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

Xin Dong, MD, Dong Lin, MD, PhD,*# Chris Low, MSc,* Emily A. Vucic, BSc,† John C. English, MD,‡ John Yee, MD,§ Nevin Murray, MD,‖ Wan L. Lam, PhD,† Victor Ling, PhD,† Stephen Lam, MD,† Peter W. Gout, PhD,* and Yuzhuo Wang, PhD*¶#*

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

Departments of *Experimental Therapeutics and †Integrative Oncology, BC Cancer Agency, Cancer Research Centre, Vancouver, BC, Canada; Departments of ‡Pathology, §Surgery, ‖Medical Oncology, and ¶Urologic Sciences, University of British Columbia, Vancouver, BC, Canada; and #The Vancouver Prostate Centre at Vancouver General Hospital, Vancouver, BC, Canada.

Disclosure: The authors declare no conflict of interest.

Xin Dong and Dong Lin contributed equally to this work and should be considered joint first authors.

Address for correspondence: Wang YZ, PhD, Department of Experimental Therapeutics, BC Cancer Agency,Cancer Research Centre, 675 West 10th Avenue, Vancouver, BC, Canada V5Z 1L3. E-mail: ywang@bccrc.ca

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