The role of aldehyde dehydrogenase 1A1 in B-cell non-Hodgkin's lymphoma

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Abstract. Previously we showed that aldehyde dehydrogenase 1A1 (ALDH1A1) is a new mediator for resistance of DLBCL to CHOP and a facility predictor of clinical prognosis. In the present study, knockdown and inhibitor of ALDH1A1 were applied to identify the role of ALDH1A1 in Raji cells. CCK-8 and clone formation assay were applied to determine the CHOP sensitivity and clone formation ability. Caspase colorimetric assay and Annexin V/FITC staining was performed to determine the degree of apoptosis. Western blot analysis was used to detect the NF-κB/STAT3 signaling proteins and apoptotic-associated proteins. Real-time quantitative PCR (RT-PCR) was used to identify the differential expression of ALDH1A1 between NHL patients and healthy donors. We demonstrated that inhibition of ALDH1A1 increased the sensitivity of Raji cells to CHOP, as indicated by increased cytotoxicity, reduced clonogenicity, activated caspase-3/-9, decreased NF-κB/STAT3 signaling and increased pro-apoptosis signaling, ad increased apoptosis rate. Moreover, we found high ALDH1A1 expression was associated with poor prognosis in NHL patients. Our data revealed the critical role of ALDH1A1 in NHL and provides a theoretical basis for the use of ALDH1A1 inhibitors in NHL patients.

Introduction

Non-Hodgkin's lymphoma (NHL) consists of many histologically and biologically unique lymphoid malignancies (1). In Western countries, 85% of NHL is of B-cell origin (2). Unfortunately, little progress has been made in improving the survival of NHL patients receiving standard therapy, largely due to insensitivity or resistance of the cancer cells to treatment. Many patients have short complete remission or early relapse after treatment, which shortens survival and causes tremendous psychological and physical pain.

Recently, *in vitro* studies have shown that chemotherapy response depends on activation of the apoptosis cascade (3). Moreover, the most important factor in chemotherapy resistance is suppression of the apoptosis pathway, which leads to disease recurrence in NHL patients. Imbalances in apoptosis regulation are associated with the abnormal activation of growth signal transduction pathways, and constitutive activation of these pathways, including NF-κB and STAT3, has been shown to occur in NHL tissue and cell lines. Activation of these pathways is thought to be the major cause of cancer cell resistance to chemotherapeutics via downstream alterations in apoptosis pathway regulation (4,5).

Accumulating data suggest that aldehyde dehydrogenase 1A1 (ALDH1A1) is also involved in the chemotherapy resistance of tumor cells (6-8). ALDH1A1 is overexpressed in a variety of solid tumors and leukemias, and it is a newly discovered cancer stem cell (CSC) marker (9-11). Hodgkin's lymphoma cells with high expression of ALDH1A1 possess the characteristics of stem cells (12). Notably, p-STAT3 plays a role in maintaining CSC characteristics in colon cancer (13), whereas NF-κB plays a similar role in pancreatic cancer (14). Recently, we demonstrated that knockdown or inhibition of ALDH1A1 increases chemosensitivity in diffuse large B-cell lymphoma (DLBCL) Farage cells, potentially via modulation of NF-κB/STAT3 signaling (15); however, in contrast, Fujita *et al* demonstrated by immunohistochemistry that ALDH1 is not expressed in DLBCL (16). Subsequently, we found that ALDH1A1 confers chemoresistance in DLBCL Pfeiffer cells, and that its expression is associated with poor prognosis in DLBCL patients (17).

In the present study, we analyzed *ALDH1A1* expression in human NHL patient samples, and we assessed the relationship between *ALDH1A1* expression and B-cell NHL patient prognosis. Furthermore, we choose the Raji cell line, a Burkitt's lymphoma cell line as a model since Raji cells with mutant p53, constitutively activated NF-κB and increased BCL-2 expression which are commonly present in patients with NHL and are considered a source of chemotherapy failure in patients whose disease are chemoresistant in B-cell NHL (18). we used the Raji cell line to explore the role of ALDH1A1

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in chemotherapy resistance, via modulation of NF-κB/STAT3 signaling and apoptosis, in B-cell NHL.

Patients and methods

Patient characteristics. The samples were obtained from 112 patients treated in the Xiang-Ya Hospital of Central South University (Hunan, China) after being diagnosed with B-cell NHL according to the WHO (2008) classification, and was confirmed by pathological histology, from 2013 to 2014. Indolent lymphoma defined follicular lymphoma, marginal zone lymphoma, mucosa associated lymphoid tissue type, unclassified small B cell lymphoma. Progressive lymphoma contained diffuse large B cells, mantle cells and Burkitt's lymphoma. For comparison, we obtained samples from 24 healthy donors as the normal controls. All patients were enrolled following approval from the Ethics Committee, and they all provided informed consent. From each patient, 3- to 5-ml peripheral blood samples were collected in sterile tubes containing anticoagulant (heparin sodium) before they received treatment. Mononuclear cells (MNCs) were enriched by density centrifugation over Ficoll-Paque (TBD Science, Tianjin, China) and stored at -80˚C.

RNA isolation and real-time PCR. Cells were lysed, and the total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (1 μg) was used in cDNA synthesis. Reverse transcription of RNA was carried out with a PrimeScript^{TM} RT reagent kit (Takara Bio, Inc., Otsu, Japan). Synthesized cDNA was subjected to quantitative real-time (qRT)-PCR for the detection of *ALDH1A1* and *GAPDH* using the SYBR-Green fluorescence-based Assay kit (Takara). The following primers were used: 5'-TGTTAGCTGATGCCGACTTG-3' and 5'-TTC TTAGCCCGCTCAACACT-3' for *ALDH1A1*; and 5'-ACC ACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTG TTGCTGTA-3' for *GAPDH* (Sangon Biotech, Shanghai, China). The following amplification conditions were used: pre-denaturation at 95˚C for 30 sec, 40 cycles of denaturation at 95˚C for 5 sec, annealing at 60˚C for 34 sec, and elongation at 72˚C for 60 sec, and a final extension at 72˚C for 10 min. The relative quantification (RQ) of *ALDH1A1* was calculated based on the threshold cycle (Ct) values as follows: $RQ = 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ = [Ct(*ALDH1A1*) - Ct(*GAPDH*)] sample (patients) - [Ct (*ALDH1A1*) - Ct(*GAPDH*)] sample (healthy controls). The qRT-PCR was performed using the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA).

Cell culture. Raji cells [human Burkitt's lymphoma cell line, ATCC CCL-86) (ATCC; American Type Culture Collection, Manassas, VA, USA)] were grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin and incubated at 37˚C in 5% CO_2.

Chemical treatment and cell survival rate assay. Cells (5,000) were seeded on a 96-well plate in a volume of 100 μl. CHOP treatment was performed using cyclophosphamide, vincristine, adriamycin and prednisone at a clinical ratio of 80/5.5/0.16/11.1, respectively, with dosages ranging from 5-1,280 ng/ml (19). Cytotoxicity was assessed using the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's instructions (Dojindo, Kumamoto, Japan). Forty-eight hours after drug treatment, CCK-8 was added to each well, and the OD at 450 nm of each sample was determined using a microplate spectrophotometer (Bio-Rad, Hercules, CA, USA).

Colony formation assay. Cells were seeded into 6-well culture plates at a density of 1,000 cells/well in triplicate, with 2 ml of methylcellulose (stem cell) mixture containing Dulbecco's modified Eagle's medium (DMEM) and FBS supplemented with 10% fetal bovine serum (FBS), with or without 400 ng/ml CHOP. The numbers of colonies were counted on day 14.

ALDH1A1 knockdown by shRNA. Lentiviral vectors expressing short hairpin RNAs (shRNA) against *ALDH1A1* (GenBank accession no. NM_000689) were obtained from GeneChem Co., Ltd. (Shanghai, China), and synthesized with the following strand sequences: forward, 5'-tcgGGCT AAGAAGTATATCCTTctcgagAAGGATATACTTCTTAGC Ccgttttttc-3' and reverse, 5'-TCGAGAAAAAA cgGGCTA AGAAGTATATCCTTCTCGAGAAGGATATACTTCTTA GCCCGA-3'. The lentivirus was transfected according to the Lentiviral Vector Particle operation manual instructions as previously described (15). Validation of the knockdown was performed at the protein level by western blotting, and at the messenger RNA (mRNA) level by relative qRT-PCR.

Detection of active caspase-3 and -9. Caspase activity was assayed using the Caspase Colorimetric Assay kit (KeyGen Biotech, Nanjing, China) according to the manufacturer's protocol. Briefly, cells were harvested and lysed for 30 min. Then, 50 μl samples were mixed with reaction buffer and the caspase-3/-9 substrate and incubated for 4 h at 37˚C in the dark. The percentage of A405 values for the test samples vs. those for the control samples indicated the percentage of caspase activity.

Flow cytometric assay for apoptosis. Cell apoptosis was assayed using the Annexin V/FITC apoptosis detection kit (Beijing Biosea Biotechnology Co., Ltd., Beijing, China) according to the manufacturer's protocol. Data acquisition and analysis were performed using a flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Western blotting. Western blotting was carried out as previously described (15). Total protein (20 μg) was loaded per well. The following antibodies and dilutions were used: anti-ALDH1A1 (1:500; Abcam, Cambridge, MA, USA), anti-NF-κB (1:1,000), anti-p-NF-κB (1:1,000), anti-STAT3 (1:1,000), anti-p-STAT3 (1:1,000), anti-BCL-2 (1:1,000), anti-BAX (1:1,000), anti-caspase-3 (1:1,000), anti-caspase-9 (1:1,000) (all from Cell Signaling Technology, Inc., Danvers, MA, USA). Anti-GAPDH (1:2,000; Goodhere Biotechnology Co., Ltd., Hangzhou, China) served as a loading control.

Statistical analysis. All data shown represent the results of at least three independent experiments. The calculations were analyzed using the Statistical Package for the Social Sciences (SPSS) software. For analysis of the survival data, the Kaplan-Meier model was applied, and the log-rank test

Table I. Correlation between ALDH1A1 expression and clinicopathological parameters in 112 NHL patients.

	Low expression		High expression			
Clinical features	N	%	N	%	χ^2	P-value
Age (years)					0.801	0.502
≤60	45	52.33	41	47.67		
>60	11	42.31	15	57.69		
Sex					1.885	0.239
Female	24	58.54	17	41.46		
Male	32	45.07	39	54.93		
B symptom					0.237	0.703
Negative	32	51.61	30	48.39		
Positive	23	46.94	26	53.06		
LDH					7.009	0.014
Normal	36	62.07	22	37.93		
High	20	37.04	34	62.96		
PS					7.467	0.011
<2	42	51.22	28	48.78		
≥2	14	33.33	28	66.67		
Ann Arbor stage					14.756	0.000
I-II	33	71.74	13	28.26		
III-IV	23	34.85	43	65.15		
IPI score					14.583	0.000
0-2	42	65.63	22	34.36		
3-5	14	29.17	34	70.84		
Lymphoma category					12.341	0.001
Indolent	30	71.43	12	28.57		
Progressive	26	37.14	44	62.86		

χ^2 test was used to compare the distribution of clinical features between ALDH1A1 low and high level expression groups. A P-value of <0.05 was considered significant. Low ALDH1A1 was <0.326, high ALDH1A1 was >0.326. ALDH1A1, aldehyde dehydrogenase 1A1; NHL, Non-Hodgkin's lymphoma; LDH, lactate dehydrogenase; PS, performance status; IPI, International Prognostic Index. Indolent lymphoma contained follicular lymphoma (n=18), marginal zone lymphoma (n=9), mucosa-associated lymphoid tissue type (n=11), unclassified small B cell lymphoma (n=4). Progressive lymphoma contained diffuse large B cell lymphoma (n=58), mantle cell lymphoma (n=9), Burkitt's lymphoma (n=3).

was performed. The association between *ALDH1A1* expression and clinicopathological features was studied using the χ^2 test. Differences between the results of experimental treatments and the average cloning number were assessed by one-way analysis of variance. Differences were two-tailed and considered significant at values of P<0.05. The diagrams were generated using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

High levels of ALDH1A1 expression are associated with an unfavorable prognosis in NHL patients. Quantitative real-time (qRT)-PCR analysis of *ALDH1A1* expression was conducted in peripheral blood samples from 112 NHL and 24 healthy control patients. The median relative quantification (RQ) values of *ALDH1A1* in NHL and control patients were 0.326 (range, 0.010-5.918) and 0.041 (range, 0.010-0.492), respectively; thus, *ALDH1A1* levels were significantly higher in NHL patients than in controls (P<0.05; Fig. 1A).

Next, we used the median RQ value of *ALDH1A1* to separate the patients into a high *ALDH1A1* group (>0.326) or a low *ALDH1A1* group (<0.326). The 56 patients with high expression had a median *ALDH1A1* RQ of 0.846 (range, 0.336-5.92), whereas those with low expression had a median *ALDH1A1* RQ of 0.148 (range, 0.010-0.316). Baseline *ALDH1A1* levels were correlated with patient lactate dehydrogenase (LDH) levels (P=0.014), performance status (PS) (P=0.011), Ann Arbor stage (P>0.05), International Prognostic Index (IPI) score (P>0.05), and lymphoma category (P=0.001), but not with other factors (Table I). Importantly, patients in the high *ALDH1A1* group showed shorter cumulative survival than those in the low *ALDH1A1* group (P<0.0001; Fig. 1B). The expression of ALDHA1 in indolent lymphoma

Table II. Multivariate analysis of factors contributing to overall survival in NHL patients.

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age, years (<60 vs. ≥60)	1.597 (0.756-3.376)	0.220	-	-
Sex (female vs. male)	1.568 (0.725-3.391)	0.253	-	-
B symptom (negative vs. positive)	1.352 (0.676-2.705)	0.393	-	-
LDH (normal vs. high)	1.670 (0.823-3.387)	0.155	-	-
PS (<2 vs. ≥2)	2.694 (1.334-5.440)	0.006	1.175 (0.520-2.657)	0.698
Ann Arbor stage (I-II vs. III-IV)	2.880 (1.29-6.428)	0.010	0.694 (0.230-2.096)	0.517
IPI score (0-2 vs. 3-5)	5.135 (2.340-11.266)	0.000	3.814 (1.262-11.523)	0.018
Lymphoma category	4.905 (1.719-13.993)	0.003	3.714 (1.254-11.005)	0.018
ALDH1A1	0.196 (0.089-0.431)	0.000	0.393 (0.160-0.968)	0.042

Univariate and multivariate analysis of prognostic factors in 112 NHL patients included in the survival analysis. Statistical analyses were performed by Cox proportional hazards regression. A P-value of <0.05 was considered significant. NHL, Non-Hodgkin's lymphoma; HR, hazard ratio; CI, confidence interval; LDH, lactate dehydrogenase; PS, performance status; IPI, International Prognostic Index.

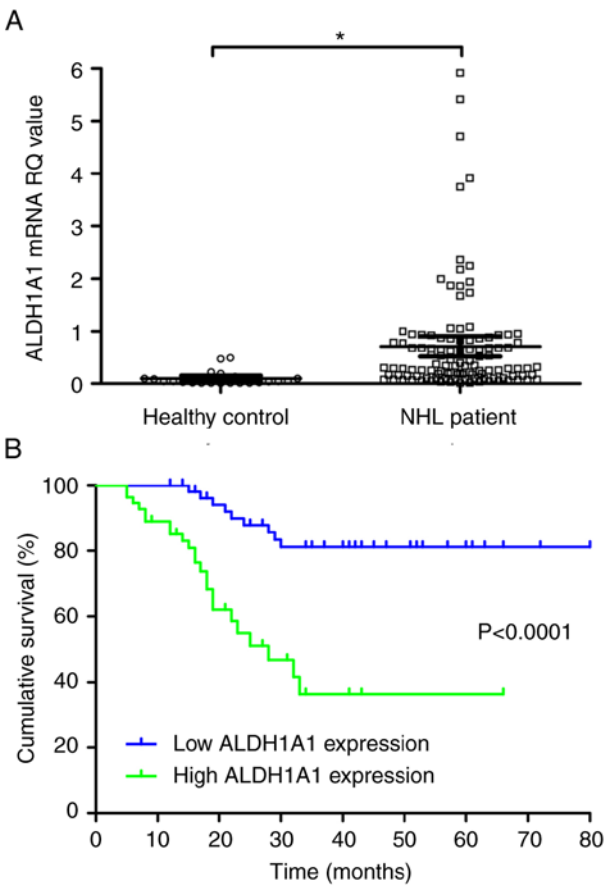

Figure 1. ALDH1A1 mRNA levels were associated with prognosis in NHL patients. (A) ALDH1A1 mRNA levels in healthy controls (n=24), and NHL patients (n=112). The ALDH1A1 mRNA levels of NHL patients was significantly higher than healthy controls. (B) The median value of ALDH1A1 mRNA RQ was used to stratify patients into high and low ALDH1A1 expression groups. The cumulative survival of patients with low ALDH1A1 (<0.326) and high ALDH1A1 (>0.326) was determined by the Kaplan-Meier method.

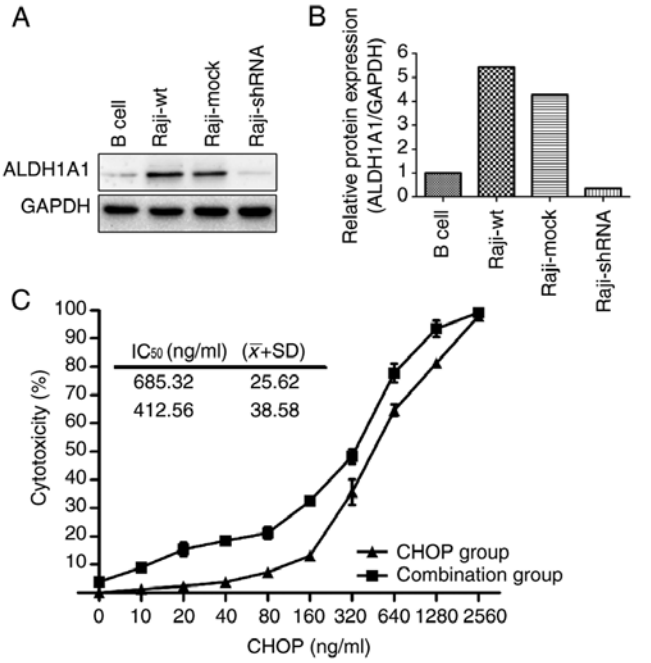

Figure 2. (A and B) ALDH1A1 upregulated expression in Raji cells, shRNA silencing efficiency was confirmed by western blotting. The expression of ALDH1A1 in the Raji cells was upregulated 5.44-fold compared to B-cell. (A and B) The expression of ALDH1A1 in Raji-shRNA decreased 0.085-fold compared to Raji-mock after transfection with lentivirus particle shRNA. The IC_{50} values of CHOP were determined by CCK-8 assay. Cells were treated with increasing concentrations of CHOP for 48 h. The IC_{50} of Raji cells to CHOP regimen decreased from 685±25.62 to 412.56±38.58 ng/ml (P=0.013) using DEAB.

was significantly lower than in progressive lymphoma. Although in the experimental group of 42 cases of indolent lymphoma patient's data showed that ALDH1A1 difference in expression related to difference cumulative survival rate

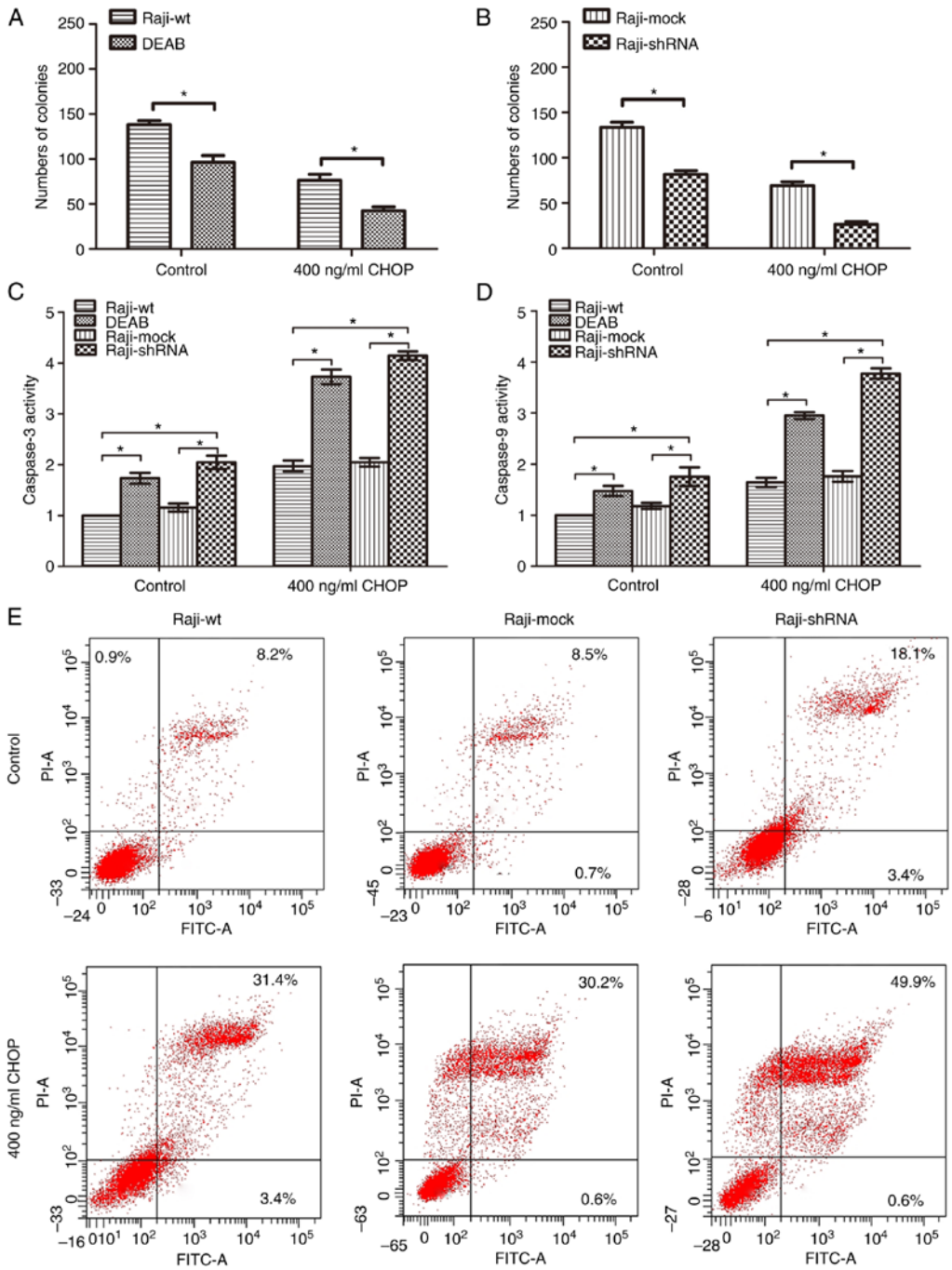

Figure 3. (A) After DEAB treatment, the numbers of colonies were significantly decreased whether adding CHOP or not (P<0.05). (B) ALDH1A1 shRNA Raji cells had a defect in clonogenic capacity compared with the mock-treated cells whether adding CHOP or not (P<0.05). (C) Caspase-3 activity assay showed that knockdown or inhibition of ALDH1A1 significantly increased apoptosis compared with the control or mock groups whether adding CHOP or not (P<0.05). (D) Caspase-9 activity assay showed knockdown or inhibition of ALDH1A1 significantly increased apoptosis compared with the control or mock groups whether adding CHOP or not (P<0.05). Annexin V and PI staining revealed that both knockdown of ALDH1A1 and drug treatments induced apoptosis. The upper left quadrant indicates late apoptosis, the lower left quadrant indicates early apoptosis. The apoptosis cells contained the early apoptosis and late apoptosis cells. The apoptosis rate of shRNA group was distinctly increased compared with wt or mock group (18.3±0.6 vs. 8.7±0.7/8.4±0.8%; P<0.01). (E) Moreover, shRNA group was significantly increased and the apoptosis rate after 400 ng/ml CHOP treatment for 24 h (50.1±1.1 vs. 18.3±0.6%; P<0.01).

(P=0.031), but in multivariate analysis ALDH1A1 was not an independent prognostic indicator (P=0.053). The number of indolent lymphoma patients was too small to get a convinced conclusion. Whether ALDH1A1 was also suitable for inactive lymphoma is unknown and required more evidence to validate it. Moreover, among all NHL patients, IPI score, lymphoma category and *ALDH1A1* levels were independent prognostic indicators (Table II).

Inhibition of ALDH1A1 resensitizes Raji cells to the CHOP regimen. Next, we performed western blot analysis and showed that ALDH1A1 expression was higher in human Burkitt's lymphoma Raji cells than in human B-cells (negative control) (Fig. 2A and B). To determine whether ALDH1A1 mediates resistance to CHOP chemotherapy in Raji cells, the ALDH1A1 inhibitor DEAB was applied to Raji cells in combination with CHOP treatment, and the resulting

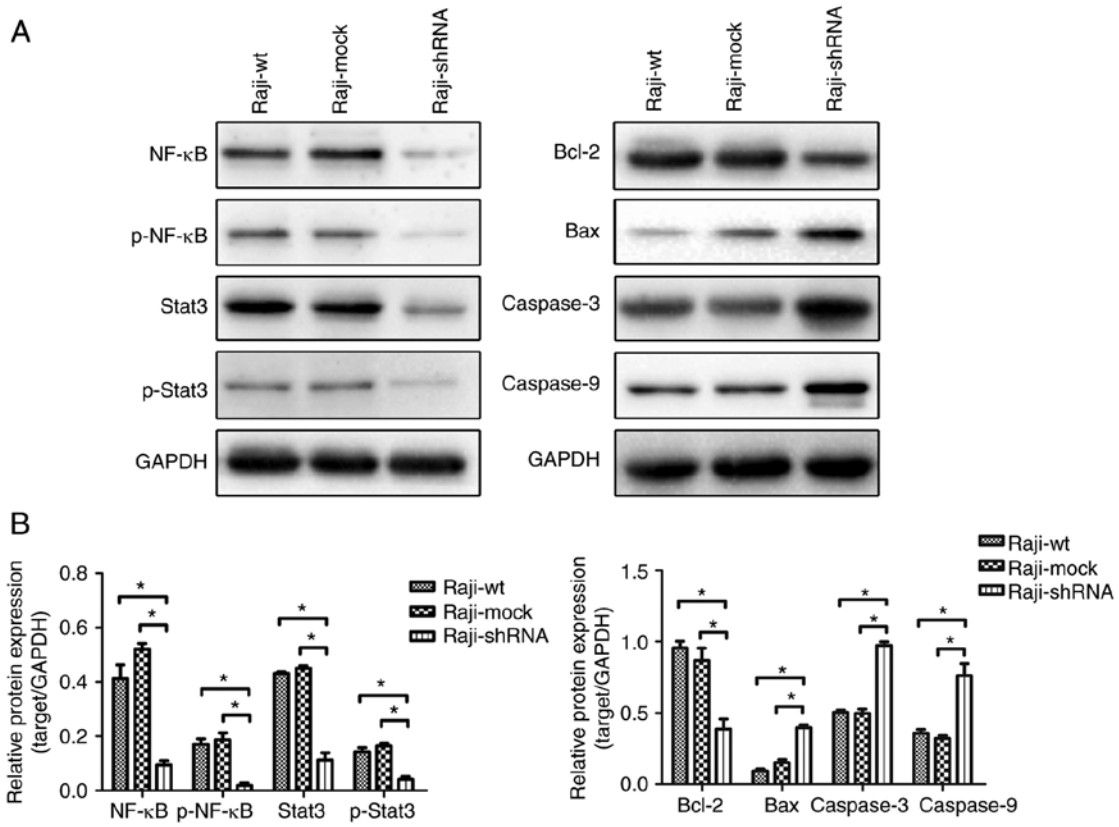

Figure 4. Western blotting showed the total Stat3, NF-κB and phosphorylation-Stat3, phosphorylation-NF-κB (A), Bcl-2, Bax, caspase-3, caspase-9 (A) expressed in Raji cells. Knockdown or inhibition of ALDH1A1 had concordant trend. Both of them significantly decreased the total Stat3, NF-κB and phosphorylation-Stat3, phosphorylation-NF-κB, Bcl-2 levels and increased Bax, Caspase-3, Caspase-9 protein (B).

cytotoxicity was assessed. The cytotoxicity in the combination group (CHOP plus DEAB) was higher than in the CHOP group alone at each concentration (Fig. 2C). Moreover, the IC_{50} values of the Raji cells to the CHOP regimen decreased from 685±25.62 to 412.56±38.58 ng/ml (P=0.013) in the presence of DEAB (Fig. 2C).

Knockdown or inhibition of ALDH1A1 reduces clonogenic capacity and increases apoptotic activity in Raji cells. To further determine the mechanisms of ALDH1A1 action, we performed loss-of-function studies, via ALDH1A1 inhibition or knockdown, in Raji cells. First, we confirmed successful shRNA-mediated knockdown of ALDH1A1 by western blot analyses (Fig. 2A and B). In colony formation assays, the numbers of colonies in the DEAB treatment group was significantly less than those of the Raji-wt control group, both in the absence or presence of CHOP treatment (Fig. 3A). Similarly, the numbers of colonies in the Raji-shRNA group was significantly less than those in the Raji-mock group, both in the absence or presence of CHOP treatment (Fig. 3B). These data demonstrated that ALDH1A1 loss-of-function reduced the clonogenic capacity of Raji cells.

Regarding apoptotic effects, colorimetric caspase assays showed that caspase-3 activity was increased in the DEAB and shRNA groups compared with those of their respective control groups, both in the absence or presence of CHOP treatment (Fig. 3C). Consistent with these data, caspase-9 activity was increased in the DEAB and shRNA groups compared with those of their respective control groups, both in the absence or presence of CHOP treatment (Fig. 3D). Finally, Annexin V and PI staining revealed an increased apoptosis rate in the shRNA group compared those of the wt or mock groups (Fig. 3E). Moreover, the shRNA group showed an even greater apoptosis rate after 400 ng/ml CHOP treatment for 24 h (Fig. 3E).

Knockdown or inhibition of ALDH1A1 decreases NF-κB/STAT3 signaling and increases pro-apoptosis signaling. Following ALDH1A1 knockdown, we also observed decreased levels of total NF-κB and STAT3, and phospho-NF-κB and -STAT3, compared with those in the control groups (Raji-wt and Raji-mock) (Fig. 4A and B, left panel and graph). Moreover, ALDH1A1 knockdown reduced BCL-2 levels and increased BAX, caspase-3 and -9 levels compared with those in the control groups (Raji-wt and Raji-mock) (Fig. 4A and B, right panel and graph). Inhibition of ALDH1A1 showed similar results (data not shown).

Discussion

There is increasing evidence that ALDH1A1 expression is associated with poor prognosis in a variety of cancers. In a meta-analysis of 38 studies involving 6,057 patients, ALDH1A1 expression was significantly associated with lymph node metastasis, histological differentiation and clinical stage in lung and breast cancer (20). In the present study, *ALDH1A1* was differentially expressed in peripheral blood samples from 112 NHL patients, compared with those in controls, and the median RQ level was 0.3263. Importantly, we further showed

that high *ALDH1A1* expression was associated with elevated levels of LDH, higher frequencies of >2 ECOG performance status and stage III/IV disease, higher IPI scores and a more invasive lymphoma category. Moreover, the overall survival time was significantly shorter in the high *ALDH1A1* expression group than in the low *ALDH1A1* expression group. Multivariate survival analysis further showed that the IPI score, lymphoma pathologic type and *ALDH1A1* expression level were independent prognostic factors.

ALDH1A1 is now recognized as a CSC marker that is associated with malignant behavior and drug resistance in tumor cells. For instance, among Hodgkin's lymphoma cells, there is a subset of clonal $CD27^{+}/ALDH1A1^{high}$ cells that are thought to be the initiating cells for HL (21). In addition, ALDH1 expression is higher in Epstein-Barr virus (EBV)-associated T/natural killer (NK)-cell lymphoproliferative disorder in children and young adults (TNKLPDC) than in extranodal nasal NK/T-cell lymphoma, and it is correlated with the biological characteristics of stem cells (22). There is also a subset of clonogenic $ALDH^{+}$ cells in mantle cell lymphoma that are associated with multiple drug resistance (23). Our previous study demonstrated that ALDH1A1 mediates resistance of DLBCL cell lines Farage and Pfeiffer cells to CHOP treatment, which included cyclophosphamide, doxorubicin, vincristine and prednisone (15,17). Consistent with these previous studies, in the present study, we have similar conclusions. ALDH1A1 was upregulated in Raji cells, and inhibition of ALDH1A1 activity by DEAB increased the sensitivity of Raji cells to CHOP drugs. Furthermore, shRNA-mediated knockdown of *ALDH1A1* decreased clonogenic ability, and increased apoptotic activity, in Raji cells. Next we should verify the conclusion in another non-aggressive NHL cell line to validate the effect of ALDH1A1.

NF-κB plays an important role in the development of B-cell NHL, and constitutive NF-κB activation is a major cause of drug resistance in relapse-refractory DLBCL patients. In a study using co-cultured engineered CD20-specific T cells with Raji cells, the T cells exerted antitumor activity against, and decreased the levels of p-STAT3 and BCL-2 in Raji cells potentially via inhibition of the NF-κB pathway (24). Moreover, invasive B-cell NHL is characterized by constitutive activation of NF-κB signaling; thus, targeting NF-κB is an attractive therapeutic strategy (25). NF-κB and STAT3 pathways interact with each other, and there are complex regulatory mechanisms between these signaling pathway networks. For instance, transglutaminase (TG2)/NF-κB and interleukin-6 (IL6)/STAT3 signaling cascades interact to promote autophagy and survival in mantle cell lymphoma, and blocking these pathways increases antitumor activity (26). Similarly, in DLBCL inhibition of an NF-κB/IL10/STAT3 autocrine loop is the main mechanism of drug-induced apoptosis (27). In the present study, we found that the levels of NF-κB/STAT3 pathway members and BCL-2 were decreased following *ALDH1A1* knockdown, and concomitantly, the levels of the apoptosis-related proteins BAX, caspase-3 and -9 were increased.

In summary, the present study demonstrated that ALDH1A1 was associated with poor prognosis in NHL, and importantly, our data suggested that ALDH1A1 may be an independent prognostic indicator and a new molecular biomarker for diagnosis in NHL. Furthermore, we showed that inhibition of ALDH1A1 increased the sensitivity of NHL cells to chemotherapeutic drugs, further supporting the validity of ALDH1A1 as a potential therapeutic target in NHL treatment. Several inhibitors of ALDH1A1 could be used in the clinic (28); however, disulfiram (DSF), which is an oral drug that was formerly used in the treatment of chronic alcoholism, is particularly attractive since its antitumor effects have been confirmed in prostate, breast, lung and glioma (29-32). Recently, DSF has been applied in a phase II clinical study to treat prostate and lung cancer (ClinicalTrials.gov Identifier: NCT01118741, NCT00312819). Thus, these clinical studies by others provide evidence of the feasibility, and the present study provides evidence of the theoretical basis, for the promising use of ALDH1A1 inhibitors to treat NHL patients.

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Competing interests

The authors declare that they have no competing interests.

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