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Glycolytic enzyme hexokinase II is a putative therapeutic target in B-cell malignant lymphoma

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Hexokinase II (HXKII) is a key regulator of glucose metabolism that converts glucose to glucose 6-phosphate. Furthermore, HXKII blocks mitochondria-dependent apoptosis by inhibiting the release of cytochrome c. HXKII overexpression is frequently observed in several types of cancer and confers chemoresistance to cancer cells. In the present study, we found that compared with cell lines generated from diffuse large-B-cell lymphoma (DLBCL) patients, cell lines with features of Burkitt lymphoma have higher levels of HXKII because of the activation of both c-MYC and HIF-1. Under normoxia, HXKII levels were correlated with the growth ability of each B-cell lymphoma cell line. HXKII levels were further enhanced when the B-cell lymphoma cells were cultured under hypoxia. The high levels of HXKII induced by hypoxia conferred cisplatin resistance in all tested B-cell lymphoma cell lines. The HDAC inhibitor panobinostat significantly suppressed HXKII expression under both normoxic and hypoxic conditions. Importantly, panobinostat reversed the anti-lymphoma action of cisplatin, and this effect was diminished by hypoxia. These data suggest that HXKII plays different roles, including in the regulation of glycolysis and inhibition of apoptosis, depending on its expression levels. Furthermore, inhibition of HXKII expression by panobinostat may represent a new and attractive strategy to overcome cisplatin resistance.

Malignant lymphoma cells tend to use glycolysis to produce ATP even when a substantial oxygen supply is available, a process known as aerobic glycolysis, as illustrated by the high uptake of [^{18}F]fluorodeoxyglucose. Hexokinases (HXKs) are a family of enzymes that catalyze the first step in glucose metabolism. There are four major HXKs: HXK I, II, III, and IV. Among these members, HXKII uniquely exhibits abnormal elevations in numerous types of cancer and is thought to play crucial roles in aerobic glycolysis in cancer cells. [1]

Recent studies have found that HXKII also plays crucial roles in the pathology of B-cell malignant lymphoma. Bhalla et al. [2] reported that a hypoxia-induced elevation of HXKII expression facilitated the development of diffuse large B-cell lymphoma (DLBCL) [2]. They reported that inhibition of HXKII expression in B-cell lymphoma cells using a short hairpin (sh) RNA significantly suppressed the in vivo growth of these cells. Furthermore, a clinical study by Gu et al. [3] revealed that upregulation of HXKII expression contributed to rituximab resistance in B-cell lymphoma.

Under physiological conditions, HXKII expression is regulated mainly by hypoxia-inducible factor (HIF)-1. Previously, we revealed that aberrant activation of NF-κB caused activation of HIF-1 pathways in B-cell lymphoma [4]. Abnormal activation of NF-κB is widely accepted as an important hallmark of B-cell lymphoma. These findings suggest that aberrant activation of the NF-κB pathway may contribute to enhanced expression

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of HXKII in B-cell lymphoma through activation of HIF. c-MYC is another important molecule in the development of B-cell lymphoma, including Burkitt lymphoma and some types of DLBCL. Interestingly, c-MYC is also an important regulator of HXKII. Kim et al. [5] first reported that HIF-1 and c-MYC cooperatively regulate HXKII in B-cell lymphoma. Recently, Mushtaq et al. [6] reported that c-MYC is responsible for the expression of HXKII in Burkitt lymphoma cells, whereas HXKII is regulated mainly by HIF-1 in lymphoblastoid lymphoma cells.

Cisplatin is one of the most important chemotherapeutic drugs for the treatment of malignant lymphoma, especially for patients who are resistant to initial therapy and for relapsed patients. Several established salvage treatment regimens for malignant lymphoma contain cisplatin. However, relapsed lymphoma cells frequently exhibit resistance to these treatments, and the outcomes of these patients are usually poor. Therefore, investigating the molecular mechanisms that reduce the antitumor activity of cisplatin and identifying strategies to overcome these mechanisms are important tasks. A number of molecular mechanisms are involved in the acquisition of cisplatin resistance, that is, increased DNA repair function, altered cellular incorporation of the drug, and enhanced inactivation of cisplatin [7]. Intriguingly, recent studies have indicated that both HIF-1 and HXKII are important contributors to cisplatin resistance in many types of cancer cells. HIF-1 protects ovarian cancer cells from cisplatin-induced cell death by modulating glucose metabolism [8]. HIF-1 also mediates hypoxia-induced cisplatin resistance in nonsmall cell lung carcinoma cells [9]. HXKII overexpression confers resistance to cisplatin in ovarian cancer cells [10]. HXKII also blocks the cytotoxicity of cisplatin in colon cancer cells [11].

Given these findings, we speculated that aberrant activation of c-MYC and/or HIF-1 may induce cisplatin resistance through upregulation of HXKII expression in B-cell lymphoma cells. Furthermore, we explored the possibility that HXKII may be a molecular target that can be exploited to overcome cisplatin resistance in B-cell lymphoma cells.

Methods

Cell culture

All lymphoma cells were obtained from ATCC (Osaka, Japan). The cells were cultured at 37°C with 5% CO_2 in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal calf serum, 25 U/mL penicillin, and 25 ng/mL streptomycin (Gibco/Invitrogen, Carlsbad, CA).

Reagents

2-Deoxyglucose (2DG) and panobinostat were purchased from Sigma-Aldrich. BAY11-7082 and 10058-F4 were purchased from Abcam (Cambridge).

Preparation of cell lysates and immunoblotting

Whole-cell lysates and nuclear and cytosolic fractions were prepared as described elsewhere [12]. Briefly, 10^7 cells were washed with ice-cold phosphate-buffered saline (PBS) containing 2 mmol/L Na_3VO_4; resuspended in a hypotonic buffer (20 mmol/L Hepes [pH 7.5], 10 mmol/L KCl, 1 mmol/L $MgCl_2$, 10% glycerol, 0.5 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg of aprotinin/mL, 10 μg of leupeptin/mL, 2 mmol/L benzamide, 10 mmol/L NaF, and 2 mmol/L Na_3VO_4 with 0.2% Nonidet P-40); and homogenized. After the cells were centrifuged at 1,000 *g* for 5 min, the supernatant was separated from the nuclear pellet. The nuclear pellet was then resuspended in a hypotonic buffer with 300 mmol/L NaCl. The debris was removed by centrifugation (14,000 *g* for 20 min), and the supernatants were collected and defined as the nuclear extracts. The lysate proteins were fractionated by size by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted onto polyvinylidene difluoride (PVDF) membranes. The blots were probed with the indicated antibodies, which are listed in Supplementary Table E1 (online only, available at www.exphem.org). The blots were visualized using a chemiluminescence detection kit (Cell Signaling Technology, Danvers, MA) according to the manufacturer's instructions.

Immunohistochemical analysis

This study was approved by the institutional review board of the University of Yamanashi. Written informed consent was obtained from each patient. The clinical information for each patient is provided in Supplementary Table E2 (online only, available at www.exphem.org). Lymph node biopsy samples were obtained at the time of diagnosis. Formalin-fixed, paraffin-embedded tissue sections were prepared and then immunostained with anti-HXKII antibodies.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U* test. We used analysis of variance (ANOVA) with Tukey's post hoc test to compare differences among three or more groups. The statistical analyses were performed using KaleidaGraph 4.5 (Synergy Software, Reading, PA) software.

Results

Expression levels of HXKII were associated with c-MYC and NF-κB activation

To explore the role of HXKII, we initially investigated the protein expression levels of HXKII in a series of B-cell lymphoma cell lines. We used Burkitt lymphoma cell lines (Raji, Ramos, Namalwa, and Daudi) and cell lines derived from DLBCL patients (Noa. 2289, 2631, and 2632 and HT). All DLBCL cell lines express CD10; therefore, these cells belong to the germinal center B (GCB) type of DLBCL [13–15]. Raji, Namalwa, and Daudi cells are known to contain Epstein–Barr virus DNA. As illustrated in Figure 1A, the expression levels of HXKII under basal conditions differed between these cell lines. Raji, Ramos, and Namalwa cells had higher levels of HXKII expression

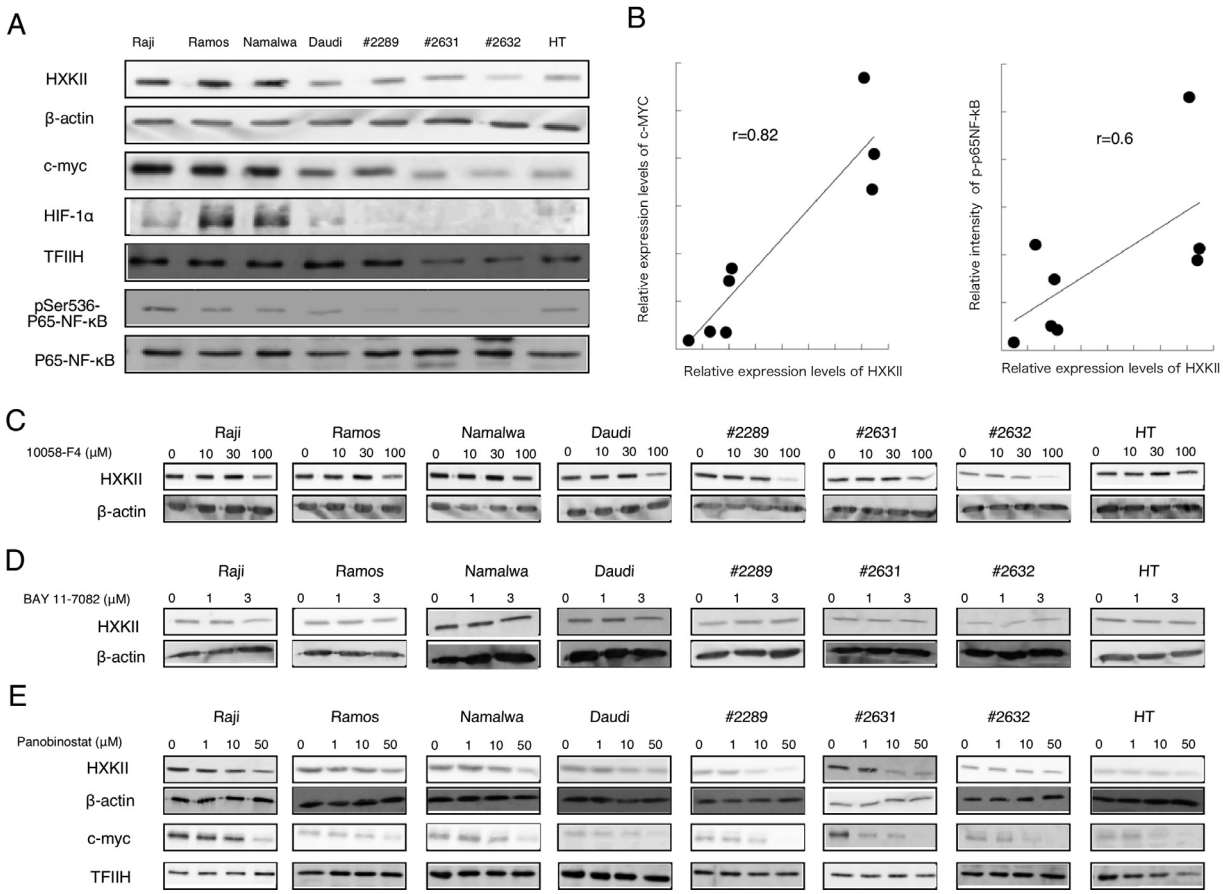

Figure 1. HXKII expression and regulatory mechanisms in B-cell malignant lymphoma cells. **(A)** Each lymphoma cell line was cultured at a density of 3×10^5 cells/mL for 24 hours, and the nuclear and cytoplasmic cell fractions were prepared for Western blotting. The expression levels of the indicated proteins were analyzed. **(B)** *Left:* Scatterplot of the HXKII and c-MYC expression levels. *Right:* Scatterplot of the HXKII and phosphorylated NF-κB p65 levels. r = Pearson's correlation coefficient. **(C)** Each lymphoma cell line was treated with the indicated concentrations of 10058-F4 for 48 hours. Then, cell lysates were prepared, and the expression levels of HXKII were analyzed. The membranes were also blotted with an antibody against β-actin as a control. **(D)** The cells were treated with the indicated concentrations of BAY11-7082 for 48 hours, and then, the nuclear and cytoplasmic fractions of the cell lysates were prepared. The expression levels of HXKII were analyzed. **(E)** The cells were cultured with the indicated concentrations of panobinostat for 48 hours, and then cell lysates were prepared. The HXKII and c-MYC expression levels were analyzed by Western blotting. For the internal control, the membranes were also blotted with anti-β-actin or anti-TFIIH antibodies.

than the other cell lines. In contrast, most of the DLBCL cell lines had relatively low levels of HXKII.

To determine the molecular mechanisms that induce HXKII expression, we analyzed the expression levels of c-MYC and HIF-1α, both of which are the main regulators of HXKII transcription [5]. Although the expression levels differed among cell lines, c-MYC expression was detected in all cell lines tested (Figure 1A). We found a positive correlation between HXKII levels and c-MYC expression ($r = 0.8$), as illustrated in Figure 1A and B. In contrast to c-MYC, HIF-1α expression was detected only in the Burkitt lymphoma cell lines and not in the DLBCL cell lines. Because we previously found that NF-κB controls HIF-1α in lymphoma cells, we analyzed NF-κB activation in these cells. As illustrated in Figure 1A, we found relatively higher phosphorylation levels of NF-κB p65 in Burkitt lymphoma cell lines. The levels of phosphorylated NF-κB p65 were also associated with HXKII levels; however, this association did not reach statistical significance ($r = 0.6$). Next, we investigated whether c-MYC and/or HIF-1α was responsible for HXKII expression in lymphoma cells. Therefore, we treated these cell lines with inhibitors of each pathway. Treatment of the cells with 10058-F4, an inhibitor of c-MYC, clearly suppressed the expression of HXKII in all cell lines (Figure 1C). To block HIF-1α, we used BAY11-7082, an inhibitor of NF-κB. In our previous studies, we confirmed that BAY11-7082 clearly blocks HIF-1α expression in Raji, Namalwa, and Ramos cells [4]. Consistent with our previous results, we confirmed that treatment with BAY11-7082 suppressed HIF-1α levels in all Burkitt lymphoma cell lines (Supplementary Figure E1A, online only, available at www.exphem.org). As illustrated in Figure 1D, BAY11-7082 reduced HXKII levels in Burkitt lymphoma cell lines but not in DLBCL cell

lines, which did not exhibit activation of HIF-1α under normoxia (Figure 1D). For further study, we treated cells with the histone deacetylase (HDAC) inhibitor panobinostat, which is known to block HIF-1α and c-MYC expression in several cancer cells [16,17]. Panobinostat significantly blocked HXKII expression in all cell lines tested. We also found that treatment with panobinostat clearly suppressed c-MYC levels in all lymphoma cell lines. Furthermore, panobinostat suppressed HIF-1α levels in Burkitt lymphoma cells (Supplementary Figure E1B). These results suggested that c-MYC plays a central role in regulating HXKII in both Burkitt and DLBCL cell lines. In addition, NF-κB/HIF axes are also responsible for HXKII expression in Burkitt lymphoma cell lines. These differences in regulation may cause the increased expression of HXKII in the Burkitt lymphoma cell lines compared with that in the DLBCL cell lines. Next, we analyzed HXKII levels in clinical samples (five GCB-type DLBCL, three non-GCB-type DLBCL, and three BL patients) and calculated the ratio of HXKII-positive cells. Immunohistological analysis of biopsy samples revealed that Burkitt lymphoma cells exhibited higher HXKII expression than GCB-type DLBCL cells (Figure 2A, B). Non-GCB-type DLBCL cells also exhibited significantly higher levels of HXKII compared with GCB-type DLBCL cells (Figure 2A, B). Notably, all DLBCL cell lines used in this study had the characteristics of GCB-type DLBCL.

Correlation of HXKII levels with cell growth

Next, we investigated the biological significance of HXKII levels in B-cell lymphoma cells. As illustrated in Figure 3A and B, we found a strong correlation between cell growth ability and HXKII protein levels in these cell lines ($r = 0.9$). Because HXKII is a master regulator of glycolysis, we blocked glycolysis using 2DG and analyzed the effects on cell growth. A strong inverse correlation was found between the inhibitory effects of 2DG and the expression of HXKII (Figure 3C, D) ($r = 0.8$). These results suggested that elevated HXKII levels at baseline enhanced glycolysis, leading to increased cellular growth, and cells with enhanced baseline expression were more sensitive to glycolysis inhibitors.

HXKII expression levels under normoxia were not correlated with anti-apoptotic action against CDDP

In addition to controlling glycolysis, HXKII directly blocks the mitochondria-dependent apoptosis induction pathway by binding and inhibiting voltage-dependent anion-selective channels (VDACs) on the mitochondria [11]. Therefore, we investigated whether HXKII confers resistance to apoptosis in lymphoma cells. Therefore, we used cisplatin, which is known to trigger DNA damage, leading to the induction of caspase 3-dependent apoptosis. Initially, we examined the growth-inhibitory effects of cisplatin on our B-cell lymphoma cell lines under baseline conditions. As illustrated in Figure 4A and B, the growth-inhibiting effects of cisplatin

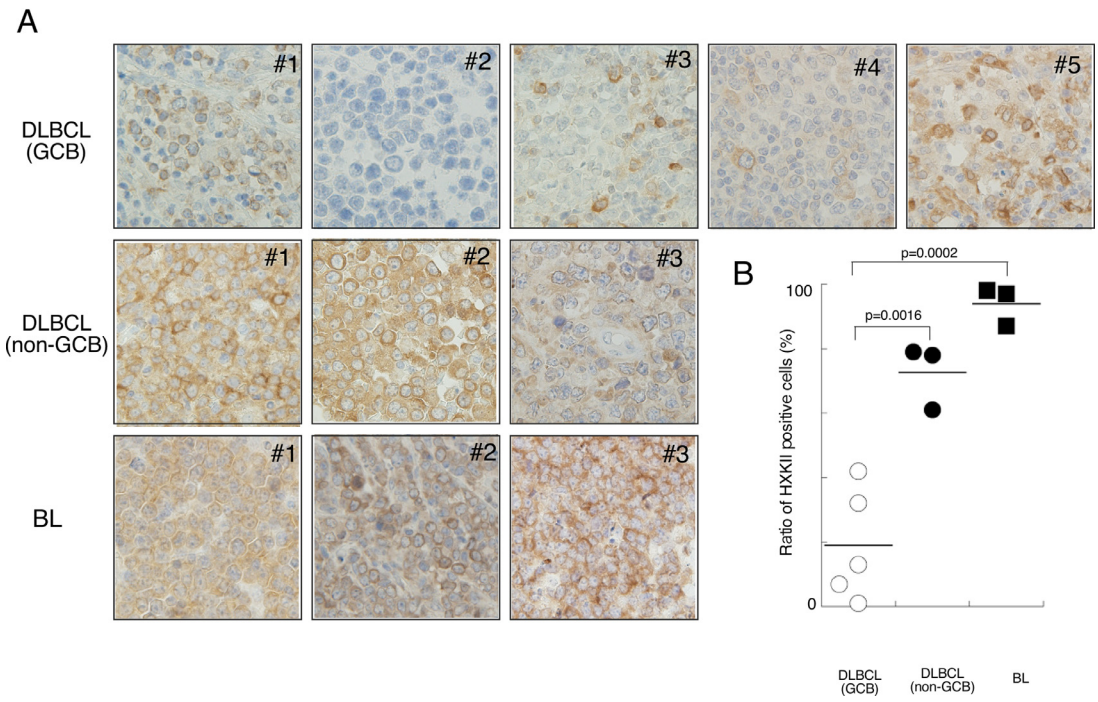

Figure 2. HXKII expression in primary lymph node biopsy samples. **(A)** The expression of HXKII in lymph node biopsy samples collected from the patients at the time of diagnosis was analyzed by immunohistochemical methods. A list of the patients is provided as Supplementary Table E2. **(B)** The ratio of HXKII-expressing cells was calculated, and the results are illustrated as a scatterplot. The horizontal line in each column indicates the average. Statistical analysis was performed using ANOVA with Tukey's post hoc test.

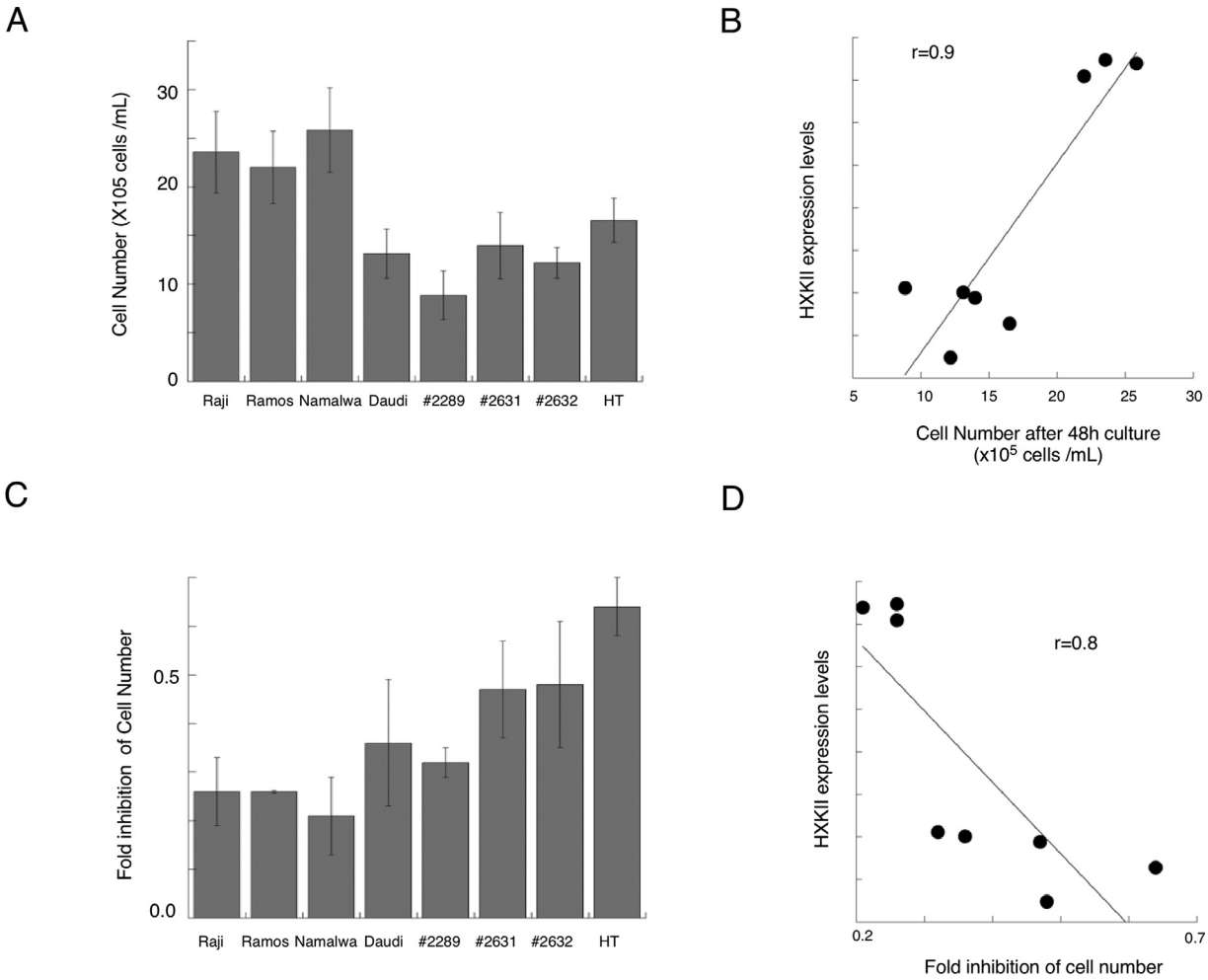

Figure 3. Biological significance of the HXKII expression levels in B-cell lymphoma cells. **(A)** Each lymphoma cell line was cultured from a starting concentration of 3×10^5 cells/mL for 48 hours. Then, the cell numbers were counted. The bars represent the averages of three independent experiments and the standard deviation. **(B)** Scatterplot of the HXKII level and cell number after 48 hours of culture. r = Pearson's correlation coefficient. **(C)** Each lymphoma cell line was cultured with or without 3 mmol/L 2DG for 48 hours. The fold decrease in the number of cells treated with 2DG compared with that of the control cells is shown. The bars represent the averages of three independent experiments and the standard deviation. **(D)** Scatterplot of the HXKII levels and the fold decrease in the cell number in cells treated with 2DG. r = Pearson's correlation coefficient.

on the B-cell lymphoma cell lines were not correlated with baseline HXKII levels. In addition, cisplatin had the same effect on caspase 3 activation in all lymphoma cell lines (Figure 4C). These results indicated that the differences in HXKII protein levels observed among the lymphoma cells under normoxic conditions did not affect DNA damage-induced apoptosis.

Hypoxia-induced HXKII expression and conferred cisplatin resistance in B-cell lymphoma cells

Next, we investigated the anti-apoptotic role of HXKII under hypoxic conditions, as hypoxia is a strong inducer of HXKII expression, and HXKII has been reported to confer resistance to cisplatin in several solid cancers [10]. Compared with normoxic conditions, hypoxic conditions clearly increased the expression of HIF-1α and HXKII in all cell lines (Figure 5A). In contrast, the protein levels of other putative HIF-1-regulated glycolytic enzymes, that is, glucose transporter 1 (GLUT1) and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), were not changed (Supplementary Figure E2, online only, available at www.exphem.org). In addition, the expression of the HIF-1-regulated anti-apoptotic proteins Bcl-XL and MCL-1 was not induced by the hypoxic conditions (Supplementary Figure E2).

Under hypoxic conditions, the growth rates of all lymphoma cell lines were decreased, especially those of Burkitt lymphoma cell lines. However, the ratio of viable cells was not changed (data not shown). Furthermore, caspase 3 activation was not increased after culture under hypoxic conditions (data not shown).

Hypoxia significantly decreased the cell growth-suppressing effects of cisplatin in all of the lymphoma cell

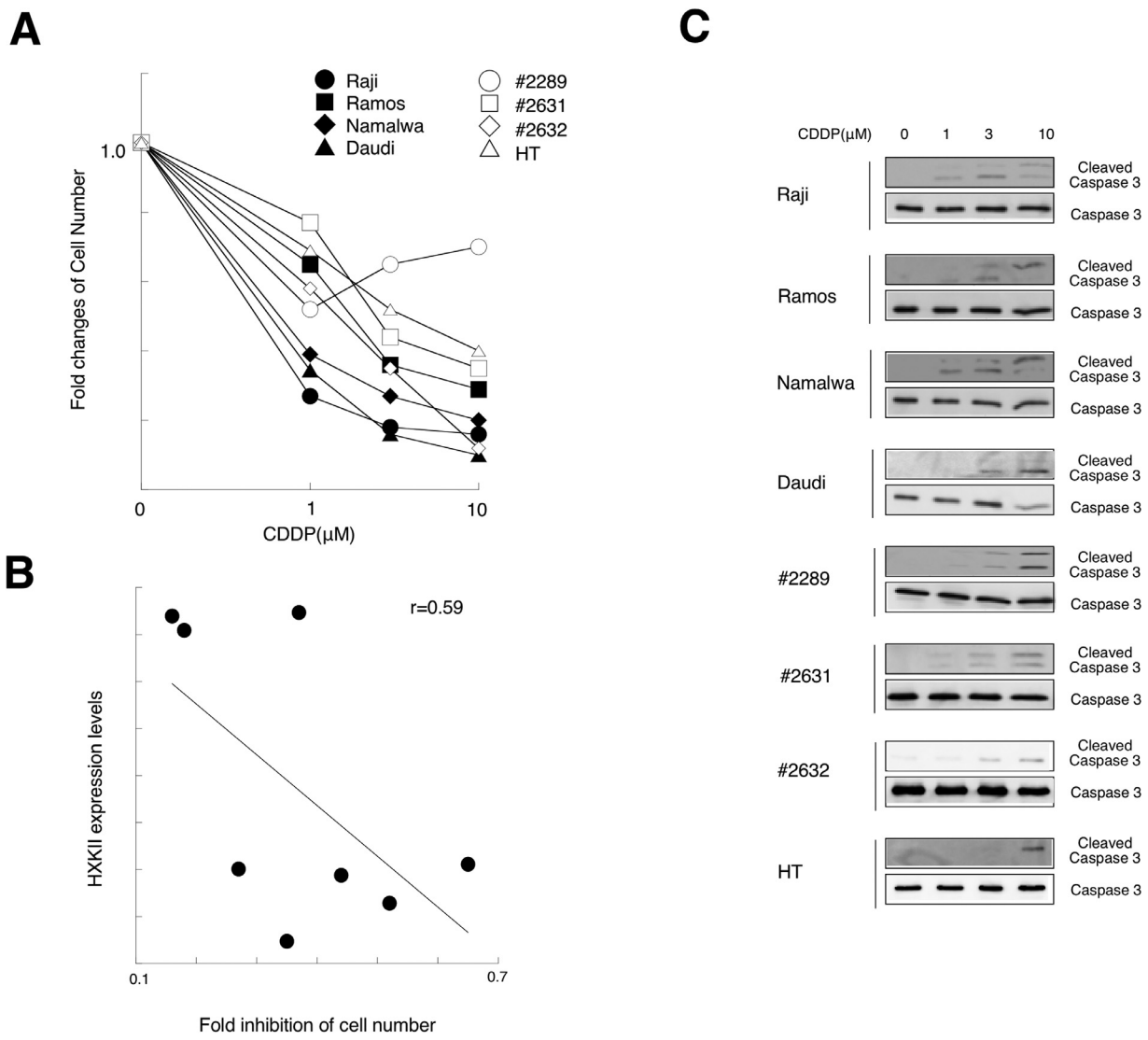

Figure 4. Basal HXKII expression levels were not associated with CDDP-induced apoptosis. **(A)** Each lymphoma cell line was cultured at a density of 3 × 10^5 cells/mL in various concentrations of cisplatin for 48 hours. The fold decrease in the number of treated cells compared with that of control cells is indicated. The bars represent the averages of three independent experiments and the standard deviation. **(B)** Scatterplot of the HXKII levels and the fold decrease in cell number in the cells treated with 3 μmol/L CDDP. r = Pearson's correlation coefficient. **(C)** Each lymphoma cell line was cultured with the indicated concentrations of CDDP for 48 hours, and activation of caspase 3 was analyzed by Western blotting.

lines tested (Figure 5B). Furthermore, the activation of caspase 3 by cisplatin was decreased under hypoxic conditions (Figure 5C). These results indicated that a hypoxic environment confers cisplatin resistance to lymphoma cells.

Panobinostat restored the cytotoxic effects of cisplatin under hypoxic conditions

As illustrated in Figure 5A and the Supplementary Figure E2, hypoxia selectively induced HXKII expression. Thus, we speculated that HXKII is responsible for the hypoxia-induced cisplatin resistance observed in our lymphoma cell models, as this mechanism has been reported in other cancer cells [10]. As mentioned, HXKII has dual functions, acting as a glycolytic enzyme and a VDAC inhibitor; therefore, we investigated whether hypoxia-induced HXKII expression protects lymphoma cells by enhancing glycolysis or blocking the release of cytochrome c. First, we treated lymphoma cells with 2DG under hypoxia and then counted cell numbers. We found that 2DG did not restore the cytotoxicity of cisplatin in any of the lymphoma cell lines tested (data not shown). This result indicated that glycolytic enzyme activity does not contribute to protecting lymphoma cells from cisplatin-induced apoptosis under hypoxic conditions. Next, we treated lymphoma cells with panobinostat because we previously found that panobinostat reduced the

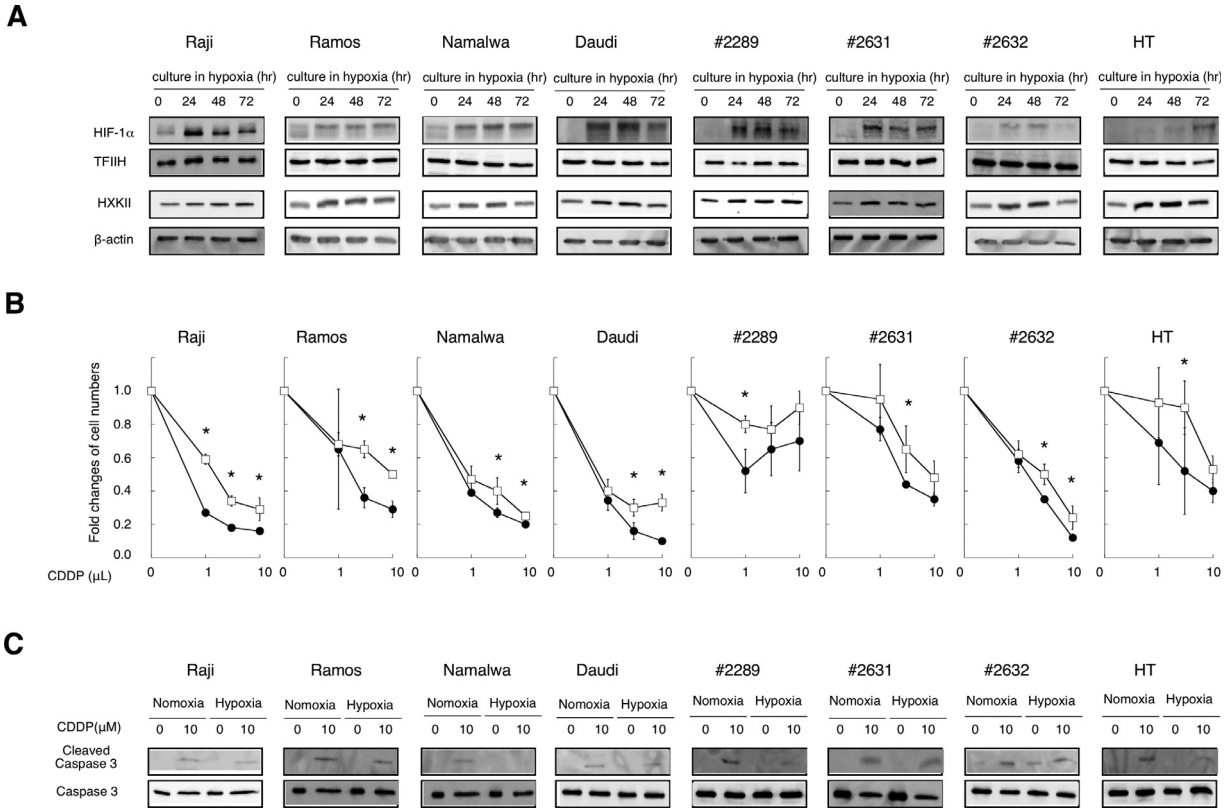

Figure 5. Hypoxia suppressed the cytotoxic effect of CDDP on lymphoma cell lines. **(A)** Each cell line was cultured under hypoxic conditions for the indicated periods. Then, the nuclear fraction and cytoplasmic fraction of the cell lysates were prepared. The induction of HIF-1α expression was analyzed by Western blotting with the nuclear fractions. Changes in HXKII expression levels in the cytoplasmic fractions were also analyzed. Expression levels of TFIIH and β-actin were analyzed as internal controls for the nuclear fraction and cytoplasmic fraction, respectively. **(B)** Each lymphoma cell line was cultured at a density of 3×10^5 cells/mL with the indicated concentration of CDDP for 48 hours under either normoxic or hypoxic conditions. Then, cells were counted. The fold reduction calculated at each point by comparing the experimental condition with the control is indicated. Each point indicates the average of three independent experiments and the standard error. The *black circles* represent normoxic conditions; the *white squares* represent hypoxic conditions. The difference in fold reduction between two conditions was statistically analyzed using the Mann–Whitney *U* test. $*p < 0.05$. **(C)** Each lymphoma cell line was cultured with 10 μmol/L CDDP for 48 hours under either normoxic or hypoxic conditions. Then, cell lysates were prepared, and caspase 3 cleavage was analyzed by Western blotting. For the control, the membranes were reblotted with anti-total caspase 3 antibodies.

expression of HXKII in all B-cell lymphoma cell lines tested (Figure 1E). We confirmed that panobinostat suppressed hypoxia-induced HXKII expression (Figure 6A). As expected, combining panobinostat with cisplatin significantly enhanced the inhibitory effect of cisplatin on lymphoma cell growth (Figure 6B). Finally, the addition of panobinostat enhanced the cisplatin-induced cleavage of caspase 3 under hypoxic conditions (Figure 6C). These results suggested that hypoxia-induced HXKII expression protects lymphoma cells from cisplatin-induced apoptosis by blocking caspase 3 activation.

Discussion

In the present study, we revealed that the glycolytic enzyme HXKII supported the proliferation of B-cell lymphoma, especially Burkitt lymphoma, under normoxic conditions. Burkitt lymphoma cells had higher levels of HXKII than the DLBCL lymphoma cell lines, and the Burkitt lymphoma cells were more sensitive to growth inhibition by 2DG, an inhibitor of the glycolytic function of HXKII. Both c-MYC and HIF-1 are responsible for the overexpression of HXKII in Burkitt lymphoma cells, but only c-MYC was required for the induction of HXKII expression in DLBCL lymphoma cell lines. Our observations also suggested that the hypoxic tumor microenvironment may affect the expression of HXKII in addition to affecting cell intrinsic signals. Culturing B cell lymphoma cell lines under hypoxic conditions significantly enhanced HXKII levels. Furthermore, hypoxia-enhanced HXKII levels resulted in resistance to the chemotherapeutic drug cisplatin. The inhibition of HXKII expression by panobinostat restored the anti-lymphoma activity of cisplatin.

Enhanced glycolysis, even under aerobic conditions, is one of the malignant hallmarks of B-cell malignant lymphoma. Pathological analysis indicated that many types of

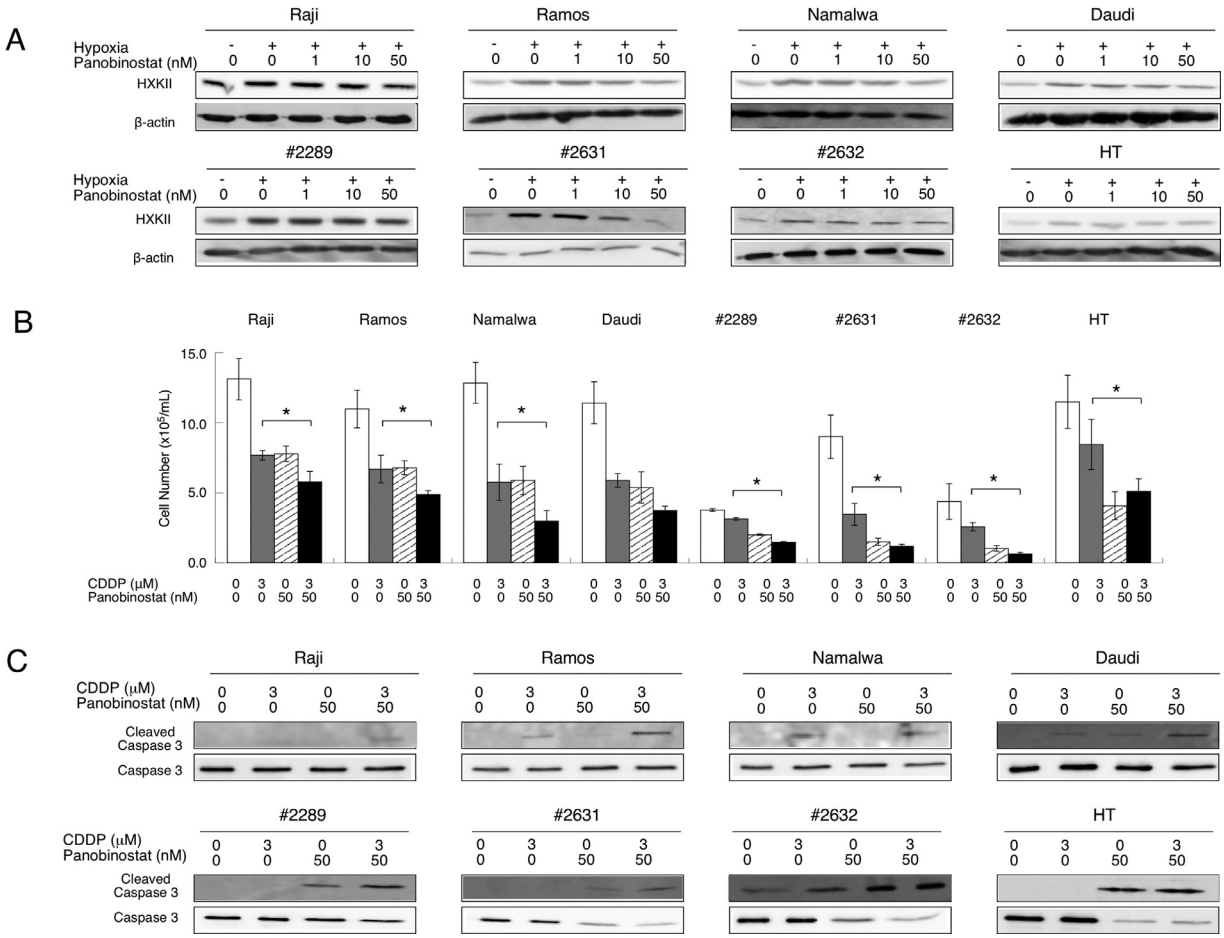

Figure 6. Panobinostat restored the cytotoxic activity of CDDP under hypoxic conditions. **(A)** Each lymphoma cell line was cultured under normoxic or hypoxic conditions with the indicated concentrations of panobinostat for 48 hours. Then, cell lysates were prepared, and the expression level of HXKII was analyzed by Western blotting. As a control, the membranes were reblotted with an anti-β-actin antibody. **(B)** Each lymphoma cell line was cultured at a density of 3×10^5 cells/mL under hypoxia with the indicated combination of CDDP and panobinostat for 48 hours, and then the cells were counted. The bar indicates the average of three independent experiments and the standard deviation. Differences between the conditions were analyzed by ANOVA with Tukey's post hoc test. **(C)** Each lymphoma cell line was cultured with the indicated combination of CDDP and panobinostat for 48 hours under hypoxia. Then, cell lysates were prepared, and caspase 3 cleavage was analyzed by Western blotting. For the control, membranes were reblotted with anti-total caspase 3 antibodies.

primary B-cell lymphoma exhibit elevated expression of GLUT1, GLUT3, and HXKII [18], all of which play important roles in glucose metabolism. More recently, Bhalla et al. [2] revealed that HXKII directly contributes to the pathogenesis of DLBCL. Using a mouse model, they demonstrated that knockdown of HXKII expression in DLBCL cells using a shRNA resulted in reduced tumor formation. They also found that hypoxia selectively enhanced HXKII levels in B-cell lymphoma cells, supporting our results illustrated in Figure 5A and Supplementary Figure E2.

The molecular mechanisms controlling HXKII expression elevation in B-cell lymphoma cells have been studied, with the results revealing that c-MYC and HIF-1 cooperate to elevate HXKII expression [2,5,6,19]. Forced activation of both HIF-1 and c-MYC significantly enhances HXKII levels in Burkitt lymphoma cell lines [5]. Another report indicated that c-MYC and HIF-1 differentially regulate HXKII levels depending on the type of lymphoma [6]. c-MYC is required for the induction of HXKII expression in Burkitt lymphoma cells, whereas HXKII expression is dependent on HIF-1 in lymphoblastoid cell lines [6]. In the present study, we found a strong correlation between HXKII levels and c-MYC levels in a series of B-cell lymphoma cell lines. The inhibition of c-MYC by a chemical inhibitor clearly decreased HXKII levels. In addition, Burkitt lymphoma cell lines exhibited higher expression levels of HXKII and activation of NF-κB and HIF-1 compared with DLBCL cell lines. Based on these results, we proposed the model illustrated in Supplementary Figure E3 (online only, available at www.exphem.org). In Burkitt lymphoma cells, activation of both the c-MYC and NF-κB/HIF-1 axes

resulted in high levels of induced HXKII expression. However, only c-MYC is involved in the regulation of HXKII expression in DLBCL cell lines. Importantly, these notions were supported by the analysis of clinical samples. Immunohistological analysis revealed that Burkitt lymphoma cells had higher levels of HXKII compared with GCB-type DLBCL cells. Interestingly, non-GCB-type DLBCL patients had high levels of HXKII compared with GCB-type DLBCL patients (Figure 2A). Aberrant activation of NF-κB is widely known to occur frequently in non-GCB-type DLBCL. Although speculative at present, our results suggest that abnormal activation of the NF-κB pathway may cause higher HXKII expression in non-GCB-type DLBCL as in Burkitt lymphoma.

In addition to these cell intrinsic mechanisms, our results revealed that the hypoxic tumor microenvironment could trigger HXKII expression through strong activation of HIF-1 in both Burkitt lymphoma and DLBCL cell lines and conferred the ability to block apoptosis induced by cisplatin, as illustrated in Supplementary Figure E3.

Platinum-based chemotherapeutic regimens, that is, GDP, DHAP, ESHAP, and ICE, are the standard salvage therapies in patients with refractory/relapsed B-cell lymphoma [20]. However, the treatment outcomes of patients on these regimens are not satisfactory. Therefore, identifying a strategy to improve the treatment outcomes of platinum-based chemotherapeutic regimens is very important. Cancer cells acquire resistance to cisplatin through a number of mechanisms [7,21]. Interestingly, HXKII also contributes to cisplatin resistance through inhibition of caspase activity. HXKII bound to VDACs on the mitochondrial outer membrane block the release of cytochrome c, leading to protection from apoptosis. Shulga et al. [11] reported that detachment of HXKII from the mitochondria sensitized cancer cells to cisplatin. In the present study, we found that hypoxia conferred cisplatin resistance to the B-cell lymphoma cell lines. In addition, we found that HXKII expression was selectively elevated under hypoxic conditions. Therefore, we speculated that increased HXKII expression may be responsible for hypoxia-induced cisplatin resistance. Our observation also suggested that elevated HXKII expression may protect B-cell lymphoma cells by blocking VDAC activity but not by enhancing glycolysis.

The impairment of TP53 is a crucial mechanism of cisplatin resistance in cancer cells [22,23]. Notably, TP53 mutations are frequently found in B-cell malignancies [24,25], especially Burkitt lymphoma, and are often associated with poor prognosis [26]. Interestingly, Wang et al. [27] found that TP53 gene deficiency leads to the upregulation of HXKII in prostate cancer cells by blocking the biogenesis of miR-143, which destabilizes HXKII mRNA. It will be interesting to determine whether therapeutic approaches targeting HXKII confer an alternative form of p53-mediated cisplatin resistance in B-cell malignancies.

In this study, the inhibition of HXKII expression by panobinostat significantly restored cisplatin-induced apoptosis. Consistent with our results, Fischer et al. [28] determined that panobinostat overcomes hypoxia-induced cisplatin resistance in non-small cell lung carcinoma cells. Although they did not discuss HXKII levels after panobinostat treatment, they reported that cotreatment with cisplatin and panobinostat enhanced the ratio of apoptotic cells. Together with our present results, their results suggest that panobinostat may be an attractive drug to overcome cisplatin resistance in malignant cells. Currently, panobinostat is approved for the treatment of multiple myeloma, another type of B-cell-origin malignancy, in several countries, including Japan. The anti-lymphoma effects of panobinostat have also been confirmed in both preclinical [29] and clinical studies [30]. Taken together, our present results suggest that panobinostat may be an attractive candidate for combination treatment of relapsed and refractory B-cell lymphoma by inhibiting the expression of HXKII.

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Conflict of interest disclosure

The authors declare that they have no competing financial interest

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Supplemental Table 1.

name of antibody	name of the provider	dilution
anti-HXKII	Santa Cruz Biotechnology, Dallas, TX, USA	1:200
anti-c-MYC	Cell Signaling Technology, Danvers, MA, USA	1:1000
anti-HIF-1α	BD Biosciences, San Jose, CA, USA	1:500
anti-phospho-NF-kBp65	Cell Signaling Technology, Danvers, MA, USA	1:1000
anti-NF-kBp65	Cell Signaling Technology, Danvers, MA, USA	1:1000
anti-Bcl-xL	Cell Signaling Technology, Danvers, MA, USA	1:1000
anti-MCL1	Santa Cruz Biotechnology, Dallas, TX, USA	1:500
anti-GLUT1	Santa Cruz Biotechnology, Dallas, TX, USA	1:500
anti-PFKFB3	Cell Signaling Technology, Danvers, MA, USA	1:1000
anti-cleaved caspase3	Cell Signaling Technology, Danvers, MA, USA	1:1000
anti-caspase3	Cell Signaling Technology, Danvers, MA, USA	1:1000
anti-b-actin	Cell Signaling Technology, Danvers, MA, USA	1:1000

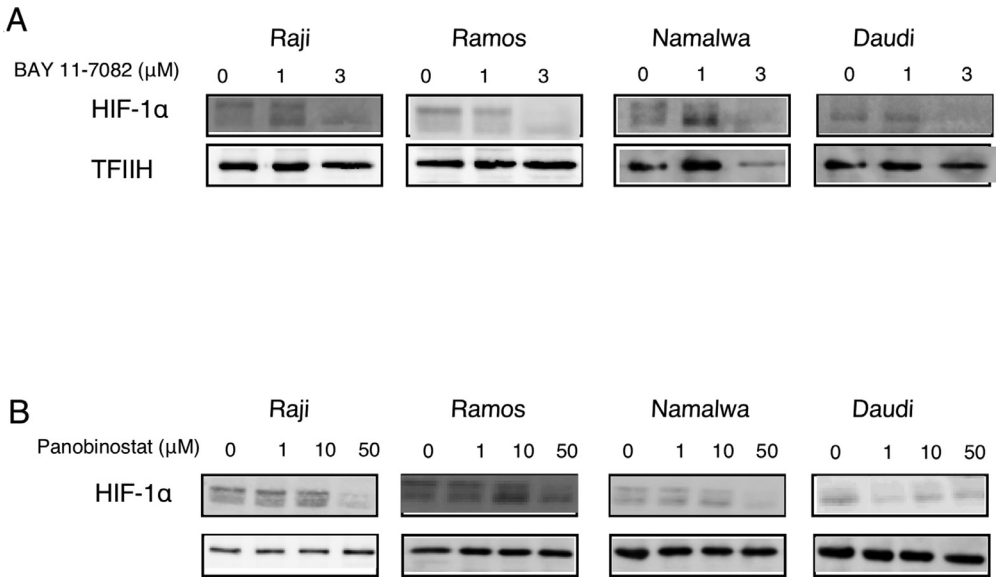
Supplemental Table 2.

List of DLBCL patients

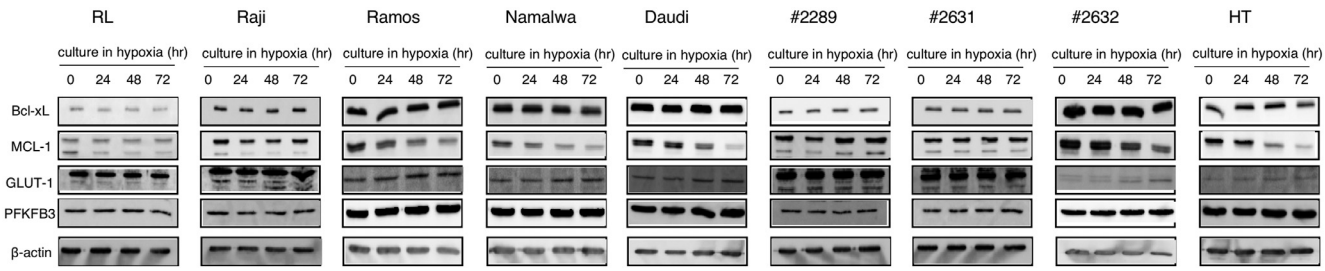
Type		Age	Sex	CD10	Bcl-6	MUM1
GCB	1	63	F	+	+	–
	2	52	M	+	–	–
	3	57	M	+	+	–
	4	80	F	+	+	+
	5	66	F	+	+	–
non-GCB	1	56	F	–	+	+
	2	80	M	–	–	+
	3	81	M	–	+	–

List of BL patients

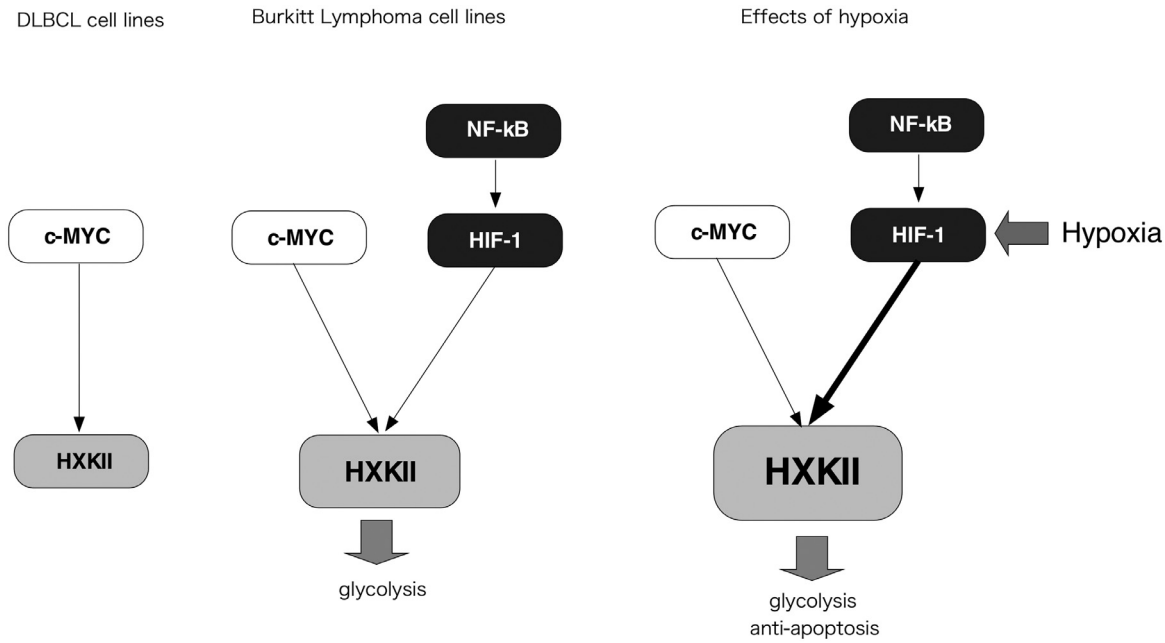
		Age	Sex
BL	1	59	M
	2	73	F
	3	77	M



Supplementary Figure E1. Effects of BAY11-7082 and panobinostat on HIF-1α expression. **(A)** Raji, Ramos, Namalwa, and Daudi cells were cultured with the indicated concentrations of BAY11-7082 for 48 hours. Then, expression levels of HIF-1α were analyzed by Western blotting. The membranes were also blotted with an antibody against TFIIH as a control. **(B)** Raji, Ramos, Namalwa, and Daudi cells were cultured with the indicated concentrations of panobinostat for 48 hours. Then, expression levels of HIF-1α were analyzed by Western blotting. The membranes were also blotted with an antibody against TFIIH as a control.



Supplementary Figure E2. Effects of hypoxia on the expression of putative HIF-1α-regulated proteins in lymphoma cells. Lymphoma cell lines were cultured under hypoxic conditions for the indicated periods. Then, whole-cell lysates were prepared, and expression of Bcl-XL, MCL-1, GLUT-1, and PFKFB3 was analyzed. As the internal control, expression of β-actin was also analyzed.



Supplementary Figure E3. Proposed model of the regulation and function of HXKII in B-cell lymphoma. In DLBCL cells, c-MYC regulates HXKII levels. In contrast, HXKII and HIF-1 cooperate to induce HXKII expression in Burkitt lymphoma cells. At basal levels, HXKII functions mainly as a glycolytic enzyme. Under hypoxia, the strong activation of HIF-1 induces substantially higher HXKII expression to block apoptosis.