Elevated Expression of BIRC6 Protein in Non–Small-Cell Lung Cancers is Associated with Cancer Recurrence and Chemoresistance

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Introduction: Non–small-cell lung cancer (NSCLC) is an aggressive, highly chemoresistant disease. Reliable prognostic assays and more effective treatments are critically required. BIRC6 (baculoviral inhibitors of apoptosis proteins repeat-containing 6) protein is a member of the inhibitors of apoptosis protein family thought to play an important role in the progression or chemoresistance of many cancers. In this study, we investigated whether BIRC6 expression can be used as a prognostic marker or potential therapeutic target for NSCLC.

Methods: In a retrospective analysis, BIRC6 protein expression was determined for 78 resected primary NSCLCs and nine benign lung tissues. Twenty-nine chemoresistant or chemosensitive subrenal capsule NSCLC tissue xenografts were assessed for BIRC6 expression, using immunohistochemistry, and 13 of them for *BIRC6* gene copy number, using array comparative genomic hybridization analysis. The effect of small interfering RNA–induced *BIRC6* knockdown on the growth of human NSCLC cell cultures and apoptosis (in combination with cisplatin) was investigated.

Results: Elevated BIRC6 protein expression in NSCLC tissues was associated with poor 3-year relapse-free patient survival, lymph node involvement, and advanced pathological tumor, node, metastasis stage. In patient-derived lung squamous cell carcinoma xenografts, chemoresistance was associated with elevated BIRC6 expression and increased gene copy number. Small interfering RNA–induced *BIRC6* down-regulation inhibited growth of the NSCLC cells and sensitized the cells to cisplatin.

Conclusions: BIRC6 may play an important role in the malignant progression and chemoresistance of NSCLC. Elevated BIRC6 protein expression may serve as a predictive marker for chemoresistance of NSCLCs and a poor prognostic factor for NSCLC patients. Down-regulation of the *BIRC6* gene as a therapeutic approach may be effective, especially in combination with conventional chemotherapeutics.

Key Words: Baculoviral inhibitors of apoptosis proteins repeat-containing 6, Prognostic factor, Non–small-cell lung cancer, Recurrence, Patient survival, Chemoresistance.

(*J Thorac Oncol.* 2013;8: 161–170)

Lung cancer is the most common cause of cancer-related deaths in both developing and developed countries, leading to more than 1.1 million deaths worldwide in 2008.[1] Approximately 85% of all lung cancer cases are non–small-cell lung cancers (NSCLC).[2,3] Despite progress in the diagnosis and treatment of NSCLC, patient survival is poor (~15% at 5 years).[4,5] Even in the case of surgically resectable stage I–III NSCLCs, approximately 50% of patients die within 5 years. In general, NSCLCs are highly chemoresistant, and postoperative conventional chemotherapy has only a marginal benefit, rendering an absolute improvement of 4% in survival at 5 years.[6]

Chemoresistance of cancers, including NSCLCs, is thought to be primarily due to resistance to drug-induced apoptosis, and overcoming such a hurdle represents an important strategy for cancer therapy.[7] Resistance to apoptosis is thought to occur through up-regulation of antiapoptotic genes and their products. A family of proteins, known as the inhibitors of apoptosis proteins (IAP), is of particular importance. The IAPs have been shown to bind to and inhibit a variety of proapoptotic factors (e.g., apoptosis-promoting caspases), thereby effectively suppressing drug- and radiation-induced apoptosis. The IAPs characteristically contain one to three copies of a baculoviral IAP repeat (BIR) domain, which plays a crucial role in this process.[8–11] Some IAPs, including X-linked inhibitor of apoptosis protein and survivin, have been reported to have therapeutic potential or prognostic value for lung cancers.[12–15]

BIRC6 (baculoviral IAP repeat-containing 6) protein is a relatively large IAP (528 kDa) containing a single N-terminal BIR domain and a unique C-terminal ubiquitin-conjugating

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Disclosure: The authors declare no conflict of interest.

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ISSN: 1556-0864/12/0802-161

(UBC) domain. Similar to other IAPs, it is able to bind directly to caspases-3, 6, 7 and 9 and inhibit their activities through its BIR domain. In addition to its IAP activity, BIRC6 can ubiquitylate the proapoptotic proteins Smac/Diablo, active caspase-9 and HTRA2/OMI through its UBC domain.[16–18] Recent evidence supports a significant role for BIRC6 in conferring apoptosis resistance to cancer cells, as reported for cell lines derived from human brain and breast cancers.[19,20] Elevated expression of *BIRC6* messenger RNA (mRNA) in childhood de novo acute myeloid leukemia has been associated with poor patient survival.[21,22] However, a role for BIRC6 in lung cancer has not yet been established.

In the present study, we assessed the prognostic relevance of BIRC6 in NSCLC by examining the levels of BIRC6 protein in preserved, surgically resected NSCLC tissues for correlations with clinicopathological features and patient outcomes. We also looked for correlations between BIRC6 protein expression and chemoresistance, using preserved tissues from patient-derived NSCLC tissue xenografts that had been evaluated for chemosensitivity. To determine whether BIRC6 could provide a chemotherapeutic target for NSCLC, the effect of BIRC6 knockdown was determined on the growth and viability of human lung cancer cell cultures using *BIRC6*-targeting small interfering RNA (siRNA) alone and in combination with an established anticancer drug (cisplatin).

MATERIALS AND METHODS

Materials

Chemicals, solvents and solutions were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada), unless otherwise indicated.

Patient Tumor Tissues

Tumor tissues from 78 patients, obtained through surgical resection of primary NSCLCs, had been fixed in 10% neutral-buffered formalin and embedded in paraffin blocks at the Vancouver General Hospital (2005–2006). None of the patients had received preoperative chemo- or radiotherapy. The clinical characteristics of the patients, including sex, age, histopathology, tumor size, lymph node involvement and pathological TNM (pTNM) cancer stage are summarized in Table 1. Three-year relapse-free survival data of the patients, calculated from the day of surgical resection, were obtained from the British Columbia Cancer Agency (BCCA) Cancer Registry and from hospital charts at the BCCA. Histologic classification of the tumors was based on World Health Organization classification. TNM staging was based on the staging system proposed by the International Association for the Study of Lung Cancer in 2009.[23]

Patient-Derived NSCLC Xenograft Tissues

Paraffin-embedded tissues of first-generation subrenal capsule NSCLC tissue xenografts, derived from 29 patients, had been generated in a previous study[24] in which the xenografts were established and evaluated for response to treatment with cisplatin plus vinorelbine in vivo (i.e., chemosensitive versus chemoresistant). Sections (5-μm thick) were cut from all 29 xenograft tissue blocks for immunohistochemical (IHC) staining of BIRC6 protein (see below).

IHC Staining and Scoring

Sections of the archival NSCLC tissues were cut on a microtome (5 μm thick) and mounted on glass slides. Sections were dewaxed in xylene and then hydrated in graded alcoholic solutions and distilled water. For histopathologic analysis, routine hematoxylin and eosin (H&E) staining was carried out. For IHC staining, sections were subjected to antigen retrieval by boiling it in antigen-unmasking solution for 10 minutes (Vector Laboratories Inc., Burlingame, CA). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 minutes followed by washing with phosphate-buffered saline (PBS [pH 7.4]) and nonspecific binding blocked, using SuperBlock blocking buffer (Thermo Scientific, Rockford, IL) in Tris-buffered saline (pH 7.4) for 60 min. The sections were incubated with rabbit polyclonal anti-BIRC6 antibody (Novus Biologicals, Littleton, CO) recognizing epitopes between residue 4775 and 4829 (C-terminus) of BIRC6 protein (Swiss-Prot entry Q9NR09; GeneID 57448) at a 1:100 dilution at 4°C overnight. Sections were then washed with PBS and incubated with goat antirabbit secondary antibodies for 30 minutes at room temperature. Sections were washed in PBS (five 5-minute washes), and incubated with avidin–biotin complex (Vector Laboratories, Foster City, CA) for 30 minutes at room temperature. After a further 25-minute washing in PBS, immunoreactivity was visualized using 3′, 3′-diaminobenzidine in PBS and 3% hydrogen peroxide. Sections were counterstained with 5% (w/v) Harris hematoxylin and dehydrated in graded alcohols.

Staining of cytoplasm-associated BIRC6 protein was evaluated independently by two investigators in blinded analyses. Areas of positive staining were given an intensity score of 0, 1, 2, or 3, representing a range from negative staining to heavy staining. The percentage of tumor cells showing positive for a given intensity (percent positive areas, P) was estimated (P1, P2 or P3). Values ranged from 0% to100%. The final staining score of each tissue section was determined by combining score intensity and percent positive areas using the formula: $1\times P1 + 2\times P2 + 3\times P3$.[25–28]

Microdissection and DNA Extraction of Patient-Derived First-Generation NSCLC Tissue Xenografts

From the paraffin blocks of seven squamous cell carcinoma and six adenocarcioma subrenal capsule xenografts, 23 sections (7-μm thick) were cut, using a microtome, and mounted on glass slides. Routine H&E staining was carried out on sections 1, 12, and 23. The H&E slides were reviewed by pathologists and cancerous areas encircled (>70% cancer cells by area). On the basis of the pathological reviews, manual microdissection of cancerous areas on sections 2 to 11 and 13 to 22 was performed using sterile scalpel blades. The remaining tissues on the slides were stained with H&E to confirm that the microdissection of cancerous areas had been carried out correctly. DNA extraction was performed according to a standard phenol-chloroform extraction

TABLE 1. BIRC6 Protein Levels in Primary NSCLCs (Immunohistochemistry Score)

Clinicopathologic Factors	*n*	BIRC6 Score (Mean ± SD)	*p*
Sex			
Male	34	1.35 ± 1.12	0.448
Female	44	1.12 ± 0.98	
Age, yrs			
≤65	30	1.28 ± 1.06	0.768
>65	48	1.18 ± 1.04	
Pathology			
Adenocarcinoma	49	1.08 ± 0.98	0.566[a]
Squamous cell carcinoma	22	1.47 ± 1.17	
Large-cell carcinoma	5	1.4 ± 1.11	
Other subtypes	2	1.5 ± 2.12	
Tumor size			
T1	14	1.03 ± 0.27	0.394[b]
T2	55	1.29 ± 0.47	
T3	3	0.36 ± 0.56	
T4	6	1.42 ± 0.96	
Lymph node involvement[c]			
N_{neg}	44	0.99 ± 1.03	0.023
N_{pos}	33	1.50 ± 1.02	
pTNM stage[c]			
I	38	0.98 ± 1.04	0.037[d]
II	24	1.42 ± 1.04	
III	14	1.40 ± 1.01	
IV	1	2.60	

[a]Adenocarcinoma versus other types.
[b]T1 versus T2–4.
[c]Information of one patient could not be assessed.
[d]Stage I versus stage II–IV.
BIRC6, baculoviral inhibitors of apoptosis proteins repeat-containing 6; pTNM, pathological TNM.

protocol.[29] DNA was quantified using a Nanodrop ND1000 spectrophotometer (Nanodrop Products, Wilmington, DE) and verified by gel electrophoresis.

Array Comparative Genomic Hybridization and Analysis

For patient-derived subrenal capsule xenografts, genome-wide copy number analysis was performed using a whole genome tiling-path array (the SMRT array v.2) against single male reference genomic DNA.[30] Fluorescent labeling, hybridization, scanning, and washing were performed as previously described.[31–34] Array images were analyzed using SoftWoRx Tracker Spot Analysis software (Applied Precision, Issaquah, WA) and systematic biases removed using a stepwise normalization algorithm, as previously described.[35,36] Normalized files were imported into *SIGMA2* to combine duplicate spot data, display $\log_2$ signal intensity ratios in relation to genomic locations (NCBI Build 36.1), and exclude data points with a standard deviation greater than 0.075 and signal to noise ratio less than three in either channel.[37] Fast Algorithm for Calling After Detection of Edges, which uses edge detection in combination with nonparametric statistics, was used to segment and call-filtered $\log_2$ signal intensity ratio data, using the following recommended settings—breakpoints = 2000, amplifications = $\log_2$ signal intensity ratio > 0.8, deletion = $\log_2$ signal intensity ratio < −0.5, 5 level output, *p* value 0.05, $\log_2$ ratio delta: 0.1.[38] Copy number data were thus scored as: gain or amplification = 1, neutral or retention = 0, or loss or deleted = −1, for each individual sample. The occurrence of alterations (gain, loss, and retention) was then statistically compared between chemoresistant and chemosensitive tumors, using a Fisher exact test, performed in the *R* statistical computing environment, on a 3 × 2 contingency table with a *p*-value threshold of 0.05.[34] Adjacent regions within 1 Mb, matching direction of copy number difference and statistical significance, were then merged.

Western Blotting

Cultured cells were lysed using cell lysis buffer (1% NP-40, 0.5% sodium deoxycholic acid) supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland); total lysate protein was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL). Whole-cell lysates (8 μg) were run on a 5% to 12.5% sodium dodecyl sulfate polyacrylamide gel at 80 volts for 2.5 hours. The BIRC6 protein band (528 kD) was electrotransferred to a polyvinylidene fluoride membrane at 25 volts for 3 hours in transfer buffer (Tris 25 mM, glycine 191.5 mM, methanol 10%, sodium dodecyl sulfate 0.05%) using a semidry transfer apparatus. Equal loading of the sample was assessed by separately electrotransferring actin (42 kDa) to a polyvinylidene fluoride membrane at 100 volts for 1 hour at room temperature with transfer buffer (Tris [25 mM], glycine [191.5 mM], methanol [15%]), using standard wet transfer apparatus. The membranes were then blocked for 1 hour in 5% bovine serum albumin in Tris-buffered saline + Tween 20 (0.5 mM Tris-HCl, 45 mM NaCl, 0.075% Tween 20 [pH 7.4]). The membranes containing the whole-cell lysate proteins were then probed with rabbit polyclonal anti-BIRC6 antibody at 1:500 dilution (Novus Biologicals, Littleton, CO) and the actin-carrying membranes with rabbit antiactin polyclonal antibody at 1:2000 dilution overnight at 4°C. Subsequently, the membranes were probed with a secondary horseradish peroxidase-conjugated goat antirabbit antibody (Thermo Scientific, Rockford, IL). The protein bands were detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL). A human prostate cancer cell line, PC3, was used as a positive control.

siRNA and Cell Transfection

SiRNA targeting *BIRC6* and nontargeting siRNAs were purchased from Dharmacon (Lafayette, CO). The *BIRC6*-targeting siRNA was sense, 5′-GUU UCA AAG CAG GAU GAU G-dTdT-3′.[39] Vehicle and nontargeting siRNA (siGENOME nontargeting siRNA #3 D-001210-03-05, Dharmacon) were used as controls. To examine the effect of the siRNAs on *BIRC6* expression, human lung adenocarcinoma cells (A549) and squamous cell carcinoma cells (H226), acquired from the American Type Culture Collection (Manassas, VA),

were plated in six-well plates in antibiotic-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (FBS; 10%). After 20 hours, the cells were transfected with 80 nM siRNA in Lipofectamine 2000 reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions. Briefly, a complex of Lipofectamine 2000 and siRNA in Opti-MEM Reduced Serum Media (500 µl) was gently added to each well. After 6 hours, the transfection mixture was removed and fresh antibiotic-free DMEM supplemented with FBS (10%) was added. Vehicle (Lipofectamine 2000) and nontargeting siRNA were applied in separate wells. Cells were incubated for 24 or 72 hours for subsequent Western blotting, measurement of cell viability, or treatment with cisplatin.

Treatment of NSCLC Cell Cultures with Cisplatin in Combination with BIRC6-Targeting siRNA

A549 and H226 cells were transfected with Lipofectamine 2000, *BIRC6*-targeting or nontargeting siRNAs for 6 hours, and the transfection medium was replaced with fresh antibiotic-free DMEM supplemented with FBS (10%) for a 24-hour incubation in 5% carbon dioxide atmosphere. The medium was then replaced by DMEM-10%FBS medium containing cisplatin with a final concentration of 20 µM (A549) or 25 µM (H226) for another 24-hour incubation. This was followed by a 24-hour incubation in drug-free DMEM-10%FBS medium and cell harvesting.

Diphenyltetrazolium Bromide Cell Viability Assay

Inhibition of NSCLC cell culture growth was determined by the 3-(4-, 5-dimethylthiazol-2-yl) 2-, 5-diphenyltetrazolium bromide (MTT) assay. In brief, 20 µl of MTT (5 mg/ml) was added to cell cultures in 48-well dishes (cell density: 10^3–10^5 cells/well). Cells were incubated for another 4 hours at 37°C in 5% carbon dioxide or air atmosphere before the medium was aspirated, and the precipitated formazan was dissolved by adding 150 µl of dimethylsulfoxide and incubating for 10 to 20 minutes at room temperature. Samples (100 µl) were then transferred to 96-well plates. Absorbance was measured at 570 nm using a microplate reader.

Flow Cytometric Apoptosis Assays

Apoptosis was measured by fluorescence-activated cell sorter analysis with annexin-V conjugated with fluorescein isothiocyanate (Annexin-V-FITC) (BD Biosciences PharMingen, San Diego, CA) for early apoptosis and 7-amino-actinomycin D (7-AAD) for late apoptosis staining according to the manufacturer's protocol. Cells were cultured in DMEM-10% FBS medium, treated with *BIRC6* siRNA and, subsequently, with cisplatin as described above. Twenty-four hours after treatment with cisplatin, cells were harvested, washed with cold PBS and then resuspended in 1X Binding Buffer (BD Biosciences PharMingen) at a concentration of approximately 1×10^6 cells/ml. Cell suspensions (100 µl; ~1×10^5 cells) were transferred to 5-ml culture tubes, and 5-µl Annexin V–FITC and 5-µl 7-AAD aliquots were added. The cultures were incubated in the dark for 15 minutes at 21°C. Annexin–FITC fluorescence was measured in the FL1 channel (using a 530/30 band pass filter) and 7-AAD in FL3 channel (660/20 BP filter band pass filter). Ten thousand events were collected. In the fluorescence-activated cell sorter graph, nonapoptotic (viable) cells were in the lower left quadrant (Q4: AnV−/7AAD−), apoptotic cells in the upper and lower right quadrants (Q3: AnV+/7AAD−, early apoptotic cells; Q2: AnV+/7AAD+, late apoptotic cells) and necrotic cells in the upper left quadrant (Q1: AnV−/7AAD+).

Statistics

The Mann–Whitney *U* test was used to determine the association of BIRC6 protein expression with demographic factors. The Kaplan–Meier method was used to estimate curves for relapse-free survival for subgroups of BIRC6 expression levels and comparisons were made with the use of the log-rank test. Hazard ratios were calculated using Cox proportional hazard models. The Student's *t* test was used for comparison of in vitro studies. All tests of significance were two sided, and differences were considered statistically significant with p values less than 0.05. SPSS software was used for all the analyses.

RESULTS

Elevated BIRC6 Protein Expression in Clinical NSCLC Samples is Associated with Regional Lymph Node Metastasis and Advanced pTNM Stages

In a retrospective study, using preserved clinical NSCLC samples, correlations were sought between degree of BIRC6 protein expression and malignant progression of the cancers. As shown in Table 1, the study involved specimens from 78 patients with an average age of 69.09 ± 9.52 (mean ± SD) years; the majority of the patients had T2 (70.51%) and N0 (56.41%) status, and 38 (49%) were graded as pTNM stage I disease. Immunohistochemical analysis of BIRC6 expression was performed on all patients' NSCLC samples and also on nine benign pulmonary parenchymal tissues. Benign epithelium cells showed weak to negative BIRC6 protein expression (mean ± SD: 0.18 ± 0.29, Fig. 1*A*). In contrast, the NSCLC cells showed approximately sixfold higher BIRC6 expression with an average score of 1.21 ± 1.05 ($p < 0.001$, Fig. 1*B–D*). Elevated expression of BIRC6 protein in the NSCLC samples was associated with metastasis to regional lymph nodes (N_{neg} versus N_{pos}, $p = 0.023$). Furthermore, there was a significant correlation between elevated BIRC6 protein expression and advanced pTNM stages ($p = 0.037$). Since adenocarcinoma and squamous cell carcinoma are the major histologic types of NSCLCs, the associations of BIRC6 expression with stage and lymph node status of these cancers were further determined. For both types, higher expression levels of BIRC6 were observed in cancers with lymph node involvement and advanced stages, although the increases lacked statistical significance (see Supplemental Table 1, Supplemental Digital Content 1, http://links.lww.com/JTO/A377).

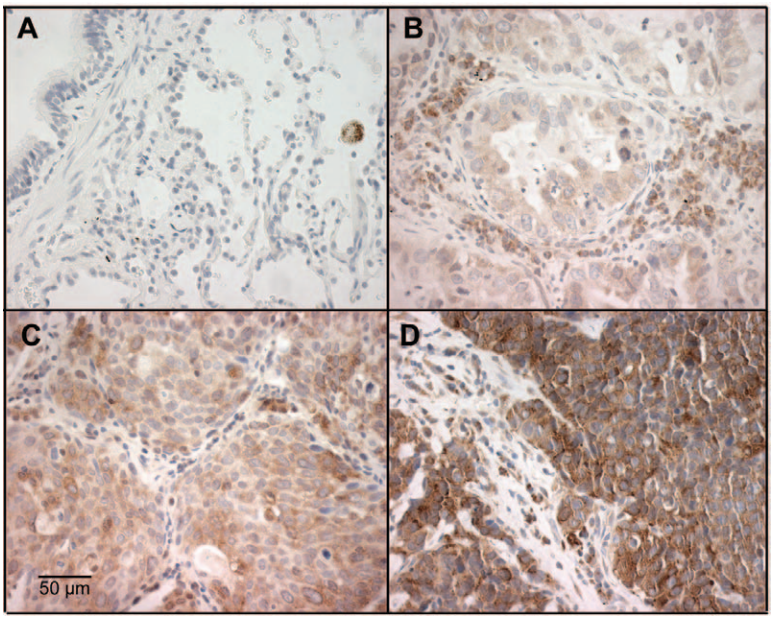

FIGURE 1. Immunohistochemical staining of BIRC6 protein in patients' *(A)* benign and (*B–D*), NSCLC tissues. Whereas the benign tissue lacked BIRC6 expression (score, A = 0), the NSCLC tissues showed elevated BIRC6 expression with scores B = 1, C = 2, and D = 3. NSCLC, non–small-cell lung cancer; BIRC6, baculoviral inhibitors of apoptosis proteins repeat-containing 6.

Elevated BIRC6 Protein Expression in NSCLCs Correlates with Poor Patient Prognosis.

Of the 78 NSCLC samples assessed for BIRC6 expression, 47.4% scored less than 1, 23.1% scored between 1 and less than 2, 21.8% scored between 2 and less than 3, and 7.7% scored 3 (Fig. 2*A*). Overall, moderate to strong BIRC6 expression (score ≥1) was observed in 52.6% of the cases. For patient survival analysis, a BIRC6 score = 1 was used to separate patients into two groups: score lesser than 1 versus score of 1 or more. Kaplan–Meier analysis showed that the group with BIRC6 score of 1 or more had markedly lower relapse-free survival (p = 0.009, Fig. 2*B*, hazard ratio = 2.458, 95% confidence interval [CI]: 1.290–4.683). For adenocarcinoma patients, BIRC6 scores of 1 or more were associated with poor relapse-free survival (Fig. 2*C*; p = 0.009, hazard ratio = 2.912, 95% CI: 1.317–6.441). There was no significant association between BIRC6 expression and 3-year relapse-free survival of patients with squamous cell carcinoma (Fig. 2*D*; p = 0.348). As shown in Table 2, Cox regression analysis of multivariates, consisting of sex, age, histopathologic types, tumor size, lymph node status, pTNM stage and BIRC6 score, indicates that elevated BIRC6 expression has a relative risk of 2.099 for 3-year relapse-free patient survival (95% CI: 0.992–4.441) with a p = 0.052. A greater statistical significance (p = 0.030) was obtained through Cox regression analysis of only three variables, that is, histopathologic types, pTNM stage, and BIRC6 score (Table 3).

Elevated BIRC6 Protein Expression in Patient-Derived NSCLC Xenografts Associated with Increased Chemoresistance.

In a pilot study, patient-derived subrenal capsule cancer tissue xenografts of seven lung squamous cell carcinomas and six lung adenocarcinomas, showing resistance or sensitivity to treatment with cisplatin plus vinorelbine in vivo, were subjected to array-based, comparative genomic hybridization. As indicated in Table 4, chemoresistant squamous cell carcinoma xenografts showed *BIRC6* gene copy number gain, in contrast to chemosensitive xenografts that did not show a change in this parameter. This difference was statistically significant using a Fisher exact test (p = 0.029). This finding was validated at the protein level by immunohistochemical analysis of tissue sections of the xenografts, showing elevated BIRC6 protein expression in the chemoresistant xenografts except 06L13 (Table 4; Fig. 3*A*, *B*) and weak to negative BIRC6 protein expression in chemosensitive xenografts (Fig. 3*C*, *D*). An association between BIRC6 gene copy number gain and chemoresistance was not observed for lung adenocarcinoma xenografts (data not shown).

We next investigated BIRC6 protein expression in tissue sections of first-generation NSCLC xenografts from 29 patients, including the above seven patients with squamous cell carcinomas and six patients with adenocarcinomas, that had been assessed for sensitivity to treatment with cisplatin plus vinorelbine in vivo. They were distributed into eight chemosensitive and 21 chemoresistant xenografts. As shown in Figure. 3*E*, expression of BIRC6 protein was markedly elevated in the chemoresistant xenografts (p = 0.012).

Down-Regulation of the *BIRC6* Gene Inhibits Proliferation of NSCLC Cells

Five of six NSCLC cell lines showed strong expressions of BIRC6 protein as demonstrated by Western blot analysis (data not shown). To determine whether targeting the *BIRC6* gene could be used as a potential therapeutic strategy, we

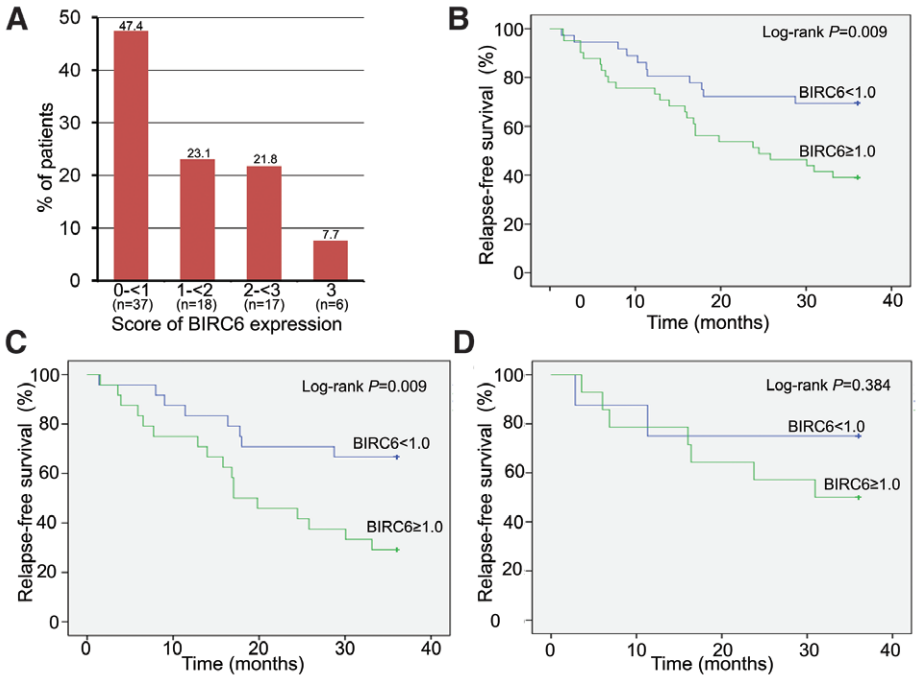

FIGURE 2. Correlation between elevated BIRC6 expression in resected NSCLCs and poorer 3-year relapse-free survival of patients. *A*, Percentage of NSCLC patients at increasing BIRC6 scores. *B*, Kaplan–Meier analysis of relapse-free survival of 78 patients grouped on the basis of BIRC6 scores of their resected tumors (BIRC6 score <1 versus score ≥1, log-rank p =0.009. Hazard Ratio=2.458; 95% CI 1.290–4.683). *C*, Kaplan–Meier analysis of relapse-free survival of 49 patients with resected adenocarcinoma (BIRC6 score <1 versus score ≥1, log-rank p =0.009. Hazard Ratio=2.912; 95% CI 1.317–6.441). *D*, Kaplan–Meier analysis of relapse-free survival of 22 patients with resected squamous cell carcinoma (BIRC6 score <1 vs. score ≥1, log-rank p =0.348). BIRC6, baculoviral inhibitors of apoptosis proteins repeat-containing 6;NSCLC, non–small-cell lung cancer; CI, confidence interval.

tested the effects of siRNA-induced *BIRC6* down-regulation on A549 cell proliferation. The gene silencing efficacy of *BIRC6* siRNA was confirmed by Western blotting. After transfection of A549 cells with *BIRC6* siRNA (80 nM/6 hour) and subsequent culturing, there was a decrease in the amount of cellular BIRC6 protein at 48 hours and essentially no BIRC6 protein at 72 hours, compared with transfection with Lipofectamine only, or nontargeting siRNA (Fig. 4*A*). The proliferation of *BIRC6* siRNA-transfected A549 cells was determined using an MTT assay. As shown in Figure 4*B*, transfected A549 cultures showed decreased growth starting at 48 hours after transfection, resulting in a 28% lower cell number at 96 hours relative to controls, that is, cells treated with Lipofectamine only or nontargeting

TABLE 2. Multivariate Cox Regression Analysis of Relapse-Free Survival of NSCLC Patients

Variables	Relative Risk	95% CI	p
Sex			
Female vs. male	1.175	0.53–2.606	0.692
Age, yrs			
<65 vs. ≥65	1.344	0.654–2.766	0.421
Histopathologic types			
Adenocarcinoma vs. other	0.601	0.296–1.345	0.215
Tumor size			
T1 vs. T2–T4	1.406	0.488–4.056	0.528
Lymph node status			
N_{neg} vs. N_{pos}	0.785	0.243–2.536	0.686
pTNM stage			
Stage I vs. stage II–IV	4.744	1.328–16.946	0.017
BIRC6 score			
<1 vs. ≥1	2.099	0.992–4.441	0.052

Cox regression analysis using seven variables.
CI, confidence interval; NSCLC, non–small-cell lung cancer; BIRC6, baculoviral inhibitors of apoptosis proteins repeat-containing 6.

TABLE 3. Multivariate Cox Regression Analysis of Relapse-Free Survival of NSCLC Patients

Variables	Relative Risk	95% CI	p
Histopathologic types			
Adenocarcinoma vs. other	0.646	0.322–1.2985	0.222
pTNM stage			
Stage I vs. stage II–IV	2.567	1.277–5.167	0.009
BIRC6 score			
<1 vs. ≥1	2.233	1.083–4.604	0.030

Cox regression analysis using three variables.
CI, confidence interval; pTNM, pathological TNM; BIRC6, baculoviral inhibitors of apoptosis proteins repeat-containing 6.

TABLE 4. Gains in BIRC6 DNA Copy Number and Elevated Scores of BIRC6 Protein in Patient-Derived Subrenal Capsule Lung Squamous Cell Carcinoma Xenografts Resistant to Treatment with Cisplatin Plus Vinorelbine

Case	DNA Copy Number Change	BIRC6 Protein (IHC Score)	Average IHC Score (Mean ± SD)
Chemoresistant			2.02±0.79
05L48	Gain	3.0	
06L13	Gain	0.7	
05L1	Gain	2.3	
Chemosensitive			0.15±0.23
L7	None	0	
05L8	None	0.01	
05L42	None	0.25	
06L28	None	0	

BIRC6, baculoviral inhibitors of apoptosis proteins repeat-containing 6; IHC, immunohistochemistry.

siRNA ($p < 0.001$). Similar results were obtained using a squamous cell carcinoma cell line, H226 (see Supplemental Fig. 1A, Supplemental Digital Content 1, http://links.lww.com/JTO/A377).

Effect of Down-Regulating *BIRC6* Expression on Apoptosis of NSCLC Cells: Enhancement of Cisplatin-Induced Apoptosis

To investigate the effect of *BIRC6* down-regulation on apoptosis, A549 cells were transfected with *BIRC6* siRNA (80 nM/6 hours), Lipofectamine or nontargeting siRNA and incubated for 48 hours. Apoptotic cells were detected through flow cytometric analysis of annexin V or 7-AAD stained cells. As shown in Figure 4*C-E*, *BIRC6* siRNA did not significantly increase apoptosis compared with controls ($p > 0.05$).

To investigate whether down-regulation of *BIRC6* expression has the potential to sensitize lung cancer cells to chemotherapy, three groups of A549 cell cultures, that is,

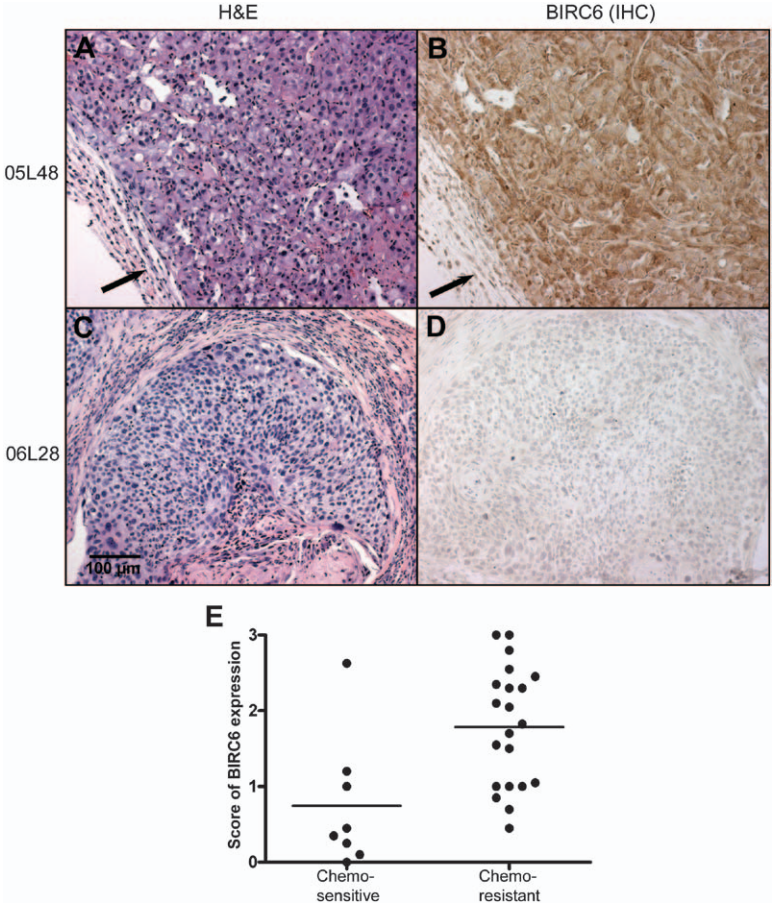

FIGURE 3. Patient-derived subrenal capsule NSCLC tissue xenografts resistant to treatment with cisplatin + vinorelbine (*A*, *B*) showed strong IHC staining (brown) of BIRC6 protein, compared with a sensitive xenograft (*C*, *D*). Arrows point at the renal capsules of the hosts (×200). *E*, Shows significant difference in BIRC6 IHC score between chemosensitive and chemoresistant xenografts ($p = 0.012$). NSCLC, non–small-cell lung cancer; IHC, immunohistochemistry. BIRC6, baculoviral inhibitors of apoptosis proteins repeat-containing 6.

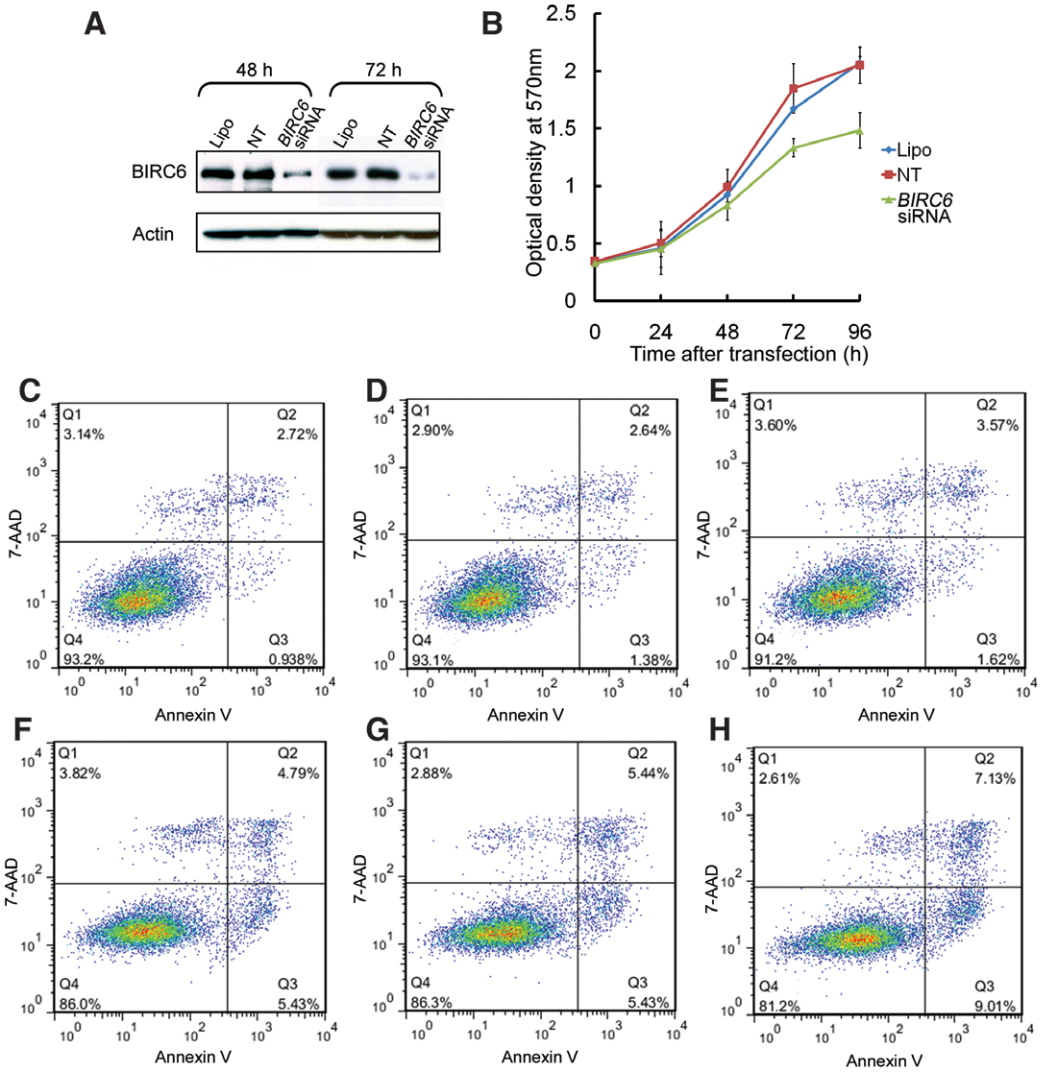

FIGURE 4. *A*, Western blot analysis confirming the gene silencing efficacy of *BIRC6* siRNA. After transfection of A549 cells with *BIRC6* siRNA (80 nM/6 hrs) and subsequent culturing, there was a substantial decrease in the amount of cellular BIRC6 protein, especially at 72 hours, compared with cells transfected with Lipofectamine only (Lipo) or nontargeting siRNA. *B*, MTT assay showed that transfection with *BIRC6* siRNA (80 nM/6 hrs) led to a decrease in the proliferation of A549 cells within 96 hours, compared with controls (Lipo or NT) ($p < 0.001$). *C–E*, Apoptosis of A549 cells transfected with (*C*) Lipo, (*D*) NT, or (*E*), *BIRC6* siRNA (80 nM/ 6 hrs) and subsequently incubated for 48 hours, as assessed by flow cytometric analysis of Annexin V/7-aminoactinomycin D stained cells. On the basis of Q2+Q3 values (apoptotic cell populations), *BIRC6* siRNA slightly increased apoptosis compared with controls, but without statistical significance. (*F–H*), A549 cells were transfected with (*F*) Lipo, (*G*) NT, or (*H*) *BIRC6* siRNA (80 nM/6 hrs), incubated for 24 hours, and subsequently treated with 20 μM cisplatin for another 24 hours. *BIRC6* siRNA markedly enhanced cisplatin-induced apoptosis of A549 cells compared with Lipo ($p = 0.034$) or NT ($p = 0.030$). (Data are representative of 3 independent experiments). BIRC6, baculoviral inhibitors of apoptosis proteins repeat-containing 6; siRNA, small interfering RNA; MTT, diphenyltetrazolium bromide assay.

pretreated with *BIRC6* siRNA, Lipofectamine, or nontargeting siRNA controls, were incubated with cisplatin to trigger apoptosis. As shown in Figure 4*F–H*, *BIRC6* siRNA markedly increased cisplatin-induced apoptosis of A549 lung adenocarcinoma cells compared with controls ($p = 0.03$ in each case). Similar results were obtained using a squamous cell carcinoma cell line, H226 (see Supplemental Fig. 1B–D, Supplemental Digital Content 1, http://links.lww.com/JTO/A377).

DISCUSSION

NSCLC is an aggressive disease characterized by rapid progression and very poor patient survival. The majority of NSCLCs exhibit high resistance to conventional first-line chemotherapy.[5] Prognostic factors for NSCLC progression and more effective therapies are, therefore, critically needed for improved management of the disease. The present study focused on BIRC6 protein, a member of the IAP family

thought to play an important role in the malignant progression and chemoresistance of cancers.[40–43] Although the clinical relevance of BIRC6 remains largely unknown, elevated expression of BIRC6 mRNA or protein has been detected in a number of cancers.[21,22,44,45] In the case of childhood acute leukemia, elevated expression of BIRC6-mRNA was found to be associated with poor relapse-free patient survival.[21,45] This is consistent with the correlation found in the present study between elevated BIRC6 protein expression and poor 3-year relapse-free survival of patients with resectable NSCLCs (Fig. 2*B*), indicating the potential for elevated BIRC6 protein expression to be used as a poor prognostic factor for patients with NSCLC. As indicated by Cox regression analysis of seven variates (Table 2), elevated BIRC6 expression may have independent prognostic value with a $p = 0.052$. This borderline significance could be a result of the relatively small number of samples ($n = 78$) used. A Cox regression analysis of only histologic type, stage, and BIRC6 score shows much higher significance for BIRC6 score ($p = 0.030$, Table 3). Notably, 49% of the cases in this study were at stage I. Patients with stage I NSCLCs in general have an estimated 28% to 45% chance of recurrence. If the patients who are likely to have recurrences after surgery could be identified, postoperative adjuvant chemotherapy could be recommended. Elevated BIRC6 protein expression could potentially serve as a marker for identifying stage I NSCLC patients with increased risk of postoperative relapse who would likely benefit from adjuvant therapy. However, in the current study, the number of patients with stage I NSCLC receiving adjuvant therapy was rather small to properly validate this suggestion ($n = 10$; all were relapse-free upon 3-year follow-up; data not shown).

The elevated expressions of BIRC6 protein in pTNM stages II–IV, relative to pTNM stage I, and in cases of lymph node involvement (Table 1, Fig. 2*A*) suggest that BIRC6 protein is involved in the malignant progression of NSCLC. The findings are echoed by those reported in the case of childhood acute leukemia, where the median level of BIRC6 expression was significantly increased in patients with extramedullary involvement.[45]

Molecular mechanisms underlying the chemoresistance of NSCLC can be studied with patient-derived, subrenal capsule NSCLC tissue xenograft models that closely resemble the disease in the clinic.[24] Chemoresistance of such xenografts was found to be associated with elevated BIRC6 protein expression and *BIRC6* copy number gains (Fig. 3, Table 4), suggesting that BIRC6 plays a role in conferring chemoresistance to NSCLCs and that its expression may be driven at the level of gene dosage. This is in agreement with a role for BIRC6 in chemoresistance reported for other cancer types where elevated expression of BIRC6 protein has been linked to poor chemoresponse in childhood leukemia,[21,45] and down-regulation of *BIRC6* expression increased sensitivity of various types of cancer cells to conventional anticancer drugs.[19,46,47] Elevated BIRC6 protein, therefore, represents a promising predictive marker for chemoresistance of NSCLCs. The disparity between BIRC6 copy number and intensity of IHC staining observed in the 06L13 xenograft (Table 4) could be a result of alterations of BIRC6 expression at the epigenetic level, for example by chromosomal methylation or histone acetylation.[48]

The present study also provides preliminary evidence suggesting that targeted inhibition of *BIRC6* may represent a novel potential target for NSCLC therapy, as indicated by the inhibition of NSCLC cell proliferation resulting from siRNA-induced down-regulation of *BIRC6* (Fig. 4*B*, Supplemental Fig. 1*A*, Supplemental Digital Content 1, http://links.lww.com/JTO/A377). Furthermore, down-regulation of *BIRC6* led to sensitization of NSCLC cells to cisplatin, as shown by increased apoptosis (Fig. 4*F-H*, Supplemental Fig. 1, *B–D*, Supplemental Digital Content 1, http://links.lww.com/JTO/A377). Similar chemosensitizing effects of *BIRC6* down-regulation have been reported for other types of cancer.[17,46] This suggests that down-regulation of the *BIRC6* gene as a therapeutic approach may be especially effective in combination with conventional chemotherapeutics. However, given the diverse mechanisms underlying anticancer effects of conventional chemotherapeutic drugs, more studies are needed to validate this effect and determine the most effective combinations.

In conclusion, the present study suggests that BIRC6 may play an important role in the malignant progression and chemoresistance of NSCLC, and that its elevated expression provides a novel and potentially useful predictive marker for chemoresistance and poor prognosis for NSCLC patients. BIRC6 may also provide a potentially effective therapeutic target for chemosensitization and treatment of NSCLC.

ACKNOWLEDGMENTS

This study was supported by Genome Canada and the Canadian Institutes of Health Research (YZW). The authors thank Rebecca Wu, Bradley Coe, and Kelsie Thu for excellent technical assistance.

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