14-3-3ζ Mediates Resistance of Diffuse Large B Cell Lymphoma to an Anthracycline-based Chemotherapeutic Regimen

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Patients presenting with diffuse large B cell lymphoma (DLBCL) are treated with a standard anthracycline-based chemotherapy mixture consisting of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP). Half of DLBCL patients will develop chemo-refractory tumors due to the emergence of CHOP-resistant DLBCL cells. We isolated DLBCL cells that were resistant to CHOP as a model system to investigate the molecular basis of CHOP resistance. Resistant cells emerged from CHOP-sensitive DLBCL populations after repeated cycles of on-off exposure to stepwise increased dosages of CHOP. A proteomic analysis of CHOP-sensitive and -resistant DLBCL cells identified the ζ isoform of the 14-3-3 family as a differentially expressed protein. CHOP-sensitive cells showed reduced expression of 14-3-3ζ protein in the presence of high-dose CHOP relative to control cells. In contrast, CHOP-resistant cells expressed markedly higher levels of 14-3-3ζ regardless the presence of high-dose CHOP. Because 14-3-3ζ is known to exert anti-apoptotic influences and chemoresistance in lung, colon, and prostate carcinoma, we hypothesized that 14-3-3ζ promotes survival of DLBCL cells in CHOP. In support of our hypothesis, knockdown of 14-3-3ζ by small interfering RNA restored the sensitivity of resistant DLBCL to CHOP-induce apoptosis. In addition, 14-3-3ζ expression was highly up-regulated in a resected DLBCL lymph node relative to a normal lymph node by Western blot analysis. Furthermore, more than half of 35 DLBCL tissues showed elevated 14-3-3ζ expression relative to normal lymph tissue by immunohistochemical analysis. Our study implicates 14-3-3ζ in the pathogenesis of DLBCL and suggests a promising combination strategy with a 14-3-3 inhibitor for the treatment of refractory DLBCL.

Diffuse large B-cell lymphoma (DLBCL) is one of the most common subtypes of non-Hodgkin lymphoma, accounting for about 30% of all cases (1, 2). DLBCL has an incidence rate of 20,000–30,000 new cases annually. The disease comprises an aggressive lymphoma composed of large, transformed B cells with a diffuse growth pattern and a high proliferation fraction. Combinatorial cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) chemotherapy has been the standard systemic therapy for DLBCL with a cure rate of 40–50%. Although a subset of DLBCL patients is cured with CHOP regimens, many succumb to chemorefractory disease (3). More recent combinatorial therapeutic strategies to high risk DLBCL patients have primarily employed high doses of CHOP and bone marrow transplantations. Recently, the addition of rituximab (monoclonal antibody to CD20) to combinatorial CHOP has been accepted as the new standard of treatment, which has resulted in the first major improvement in therapy in more than two decades. Although treatment outcomes have significantly improved, resistance to the CHOP anthracycline-based regimen continues to constitute a serious problem for curing DLBCL (1). The identification of more rational, molecularly defined approaches to treatment is urgently needed to improve the quality of patient care and effectiveness of CHOP therapies.

In this report we provide evidence for a role of a member of the 14-3-3 family in mediating resistance of DLBCL cells to CHOP. 14-3-3ζ proteins function in multiple cellular processes including the maintenance of cell cycle checkpoints, DNA repair, and the prevention of apoptosis (4–7). 14-3-3ζ is a family of acidic dimeric proteins that are highly conserved and ubiquitously expressed in human cells. Seven 14-3-3 isoforms (β, γ, ζ, ε, σ, τ, η) are found in human cells. 14-3-3ζ proteins act primarily by binding to defined phosphoserine/phosphothreonine-containing motifs in protein targets. These motifs include the well characterized consensus sequences RXSxyXp and RXxpyXp (p = phosphoserine). 14-3-3 inhibits multiple proapoptotic proteins in part by coordinating with survival kinases such as Akt.

One mechanism whereby 14-3-3ζ proteins can exert anti-apoptotic activity is through the direct interference with the function of BH3-only proteins comprising the mitochondrial core proapoptotic machinery. 14-3-3ζ binds and sequesters Bad in the cytoplasm, allowing Bcl-XL to bind Bax, which neutralizes the ability of Bax to induce apoptosis (8). The Akt kinase phosphorylates Bad, which promotes its binding to 14-3-3 (9, 10). The dephosphorylation of Bad by phosphatases such as phosphatase 2A that are activated by proapoptotic signals triggers its release from 14-3-3 (11). Another BH3-only protein designated Bim is also regulated by 14-3-3 and Akt (12). Phosphorylation of Bim enhances its binding to 14-3-3, thus attenuating its proapoptotic activity.

Overexpression of 14-3-3ζ has been found in specific cancers. Increased expression of 14-3-3ζ has been reported in lung cancers (13–15), oral squamous cell carcinomas (16), stomach cancers (17), breast cancer (18), and in Papillomavirus-induced...
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carcinomas (19). 14-3-3γ was also overexpressed in chemoresistant melanomas (20). Overexpression of 14-3-3ζ is implicated as an early event in oral tumorigenesis (21). Increased 14-3-3ζ expression positively correlated with a more advanced pathologic stage and grade of non-small cell lung carcinoma (22). Overexpression of 14-3-3γ in nonsmall cell lung carcinoma H322 cells resulted in abnormal DNA replication and polypliodization (23). Up-regulation of 14-3-3β promoted mitogen-activated protein kinase-dependent tumor formation in nude mice (24). The up-regulation of 14-3-3 proteins in cancers implicate not only an anti-apoptotic role but also a tumor-promoting role as well (25).

14-3-3ζ has been validated as a potential molecular target for anticancer therapeutic development. Down-regulation of 14-3-3ζ sensitized tumor cells to apoptosis induced by ionizing radiation and chemotherapeutic agents (22, 25, 26). Knock-down of 14-3-3ζ restored the sensitivity of A549 lung carcinoma cells to anoikis and inhibited their anchorage-indepen-dent growth (15). Ectopic up-regulation of 14-3-3ζ reduced 9-nitrocamptothecin-induced apoptosis in prostate carcinoma cells (27). Recent clinical data associate 14-3-3ζ expression with advanced disease grade and poor survival outcome of lung cancer patients (22).

In this manuscript we describe our novel in vitro system to study the process of multidrug chemoresistance in DLBCL. We generated DLBCL cells resistant to the standard anthracycline-based chemotherapeutic mixture (CHOP) by repeated on-off exposures of sensitive DLBCL cell populations to stepwise increasing dosages of CHOP, which is similar to standard clinical treatment regimens. Proteomics revealed the 14-3-3ζ protein as a differentially expressed protein in CHOP-sensitive and -resistant cells. 14-3-3ζ was repressed in CHOP-sensitive but not -resistant cells when exposed to CHOP. siRNA-mediated knockdown of 14-3-3ζ in CHOP-resistant cells restored sensi-tivity to CHOP. Moreover, 14-3-3ζ protein was markedly up-regulated in more than one-half of 35 DLBCL tissues relative to normal lymph node tissue. Our observations implicate 14-3-3ζ as a potential therapeutic target in chemo-refractory DLBCL. To our knowledge this is the first report describing a role for a 14-3-3 protein in mediating a multidrug-resistant phenotype in DLBCL.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Diffuse large B cell lymphoma lines CRL2631 and CRL2289 were obtained from the American Type Culture Collection. Cell lines were propagated in RPMI 1640 supplemented with 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. Cells were passaged every 2–3 days to main-tain a density between 1–2 × 10⁶ cells/ml.

**Isolation of CHOP-resistant DLBCL Cell Lines**—Four to five T75 flasks each containing a total of 3 × 10⁶ DLBCL cells in 30 ml of medium (1 × 10⁶ cells/ml) were treated to on and off cycles of CHOP exposure. The composition of CHOP consisted of cyclophosphamide, doxorubicin, vincristine, and predni-sone at the clinical ratio of 80/5.5/0.16/11.1, respectively (28), with the highest combined CHOP concentration set at 1280 ng/ml and the lowest set at 5 ng/ml. The various dosages are also designated A through I (see Table 1). All reagents were purchased from Sigma. Cyclophosphamide and doxorubicin were dissolved in Millipore-purified water, vincristine was dis-solved in methanol, and prednisone was dissolved in chloro-form/ethanol (1:1). CHOP reagents were stored at −80 °C.

The proportion of the four components used in CHOP treatment of DLBCL was consistent with the standard dosage CHOP therapy (28). Similar to patient CHOP regimen, cells were subjected to cycles of 5 days of CHOP treatment followed by 21 days of recovery in the absence of CHOP. Cells were initially selected with several cycles of 80 ng/ml CHOP. Greater than 99.9% of the cells died within 5 days of the first CHOP cycle, but a few cells were able to grow and proliferate. After several on-off cycles in 80 ng/ml CHOP, the viable cells were treated with several on-off cycles of a higher CHOP dose (160 ng/ml). Cycling with CHOP was continued until a cell popula-tion emerged that could survive and recover from 5-day expos-ures to 640 ng/ml CHOP. Three independently derived CHOP-resistant populations (designated G1, G2, G3) were derived from the CRL2631 line, and one (designated S5H) was derived from the CRL2289 line.

**Antibodies and Western Blotting**—The p53 (Bp53-12) and Bcl-2 (Ab-1) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), the actin monocolonal antibody (N350) was from Amersham Biosciences, the rabbit 14-3-3ζ antibody (ab32622) was from Abcam, and the PARP monoclonal antibody (66401A) and caspase-3 rabbit polyclonal antibody (65906E) were from Pharmingen. Western blotting was performed by using the SuperSignal immunodection system (Pierce) as previously described (29). Western blotting was conducted using a 1:2000 dilution of antibody in PBS containing 0.3% Tween 20 and 5% dried milk. Immune reactions were visualized using a secondary horseradish pero-xidase-conjugated anti-rabbit or mouse IgG obtained from Pierce.

**Generation of 14-3-3ζ Knockdown DLBCL Cell Lines**—The generation of 14-3-3ζ siRNA retroviruses is described else-where (15). G3 cells were infected with siRNA retroviruses in the presence of 5 µg/ml Polybrene for 72 h. One week later the infected cells expressing siRNA were selected by propagation in puromycin (1.6 µg/ml) for 3 weeks. A knockdown control con-sisted of a scrambled siRNA retrovirus.

**Two-dimensional Gel Proteomics**—Cells (1.5 × 10⁷) were pelleted at 500 × g from the cell medium and washed once in ice-cold PBS. Cell pellets were flash-frozen in liquid nitrogen and stored at −80 °C. Frozen cell pellets were lysed by thawing and pipetting on ice in 1 ml of lysis solution I (0.3% SDS, 200 mM dithiothreitol, 50 mM Tris, pH 7.5, broad range protease inhibi-tors (GE Healthcare), and HALT protein phosphatase inhibi-tors (Pierce)). Proteins were solubilized by heating at 100 °C for 10 min followed by incubating on ice for 5 min. Lysates were sonicated with a 15-s burst (amplitude setting 60) on ice using a Sonic's Vibracell sonicator to further fragment DNA and cytoskeletal structures. Nucleic acids were digested by adding DNase/RNase mix (GE Healthcare) and rotating the lysates at 4 °C for 45 min.

Lysates were delipidated in chloroform-methanol by adding 4 ml of methanol and vortexing for 30 s followed by adding 1 ml
of chloroform and vortexing for 30 s, and finally, by adding 3 ml of Millipore-purified water and vortexing for 60 s. Samples were rotated at room temperature for 15 min, transferred to Corex glass tubes, and centrifuged at 6500 rpm in a Sorvall JA20 rotor for 20 min at room temperature. The interphase-containing proteins were transferred to a microcentrifuge tube and mixed with 0.5 ml of methanol. After spinning for 10 min in a microcentrifuge, the pellets were resuspended in 0.6 ml of water, and proteins were re-precipitated by adding a 0.8 volume of ice-cold acetone and incubating on ice for 15 min. Proteins were pelleted by spinning in a microcentrifuge for 15 min and solubilized in 100 µl of a two-dimensional gel proteomic solubilization buffer (9.9 M urea, 4% CHAPS, 15.2% SDS, 10 mM Tris, pH 7.5, 40 mM dithiothreitol). Proteins were allowed to solubilize for 1 h by rotating at room temperature and then desalted through microspin Pierce desalting columns into desalting solution I (9.9 M urea, 4% CHAPS). Protein concentrations were determined by the BCA protein assay after pretreating and re-precipitating an aliquot of each sample with BCA compatibility reagent and Compat-Able protein assay preparation set (Pierce catalog #23250 and #23215, respectively) to effectively remove interference by residual thiourea.

Desalted protein lysates were mixed with 400 µl of Destreak Rehydration sample buffer (GE Healthcare) containing 0.077 mg of dithiothreitol. Protein samples were further solubilized by rotating for 1 h at room temperature. After adding another 0.077 mg of dithiothreitol and ampholytes (pH 3–6, Bio-Rad Bio-Lyte 100× solution), samples were rotated for 30 min at room temperature and then allowed to rehydrate Immobiline dry-strip gel strips (GE Healthcare) for 18 h. Samples were subjected to isoelectric focusing at 150 V for 6 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for 6 h in an Etten IPGphorII unit. Proteins were separated in the second dimension on large format (24 × 21 cm) 8–16% gradient SDS-polyacrylamide gels at 5 watts/gel for 9.5 h. Protein gels were stained with Sypro Red for 6 h and destained for 9.5 h. Protein gels were scanned and imaged in a Typhoon 9200 laser scanner. Two-dimensional gel protein images were analyzed by Decyder (GE Healthcare) and ImageMaster (Amersham Biosciences) programs.

**Fluorescence-activated Cell Sorting (FACS)**—To detect CD20 and the cancer stem cell markers CD44 and CD133, we incubated phycoerythrin (PE)-conjugated antibodies to intact, viable cells followed by flow cytometry (FACS) to detect binding. The PE-conjugated monoclonal CD133 (AC133 catalog #130-080-801) and CD20 (catalog #130-091-109) antibodies were purchased from Miltenyi Biotec (Auburn, CA), and PE-conjugated CD44v6 monoclonal antibody (catalog #FAB3660P) was obtained from RD Systems (Minneapolis, MN). Cells (1 × 10⁷) were washed twice in PBS and suspended in 80 µl of flow cytometry buffer (0.5% bovine serum albumin and 2 mM EDTA in PBS). Nonspecific binding by Fc receptors was blocked by adding 20 µl of FcR Blocking Reagent (Miltenyi Biotec) and incubating for 2 min at room temperature. The cells were washed once in flow cytometry buffer, suspended in 1 ml of flow cytometry buffer, and immediately subjected to flow cytometric analysis.

**Apoptosis Assays**—Trypan blue viability stain was used to quantitate cell death in lymphoma cell suspension cultures by adding and mixing an equal volume of 0.4% trypan blue in PBS to the cell suspension growth medium. Flow cytometry to quantitate apoptosis and cell cycle phases was performed on ethanol-fixed and -permeabilized cells that were stained with propidium iodide according to a previous published protocol (30). Apoptotic cells were quantitated in the sub-G₁ DNA content portion of the flow cytometric scans. Annexin V staining was performed as previously described (31). Apoptotic cells were also identified by morphological criteria, which included cell blebbing, fragmented and shrunken nuclei, and apoptotic bodies. To examine and quantify apoptotic nuclei, cells were fixed by paraformaldehyde on glass slides and stained with 4′,6-diamidino-2-phenylindole nuclear stain (0.5 µg/ml) as described (32). Imaging and quantitation of apoptotic nuclei were performed with a Nikon TE2000 ultraviolet microscope using the MetaMorph Imaging System software (Molecular Devices Corp., Chicago IL).

**Reverse Transcription (RT) PCR**—Total RNA was extracted from DLBCL cells using the Absolutely RNA Microprep kit (Stratagene). RT-PCR on the purified total RNA was conducted with the AccuScript High Fidelity RT-PCR kit (Stratagene). PCR primers to detect 14-3-3ζ mRNA expression were as follows: forward, 5′-TTTCACAGGTGCAACAGATTAC-3′; reverse, 5′-GGATGAGGGAAATAAGCTCGTG-3′. Inclusion of glyceraldehyde-3-phosphate dehydrogenase primers in the RT-PCR reaction were for a quantitative reaction control: forward, 5′-ACCACAGTCCATGCCATC-3′; reverse, 5′-TCCACACCCCTGTGTCTGTA-3′.

**Preparation of DLBCL Tissue Extracts for Western Blotting**—Normal and DLBCL lymph node tissues were procured through Folio Biosciences. Frozen tissue was ground to a powder in liquid nitrogen in a pestle and mortar. The tissue powder (50 mg) was vortexed vigorously into 1 ml of SDS lysis buffer (2% SDS, 0.125 M Tris, pH 6.8, 10% β-mercaptoethanol, 10% glycerol). The lysates were heated at 100 °C for 10 min and then sonicated with a 15-s burst. Tissue extracts were microcentrifuged for 15 min and either subjected to Western blotting or stored in aliquots at −20 °C.
Immunohistochemistry Analysis—Cancer tissue array slides with 35 cases of non-Hodgkin lymphoma in duplicates and 4 normal lymph node tissues were from Folio Biosciences and stained for 14-3-3\textsubscript{\textgamma}/H9256 expression with the immunohistochemistry technique using the VECTASTAIN kit (Vector Laboratories, Inc.). Antibodies specific for 14-3-3\textsubscript{\textgamma} and the immunohistochemistry procedures used were as previously described (15). Briefly, slides were deparaffinized and processed by boiling in citrate buffer (0.01M) followed by washing. The treated slides were incubated with anti-14-3-3\textsubscript{\textgamma} antibody followed by hydrogen peroxidase-conjugated secondary antibodies. The stained slides were then counterstained with hematoxylin and scored by an experienced pathologist.

RESULTS

Isolation of CHOP-resistant DLBCL—The proportion of the four components used in CHOP treatment of DLBCL cells in this study were consistent with the standard dosage CHOP therapy (28). The dosages used ranged from 5 to 1280 ng/ml in the growth medium and are designated as A to I (see Table 1). CRL2631 cells showed a dosage-response to increasing CHOP in the growth medium from B to G concentrations with 90% cell death occurring at the G dosage after 3 days of exposure (Fig. 1). However, some cell growth did occur at the CHOP E concentration from 48 to 72 h of exposure, so we decided to begin our selection for resistant cells at this dose of CHOP.

We subjected CRL2631 cells to cycles of on-off CHOP treatment followed by periods of recovery in the absence of CHOP, which is similar to the standard clinical CHOP regimen. CRL2631 cells were treated with CHOP dosage E for 5
days followed by removal of the cells from CHOP to allow for recovery of any survivors. After another three cycles of on-off exposure to CHOP dosage E, a cell population emerged that could grow in the presence of CHOP. We subjected these CHOP-resistant cells to 5 cycles of on-off exposures to CHOP dosage F followed by five cycles of on-off treatments with CHOP dosage G. At the end of the CHOP cycling process, cells emerged that could survive and recover after 7 days of exposure to CHOP dosage H. Three independently derived populations were isolated from the CRL2631 cells by repeated on-off exposures to CHOP. Shown in Fig. 2B are growth curves for two of these CRL2631-derived CHOP resistant cells, which exhibited cell growth up to 72 h in the presence of 640 ng/ml CHOP, whereas the parental CRL2631 cells progressively died.

Cluster of Differentiation (CD) Marker Expression on CHOP-sensitive and -resistant Cells—Approximately 80–85% of non-Hodgkin lymphomas are of B-cell origin, and 95% of these cells express the surface CD20 marker (33, 34). We investigated the expression of CD20 on the CHOP-resistant cells to confirm...
that they were of B-cell origin. As shown in Fig. 3, strong expression of the CD20 B cell marker was detected by flow cytometry on both CHOP-sensitive (CRL2631) and -resistant (G3) cells, as indicated by the fluorescence in the M1 region of the FACscan.

In recent years, chemoresistance has been proposed to arise from a small subpopulation of cancer stem cells that are intrinsically multidrug-resistant (35). Cancer stem cells are defined as a small subset of cancer cells within a cancer that constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor (36, 37). Stem cells isolated from cancers arising in different organs show phenotypic differences in their cell surface epitopes. Cancer stem cells isolated from a variety of cancers express CD44 and CD133 (38). FACS analysis of the CHOP-resistant cells did not show any detectable expression of the CD44 or CD133 stem cell markers (Fig. 3).

**Identification of the ζ Isoform of the 14-3-3 Family As a Differentially Expressed Protein in CHOP-sensitive and -resistant DLBCL Cells**—We conducted two-dimensional gel proteomics to identify CHOP-responsive proteins in CRL2631 cells treated with CHOP for 24 h. As shown in the two-dimensional gel images in Fig. 6, a protein with an apparent pI of 4.7 and 25 K relative Mr decreased in expression with increasing concentrations of CHOP. Mass spectrometry identified the CHOP-responsive protein as the 14-3-3ζ isoform. Western blot confirmed the proteomics analysis showing the decreased expression of 14-3-3ζ in CRL2631 cells treated with CHOP for 24 h. As shown in the two-dimensional gel images in Fig. 6, a protein with an apparent pI of 4.7 and 25 K relative Mr decreased in expression with increasing concentrations of CHOP. Mass spectrometry identified the CHOP-responsive protein as the 14-3-3ζ isoform of the 14-3-3 protein family.

Western blot confirmed the proteomics analysis showing the decreased expression of 14-3-3ζ in CRL2631 cells treated with CHOP G and H concentrations as compared with the control cells (Fig. 7A, CRL2631, compare lane 0 with lanes G and H). When compared with CRL2631 cells, expression of 14-3-3ζ was higher in G1 CHOP-resistant cells in the presence or absence of CHOP (Fig. 7A, G1, lanes 0, F, G, and H). The two other CHOP-resistant cell lines, G2 and G3, also exhibited similar 14-3-3ζ expression profiles as G1 in the presence or absence of CHOP (Fig. 7B). A different lymphoma cell line, CRL2289, was subjected to CHOP selection, and a CHOP-resistant variant was isolated (S5H). 14-3-3ζ expression in the S5H variant was sim-
ilar to that observed in the CRL2631 CHOP-resistant variants (G1, G2) in that 14-3-3ζ was highly expressed in the presence or absence of CHOP (Fig. 7C). No changes in the amounts of 14-3-3ζ transcripts were detected by RT-PCR in CRL2631 cells in the presence or absence of CHOP (Fig. 7D), indicating that the reduced amount of 14-3-3ζ in CHOP-treated CRL2631 cells is likely the result of a posttranslational mechanism.

Knockdown of 14-3-3ζ Restores CHOP Sensitivity to CHOP-resistant DLBCL Cells—G3 cells stably transfected by 14-3-3ζ siRNA retrovirus were investigated for expression of 14-3-3ζ by Western blotting and for growth in CHOP H from 0 to 162 h. Substantial knockdown of 14-3-3ζ protein expression was observed in two 14-3-3ζ siRNA G3 cell populations (Fig. 8A, Kd2-A and Kd2-B) as compared with the scrambled siRNA control cells (Fig. 8A, KdC-A and KdC-B) and the actin control. The growth of 14-3-3ζ knockdown cells was markedly inhibited in the presence of CHOP dose H over the time period up to 120 h as compared with the control cells (Fig. 8B). Knockdown of 14-3-3ζ led to the accumulation of G3 cells in the G2-M cell-cycle phase (Fig. 8C).

14-3-3ζ Is Up-regulated in DLBCL Tissues Relative to Normal Lymph Nodes—The up-regulation of 14-3-3ζ in CHOP-resistant cells isolated from two independent DLBCL cell lines suggested that we might potentially observe up-regulation of 14-3-3ζ in some DLBCL-diseased lymph nodes. We, thus, compared the expression of 14-3-3ζ in a metastatic DLBCL (T1) and a non-metastatic DLBCL (T2)-diseased lymph node relative to a normal lymph node. SDS-extracted proteins from the lymph nodes were prepared as described under “Experimental Procedures” and immunoblotted with a 14-3-3ζ antibody. As shown in Fig. 9, the metastatic T1 DLBCL lymph node tissue expressed abundant levels of 14-3-3ζ. In contrast, considerably less 14-3-3ζ expression was

**FIGURE 6.** Two-dimensional gel separation of proteins in CHOP-sensitive CRL2631 cells. CRL2631 cells were incubated in growth medium containing CHOP at concentrations of 0 (No CHOP), 80 ng/ml (CHOP E), 160 ng/ml (CHOP F), or 320 ng/ml (CHOP G). After 36 h of incubation in the presence of CHOP, cells were extracted, and proteins were separated in the first dimension in a pH 3–6 isoelectric focusing gel and then subjected to slab gel electrophoresis in the second dimension on large format 8–16% gradient SDS-polyacrylamide gels. Proteins were visualized by staining with Sypro red and imaging with a Typhoon scanner. A, a typical representative two-dimensional gel image of the CRL2631 proteome. Migration of protein standards of known pl and relative molecular mass are designated by the red crosses. The rectangle outlined by dashed lines demarcates the region where a CHOP-responsive protein was detected. The CHOP-responsive protein is designated by the arrow. B, the selected gel region shown in panel A from multiple, independent gels is shown. A CHOP-responsive protein (designated by arrows) underwent decreases in expression in CHOP F and G gels relative to the No CHOP control gel. The image inset shows an overlay of a blue-colored image of the CHOP G gel and a red-colored image of the No CHOP control, which illustrates the differential expression of the candidate CHOP-responsive protein that was identified as 14-3-3ζ by MALDI-TOF mass spectrometry.
detected in the non-metastatic (T2; localized) DLBCL tissue and a normal lymph node. Low levels of 14-3-3 were detectable in the normal lymph node and the localized T2 tissue after prolonged overexposures of the Western blot shown in Fig. 9. The differential expression of 14-3-3 protein in normal and DLBCL-diseased lymph nodes suggested a potential role for 14-3-3 in the pathogenesis of DLBCL and, thus, warranted further investigation into the expression of 14-3-3 in DLBCL patient tissues.

Based on the Western blot data above, we extended our analysis of 14-3-3 expression in DLBCL patients by immunohistochemical staining of a tissue microarray containing 35 cases of non-Hodgkin B-cell lymphoma and 4 normal lymph node tissue sections. The majority of the lymphoma cases (57%; 20 of 35 cases) showed positive expression of 14-3-3 with 9% (3/35) of relatively high expression (Fig. 10). In contrast, all of the normal lymph cases showed only weak or completely negative staining. These data suggest that up-regulated 14-3-3 may be a common event in the majority of lymphoma cases, which may contribute to their resistance to therapies.

**DISCUSSION**

The addition of rituximab (monoclonal antibody to CD20) to CHOP generated the first major improvement in anti-DLBCL therapy in two decades. Although patient outcomes have significantly improved to a greater than 40% cure rate, resistance to the CHOP anthracycline-based regimen continues to pose a problem in managing or curing DLBCL. It is clear that the identification of more rational, molecularly defined approaches to treatment is urgently needed to improve the quality of patient care and effectiveness of CHOP therapies. We developed a CHOP-resistant DLBCL model to identify gene targets that are involved in the emergence and maintenance of resistance to CHOP. Two-dimensional gel proteomics identified the 14-3-3 member of the 14-3-3 protein family as a differentially expressed gene in CHOP-treated DLBCL cells. CHOP-sensitive cells expressed markedly less 14-3-3 protein than CHOP-resistant cells in the presence of CHOP. Small-interfering RNA-mediated repression of 14-3-3 restored sensitivity of resistant cells to CHOP, implicating up-regulation of the protein in the emergence and/or maintenance of CHOP chemoresistance in DLBCL.

Knockdown of 14-3-3 resulted in the accumulation of CHOP-resistant cells in G2-M of the cell cycle in the presence of CHOP, which might involve the Bad proapoptotic protein. Bad has been reported to dissociate from 14-3-3 at each G2-M phase of proliferating lymphoid cells, resulting in a G2-M arrest (39). The cell cycle-dependent dissociation of Bad and 14-3-3 is associated with phosphorylation at Ser-128 of Bad (9, 10). Likewise, the siRNA-mediated knockdown of 14-3-3 in CHOP-resistant DLBCL cells might lead to more unseques-tered Bad, resulting in a G2-M block. CRL2631 cells also

**FIGURE 7.** 14-3-3 expression in CHOP-sensitive and -resistant cells in the absence or presence of CHOP. A, CRL2631 CHOP-sensitive and G1 CHOP-resistant cells were incubated in the absence (lanes 0) or presence of CHOP concentration F, G, or H. After 48 h, cells were subjected to Western blotting using actin and 14-3-3 antibodies. B, Western blot comparison of 14-3-3 expression in CHOP-sensitive (CRL2631) and CHOP-resistant (G1, G2, G3) cells in the absence (lanes 0) or presence of CHOP concentration (lanes H). C, Western blot of 14-3-3 in CHOP-sensitive (CRL2631) and CHOP-resistant G1, G2, and SSH cells. SSH is a CHOP-resistant cell line derived from lymphoma cell line CRL2289. D, RT-PCR of CRL2631 cells in the presence (H) or absence (0) of CHOP H. GAPDH, glyceraldehyde phosphate dehydrogenase.
undergo a G2-M arrest in the presence of CHOP that is associated with repressed expression of 14-3-3/H9256.

A number of studies have documented roles for 14-3-3 proteins in both chemoresistance and tumorigenesis. For example, 14-3-3 proteins have been implicated in the chemoresistant response of esophageal adenocarcinoma, lung carcinoma, and colorectal carcinoma to platinum-based agents and nonsteroidal anti-inflammatory drugs (22, 40, 41). Repression of 14-3-3 sensitizes cells to apoptotic signals, and overexpression of 14-3-3 renders a variety of cancer cells resistant to apoptotic signals (22, 42–45). Treatment of medulloblastoma cancer cell lines with 2-methoxyestradiol resulted in decreased 14-3-3 expression in parallel with the induction of apoptosis, decreased cell growth, and cell cycle arrest (46). A 14-3-3 antagonist peptide, difopein, competes with cellular 14-3-3 targets for 14-3-3 binding, effectively sequestering endogenous 14-3-3 (47). Treatment of various cancer cell lines with difopein increased apoptosis and their sensitivity to cisplatin. A similar 14-3-3 antagonist peptide (R18) sensitized Bcr-Abl-transformed cells to inhibition with anticancer agents such as the mTOR inhibitor rapamycin. Targeting 14-3-3 also induced apoptotic cell death in cells expressing diverse imatinib-resistant Bcr-Abl mutants, which was further enhanced in combination with U0126 and rapamycin treatment (44). These data support a proof-in-concept that compounds that reduce functional 14-3-3 can sensitize or potentially re-sensitize cancers to chemotherapeutic agents.

Additional support for a role for 14-3-3/H9256 in CHOP-resistant DLBCL is derived from studies demonstrating that 14-3-3/H9256 specifically participates in the chemoresistance phenotype of prostate and lung cancers. Expression of 14-3-3/H9256 was dramatically increased in DU-145 human prostate cancer cells selected for resistance to 9-nitrocamptothecin-triggered apoptosis (27). Ectopic overexpression of 14-3-3/H9256 in 9-nitrocamptothecin (9NC)-sensitive DU-145 cells decreased 9NC-induced apoptosis, suggesting a role for 14-3-3/H9256 in mediating resistance of DU-145 cells to the topoisomerase I inhibitor. A 14-3-3/H9256 antisense reduced the expression of 14-3-3/H9256 in lung cancer cell lines.
14-3-3ζ Mediates Drug Resistance in B Cell Lymphoma

We report here that 14-3-3ζ of 14-3-3ζ significant number of DLBCL tissues indicate that up-regulation positive staining for 14-3-3ζ JOURNAL OF BIOLOGICAL CHEMISTRY

resulting in increased sensitivity of the cells to ionizing radiation (26).

14-3-3ζ has not previously been identified as a gene of interest in lymphoma cDNA expression profiling studies (3, 48, 49). We report here that 14-3-3ζ is highly expressed in three different DLBCL cell lines (CRL2631, CRL2289, CRL2632) and a surgically excised DLBCL lymph node. Moreover, more than half of DLBCL tissues showed marked 14-3-3ζ expression, with 9% exhibiting high expression relative to normal lymph tissue, which showed only weak or no expression. Marked up-regulation of 14-3-3ζ in CHOP-resistant DLBCL cell lines and a significant number of DLBCL tissues indicate that up-regulation of 14-3-3ζ may be a common event that contributes to the development of resistance to chemotherapies.

The ability to restore chemosensitivity to multidrug-resistant DLBCL through modulation of a single 14-3-3 protein is significant and may very well be due to the ability of 14-3-3 proteins to interact with and neutralize multiple proapoptotic players. Additional studies on 14-3-3ζ expression in DLBCL patients are warranted to determine whether overexpression of 14-3-3ζ is associated with the metastatic and/or the chemoresistant phenotype of DLBCL.

Many chemotherapeutic agents act to promote apoptosis in the cancer cell by inducing DNA damage and oxidative stress. Unfortunately, chemotherapies often result in secondary tumors that develop resistance to chemotherapy. Decreasing 14-3-3ζ expression in cancers has been proposed as a more specific way to enhance or restore sensitivity of cancer cells to chemotherapy (6, 47). In support of that proposal, the data presented here demonstrate that CHOP-resistant DLBCL can be re-sensitized to CHOP through specific down-regulation of the ζ isoform of 14-3-3.

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FIGURE 10. Expression of 14-3-3ζ in non-Hodgkin B-cell lymphoma patient tissues. A, representative tissues showing positive and negative 14-3-3ζ expression. B, summary of tissue array data. Scoring system: 0–1c, no or nonspecific weak staining; 1c, weak positive (>5% staining); 2c, strong positive staining for 14-3-3ζ.
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