented with 2% FBS and then transfected with the PPA1 or scrambled siRNA. Approximately 72 h after transfection, 10 μL of the kit's reagent was added to each well, and the cells were incubated for 2 h at 37 °C. The optical density was measured at a wavelength of 450 nm using multiscanner autoreader (Thermo Electron Corp, Waltham, MA, USA).

Apoptosis assay

Apoptotic cells were stained with propidium iodide and Annexin V-fluorescein isothiocyanate (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Flow cytometry (BD Biosciences) was performed to detect cells positively-stained for annexin-V. This assay was performed three independent times, and data were expressed as means ± standard deviation (SD).

Fig. 1 PPA1 expression in patients with DLBCL and various cell lines. **a** Relative PPA1 expression in various tissues, including reactive hyperplasia of the lymph nodes, from patients with DLBCL as determined by immunohistochemistry. Scale bar: 50 μ m. **b** Relative PPA1 mRNA expression in HMV2.CIR and DLBCL cell lines including two ABC type DLBCL cells (SU-DHL-2, SU-DHL-8) and three GCB type DLBCL cells (OCI-Ly19, SU-DHL-4 and VAL) as measured by real-time PCR. **c** Relative PPA1 protein expression in HMV2.CIR and DLBCL cell lines as determined by western blotting. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Statistical analyses

Statistical analyses were performed using SPSS 13.0 Software (Chicago, IL, USA). All data are presented as means $\pm$ SEM. The differences in PPA1 expression between the groups were analyzed by Fisher's exact test. The correlations between the clinicopathological factors of DLBCL and PPA1 expression were examined by Pearson's Chi squared test. A value of $p < 0.05$ was used as the criterion for statistical significance. *Indicates significant difference with $p < 0.05$, **indicates significant difference with $p < 0.01$, ***indicates significant difference with $p < 0.001$.

Results

Patient characteristics and PPA1 expression

We investigated the PPA1 level in cross-sections of paraffin-embedded tissues from 65 patients with DLBCL and 20 patients with reactive hyperplasia of the lymph nodes (control). The PPA1 level was significantly higher in patients with DLBCL than in control subjects ($p < 0.05$, Fig. 1a), and the PPA1 level was high (38.5%) in 25 out of 65 patients with

DLBCL. The PPA1 level was then examined in the control HMV2.CIR cell line and five DLBCL cell lines, two of which were ABC type derived, (SU-DHL-2, SU-DHL-8), and the other three were GCB type derived (OCI-Ly19, SU-DHL-4 and VAL). Although highly heterogeneous, PPA1 expression was elevated in most DLBCL cell lines at both mRNA and protein levels (Fig. 1b, c), in agreement with previously published data (Jeong et al. 2012; Yi et al. 2012; Bodnar et al. 2016; Luo et al. 2016). These results suggest that PPA1 has a tumor-facilitating role.

The correlations between the clinicopathological features of DLBCL and PPA1 expression are described in Table 1. Elevated PPA1 expression closely associated with B symptoms and the IPI score ($p < 0.05$). However, there were no correlations between the other characteristics, such as subtype, age, sex, Ann Arbor stages, β 2-macroglobulin concentration, extra nodal status and Ki-67 expression ($p > 0.05$).

PPA1 knockdown induces DLBCL cell apoptosis

To understand the role of PPA1 in the pathogenesis of DLBCL, we assessed the effects of PPA1 on DLBCL cell apoptosis using PI-Annexin V staining. The PPA1 protein levels were measured by western blotting. The results showed that PPA1 level was decreased in PPA1-

Table 1 PPA1 expression and clinicopathological features in DLBCL patients

Characteristics	N (n = 65)	PPA1 expression levels		<i>p</i> value
		Weak (n = 40)	Strong (n = 25)	
Types				<i>p</i> = 0.783
ABC	35	21	14	
GCB	30	19	11	
Age (years)				<i>p</i> = 0.969
≥ 60	31	19	12	
< 60	34	21	13	
Gender				<i>p</i> = 0.842
Male	38	23	15	
Female	27	17	10	
Stage				<i>p</i> = 0.811
I/II	21	14	7	
III/IV	44	28	16	
B symptoms				<i>p</i> = 0.001
Absent	37	29	8	
Present	28	11	17	
IPI score				<i>p</i> = 0.016
0–2	25	20	5	
3–5	40	20	20	
Presentation				<i>p</i> = 0.603
Nodal	26	17	9	
Extranodal	39	23	16	
β2-MG				<i>p</i> = 0.698
Normal	19	11	8	
Elevated	46	29	17	
Ki-67				<i>p</i> = 0.927
≥ 80%	49	30	19	
< 80%	16	10	6	

silenced cells in comparison to control cells (Fig. 2a). FACS analysis of PI-Annexin V stained cells showed the percentage of apoptotic cells including early apoptotic cells, late apoptotic cells as well as viable non-apoptotic cells and necrotic cells, respectively, which indicates that suppression of PPA1 induced apoptosis in the DLBCL cells (Fig. 2b). Meanwhile, the protein levels of p53 and cleaved caspase-3 were higher in silenced cells than in control cells (Fig. 2a). These results indicate that PPA1 plays an important role in the regulation of DLBCL cell apoptosis.

PPA1 knockdown decreases DLBCL cell viability

To understand the role of PPA1 in cell proliferation, a cell viability assay was carried out using CCK-8. As

shown in Fig. 3A, cell viability decreased in PPA1-silenced cells compared to control cells at 72 h after transfection. Furthermore, p21 protein levels were higher in silenced cells than in control cells (Fig. 3a), whereas the Ki-67 protein level was lower in silenced cells than in control cells (Fig. 3b). Taken together, these results indicate that PPA1 enhances DLBCL cell proliferation.

Discussion

Studies have shown that abnormalities in gene expression and cell metabolism play a critical role in carcinogenesis. The identification and characterization of the deregulated genes may contribute to our

Fig. 2 PPA1 knockdown promotes apoptosis in the DLBCL cell line. **a** The western blot showed depletion of PPA1 in DLBCL cells treated with siRNA targeted to human PPA1 compared with the scrambled control siRNA. The expression of P53 and cleaved caspase-3 in DLBCL cells with PPA1 knockdown was also detected, β-actin was used as loading control. The statistical analysis of the relative expression of P53 and cleaved caspase-3 is also shown. **b** Representative apoptosis

analysis using PI-Annexin-V staining and flow cytometry of DLBCL cells treated with siRNA (si-1 (PPA1-siRNA-1), si-2 (PPA1-siRNA-2) targeted to human PPA1 and targeted to scramble control for 48 h. The statistical results of the percentage of apoptotic cells including early apoptotic cells, late apoptotic cells as well as viable non-apoptotic cells and necrotic cells are also shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

understanding of the mechanisms behind cancer progression, which may ultimately improve its diagnosis and treatment (Garnis et al. 2004).

PPA1 is a pyrophosphate hydrolyzing enzyme with an important role in the phosphate metabolism of cells. Studies have reported that PPA1 is upregulated in many types of solid cancer (Chen et al. 2002; Tomonaga et al. 2004), and it closely associates with cancer cell migration, invasion (Jeong et al. 2012) and proliferation (Giri et al. 2014), and it is a negative

prognostic marker in gastric cancer (Yi et al. 2012; Yang et al. 2015), ovarian cancer (Li et al. 2017) and colorectal cancer (Wang et al. 2017). However, little is known about the role of PPA1 in the occurrence and progression of cancer, especially in hematologic tumors.

Currently, gene expression profiling and immunohistochemistry algorithms methods are used to distinguish between GCB and no-GCB of DLBCL for predicting prognosis, our data showed that PPA1 was

Fig. 3 PPA1 knockdown decreases cell viability. **a** Cell proliferation was measured 3 days after transiently transfecting the DLBCL cell line with siRNAs specific to human PPA1 using the CCK-8 Cell Proliferation Kit. The data are presented as means $\pm$ SEM of three independent experiments. **b** The immunoblot shows the expression of p21 and Ki67 protein in DLBCL cells treated with specific PPA1 siRNA and scrambled control siRNA. β -Actin was used as the loading control. The statistical results of the relative expression of P21 and Ki67 are also shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

upregulated in tissues of patients with DLBCL and several representative DLBCL cell lines, there was no significant difference in PPA1 expression between GCB and no-GCB groups, which indicates that PPA1 dysregulation plays an important role in DLBCL. This is inconsistent with previous studies (Chen et al. 2002; Tomonaga et al. 2004). Furthermore, its overexpression is associated with B symptoms and the IPI score, indicating that it may be a novel negative prognostic molecule in routine clinical practice.

To investigate the role of PPA1 in patients with DLBCL, RNA interference was used. PPA1 knockdown significantly decreased DLBCL cell proliferation, possibly through the activation of JNK and the phosphorylation of p53 and p21. In addition, the decreased PPA1 level upregulated the cleaved caspase-3 level, revealing its potential role in the regulation of apoptosis. In agreement with our results, Luo et al. showed that PPA1 loss can induce apoptosis in various cancer cells (Luo et al. 2016). Meanwhile, Fu et al. showed that PPA1 can promote colon cancer cell proliferation and survival through the JNK signaling pathway (Wang et al. 2017). Therefore, PPA1 is a novel molecule that may be useful in the pathogenesis,

diagnosis and treatment of DLBCL in the future. Unfortunately, this study was comprised of a few cases recruited from a single center; thus, further studies are needed to validate our results and the role of PPA1 in DLBCL development, as well as to investigate the potential use of PPA1 as a therapeutic target.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest.

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