

cIAP2 Upregulated by E6 Oncoprotein via Epidermal Growth Factor Receptor/Phosphatidylinositol 3-Kinase/AKT Pathway Confers Resistance to Cisplatin in Human Papillomavirus 16/18–Infected Lung Cancer

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Abstract

Purpose: Inhibitors of antiapoptosis protein (IAP) have been implicated in the resistance to cisplatin. Therefore, verifying which pathway is involved in cIAP2 upregulation may be helpful in finding a feasible pathway inhibitor to increase the chemotherapeutic efficacy in human papillomavirus (HPV)–infected lung cancer.

Experimental Design: Specific inhibitors of different pathways were used to verify which pathway is involved in cIAP2 transcription. cIAP2 promoter fragments with various deletions and/or mutations were constructed by site-directed mutagenesis. cIAP2, epidermal growth factor receptor (EGFR), and phospho-AKT (p-AKT) expressions in 136 lung tumors were evaluated by immunohistochemistry.

Results: Our data show that two NF- κ B (–209 to –200 and –146 to –137) and one CREB (cyclic AMP–responsive element binding protein; –52 to –42) binding sites in cIAP2 promoter region were responsible for cIAP2 upregulated by E6 in TL-1 cells. Moreover, CREB was phosphorylated by EGFR/phosphatidylinositol 3-kinase (PI3K) pathway. To test the involvement of cIAP2 on cisplatin resistance, IC₅₀ was lowered to 8.6 μ mol/L in TL-1 cells with cIAP2 short hairpin RNA (shRNA) transfection and compared with 39.7 μ mol/L in TL-1 cells with nonspecific shRNA. Pretreatment with EGFR or PI3K inhibitor in TL-1 cells diminished the resistance to cisplatin. Among the tumor groups, cIAP2 expression correlated significantly with HPV16/18 E6, EGFR, and p-AKT. We followed up 46 of 136 patients who had tumor recurrence and/or metastasis and underwent chemotherapy. Tumors with cIAP2-positive immunostaining were associated with a poorer tumor response to chemotherapy compared with those with negative immunostaining.

Conclusions: cIAP2 upregulated by E6 via EGFR/PI3K/AKT cascades may contribute to cisplatin resistance, revealing that the EGFR or PI3K inhibitor combined with cisplatin may improve the chemotherapeutic efficacy in HPV-infected lung cancer. *Clin Cancer Res*; 16(21); 5200–10. ©2010 AACR.

Lung cancer in never smokers is a worldwide public health concern, with incidence and mortality similar to many other major cancer types. In the United States, it is as common a cause of death as cancer of the liver or of the esophagus, with 10% to 15% of lung cancers being in never smokers (1, 2). In Taiwan, >90% of women suffering with lung cancer are never smokers, and ~60% of female tumors have been detected to have human papillomavirus

(HPV) 16/18 infection, suggesting that HPV16/18-infected lung cancer may have a different molecular tumorigenic pathway in comparison with that of HPV-uninfected lung cancer (3).

Lung cancer has a poor prognosis. This is usually due to the fact that most patients with this disease receive a late diagnosis and experience a relapse because of the resistance to cisplatin-based chemotherapy (4, 5). To explore

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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doi: 10.1158/1078-0432.CCR-10-0020

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Translational Relevance

Lung cancer in never smokers is a different disease compared with smokers, as seen in Taiwanese women with lung cancer who have a higher prevalence of human papillomavirus (HPV) 16/18 infection but have never smoked in their lifetime. However, the chemoresistance mechanism in never smokers with this disease is unclear. In the present study, we provide evidence to show that cIAP2 upregulated by HPV E6 via the epidermal growth factor receptor (EGFR)/phosphatidylinositol 3-kinase (PI3K)/AKT cascade may play a crucial role in resistance to cisplatin in HPV16/18-infected lung cancer. Additionally, we followed up patients who underwent surgical resection and had received cisplatin-based chemotherapy for tumor recurrence and/or metastasis; we found that the patients with cIAP2-positive immunostaining had a poorer response to chemotherapy than those with cIAP2-negative immunostaining. Therefore, we suggest that the EGFR or PI3K inhibitor combined with cisplatin-based chemotherapy may improve the chemotherapeutic efficacy in lung cancer with HPV16/18 infection.

and establish an early diagnostic marker and target the molecular resistance to cisplatin is important to improve the patient's survival rate. However, information on the chemotherapeutic efficacy in lung cancer of never smokers is needed, as there is no available information in this area (6–8). Therefore, optimal treatment approaches for never smokers with lung cancer should be established.

Inhibitors of antiapoptosis proteins (IAP), including cIAP1, cIAP2, XIAP, and survivin, directly inhibit caspases to block cell apoptosis (9). It is conceivable that the overexpression of IAPs may be linked with tumor prognosis and drug resistance to chemotherapy (10). Among studies that associate IAPs with the clinical outcome, XIAP expression has been shown to have an adverse prognostic significance for patients with lung cancer (11), mesothelioma (12), and myeloid leukemias (13), but not for cervical cancer (14). In chemoresistance studies, expressions of XIAP and cIAP2 in pancreatic and prostate cancer have been shown to be associated with resistance to cisplatin and other anticancer drugs (15–17). Therefore, IAPs are considered to be a therapeutic target in cancer (18–20). However, the association of the expression of IAPs, including cIAP1, cIAP2, and XIAP, with the response to chemotherapy has not been observed in advanced non-small cell lung cancer (21).

Previous reports have indicated that cIAP2 is upregulated by E6 through the NF- κ B signaling pathway to confer resistance to apoptosis in HPV16 E6-immortalized human oral keratinocytes and primary human airway epithelial cells (22, 23). Therefore, we expect that HPV16/18-infected lung tumors and lung cancer cells may have

higher cIAP2 expression to confer resistance to chemotherapy. In the present study, we verify which signaling pathway may be responsible for the upregulation of cIAP2 by E6 in lung tumors and lung cancer cell lines. We also examine whether the optimal inhibitor can be used to reduce cisplatin resistance via blocking cIAP2 upregulation.

Materials and Methods

Chemicals and antibodies

PD153035 was obtained from Calbiochem. All other chemicals were acquired from Sigma Chemical unless otherwise indicated. Anti-EGFR (epidermal growth factor receptor), anti-total AKT, anti-CREB [cyclic AMP (cAMP)-responsive element binding protein], and anti-phospho-CREB (p-CREB) antibodies were obtained from Cell Signaling. All other antibodies were purchased from Santa Cruz Biotechnology.

RNA interferences

The short hairpin RNA (shRNA) template was constructed using two complementary oligos, which, when partially annealed, create a loop region with a sequence complementary to Ape1 mRNA (24). Two target sites from the Ape1 sequence were chosen for RNA interference. The oligos contained 21 and 19 nucleotides from the XIAP and cIAP2 sequence, respectively, as follows: forward^{shXIAP}, 5'-GATCGGTCCTGATGCAGATCTTTCITCAAGAGAT-3', reverse^{shXIAP}, 5'-AGCTAAAAAGGTCCTGATTGCAGATCTTTCITCTTGA-3'; forward^{shIAP2}, 5'-GATCGCTCGTGCTGTTTCTATTTCAAGAGAT-3', reverse^{shIAP2}, 5'-AGCTAAAAAGCTCGTCTGGTTTCATTTCTCTTGA-3'. The shRNA template was cloned into the vector pCDNA-HU6 as described. The Ape1-specific shRNA plasmid (3 μ g) was then mixed with TransFast transfection reagent (Promega) and added to 1×10^5 cells. After 24 hours, stable transfectants were selected using 300 μ g/mL gentamicin (Promega). The selection medium was replaced every 3 days for 3 weeks. Interference of cIAP2 and XIAP expression was confirmed by Western blot and reverse transcription-PCR analyses.

Protein extraction and Western blotting

Total protein was extracted from cells with a lysis buffer [100 mmol/L Tris (pH 8.0), 1% SDS], and recovered protein concentrations were determined using the Bio-Rad protein assay kit followed by separation with SDS-PAGE (12.5% gel). After the electrophoretic transfer to a polyvinylidene difluoride membrane, nonspecific binding sites were blocked with 5% nonfat milk in TBS containing 0.1% Tween 20. Signals were detected by incubating the membrane with antibodies for 16 hours at 4°C, followed by a subsequent incubation with a peroxidase-conjugated secondary antibody (1:5,000 dilution). Extensive washing with TBS/Tween 20 was done after each step of antibody incubation to remove nonspecific binding. The protein

bands were observed using enhanced chemiluminescence (NEN Life Science Products, Inc.).

Luciferase reporter assay

To construct the various cIAP2 promoter (accession number AY764389)-driven luciferase reporters, cIAP2 (-397-+1)-Luc, cIAP2(-397-+1)MutCREB-Luc, cIAP2 (-148-+1)MutNF- κ B-Luc, cIAP2(-148-+1)MutCREB-Luc, and cIAP2(-148-+1)MutNF- κ B/MutCREB-Luc were used. The following primers were provided for cloning: -397-F (GGTACCAAAGTGATGGCGGATGGAG), -148-F (GGTACCTGGAGTCCCCTAAGTCCTAAA), -148MutNF- κ B-F (GGTACCTaGAGTtCtTAAGTCCTAAA), +1-R (AGATCTTTTCCGTGCCGGAGGGCGCGAGTCT-CACGCTGTCTTTTAAATGC), +1 Mut CREB-R (AGATCTTTTCCGTGCCGGAGGGCGCGAGTCT-CACGCTGTCTTTTAAATGtATtACCC). The sites of point mutation for preventing NF- κ B and CREB binding are underlined and italicized. BamHI/BglII-treated and PCR-amplified cIAP2 promoter fragments were ligated with a BamHI/BglII-treated pGL2 vector. For the luciferase reporter assay, TL-1, SiHa, and E6-expressed TL-4 cells were transfected with an appropriate amount of reporter plasmid, pGL3-cIAP2 (-397 to +1), its derivatives, or the empty parental vector. The β -galactosidase expression plasmid was also cotransfected for normalization of transfection efficiency. Transfected cells were harvested at 48 hours after transfection, and a dual luciferase assay was done according to the manufacturer's instructions. The luciferase activity was measured with an AutoLumat LB953 luminometer (Berthold) and normalized with the cotransfected β -galactosidase activity.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) analysis was done using a published procedure with the following modifications (25). The immunoprecipitated DNA was ethanol precipitated and resuspended in 25 μ L H₂O. Total input samples were resuspended in 100 μ L H₂O and diluted 1:100 before PCR analysis. PCR amplification of immunoprecipitated DNA was carried out with diluted aliquots using the oligos 5'-GCCTGATGCTGGA-TAACTGG-3' and 5'-GCTCTTGCCAATTCTGATGG-3' as primers. PCR products were run on 2% agarose gel and analyzed by ethidium bromide staining.

MTT cytotoxicity assay

The cell lines were cultured in 96-well flat-bottomed microtiter plates supplemented with RPMI 1640 and DMEM containing 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 units/mL streptomycin in a humidified atmosphere containing 95% air and 5% CO₂ at 37°C in a humidified incubator. Before cisplatin treatment, the cells cultured in the exponential growth phase were pretreated with shRNAs for cIAP2 and XIAP knockdown for 12 hours or with various inhibitors for 2 hours. After 48 hours of incubation, the *in vitro* cytotoxic effects of these treatments were determined by MTT assay

(at 570 nm), and cell viability was expressed as a percentage of the control (untreated) cells (% of control).

Study subjects

One hundred and thirty-six lung tumors, including adenocarcinomas and squamous cell carcinomas, were collected from non-small cell lung cancer patients admitted to Taichung Veteran's General Hospital in Taiwan between 1993 and 2003. Among these, 52 female (38.2%), 84 male (61.8%), 81 nonsmoker (59.6%), 55 smoker (40.4%), 80 adenocarcinoma (58.8%), 56 squamous cell carcinoma (41.2%), 44 stage I (32.4%), 24 stage II (17.6%), and 68 stage III (50.0%) patients were enrolled for this study (Supplementary Table S1). Written informed consent was obtained from each lung cancer patient before surgery for the use of tumor specimens, as approved by the Institutional Review Board at Taichung Veteran's General Hospital. Information about each patient's smoking history was collected using a standard written questionnaire. Only the patients who reported never having smoked were categorized as nonsmokers. Tumor types and stages were determined by qualified pathologists according to the 1981 WHO classification system. The overall survival rate was calculated from the day of surgery to the date of death until December 2006. A total of 101 of the 136 cases in this study had a successful follow-up in this experiment. A postoperative follow-up was scheduled at 1 month, 2 months, and every 3 months thereafter during the first 2 years after surgery and then every 6 months thereafter, or more frequently if needed. A chest X-ray, a chest computed tomography scan, carcinoembryonic antigen analysis, and other serum analyses were obtained at every follow-up visit. The end of the follow-up period was defined as May 2004. The mean follow-up period for patients was 36.1 months (range, 3.3-68.9 months). The Institutional Review Board protocol CS07159 was approved by Chung Shang Medical University Hospital.

Immunohistochemistry

Formalin-fixed and paraffin-embedded specimens were sectioned at a thickness of 3 μ m. All sections were then deparaffinized in xylene, rehydrated through serial dilutions of alcohol, and washed in PBS (pH 7.2), the buffer used for all subsequent washes. For cyclooxygenase-2 detection, sections were heated in a microwave oven twice for 5 minutes in citrate buffer (pH 6.0) and then incubated with anti-EGFR (1:100), anti-phospho-AKT (p-AKT; 1:100), or anti-cIAP2 (1:50) for 2 hours at 25°C. The conventional streptavidin peroxidase method (LSAB Kit K675, DAKO) was done to develop signals, and the cells were counterstained with hematoxylin. Negative controls were obtained by leaving out the primary antibody. "Low" immunostaining was defined as \leq 10% of tumor cells in the lung tumor paraffin sections with positive immunostaining, and $>$ 10% of tumor cells in the lung tumor paraffin sections with positive immunostaining were defined as "high" immunostaining. The immunostaining results were determined by three observers, and two observers were pathologist.

Statistical analysis

The χ^2 test and Fisher's exact test (two-tailed) were used for statistical analysis. All analyses were done using the SPSS version 11.0 statistical package.

Results

EGFR and p-AKT expression associated with cIAP2 and XIAP expression in HPV-infected lung cancer cells

To verify whether cIAP2 and the other three IAPs (cIAP1, XIAP, and survivin) were upregulated by E6, HPV16 E6–positive TL-1 and HPV16 E6–negative TL-4 lung cancer cells, which were primarily cultured from plural effusions of nonsmoking female lung adenocarcinoma (T4N3M1) patients (26), were transiently transfected with a small hairpin E6 RNA and E6-expressing vector, respectively. As expected, the E6 protein was decreased in E6 knockdown TL-1 (TL-1/shE6) and increased in E6-overexpressed TL-4 (TL-4/E6) cells, and reversely, the p53 protein was elevated and reduced in both cells (Fig. 1A and B). Meanwhile, the

expression of cIAP2 and XIAP significantly decreased or increased in TL-1/shE6 or TL-4/E6 cells, but the levels of cIAP1 and survivin were unchanged in both cells (Fig. 1A and B; Supplementary Fig. S1A-C). The PDZ domain of E6 has been reported to be the binding site for NF- κ B in the involvement of cIAP2 upregulation (23). However, cIAP2 was still expressed in the TL cells, which were transfected with 5 μ g of E6 Δ PDZ, suggesting that cIAP2 upregulated by E6 in lung cancer cells is not only predominately regulated by NF- κ B (Supplementary Fig. S1B, right). HPV16 E6 has been shown to activate the EGFR/phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway and may contribute to cell survival (27–30). Therefore, the upregulation of cIAP2 and XIAP by E6 may be associated with EGFR and p-AKT expressions. Western blot data showed that EGFR and p-AKT expression decreased or increased in TL-1/shE6 or TL-4/E6 cells (Fig. 1A and B), and similar observations were shown in TL-2/shE6, A549/E6, and H460/E6 cells (Supplementary Fig. S2A). These results suggest that cIAP2 and XIAP upregulated by E6 may be mediated

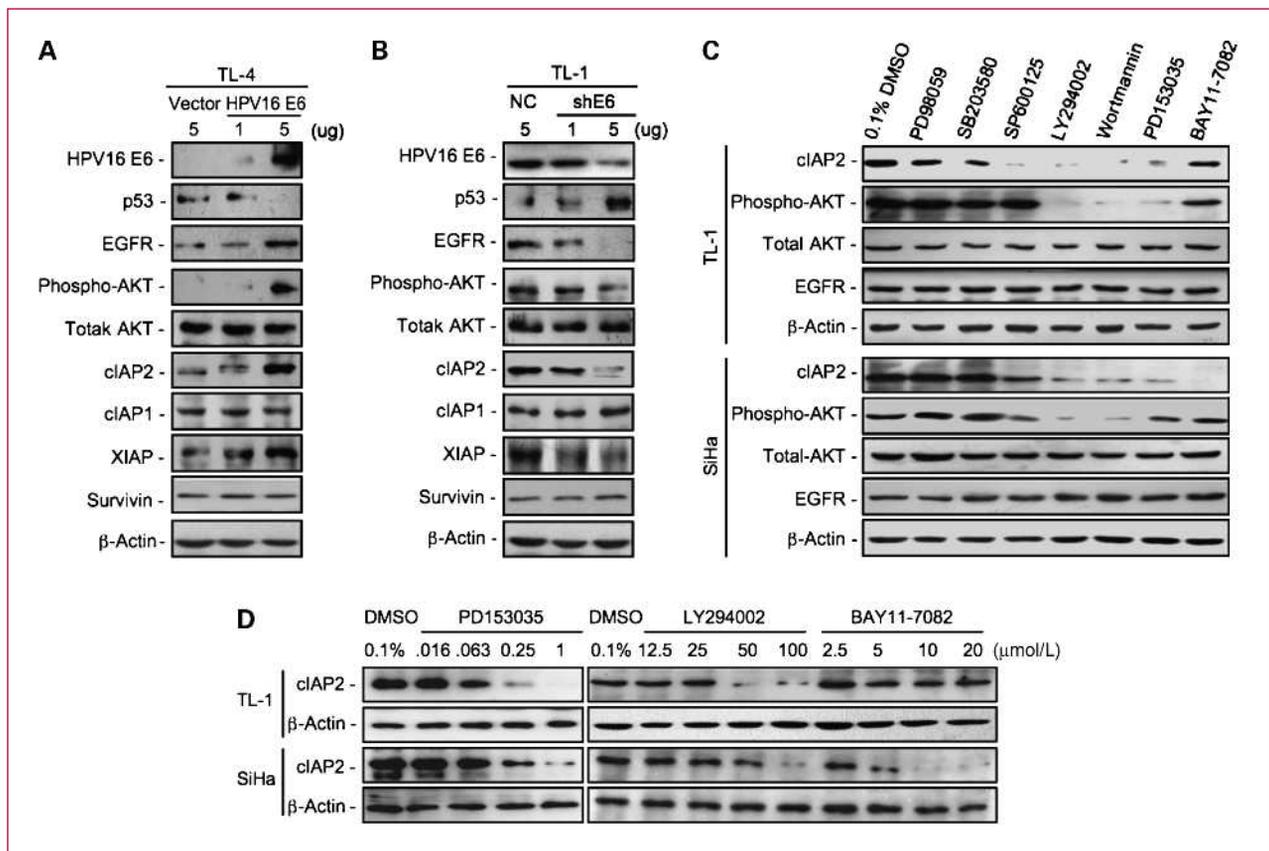


Fig. 1. Correlation of cIAP2 with EGFR and p-AKT expressions in E6-positive lung cancer cells. The cell lysates of E6 knockdown TL-1 (A) and E6-overexpressed TL-4 cells (B) were separated by SDS-PAGE, and the specific antibodies were used to evaluate HPV16 E6, p53, cIAP2, p-AKT, total AKT, and β -actin expressions by Western blot. C, TL-1 and SiHa cells were treated with ERK (20 μ mol/L PD98059), p38/MAPK (5 μ mol/L SB203580), JNK (10 μ mol/L SP600125), PI3K/AKT (25 μ mol/L LY294002; 10 μ mol/L wortmannin), EGFR (0.5 μ mol/L PD153035), and NF- κ B (20 μ mol/L BAY11-7082) inhibitors, respectively, and then the cell lysates were separated by SDS-PAGE for the evaluation cIAP2, p-AKT, total AKT, EGFR, and β -actin expression by the same specific antibodies using Western blot. D, dose-dependent inhibition of cIAP2 expression in TL-1 and SiHa cells, which were respectively treated with various concentrations of PD153035, LY294002, and BAY11-7082.

through the EGFR/PI3K/AKT pathway. To further verify whether the EGFR/PI3K/AKT pathway could contribute to the upregulation of cIAP2 by E6, a specific inhibitor with a different signaling pathway was treated with TL-1 and SiHa cells. As shown in Fig. 1C, cIAP2 expression in TL-1 cells was almost completely inhibited by c-Jun NH₂-terminal kinase (JNK; SP600125), PI3K (LY294002, wortmannin), and EGFR (PD153035) inhibitors but was relatively reduced by extracellular signal-regulated kinase (ERK; PD98059), p38 (SB203580), and NF- κ B (BAY11-7082) inhibitors (Fig. 1C), and similar findings were observed in TL-2/shE6, TL-4/E6, and A549/E6 cells treated with cisplatin plus EGFR, PI3K, and NF- κ B inhibitors (Supplementary Fig. S2B and C). However, cIAP2 expression in SiHa cells was completely or almost inhibited by NF- κ B, PI3K, and EGFR inhibitors, and was relatively reduced by JNK inhibitor but unchanged by ERK and p38 inhibitors. In dose-dependent experiments, cIAP2 expression in TL-1 cells was reduced by EGFR, and the PI3K inhibitor was more effective in SiHa cells (Fig. 1D); conversely, the cIAP2 expression inhibited by the NF- κ B inhibitor in SiHa cells was remarkably higher than in TL-1 cells (Fig. 1D). These results clearly indicate that cIAP2 upregulated by E6 via the EGFR/PI3K/AKT pathway in lung cancer cells may be more effective than via the NF- κ B pathway.

p-CREB involved in E6 upregulated cIAP2 in lung cancer cells, not in cervical cancer cells

Based on previous reports (22, 23, 31) and present results in Fig. 1, we hypothesized that the transcription of cIAP2 regulated by E6 in lung cancer cells may differ from cervical cancer cells. As shown in Fig. 2A (left), we therefore constructed a -397 to +1 promoter region of cIAP2 as pGL3-cIAP2(-397+1) for the luciferase reporter assay based on a previous report (31). NF- κ B is thought to be the major regulator of cIAP2 transcription (31). CREB, a regulatory targeting molecular of AKT (32), has been shown to be involved in cIAP2 transcription (33). However, the cAMP response element has still not been identified. In this study, a putative CRE at the -52 to -42 promoter region of cIAP2 was found by TFSEARCH software (<http://www.cbrc.jp/research/db/TFSEARCH.html>), and the reporter region containing mutant NF- κ B and/or CREB binding sites (MutNF- κ B; MutCREB) was constructed (Fig. 2A, left). In TL-1 lung cancer cells, 40% of the reporter activity was attributed to a putative CREB binding site at the -52 to -42 reporter region as pGL3-cIAP2(-397+1)MutCREB compared with the total activity of pGL3-cIAP2(-397+1), and the other two NF- κ B binding sites contributed 30% and 22% (Fig. 2A, right). A similar observation was also seen in E6-overexpressed TL-4 lung cancer cells (Fig. 2A, right). Surprisingly, the contribution of the CREB binding site in SiHa cervical cancer cells contributed only 10% of the reporter activity of pGL3-cIAP2(-397+1)MutCREB; however, the NF- κ B binding sites at -209 to -200 [pGL3-cIAP2(-148+1)] contributed 60% of the total reporter activity of pGL3-cIAP2(-397+1), and the reporter activity of the other

NF- κ B binding sites at -209 to -200 contributed 33% (Fig. 2A, right). To further verify the contribution of CREB and NF- κ B on cIAP2 upregulated by E6, ChIP analysis showed that CREB was bound to the cIAP2 promoter site in E6-overexpressed TL-4 cells, but the binding activity of CREB was diminished in E6 knockdown TL-1 cells (Fig. 2B, top). Western blot data showed that the p-CREB protein level was increased by E6 overexpression, but decreased by E6 knockdown in lung cancer cells. Interestingly, the p-CREB level was unchanged by E6 knockdown in SiHa cervical cancer cells (Fig. 2B, bottom). These results clearly indicate that p-CREB plays a more important role in cIAP2 induction by E6 in lung cancer cells than in cervical cancer cells.

CREB phosphorylation via EGFR/PI3K/AKT pathway plays a crucial role in cIAP2 upregulation by E6 in lung cancer cells

We further explored whether different signaling pathways were involved in cIAP2 upregulation by E6 between lung cancer and cervical cancer cells. ChIP analysis showed that NF- κ B binding affinity on the cIAP2 promoter region was diminished by NF- κ B, PI3K, and EGFR inhibitors in TL-1 and TL-4/E6 cells (Fig. 2C, top). Interestingly, the effectiveness of PI3K and EGFR inhibitors on NF- κ B binding affinity of the cIAP2 promoter in SiHa cells was lower than in TL-1 cells, but a similar effect was observed on NF- κ B binding affinity with NF- κ B inhibitor between lung and cervical cancer cells (Fig. 2C, top). Surprisingly, the CREB binding affinity on the cIAP2 promoter in TL-1 and TL-4/E6 cells was markedly suppressed by PI3K and EGFR inhibitors; however, the CREB binding activity on the cIAP2 promoter was not observed in SiHa cells (Fig. 2C, top). To clarify whether CREB phosphorylated by AKT was responsible for the CREB binding on the cIAP2 promoter, Western blot data showed that p-CREB protein was completely diminished by PI3K inhibitor (LY294002) in TL-1 and TL-4/E6 cells, but only slightly reduced in SiHa cells (Fig. 2C, bottom). In addition, the p-CREB protein level was not influenced by NF- κ B and EGFR inhibitors in TL-1, TL-4/E6, and SiHa cells (Fig. 2C, bottom). These results indicate that CREB phosphorylation via the EGFR/PI3K/AKT pathway in lung cancer cells plays a crucial role in the upregulation of cIAP2 by E6.

cIAP2 upregulated by E6 via the EGFR/PI3K/AKT pathway may be responsible for the resistance to cisplatin in HPV-infected lung cancer cells

To elucidate whether cIAP2 and XIAP upregulated by E6 in TL-1 cells may be responsible for the resistance to cisplatin, cIAP2 and XIAP in TL-1 cells were respectively knocked down by shRNA (Fig. 3A). The inhibition concentration of the 50% cell survival value (IC₅₀) for TL-1/shIAP2, TL-1/shXIAP, and TL-1 nonspecific shRNA control (TL-1/NC) cells was 8.6, 10.3, and 39.7 μ mol/L, respectively (Fig. 3B). When TL-1/shE6, TL-1/shIAP2, TL-1/shXIAP, and TL-1/shIAP2 + shXIAP cells were treated with 35 μ mol/L cisplatin for 48 hours, the cell survival

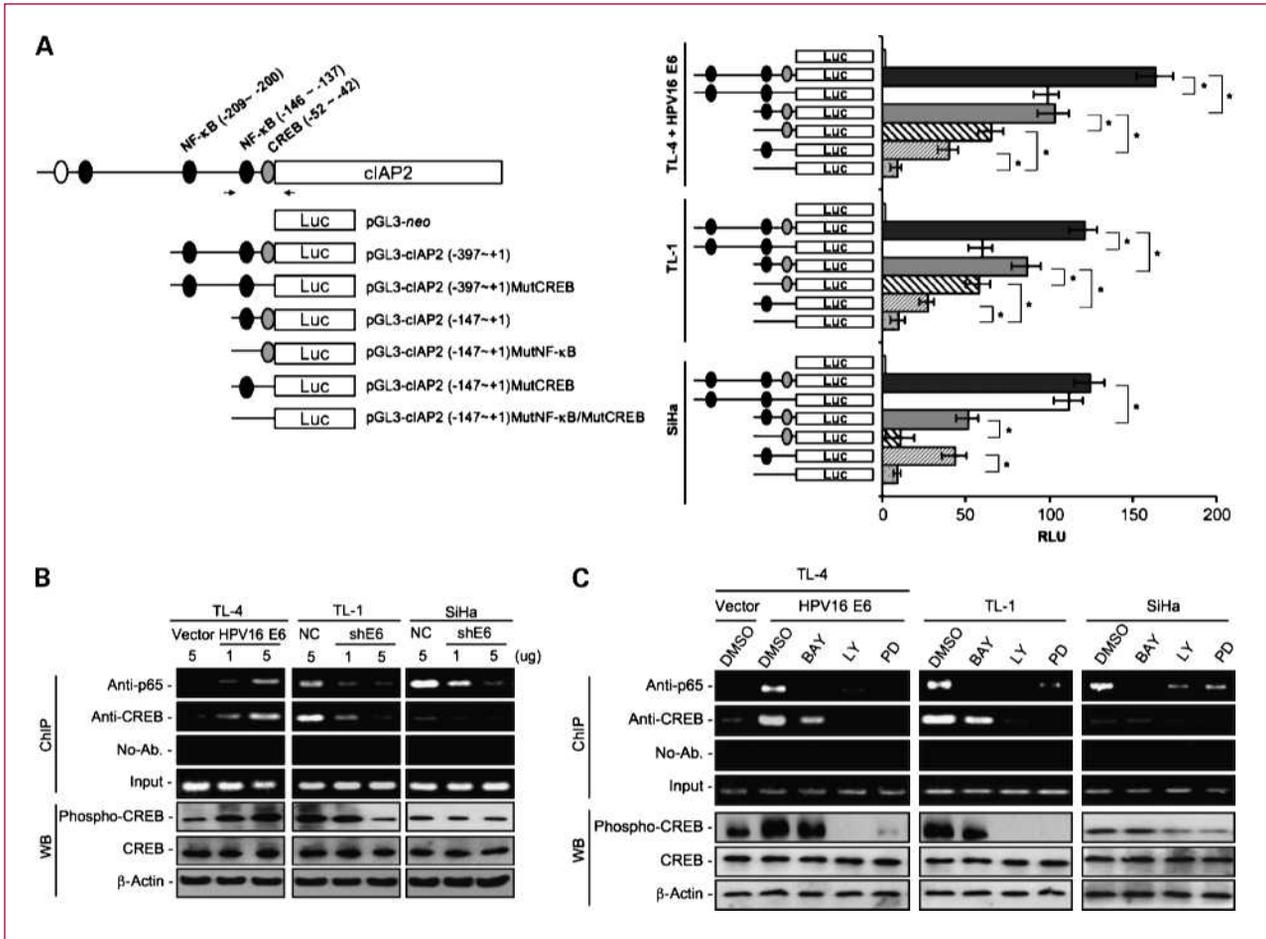


Fig. 2. Transcriptional activation of deletion and/or mutation constructs of ciAP2 promoter region by E6 via the activation of NF-κB and CREB in TL-1 cells. A, diagram summarizing the positions of potential regulatory elements relative to the structure of six deletion and/or mutation constructs used in these experiments, where 5'-deletion boundaries are indicated. Left, summary of luciferase assays carried out with extracts of TL-1, SiHa, and E6-overexpressed TL-4 cells cotransfected with 2 μg of each construct and 0.5 μg of β-galactosidase expression plasmid. Cell lysates were prepared and measured for their luciferase activities. The luciferase activity of each transfection was normalized to the β-galactosidase activity. Relative luciferase activity (RLU) is expressed as the luciferase activity of mutant transfectant divided by that of pGL3-neo. B, TL-1/shE6, TL-1/NC, and TL/E6 cells were established by transiently transfecting with shE6, nonspecific control shRNA (NC) plasmid, and E6 expression construct for 48 h. C, TL-4/E6, TL-1, and SiHa cells were treated with PD153035, BAY11-7082, and LY294002 for 24 h. ChIP assay was done to evaluate the binding affinity of NF-κB and CREB on the ciAP2 promoter region. The cell lysates were separated by SDS-PAGE, and E6, p53, ciAP2, p-AKT, total AKT, and β-actin expressions were evaluated by their specific antibodies using Western blot (WB).

percentage was 11%, 14%, 19%, and 35% compared with 54% for TL-1/NC cell survival (Fig. 3C). Similar findings were also observed in TL-4/E6 cells with shAP2, shXIAP, and shAP2/shXIAP transfection (Supplementary Fig. S3A). These results indicate that the involvement of ciAP2 in the resistance to cisplatin is more effective than XIAP. Besides, AKT has been reported to promote cisplatin resistance in human ovarian cancer cells (34). To verify whether cisplatin resistance contributes more to the upregulation of ciAP2 by E6 via the EGFR/PI3K/AKT pathway in lung cancer cells than in cervical cancer cells, a specific inhibitor was combined with cisplatin and treated with TL-1 and SiHa cells, respectively. As expected, the cell survival rate of TL-1 cells decreased remarkably with the treatment of cisplatin plus EGFR inhibitor

(14%) and cisplatin plus PI3K inhibitor (9%) compared with cisplatin plus NF-κB inhibitor (50%; Fig. 3D, left). Similar findings were also observed in TL-4/E6, A549/E6, and H460/E6 cells treated with cisplatin plus EGFR, PI3K, and NF-κB inhibitors (Supplementary Fig. S3B-D). However, the cell survival rate of SiHa cells was reduced by cisplatin plus NF-κB inhibitor (6%) compared with cisplatin plus EGFR (31%) or PI3K inhibitor (28%; Fig. 3D, right). More importantly, the IC₅₀ value obtained from the dose-dependent cell survival of the TL-1 cells after treatment with PI3K, EGFR, and NF-κB inhibitors plus various concentrations of cisplatin showed that the lowest IC₅₀ value was PI3K inhibitor + cisplatin (11 μmol/L), followed by EGFR inhibitor + cisplatin (17 μmol/L), and NF-κB inhibitor + cisplatin (38 μmol/L; Fig. 3E).

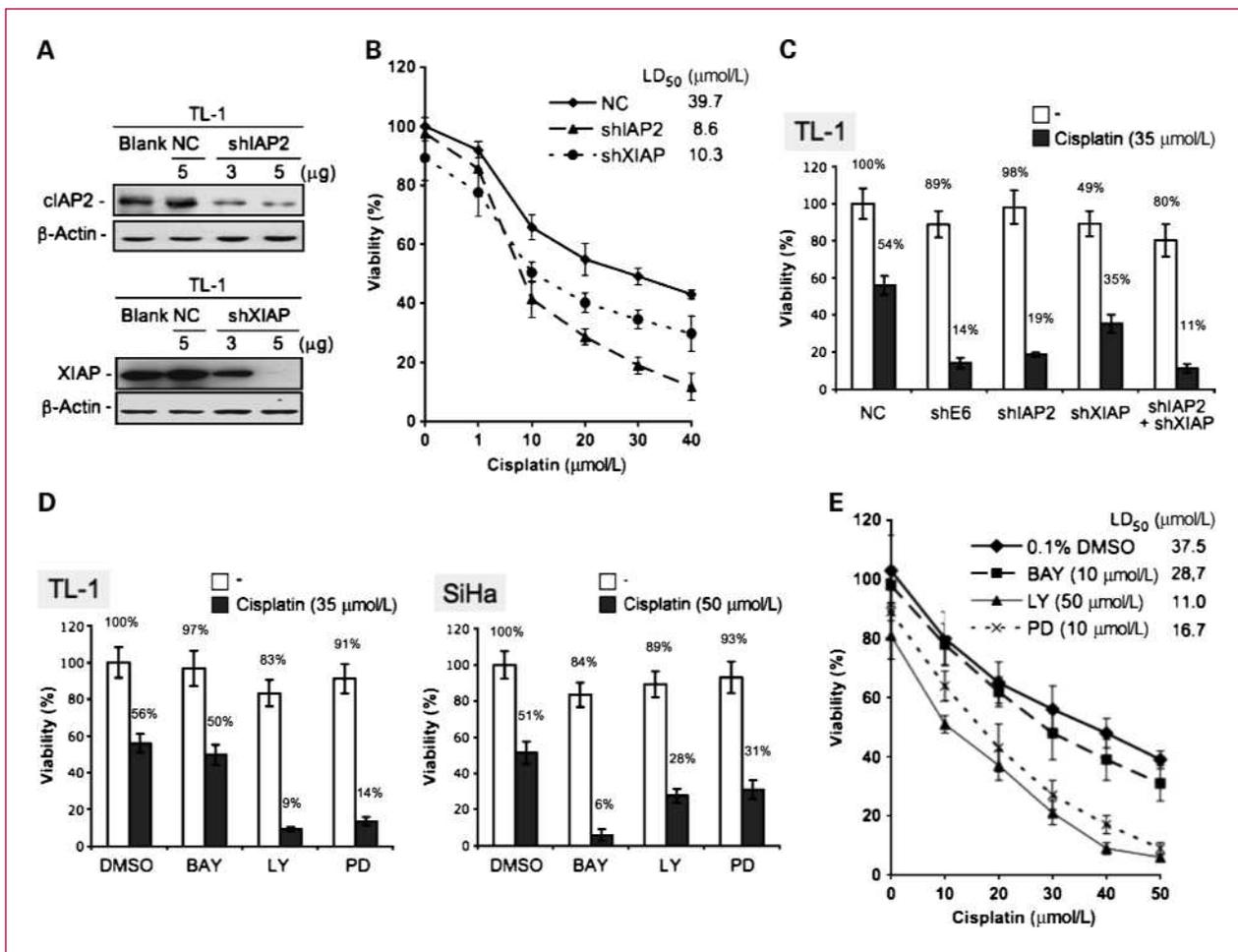


Fig. 3. The cell viability of TL-1 and SiHa cells was modulated by shIAP2 transfection and treatment with EGFR (PD153035), PI3K (LY294002), and NF- κ B (BAY11-7082) inhibitors. **A**, Western blot used for evaluating cIAP2 and XIAP expression in TL-1 cells after transfection with shIAP2 or shXIAP. **B**, the cell viability of TL-1 cells transfected with shIAP2, shXIAP, and NC was evaluated by MTT assay after the cells were treated with various concentrations of cisplatin, and the IC₅₀ value was calculated from the linear cell viability curves. **C**, the cell viability of TL-1 cells transfected with shIAP2, shXIAP, and shRNA was evaluated by MTT assay after the cells were treated with 35 μ mol/L cisplatin. **D**, TL-1 or SiHa cells were pretreated with BAY11-7082, LY294002, and PD153035 for 2 h and then the cells were treated with 35 or 50 μ mol/L cisplatin for 48 h. **E**, TL-1 cells were pretreated with BAY11-7082, LY294002, and PD153035 for 2 h and then the cell viability was evaluated by MTT assay after the cells were treated with various concentrations of cisplatin. The IC₅₀ values were calculated from the linear cell viability curves.

cIAP2 expression in HPV16/18 E6-positive lung tumors associated with EGFR and p-AKT expression

To understand whether cIAP2 expression in lung tumors is associated with HPV16/18 E6 expression, HPV16/18 E6 immunostainings of 136 lung tumors evaluated by immunohistochemistry (26) were further enrolled for detection of cIAP2 expression, and the data showed that cIAP2-positive immunostaining (>10% tumor cells with cIAP2 immunostaining) in E6-positive tumors was more prevalent than in HPV16/18 E6-negative lung tumors (68% versus 48%; $P = 0.018$; Table 1). To verify whether a higher cIAP2 expression in E6-positive lung tumors was associated with the EGFR/PI3K/NF- κ B pathway, EGFR and p-AKT protein were also evaluated by immunohistochemistry (Supplementary Fig. S4), and

the data showed that E6 immunostaining correlated significantly with p-AKT immunostaining ($P = 0.008$; Table 1). However, E6 immunostaining only related marginally with EGFR immunostaining ($P = 0.052$; Table 1). Additionally, cIAP2 immunostaining was associated with EGFR ($P < 0.001$) and p-AKT immunostaining ($P < 0.001$), respectively (Table 1). To further verify whether cIAP2 upregulated by E6 is predominately mediated through the EGFR/PI3K/AKT pathway, the association between E6 and EGFR/p-AKT, which were both positive in 77 cIAP2-positive lung tumors, was statistically analyzed. Our results indicate that in cIAP2-positive tumors, EGFR/p-AKT-positive immunostainings were more prevalent in E6-positive tumors than in E6-negative tumors (69% versus 39%; $P = 0.009$; Supplementary Table S2). We also

observed that lung tumors with EGFR mutation were more common to have positive cIAP2 expression compared with those with wild-type EGFR. These results suggest that cIAP2 expression may be associated with the activation of the EGFR/PI3K/AKT pathway in HPV-infected lung tumors.

cIAP2 expression in resected tumors correlated with the cisplatin-based chemotherapy response in recurrent and/or metastatic tumors

The 136 lung cancer patients enrolled in this study were followed up for 36.1 months, and 46 patients had tumor recurrence and/or metastasis after surgical therapy for cisplatin-based chemotherapy. We examined whether the cIAP2 expression in the tumors of these 46 patients could have had a poorer response to cisplatin-based chemotherapy compared with the tumors without cIAP2 expression. Our data show that the prevalence of stable disease plus progressive disease in patients with cIAP2-positive tumors was significantly higher than in patients with cIAP2-negative tumors (75% versus 44%; $P = 0.036$; Table 2). On the contrary, the prevalence of complete response plus partial response in patients with cIAP2-negative tumors was more common than in patients with cIAP2-positive tumors (56% versus 25%; Table 2). The tumor response to cisplatin-based chemotherapy in these patients seems to support observations from *in vitro* cell experiments.

Discussion

The EGFR/PI3K/AKT cascade has been shown to confer cancer cell survival and to enhance resistance to chemotherapy. Therefore, EGFR/PI3K/AKT inhibitors have been developed to act as potential molecular targeting drugs for cancer therapy or have been used in combination with cytotoxic drugs to enhance the chemotherapeutic response. For example, cisplatin combined with EGFR or PI3K inhibitors may reduce the resistance to cisplatin in gliomas (35), pancreatic cancer (36), ovarian cancer (37), and lung cancer cells (38) *in vitro*. Consistent results were shown in the present study, indicating that EGFR or PI3K inhibitor combined with cisplatin may synergistically enhance the cytotoxicity of lung cancer cells, where cIAP2 is induced by E6 via the EGFR/PI3K/AKT cascade (Fig. 3C). However, the chemotherapeutic response enhanced by EGFR inhibitor plus cisplatin-based chemotherapy in cancer patients has failed (39), showing that a different molecular pathway may be responsible for the resistance to cisplatin among patients, and thus, developing individual chemotherapy is needed to improve chemotherapeutic efficacy. In the present study, we provide evidence that shows that cIAP2 knockdown TL-1 cells were more sensitive to cisplatin than TL-1 parental cells (8.6 $\mu\text{mol/L}$ for TL-1/shIAP2 cells versus 39.7 $\mu\text{mol/L}$ for TL-1 cells; Fig. 3B), suggesting that cIAP2 may be partially responsible for cisplatin resistance in HPV-infected lung cancer cells. Moreover, cIAP2

Table 1. Relationships of HPV16/18 E6 with EGFR, p-AKT, and cIAP2, correlation of EGFR with p-AKT and cIAP2, and correlation between p-AKT and cIAP2 in lung tumors

	Total N	EGFR		p-AKT		cIAP2	
		Negative n (%)	Positive n (%)	Negative n (%)	Positive n (%)	Negative n (%)	Positive n (%)
HPV16 or HPV18 E6	136	61 (45)	75 (55)	51 (38)	85 (62)	59 (43)	77 (57)
Negative	79	41 (52)	38 (48)	37 (47)	42 (53)	41 (52)	38 (48)
Positive	57	20 (35)	37 (65)	14 (25)	43 (75)	18 (32)	39 (68)
P		0.052		0.008		0.018	
EGFR							
Negative	61			33 (54)	28 (46)	37 (61)	24 (39)
Positive	75			18 (24)	57 (76)	22 (29)	53 (71)
P				<0.001		<0.001	
p-AKT							
Negative	51					33 (65)	18 (35)
Positive	85					26 (31)	59 (69)
P						<0.001	
EGFR gene							
Wild-type	93					47 (51)	46 (49)
Mutant	43					12 (28)	31 (72)
P						0.013	

NOTE: P value was calculated by χ^2 test. EGFR gene mutation in exons 18 to 21 was determined by direct autosequencing.

Table 2. Association between cIAP2-positive immunostaining in lung tumors and tumor response to cisplatin-based chemotherapy in these patients with tumor recurrence and/or metastasis after surgical resection

cIAP2	Total N	Tumor response		P
		Favorable n (%)	Poor n (%)	
Negative	18	10 (56)	8 (44)	0.036
Positive	28	7 (25)	21 (75)	

NOTE: One hundred and one of the 136 patients enrolled in this study were followed up clinically. Among these patients, 63 patients were found to have tumor recurrence and/or metastasis after surgical therapy, and 46 patients were treated with cisplatin-based chemotherapy. Complete response: a complete disappearance of all the tumors; partial response: a decrease in size or number of the tumor lesions by $\geq 50\%$; progressive disease: at least 25% increase in size or number of the tumor lesions; stable disease: neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease. Therefore, the favorable response (complete and partial response) is the decrease of tumor size at least $\geq 50\%$. P value was calculated by χ^2 test.

expression in resected tumors from lung cancer patients was associated with a poorer response to cisplatin-based chemotherapy (Table 2). To the best of our knowledge, this is the first report that shows that cIAP2 expression may be associated with the resistance to cisplatin-based chemotherapy in lung cancer patients who had tumor recurrence/or metastasis after surgical therapy.

The EGFR inhibitors gefitinib and erlotinib have been shown to be potential molecular targeting drugs for EGFR-mutated lung cancer, which are frequently found in females, nonsmokers, and adenocarcinomas (40–42). Therefore, both EGFR inhibitors are recommended to be the first line of anticancer drugs in lung cancers with EGFR mutations (43, 44). In this study population, EGFR mutations associated positively with HPV16/18 E6 expression (data not shown), and cIAP2 expression correlated with EGFR mutations ($P = 0.013$; Table 1) and E6 expression ($P = 0.018$), revealing the possibility that an EGFR inhibitor not only was useful in patients with EGFR mutations but also is feasible in patients with HPV16/18 infections. In addition, an EGFR inhibitor has been shown to increase cisplatin sensitivity, particularly in EGFR-overexpressed cells (45–47). Therefore, a relatively low dosage of cisplatin may be used for chemotherapy when cisplatin sensitivity is restored by an EGFR inhibitor. An EGFR inhibitor combined with cisplatin may not only benefit chemotherapeutic efficacy but also reduce the side effects of cisplatin to improve the patient's quality of life.

The involvement of NF- κ B and CREB in cIAP2 transcription has been investigated extensively. Hong et al. (31) first reported that two NF- κ B binding sites located at –209 to –200 and –146 to –137 on the cIAP2 promoter region were responsible for cIAP2 transcription. In the present study, 50% of the cIAP2 transcription activity induced by E6 in lung cancer cells was dependent on these two NF- κ B binding sites. ChIP analysis further indicated that NF- κ B was indeed bound to these cIAP2 promoter sequences. Half of the cIAP2 transcriptional activity regulated by NF- κ B in HPV16-infected lung cancer cells was quite different from the HPV16-immortalized human oral keratinocytes and primary human airway epithelial cells, in which cIAP2 was almost completely transactivated by NF- κ B. On the other hand, the CREB binding site on the cIAP2 promoter region was located at 1.6 to 1.8 kb of 5'-flanking region (48), and this differed from the present study showing that the CREB binding site was located at –52 to –42, which was responsible for 50% of the cIAP2 transcription activated by E6 in lung cancer cells (Fig. 2A, right). More interestingly, the upregulation of cIAP2 by CREB in colon cancer cells was mediated through the ERK1/2 and p38 mitogen-activated protein kinase (MAPK) pathways (28); however, cIAP2 induced by E6 in lung cancer cells was unchanged by the addition of both pathway inhibitors (Fig. 1C). This conflicting result may be due to the fact that different CREB binding sites on the cIAP2 promoter region may be regulated by different signaling pathways between colon and lung cancer cells.

In summary, we provide evidence that shows that cIAP2 induction in HPV-infected lung cells contributes equally to CREB phosphorylation and NF- κ B activation by E6 via the EGFR/PI3K/AKT signaling pathway. The upregulation of cIAP2 by E6 in HPV-infected lung cancer cells may be responsible for the resistance to cisplatin. Additionally, tumor recurrence or metastasis, in patients who underwent tumor resection, with cIAP2-positive tumors had a poorer response to cisplatin-based chemotherapy than those with cIAP2-negative tumors. Therefore, we suggest that EGFR or PI3K inhibitor may be helpful to improve the efficacy of cisplatin-based adjuvant chemotherapy in HPV-infected lung cancer patients. Before the clinical use, we will establish E6-transgenic mice to test whether the *in vitro* observation could be further confirmed by *in vivo* animal model.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

National Health Research Institute grants NHRI96-TD-G-111-006 and NHRI97-TD-G-111-006; National Science Council of Taiwan, Republic of China grant NSC-96-2628-B-040-002-MY3; and Buddhist Tzu Chi General Hospital grant TTCRD-9710

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Received 01/05/2010; revised 04/22/2010; accepted 06/27/2010; published OnlineFirst 10/19/2010.

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