Expression of far upstream element binding protein 1 in B-cell non-Hodgkin lymphoma is correlated with tumor growth and cell-adhesion mediated drug resistance

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Abstract. Cell adhesion-mediated drug resistance (CAM-DR) remains a major obstacle to the effectiveness of chemotherapeutic treatment of lymphoma. Far upstream element binding protein 1 (FBP1) is a multifunctional protein that is highly expressed in proliferating cells of several solid neoplasms; however, its expression and biological function in B-cell lymphoma is largely unknown. FBP1 expression in both reactive lymphoid tissues and several B-cell lymphomas, including follicular lymphoma and diffuse large B-cell lymphoma were detected by immunohistochemistry analysis. FBP1 expression in B-cell lymphoma was also associated with poor survival outcomes. Functionally, small interfering RNA-mediated silencing of FBP1 was able to inhibit the proliferation of B-cell lymphoma cells, resulting in G_0/G_1 phase cell cycle arrest. Furthermore, results of a cell adhesion assay demonstrated that adhesion to fibronectin or bone marrow stromal cells induced FBP1 expression, which in turn facilitated cell adhesion. Finally, FBP1 knockdown reversed CAM-DR. These findings support a role for FBP1 in non-Hodgkin lymphoma cell proliferation, adhesion and drug resistance, and may lead to the generation of a novel therapeutic approach targeting this molecule.

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Introduction

Lymphoma is the fifth most common type of cancer; ~90% of cases of which are non-Hodgkin lymphoma (NHL) and the remaining 10% are Hodgkin lymphoma. Lymphoma is currently one of the fastest-growing cancers, with an annual increase in rate of 4-5% (1,2). NHL consists of a large group of immune system neoplasms, and represents a heterogeneous group of diseases that are characterized by the monoclonal expansion of B or T lymphocytes (3,4). The classification of NHL is diverse, and includes diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), extranodal lymphoma of mucosa-associated lymphoid tissue (MALT) and mantle cell lymphoma (5,6).

Despite marked efforts to develop novel therapeutics, and recently observed improvements in overall survival rate, which are likely due to the routine incorporation of monoclonal antibody therapy, NHL remains predominantly incurable with standard therapeutic approaches (7,8). Previous studies have demonstrated that adhesion to cultured stromal cells or ligand-coated surfaces is able to protect malignant B cells from chemotherapy-induced apoptosis; this process is known as cell adhesion-mediated drug resistance (CAM-DR) (9,10). Bone marrow stroma has long been known as a 'sanctuary site' for lymphoma cells during traditional chemotherapy (8). Two modes of CAM-DR have been described: i) Cell interaction, known as the stromal model, in which cells interact with a stromal cell monolayer, establishing CAM-DR by heterocellular cell interaction; ii) substrate interaction, known as the fibronectin (FN) model, in which CAM-DR is mediated by cell-substrate interaction. Although the initial type of interaction markedly differs between these models, the subsequently activated intracellular molecular mechanisms are often similar, or even identical (11).

Human far upstream element (FUSE) binding protein 1 (FBP1) was initially recognized as a factor that binds to

the FUSE DNA sequence, which is located upstream of the c-myc proto-oncogene promoter (12). It has previously been reported that the c-myc oncogene is associated with apoptosis, growth and proliferation, and that FBP and c-myc share the same expression pattern (13). Furthermore, FBP and c-myc are expressed in proliferating cells, but not in quiescent or differentiated cells (13). FBP1 has been reported to be a potential c-myc regulator in renal cancer, but not in prostate and bladder cancer (12). In addition, several proteins, including FBP1, have been shown to be modified in Jurkat T cells during apoptosis (14). A previous study demonstrated that knockdown of FBP1 in hepatocellular carcinoma (HCC) cells resulted in increased sensitivity to apoptotic stimuli and reduced cell proliferation (15). Undoubtedly, FBP1 is a multifunctional protein; however, its function in lymphoma remains unknown.

The present study investigated the expression of FBP1 in various histological types of human B-cell NHL, and determined its prognostic role in NHL. Since stromal cell-mediated drug resistance is a common feature of chronic B-cell malignancies, including multiple myeloma and chronic lymphocytic leukemia (16,17), the present study also investigated the role of FBP1 in CAM-DR in NHL. The results may provide a novel perspective for a better understanding of the mechanism underlying drug resistance in NHL.

Materials and methods

Pathological samples. The present study collected 99 B-cell lymphoma and 19 reactive lymphadenopathy (RL) biopsy samples, which were histopathologically and clinically diagnosed at the Affiliated Cancer Hospital of Nantong University (Nantong, China), between January 1, 1993 and April 1, 2005. Diagnoses were made according to the World Health Organization criteria (18). Written informed consent was obtained from all patients prior to obtaining specimens for the present study. All of the tissues were fixed with formalin and were embedded in paraffin and sectioned (3-4 mm) for histopathological diagnosis and immunohistochemical study. The study was approved by the ethics committee of the Affiliated Cancer Hospital of Nantong University.

Immunohistochemistry. Immunohistochemical staining was performed with a Dako Autostainer (Dako Denmark A/S, Glostrup, Denmark) using a polymer detection system. Briefly, sections were deparaffinized in xylene and were rehydrated in a graded series of ethanol. Hydrogen peroxide (0.3%) was used to block endogenous peroxide activity for 10 min. Slides were then incubated with anti-FBP1 (1:900; cat. no. sc-271241; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 4 h at room temperature. The tissue sections were then counterstained with hematoxylin, dehydrated and mounted.

For assessment of FBP1, >2,000 cells from five high-power fields in each specimen were selected randomly, and the staining was examined by light microscopy to determine the mean percentage. Tumor cell proportion was scored as follows: 0, no positive tumor cells; 1, 1-24% positive tumor cells; 2, 25-49% positive tumor cells; and 3, 50-100% positive tumor cells. Staining intensity was graded according to the following criteria: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining (19). The product of staining intensity and the proportion of positive tumor cells was used to calculate the staining index (SI) (20). Using this method, FBP1 expression was examined in lymph node tissues by calculating the SI (scores, 0, 1, 2, 3, 4, 6 or 9). With the SI, an optimal cutoff value was identified: SI score ≥4 was used to define tumors with high FBP1 expression, and SI score ≤3 was used to indicate low FBP1 expression.

Cell lines, co-culture and adhesion assay. The Daudi and OCI-LY8 human malignant lymphoma cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 2 mM L-glutamine at 37˚C in an atmosphere containing 5% CO_2. The HS-5 human bone mesenchymal stem cell line was obtained from Shanghai Bioleaf Biotech Co., Ltd. (Shanghai, China), and was cultured in RPMI F12 (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). Six-well culture dishes were coated overnight at 37˚C with a monolayer of HS-5 cells or 5 $\mu g/cm^2$ human FN (Sigma-Aldrich; Merck Millipore). Lymphoma cell lines were then allowed to adhere to the pre-established monolayer of HS-5 cells or FN, or were maintained in suspension for 12-24 h at 37˚C. Subsequently, lymphoma cells were carefully removed, and the monolayer of HS-5 cells remained intact.

The Huh-7 human HCC and L02 normal liver cell lines were purchased from the Cell Library of the Chinese Academy of Science (Beijing, China). Cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich; Merck Millipore) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin prior to use in western blot analysis to examine FBP1 expression.

The cell adhesive ability was assessed by staining lymphoma cells with calcein (Santa Cruz Biotechnology, Inc.), according to the manufacturer's protocol, for 30 min. The cells were then incubated in 96-well plates with a FN-coated surface or pre-established monolayer of HS-5 cells in the recommended media containing 10% FBS. After 2 h of culture, the non-adherent cells were washed off twice with 1 ml phosphate-buffered saline (PBS) and the number of adherent cells was measured using a fluorometer (CytoFluor; Applied Biosystems; Thermo Fisher Scientific, Inc.).

Preparation of small interfering (si)RNA and transient transfection. Daudi and OCI-LY8 cells (Jiangsu Institute of Hematology, Suzhou, China) were cultured in FBS-free RPMI 1640 medium (Sigma-Aldrich; Merck Millipore) without antibiotics. Control and FBP-siRNA (#1, 5'-GGTGCTGAC AAACCTCTT-3'; #2, 5'-CCCATATAAAGTTCAACA-3'; and #3, 5'-GCTGCTTATTACGCTCAC-3') were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China) and transfection of cells with duplex synthetic siRNA was performed with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 48 h, cells were resuspended in normal medium at 10^6/ml and processed for further experiments.

Immunoblot analysis. The OCI-LY8 and Daudi cells, and lymph node tissues were homogenized in lysis buffer [1% NP-40, 50 mmol/l Tris (pH 7.5), 5 mmol/l EDTA, 1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate,

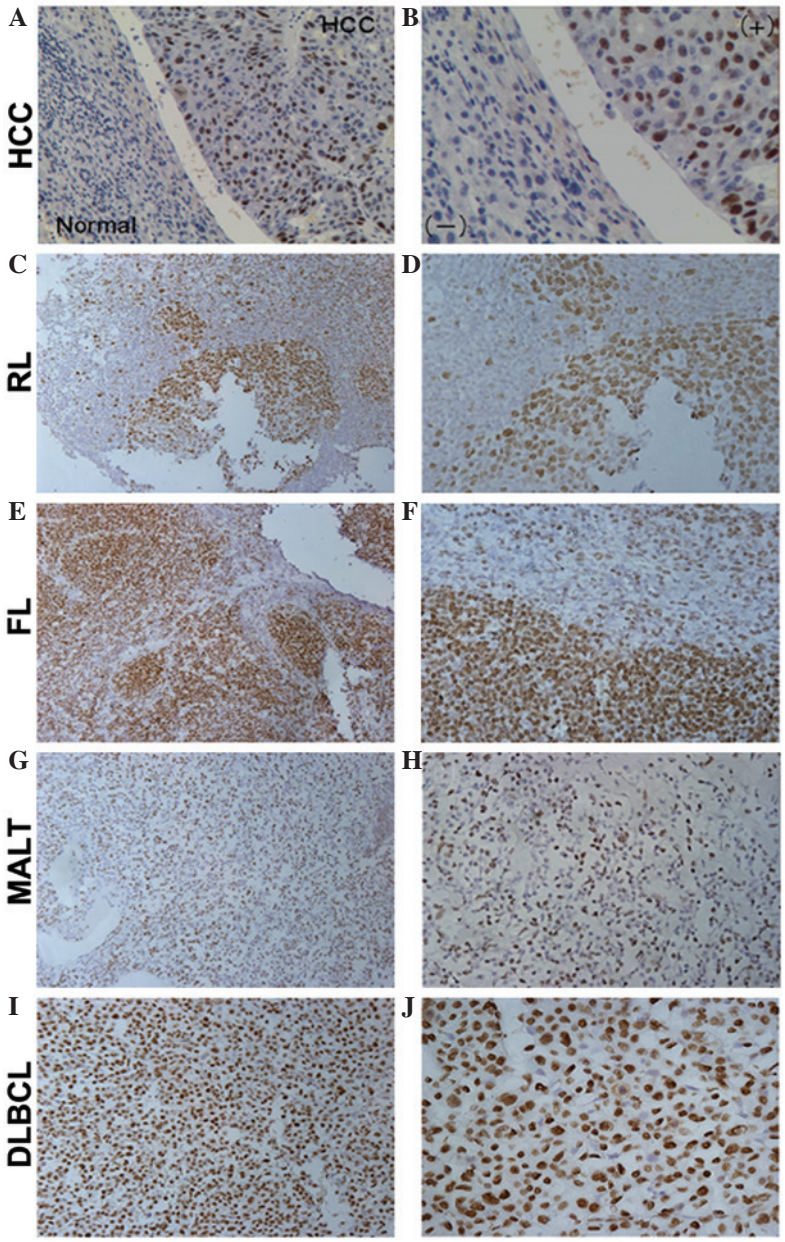

Figure 1. Immunohistochemical staining (IHC) results for far upstream element binding protein 1 (FBP1) expression in reactive lymphadenopathy (RL) and human B-cell non-Hodgkin lymphoma tissues. IHC was performed to detect FBP1 expression in (A and B) hepatocellular carcinoma (HCC) and adjacent normal tissues, as the positive and negative controls (A, x20; B, x40); (C and D) RL (C, x20; D, x40); (E and F) follicular lymphoma (FL; E, x20; F, x40); (G and H) extranodal lymphoma of mucosa-associated lymphoid tissue (MALT; G, x20; H, x40) and (I and J) diffuse large B-cell lymphoma (DLBCL; I, x20; J, x40). The FBP1 expression pattern varied between the different lymphoma types (DLBCL vs. FL vs. MALT=63.88% vs. 35.29% vs. 40.74%).

1% Triton X-100, 1 mmol/l phenylmethylsulfonyl fluoride, 10 g/ml aprotinin and 1 g/ml leupeptin] and were cleared by centrifugation at 9,388 x *g* for 20 min in a microcentrifuge at 4˚C (21). A nuclei acid analyzerwas used to determine the protein concentration and equal amounts of total protein (100 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and were electrophoretically transferred to polyvinylidene fluoride membranes (EMD Millipore, Bedford, MA, USA). The membranes were then blocked with 5% nonfat milk and were incubated with anti-FBP1 (1:1,000); anti-cyclin-dependent kinase 2 (CDK2; 1:500; cat. no. sc-163); anti-cyclin A (1:500; cat. no. sc-751;); anti-cyclin D1 (1:500; cat. no. sc-753); anti-proliferating cell nuclear antigen (PCNA; 1:1,000; cat. no. sc-790); anti-c-myc (1:1,000; cat. no. sc-764); anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1,000; cat. no. sc-32233), all obtained from Santa Cruz Biotechnology, Inc.; and anti-cleaved caspase (1:2,000; cat. no. C8487; Sigma-Aldrich; Merck Millipore) antibodies overnight at 4˚C. Following three washes with Tris-buffered saline-0.1% Tween 20, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000; cat. no. PV-6000-D; OriGene Technologies, Inc., Beijing, China) for 2 h at room temperature. The signals were visualized using an enhanced chemiluminescence detection system. Protein levels were analyzed using Image J (version 1.49; imagej.nih.gov/ij/) and normalized with GAPDH levels. The results were derived from at least three independent experiments.

Table I. FBP1 expression and clinicopathological parameters in 99 non-Hodgkin lymphoma specimens.

Parameter	Total	FBP1 expression		P-value
		Low	High	
Age (years)				<0.001[a]
≤60	59	36	23	
>60	40	11	29	
Gender				0.743
Male	56	30	26	
Female	43	17	26	
B symptoms				0.967
Absent	20	11	9	
Present	79	36	43	
Extranodal sites				0.025[b]
<2	64	35	29	
≥2	35	12	23	
Lactate dehydrogenase[b]				0.557
Normal	30	17	13	
Elevated	33	11	22	
Treatment				0.224
CHOP	55	24	31	
Other	44	22	22	
Ki-67 expression				<0.001[b]
<70%	42	33	9	
≥70%	57	11	46	
Histological type				0.482
Indolent	63	39	24	
Invasive	36	13	23	

Statistical analyses were performed using Pearson χ^2. [a]P<0.05 was considered to indicate significance. [b]Information not available in some cases. CHOP, cyclophosphamide, doxorubicin hydrochloride, vincristine, prednisolone treatment; FBP1, far upstream element binding protein 1.

Cell cycle analysis and viability assay. OCI-LY8 and Daudi cells were fixed in 75% ethanol at -20˚C overnight. Subsequently, the cells were incubated with 1 mg/ml RNase A for 30 min, and were stained with propidium iodide (PI; 50 μg/ml; BD Biosciences, San Jose, CA, USA) in PBS containing 0.5% Tween-20. Cells were analyzed using a BD FACScan flow cytometer (BD Biosciences).

Cell proliferation and viability were assessed using the standard Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD, USA), according to the manufacturer's protocol. The viability of lymphoma cells following treatment with 1 μM doxorubicin (Sigma-Aldrich; Merck Millipore) for 48 or 72 h was determined by CCK-8 assay.

Statistical analysis. SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analysis. Differences between two groups were compared using χ^2 test (Table I). Multivariate analysis was performed using Cox's proportional hazards model (Table II). The risk ratio and its 95% confidence interval were recorded for each marker. Other statistical analyses were performed using the Student's t-test. P<0.05 was considered to indicate a statistically significant difference. Each experiment consisted of at least three replicates per condition.

Results

FBP1 is expressed in RL and human B-cell NHL tissues. FBP1 is highly expressed in several solid neoplasms, including basal-like breast cancer, renal cancer, HCC, colon cancer and non-small cell lung cancer (12,22-25); however, whether this is true for hematological malignancies remains to be elucidated. Therefore, immunohistochemical analysis was performed to investigate the *in vivo* expression of FBP1 in clinical specimens of RL and lymphoma tissues, including FL, MALT and DLBCL (Fig. 1). HCC tissue was used as a positive control, whereas adjacent normal tissue was used as a negative control (15) (Fig. 1A and B). In RL tissues, FBP1 was predominantly expressed in proliferating germinal centers (Fig. 1C and D). Furthermore, in lymphoma tissues other than MALT, overexpression of FBP1 was detected. FBP1

Table II. Multivariate analysis with Cox regression model of 99 non-Hodgkin lymphoma specimens.

Parameter	Relative ratio	95% confidence interval	P-value
Age (years)			<0.01[a]
>60	1.114	0.434-0.717	
Gender			0.615
Female	0.842	0.504-0.615	
B symptoms			0.019[a]
Present	0.723	0.048-0.419	
Extranodal sites			<0.01[a]
≥2	0.967	0.157-0.514	
Lactate dehydrogenase[b]			0.045[a]
Elevated	0.924	0.011-0.404	
Treatment			0.598
CHOP	0.979	0.135-0.247	
Ki-67 expression			<0.01[a]
≥70%	1.156	0.420-0.717	
Histological type			0.006[a]
Invasive	1.949	0.096-0.447	
FBP1 expression			<0.01[a]
High	1.138	0.521-0.766	

Statistical analyses were performed using Cox regression test. [a]P<0.05 was considered to indicate significance. [b]Information not available in some cases. CHOP, cyclophosphamide, doxorubicin hydrochloride, vincristine, prednisolone treatment; FBP1, far upstream element binding protein 1.

immunoreactivity was primarily localized in the follicular mantle zones of FL (Fig. 1E and F), whereas in DLBCL tissues FBP1 was diffusely expressed (Fig. 1I and J). Compared with FL and DLBCL tissues, the expression of FBP1 in MALT tissues was much weaker (Fig. 1G and H). Furthermore, the positive rate of FBP1 expression was evaluated among the subtypes of lymphoma. FBP1 was highly expressed in several lymphoma tissues, with 63.88% positivity in DLBCL (23/36), 35.29% positivity in FL (6/17), and 40.74% positivity in MALT (11/27). Generally speaking, the FBP1 expression pattern varied among the various lymphoma types; however, there were no quantifiable differences between expression of FBP1 between tumor types, or between malignant and non-malignant specimens (P=0.482).

FBP1 expression is associated with high-risk clinical parameters in NHL. Various clinicopathological parameters were compared between patients with high or low FBP1 expression (Table I). A significant positive correlation was detected between FBP1 expression and Ki-67 (P<0.001), which is a proliferative marker. There were also significant correlations between high levels of FBP1 expression and two other adverse prognostic factors: Advanced age (P<0.001) and multiple extranodal sites (P<0.05). However, high levels of FBP1 expression were not significantly correlated with gender, serum lactate dehydrogenase (LDH) levels, histological type, chemotherapy or clinical symptoms. Survival analysis was performed on 99 patients who had follow-up data until mortality. After all variables were compared separately with survival status, FBP1 (P<0.01), Ki-67 (P<0.01), age (P<0.01), B symptoms (P=0.019), extranodal sites (P<0.01), LDH (P=0.045) and invasive histological type (P=0.006) significantly influenced survival (Table II).

FBP1 expression promotes proliferation of NHL cell lines. According to a previous study (23), FBP1 can be detected in HCC cell lines, including Huh-7; however, it is undetectable in the L02 normal liver cell line. Therefore, the present study assessed the protein expression levels of FBP1 in three cell lines: OCI-LY8, and the positive and negative control cells, Huh-7 and L02 (Fig. 2A). To further investigate the potential effects of FBP1 on NHL cell proliferation, OCI-LY8 cells were transiently transfected with FBP1-siRNA for 48 h, and the efficacy of FBP1 siRNA-mediated downregulation was confirmed by western blot analysis (Fig. 2B). Of the three siRNAs used FBP1-siRNA#1 exhibited the highest efficacy (Fig. 2B). Therefore, FBP1-siRNA#1 was chosen for subsequent assays. OCI-LY8 cells were transfected with FBP1-siRNA#1, and the expression levels of FBP1, c-myc, PCNA, CDK2, cyclin A and cyclin D1 were measured by western blotting (Fig. 2C). Previous studies have suggested that FBP1 is associated with carcinogenesis via c-myc dependent or independent pathways (15,26). The present study demonstrated that the expression levels of c-myc were higher when FBP1 expression was not suppressed. Knockdown of FBP1 resulted in a marked decrease in the expression of PCNA, which is a marker of cell proliferation. Furthermore, knockdown of FBP1 was correlated with the downregulation of several key cell cycle regulators, including

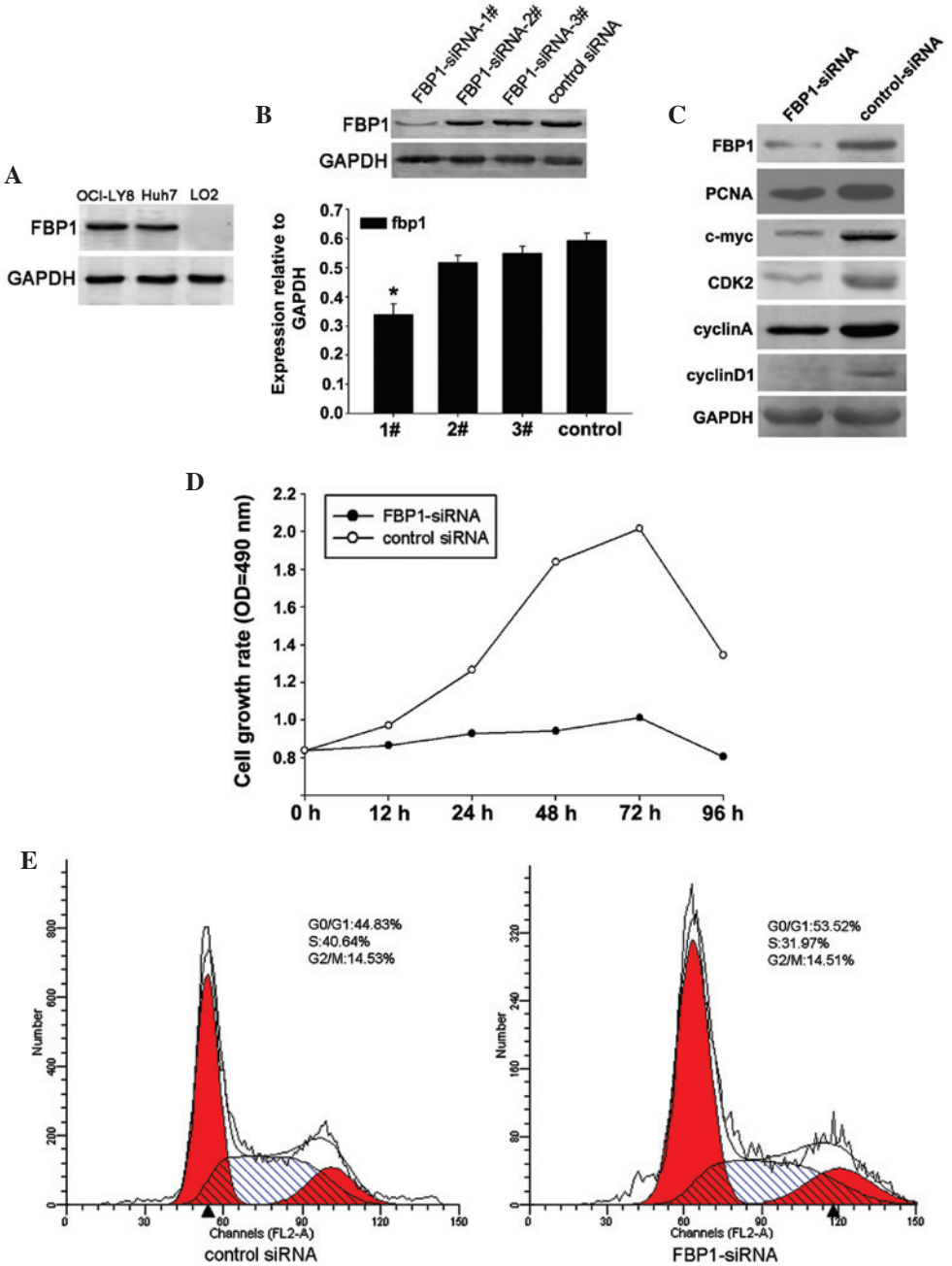

Figure 2. Far upstream element binding protein 1 (FBP1) expression promotes proliferation of OCI-LY8 cells. (A) Expression of FBP1 in various cell lines. The hepatocellular carcinoma cell line Huh-7 was used as a positive control, and the normal liver cell line L02 was used as a negative control. (B) OCI-LY8 cells were transfected with either FBP1-small interfering (si)RNA or a scrambled sequence (control siRNA). The efficacy of FBP1-siRNA-mediated downregulation was conformed by western blot analysis, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control. $^{*}P<0.05$ vs. control siRNA. (C) OCI-LY8 cells transfected with FBP1-siRNA or control siRNA were lysed and analyzed by western blotting using antibodies against FBP1, c-myc, proliferating cell nuclear antigen (PCNA), cell-dependent kinase 2 (CDK2), cyclin A, cyclin D1 and GAPDH. (D) Cell growth was examined by Cell Counting kit-8 assay at the indicated times. Each experiment was conducted at least three times. (E) Cells transfected with FBP1-siRNA or control siRNA were stained with propidium iodide for DNA content analysis by flow cytometry. OD, optical density.

CDK2, cyclin A and cyclin D1. Subsequently, a CCK8 assay was performed to determine cell viability of cells transfected with FBP1-siRNA (Fig. 2D). Knockdown of FBP1 resulted in a marked inhibition of cell growth rate. To explore the mechanism underlying FBP1-siRNA-induced decreased cell growth, cell cycle distribution was determined following transfection with FBP1-siRNA or control siRNA by flow cytometry. The percentage of cells in S phase was markedly decreased in the FBP1-siRNA group compared with in the control siRNA group (Fig. 2E), thus suggesting that FBP1 may promote G_0/G_1-S transition and accelerate cell growth. Similar results were detected in the experiments that used the Daudi cell line (data not shown).

Adhesion to FN or bone marrow stromal cells induces FBP1 protein expression, which in turn facilitates cell adhesion. It has previously been demonstrated that adhesion of hematological malignant cells to the extracellular matrix component FN reverses cell cycle arrest (27). The present study conducted an adhesion assay where lymphoma cells were adhered to

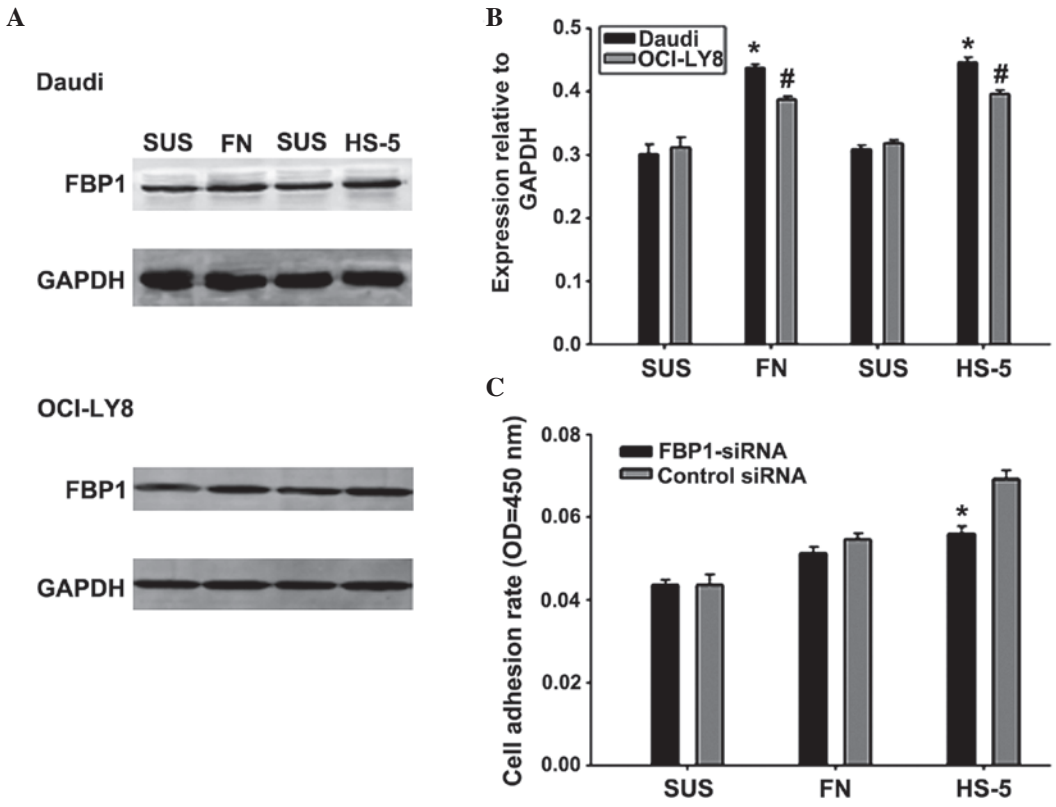

Figure 3. Adhesion to fibronectin (FN) or bone marrow stroma induces far upstream element binding protein 1 (FBP1) protein expression, which in turn facilitates cell adhesion. (A) FBP1 expression in Daudi and OCI-LY8 cells adherent to stromal cells, adeherent to FN, or in suspension (SUS). (B) Ratio of FBP1 protein to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as determined by densitometry. Each experiment was repeated at least three times. Data are presented as the mean ± standard error of the mean. *P<0.05, vs. Daudi cells in suspension; #P<0.05 vs. OCI-LY cells in suspension. (C) Cells were plated on a FN-coated surface or a pre-established monolayer of HS-5 cells and were stained with calcein. After 2 h the non-adherent cells were washed twice, and the number of adherent cells was measured. Cell adhesion rate was significantly higher in the control small interfering (si)RNA group compared with in the FBP1-siRNA group. *P<0.05 vs. control siRNA. OD, optical density.

stromal cells or FN in an attempt to elucidate the correlation between cell adhesion and the expression of FBP1. A western blot analysis was conducted to evaluate the expression of FBP1 in Daudi and OCI-LY8 cells following 24 h of adhesion to stromal cells or FN, or in suspension. FBP1 protein expression was markedly increased when lymphoma cells adhered to FN or stromal cells compared with cells in suspension (Fig. 3A and B). Subsequently, the role of FBP1 in promoting adhesion of lymphoma cells to FN or HS-5 cells was investigated using FBP1-siRNA. When cells were cultured in the presence of FN or HS-5, the adhesion rates of Daudi cells were more significantly increased compared with OCI-LY8 cells. Therefore, Daudi cells were transiently transfected with FBP1-siRNA or control siRNA for 48 h and were stained with calcein for 30 min. Subsequently, they were plated on a FN-coated surface or pre-established monolayers of HS-5 and were cultured for a further 2 h. Cell adhesion assay revealed that the cell adhesion rate was significantly reduced following knockdown of FBP1 in the HS-5 cell adhesion group (Fig. 3C). Taken together, the expression of FBP1 may promote the adhesion of Daudi cells to FN or HS-5 cell, which may lead to CAM-DR.

FBP1 knockdown reverses adhesion-mediated drug resistance. CAM-DR is considered a major mechanism by which tumor cells escape the cytotoxic effects of therapeutic agents (28,29). The present study demonstrated that FBP1 expression promotes adhesion of lymphoma cells. However, the specific association of FBP1 with drug resistance has yet to be elucidated. Therefore, FBP1-siRNA was used to knock down FBP1 expression in Daudi lymphoma cells cultured in three different conditions: Adhesion to FN, adhesion to bone marrow stromal cells, or in suspension. Subsequently, in order to evaluate the effects of FBP1 downregulation on drug resistance, a CCK8 assay was conducted following treatment with doxorubicin for 48 or 72 h (Fig. 4A and B). In a preliminary experiment, the appropriate drug concentration for treatment of lymphoma cells was determined to be 1 μM; this was the concentration at which cells were shown to be sensitive to drug-induced apoptosis. The results of the present study demonstrated that cell adhesion to HS-5 cells significantly protected Daudi lymphoma cells from the cytotoxicity of doxorubicin, as compared with cells in suspension. Conversely, this effect was partially abrogated following knockdown of FBP1. Adhesion to FN only resulted in weak drug resistance, and there was no marked downregulation of drug resistance in FN-adhered cells following FBP1-siRNA transfection. These results support a role for FBP1 in conferring drug resistance through cell-adhesion mechanisms.

It has previously been reported that FBP1 can be cleaved in the process of chemotherapy-induced apoptosis (13). Therefore, cleavage of FBP1, along with caspase-3 was evaluated by western blot analysis (Fig. 4C). Exposure of Daudi

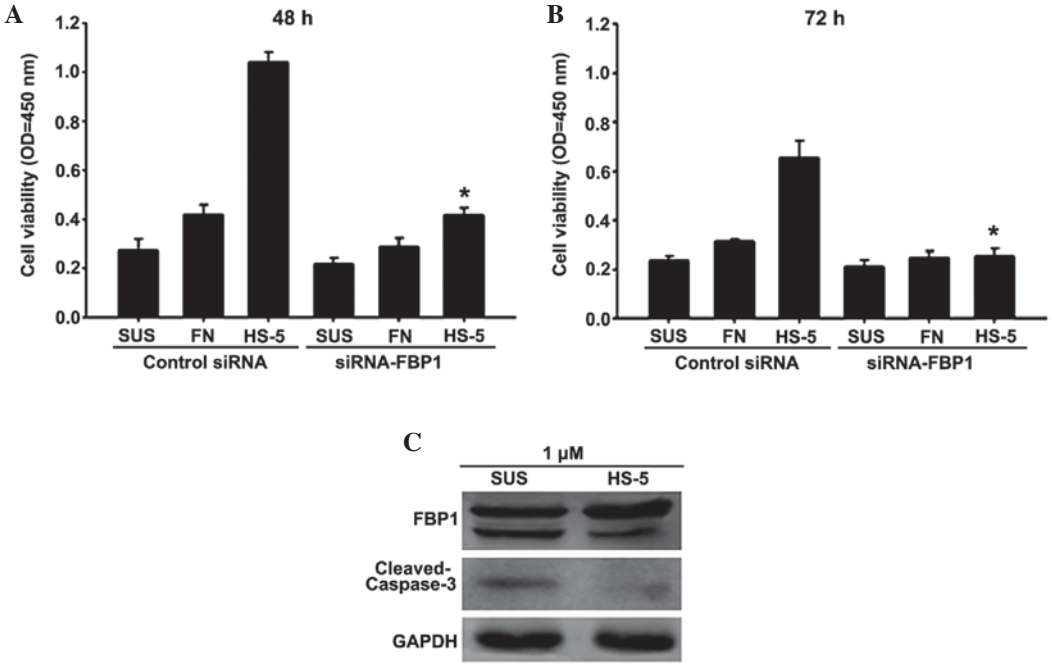

Figure 4. Far upstream element binding protein 1 (FBP1) knockdown reverses adhesion-mediated drug resistance. (A and B) Daudi cells were transfected with small interfering (si)RNA targeting either FBP1 or a scrambled sequence (control siRNA) . The cells were then cultured in three conditions: Adhesion to fibronectin (FN), adhesion to bone marrow stromal cells (HS-5), or in suspension (SUS), alongside doxorubicin treatment. Cell Counting kit-8 assay was performed to evaluate the effects of FBP1 knockdown on drug resistance following treatment with 1 μM doxorubicin for 48 or 72 h. Data are representative of three independent experiments. (C) Cleavage of FBP1 and the apoptosis marker caspase-3 in Daudi cells adhered to HS-5 cells or in suspension was evaluated by western blotting. Daudi cells were treated with 1 μM doxorubicin. Each experiment was performed at least three times. *P<0.05 siRNA-FBP1 vs. control siRNA. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OD, optical density.

lymphoma cells to 1 μM doxorubicin resulted in cleavage of FBP1 and caspase-3. Cell adhesion to HS-5 cells led to decreased cleavage of FBP1 and caspase-3, thus suggesting that stromal cell adhesion inhibited drug-induced apoptosis. Furthermore, compared with cells in suspension, adhesion to HS-5 cells increased FBP1 expression, which was consistent with previous results. These findings suggest that cell-cell interactions can substitute for cell-substrate interactions in conferring apoptotic resistance in NHL cells. FBP1 has an important role in stromal/lymphoma cell interactions and may be considered a novel therapeutic target for residual resistant lymphoma following chemotherapy.

Discussion

NHL is a disease that demonstrates a high proliferative rate and eventually becomes resistant to chemotherapy (30). Lymphoma cells are present in the bone marrow or secondary lymphoid organs, thus suggesting that the microenvironment may provide necessary components for growth and survival of tumor cells (31). FBP1, which acts as an activator of transcription for the proto-oncogene c-myc, contributes to decreased cell sensitivity to apoptotic stimuli and increased cell proliferation (23). FBP1 is a potential target of malignant cell transformation. It has been found to be upregulated in several types of cancer, including basal-like breast cancer, renal cancer, HCC, colon cancer and non-small cell lung cancer (15,22-26). These findings have implicated FBP1 expression in the progression of solid cancers; however, whether FBP1 is involved in hematological malignancies remains largely unknown. The present study detected FBP1 expression in RL tissues and several types of B-cell lymphoma tissue, including FL and DLBCL, by immunohistochemical analysis. In addition, FBP1 expression was shown to be associated with high-risk clinical parameters, and was identified as an independent prognostic factor for NHL in multivariate analysis. Therefore, evaluation of FBP1 expression may be considered an important factor in identifying poor prognosis in patients with NHL.

To explore the mechanisms underlying the effects of FBP1 on the promotion of NHL cell proliferation, knockdown of FBP1 via FBP1-siRNA transfection was performed in OCI-LY8 and Daudi cells. Downregulation of FBP1 expression significantly decreased the protein expression levels of cyclin A, cyclin D1 and CDK2, and suppressed the cell cycle progression of OCI-LY8 cells. It is well-known that G_1/S phase transition is a major checkpoint for cell cycle progression, and two complexes, cyclin D1-CDK4 and cyclin E-CDK2, function as critical positive regulators during this transition (32). Accordingly, the results of the present study indicated that the expression levels of cyclin D1 and CDK2 were decreased in FBP1-siRNA-transfected OCI-LY8 cells. These results suggested that FBP1 may be considered a promising novel target for the treatment of NHL therapy.

A better understanding regarding the biology of B-cell malignancies is required for the development of potential therapeutic agents that target specific intracellular pathways and the interaction between malignant B cells and their microenvironment (33). The 'tumor microenvironment' is a critical determinant for tumor initiation, progression, response to therapy, and drug resistance. Previous studies, including results from our own

laboratory, have reported that cell adhesion of hematopoietic tumor cell lines to stromal cells confers a multidrug-resistant phenotype, and that disruption of cell adhesion-mediated signaling may increase the efficacy of currently used cytotoxic agents (34-36). The present study investigated the effects of adhesion between stromal cells and B-cell lymphoma cells on the survival of NHL cell lines. The results indicated that FBP1 may have an important role in the adhesion and survival of NHL cells. Downregulation of FBP1 expression attenuated the observed CAM-DR. Therefore, a better understanding regarding the molecular mechanisms underlying the effects of FBP1 on stromal/lymphoma cell interactions may lead to the generation of novel therapeutic approaches for the treatment of residual resistant lymphoma following chemotherapy. However, in the process of CAM-DR, it remains unclear as to which signal pathway or targets are affected by FBP1 expression; therefore, this mechanism may warrant further investigation.

In conclusion, the present study demonstrated that FBP1 has a critical role in NHL cell proliferation, adhesion and drug resistance. These results may lead to the generation of a novel therapeutic approach that targets FBP1.

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