IL-10 Promotes Tumor Aggressiveness via Upregulation of CIP2A Transcription in Lung Adenocarcinoma

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Running head: IL-10 promotes tumor aggressiveness via CIP2A

Key words: IL-10; CIP2A; lung cancer; HPV

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Abstract

Purpose: IL-10 determines virus persistent infection and promotes viral-associated tumor progression via tumor immune escape. However, the role of IL-10 in tumor progression and prognosis in lung adenocarcinoma remains controversial.

Experimental design: To investigate how IL-10 is regulated by HPV E6, IL-10 promoter was constructed to understand which transcriptional factor could be responsible for its transcription. To verify which molecule could be responsible for IL-10-mediated soft agar growth and invasion capability, PCR array and mechanistic strategies were performed. IL-10 and CIP2A mRNA levels in lung tumors from lung cancer patients were determined by real-time RT-PCR. The prognostic value of both molecules on survival was estimated by Cox regression model.

Results: Mechanistic studies showed that IL-10 protein and mRNA expression was decreased in E6-knockdown TL1 cells and increased in E6-overexpressing TL4 cells. In addition, IL-10 transcription was predominantly regulated by E6-mediated phosphorylation of CREB and C/EBPβ via PI3K signaling pathway. IL-10-mediated tumor aggressiveness in vitro and in vivo occurs through increased CIP2A expression via PI3K signaling pathway. Among patients, IL-10 mRNA expression in lung tumors was positively correlated with CIP2A mRNA expression. Cox-regression analysis
showed that IL-10 and CIP2A mRNA levels may independently predict survival in patients with lung adenocarcinoma, especially in patients with E6-positive tumors.

Conclusion: IL-10 production from lung tumors and immune cells promotes lung adenocarcinoma aggressiveness and patients with poor survival. We thus suggest that PI3K inhibitor combined with chemotherapy may potentially enhance tumor regression and improve patients’ outcome and life quality.
Translational Relevance

The role of IL-10 in tumor progression and prognosis of lung adenocarcinoma remains controversial. In this study, we provided evidence to demonstrate that IL-10 production from E6-positive lung cancer cells via PI3K/AKT signaling pathway directly promotes cell proliferation soft agar growth, cell invasion, and xenograft tumor formation. In addition, CIP2A induced by IL-10 via PI3K/AKT pathway is responsible for IL-10-mediated cell invasion. Among lung adenocarcinoma patients, those with high IL-10 and CIP2A mRNA expression detected in tumors had poorer survival and relapse than those with low IL-10 and CIP2A mRNA detected in tumors. Therefore, we suggest that PI3K/AKT inhibitor combined with chemotherapy may be potentially useful to reduce tumor progression and consequently to improve drug sensitivity and clinical outcome in patients with high IL-10 mRNA levels.
Introduction

IL-10 belongs to Th2 cytokine for anti-inflammation and it also inhibits T cell immunity to block tumor immune surveillance (1-3). Most studies have indicated that IL-10 expression in immune cells, including macrophages, infiltrating T lymphocytes, and NK cells, promotes progression of tumors in kinds of cancer types including lung cancer (4, 5). In lung cancer cases, some reports have indicated that loss of IL-10 in lung tumors may promote tumor progression and result in poor clinical outcomes in patients; however, an opposite effect has been reported in other studies (6-8). Interestingly, the absence of IL-10 expression has been associated with poor outcome in stage I lung cancer, whereas in late-stage lung cancer, the presence of IL-10-positive macrophages at the tumor margins can be an indicator of poor prognosis (6-8). In addition, shorter survival times have been reported in advanced lung cancer patients who had high serum IL-10 levels, when compared with similar patients who had low serum IL-10 levels (8). Therefore, the role of IL-10 in lung tumorigenesis remains elusive.

The infection of human papillomavirus (HPV) 16/18 has been documented to associate with cancers of squamous epithelia. However, in Taiwan, HPV 16/18 infection rate was significantly higher in lung adenocarcinoma than in lung squamous
cell carcinoma (9, 10). A high HPV infection rate in Taiwanese lung cancer reflects the possibility that imbalanced immune function might play an important role in cancer development, especially in Taiwanese women who never smoked (never-smokers) (9). IL-10 has been shown to determine virus clearance and infection persistence (3). HPV infection is a major etiological factor in cervical carcinogenesis. IL-10 mRNA levels in cervical intraepithelial neoplasia (CIN) were significantly higher than in normal cervical tissues (11-13). The up-regulated secretion of IL-10 may inhibit immune response against HPV infection in early cervical lesions. Reports have shown that higher IL-10 could be detected in plasma of patients with CIN III and with carcinoma than in patients with CIN I and CIN II (11-13). Moreover, patients suffering from cervical cancer show higher IL-10 expression in HPV16 positive tumors than in HPV16 negative tumors, which again indicates an association between IL-10 and the carcinogenesis of HPV-associated cancer (14).

Conceivably, HPV persistent infection might cause integration of HPV DNA into host chromosomes, leading to expression of E6 and E7 oncoproteins, and consequently to promotion of tumor progression via inactivation of the p53 and Rb pathways (15, 16). Previous reports have indicated that patients with HPV-associated advanced stage cervical and oropharyngeal cancer who had high IL-10 expression in serum or plasma also had poorer survival when compared with patients with low IL-10 expression (12,
We expected that IL-10 detected in blood circulation might represent IL-10 expressed not only in immune cells but also IL-10 that derived from tumor cells. Therefore, we hypothesized that: (1) IL-10 expression in lung tumors could be elevated by E6 oncoprotein; and (2) IL-10 induced by E6 in lung tumors could be responsible for in vitro and in vivo tumor invasion. The aim of the present study was to further investigate the underlying mechanism of IL-10 in tumor aggressiveness based on these hypotheses.
Materials and Methods

Study subjects

This study consisted of 98 lung cancer patients. All patients were unrelated ethnic Chinese and residents of central Taiwan. The inclusion criteria for patients were: primary diagnosed with lung adenocarcinoma; no metastatic disease at diagnosis; no previous diagnosis of carcinoma; no neoadjuvant treatment before primary surgery; no evidence of disease within one month of primary surgery. Lung tumor specimens were collected by surgical resection, and surgically resected tissues were stored at −80°C at the Division of Thoracic Surgery, Taichung Veterans General Hospital. Patients were consecutively recruited between 1993 and 2004. This study was approved by the hospital’s Institutional Review Board (Institutional Review Board, Chung Shan Medical University Hospital. CSMUH No: CS11177). The TNM stage, tumor type and stage of each collected specimen were histologically determined according to the WHO classification system. The age of all patients was between 26 and 84 years (mean ± SD = 64.2 ± 11.2). Clinical parameters and overall survival data were collected from chart review and the Taiwan Cancer Registry, Department of Health, Executive Yuan, ROC. The survival time was defined to be the period of time from the date of primary surgery to the date of death. The median follow-up time after
surgery was 21.5 months and the median overall survival of all patients was 21.9 months. During this survey, 63 patients died. Based on follow-up data, 35 patients relapsed (15 had local recurrence, 35 had distant metastasis, and 11 had local and distant metastasis). Among these patients, tumors frequently relapsed in the lung (15 patients) and metastasized in the bone (13 patients), brain (10 patients), liver (5 patients), pleura (4 patients), chest wall (3 patients), and mediastinum (1 patient). In total, 11 patients had tumors that metastasized to more than one organ.

Cell culture

SiHa, HeLa, C33A, A549 and H1299 cancer cell lines were obtained from The American Type Culture Collection (ATCC) (18). TL1, TL2, and TL4 cells were kindly provided by Dr. Cheng YW (10). All of these three cell lines are p53 wild type. TL1 and TL2 cell lines are HPV 16 E6 positive and TL4 is HPV E6 negative. Cells were cultured and stored according to the suppliers’ instructions.

Genomic DNA extraction from tissues and cells

Genomic DNA was extracted from tissues and cell lines by conventional methods, as described previously (19).

RNA extraction and cDNA synthesis from tumor tissues and cells
RNA extraction and cDNA synthesis were performed as described previously (19).

Real-time RT-PCR

The detail method was described previously (19). Real-time RT-PCR primers were listed in Supplementary Table 1. There is no clinically defined cutoff point of IL-10 and CIP2A mRNA expression level, and the median value of both gene mRNA expression levels in lung tumors was used to category the study population into “low” and “high” expression groups.

Immunohistochemical staining

Immunohistochemical (IHC) staining to evaluate HPV 16/18 E6 expression in tumor tissues was performed as described previously (10). Briefly, formalin-fixed and paraffin-embedded specimens were sectioned at a thickness of 3 μm. Sections were deparaffinized in xylene, rehydrated through serial dilutions of alcohol, and washed in phosphate-buffered saline, the buffer which was used for all subsequent washes. Sections were heated in a microwave oven twice for 5 min in citrate buffer, and then incubated with polyclonal anti-HPV16 or HPV18 E6 antibody (Santa Cruz Biotechnology and Chemicon International, Inc.) for 90 min at 25°C. The conventional streptavidin peroxidase method (DAKO, LSAB Kit K675) was performed to develop signals and the cells were counterstained with hematoxylin.
Negative E6 immunostaining was defined to be with 0% to 10% positive nuclei, and cases with >10% positive nuclei were decided to be positive for E6 immunostaining.

Three observers independently evaluated the intensities of the signals.

Plasmid construction, transfection, and stable clone selection

IL-10 shRNA was purchased from National RNAi Core Facility, Academia Sinica, Taiwan, ROC. The expression vector of HPV16 E6 and RNA interference target sequences for HPV16 E6 shRNA have been previously verified (18). Non-specific shRNA control (NC) of scramble sequence was used as the control in the knockdown experiment and vector control (VC) was used as the control of HPV E6 overexpression. The procedures and methods were as described previously (19).

CIP2A reporter plasmid

The CIP2A-Luc plasmid was constructed by inserting a 972, 452 or 162 bps XhoI/KpnI fragment into an XhoI/KpnI-treated pGL3 vector (Promega). The primer sequences were listed in Supplementary Table 1.

Cell doubling time, Migration, invasion assay and anchorage-independent soft agar growth

The procedures and methods were as described previously (18).
anchorage-independent soft agar growth assay, the colonies larger than 150 μm in diameter were counted.

Chromatin immunoprecipitation (ChIP) assay

ChIP analysis was performed as described in previous reports (18). The primers were listed in Supplementary Table 1.

Animal model

BALB/cAnN.Cg-Foxn1nu/CrlNarl mice were maintained in a standard mouse facility at Chung Shan Medical University. When these nude mice were 7 weeks of age, NC and IL-10 knockdown TL1 stable cells were injected via the tail vein (1x10^6). The mice were then sacrificed after four months. The tumor nodules (diameter > 1 mm) on the surface of lung were counted. Some tumor nodules were frozen at −80 °C for the determination of IL-10 expression by real-time RT-PCR. The remaining lung specimens were stained with hematoxylin and eosin (H&E) and the pathology was confirmed by pathologists.

Nested-polymerase chain reaction (Nested-PCR)

Tumor genomic DNA was extracted from the tumor portion of whole-mount paraffin sections. SiHa and HeLa cervical cancer cells were used as positive controls for the
detection of HPV16 and HPV18 DNA, and PBS was used as a negative control. HPV
viral DNA was first amplified with type consensus primers MY09 and MY11
followed by a second round of amplification with type-specific primers flanking the
L1 region to identify the subtype. The detailed procedures were described previously
(9).

Protein extraction from cells and western blotting

The procedures and methods were as described previously (18).

Chemicals and antibodies

PD153035 was purchased from Calbiochem. All other chemicals were acquired from
Sigma Aldrich. Anti-IL-10Ra (C-20), anti-CIP2A (HL1916), anti-p-CREB (Ser133),
anti-CREB (Thr217) and anti-c-Myc (9E10) antibodies were purchased from Santa
Cruz Biotechnology, Inc. Anti-Akt and anti-p-Akt (Ser 473) antibodies were
purchased from Cell Signaling. Anti-human IL-10 antibody (MAB2171) was purchase
from R & D Systems, Inc. The antibody for p53 (BP53-12), β-actin and secondary
antibodies were purchased from Sigma Aldrich.

Statistical analysis

All statistical analyses were performed using the SPSS statistical software program as
described previously (version 11.0; SPSS, Inc., Chicago, IL) (18, 19). Median survival time and 5-year survival rate for the whole test set were estimated using the Kaplan-Meier product limit method. Multivariate and univariate Cox regression analysis were performed to assess the prognostic value of IL-10 and CUP2 mRNA expression level with or without adjustment of the parameters including age, gender, smoking history, and tumor stage. All statistical tests were two-sided and P values < 0.050 were considered to be statistically significant.
Results

IL-10 expression was higher in E6-positive lung cancer cells than in E6-negative lung cancer cells

We explored whether IL-10 expressed from lung tumors could promote tumor progression by enrolling a panel of lung cancer cells to evaluate IL-10 protein and mRNA expression. IL-10 protein and mRNA expression was higher in HPV16 E6-positive TL1 and TL2 than in E6-negative lung cancer cells (Figure 1A). Higher expression was also found in E6-positive SiHa and HeLa cervical cancer cells than in E6-negative C33A cervical cancer cells (Figure 1A). Therefore, E6 appeared to promote IL-10 expression.

Knockdown and overexpression of E6, by shRNA and a cDNA plasmid, respectively, were then used to examine whether IL-10 expression could be modulated by E6 in TL1 and TL4 cells. E6 expression was decreased and p53 expression was increased by E6-knockdown in TL1 cells. Conversely, E6 expression was increased and p53 expression was decreased by E6-overexpression in TL4 cells (Figure 1B). In addition, IL-10 protein expression was modulated by E6-knockdown or overexpression in a pattern consistent with the IL-10 mRNA levels found in TL1 or TL4 cells (Figure 1B).

Therefore, IL-10 induction due to E6 oncoprotein expression in lung cancer cells
might occur through transcriptional activation.

Upregulation of IL-10 by E6 is mediated through PI3K/AKT signaling pathway

We verified which signaling pathway might be linked with E6-induced IL-10 transcription by using different specific inhibitors. Western blotting data showed that IL-10 expression was significantly reduced in a dose-dependent manner in TL1 cells treated with wortmannin or LY294002 (PI3K inhibitors), and was slightly decreased by treatment with PD153035 (an EGFR inhibitor); however, IL-10 expression was not changed by treatment with PD98059 or U0126 (MEK inhibitors) or BAY11-7082 (a NFκB inhibitor) (Figure 2A, upper panel). We further tested whether IL-10 production in E6-positive cells was mediated through PI3K/AKT pathway by treating TL1 and E6-overexpressing TL4 cells with LY294002 and wortmannin. IL-10 expression was reduced in a dose-dependent manner in both cell types by LY294002 and wortmannin (Figure 2A, lower panel).

We then explored which transcriptional factor(s) might be responsible for IL-10 transcription by using software analysis to predict the putative binding sites of transcription factors (http://www.genome.jp/tools/motif/). As shown in Figure 2B, the IL-10 promoter (-858~+1) had putative binding sites of C/EBPα, C/EBPβ, CREB, and MZF-1 (Figure 2B, upper panel). Three promoter regions for the IL-10 gene (-855~+1,
-458~+1, and -349~+1) were constructed for evaluation of luciferase reporter activity. Separately transfected each of these three promoters into TL1 cells resulted in activities of the -458~+1 and -349~+1 promoters that were 95% and 38%, respectively, of the reporter activity of -855~+1 promoter. This finding suggests that C/EBPβ and CREB, located at -458~349 promoter region, might play an important role in IL-10 transcription. The luciferase reporter activity of the -458~+1 promoter in TL1 cells was markedly reduced by E6-knockdown and by the inhibitors of PI3K, but to a lesser extent by inhibitors of the EGFR signaling pathway (Figure 2B, lower panel). This suggested a crucial role for phosphorylation of CREB and C/EBPβ, via the PI3K/AKT signaling pathway, in IL-10 transcription.

*Phosphorylation of CREB and C/EBPβ by E6 via PI3K/AKT signaling pathway plays a crucial role in IL-10 transcription*

We next questioned whether phosphorylation of CREB and C/EBPβ by E6, via the PI3K/AKT pathway, could play an important role in E6-mediated IL-10 transcription. Western blotting data showed that levels of phosphorylated CREB and C/EBPβ protein were markedly reduced by PI3K inhibitors (LY294002 and wortmannin), but the total protein levels of CREB and C/EBPβ were not changed by these inhibitors. As expected, the phosphorylation of both proteins was decreased by treatment with an
EGFR inhibitor (PD98059) (Figure 2C, left panel). ChIP analysis further indicated that the DNA binding activities of CREB and C/EBPβ were diminished by PI3K inhibitors, but not by the EGFR inhibitor (Figure 2C, right panel). Therefore, phosphorylation of CREB and C/EBPβ via PI3K/AKT signaling pathway appeared to play a crucial role in E6-mediated IL-10 transcription in lung cancer cells.

*IL-10 induced by E6 is responsible for soft-agar growth, invasion, and xenograft tumor nodule formation*

We used soft-agar colony formation and Boyden chamber assays to explore whether IL-10 induced by E6 could promote anchorage independent soft-agar growth and invasiveness, respectively. As expected, IL-10 expression was reduced in IL-10-knockdown TL1 cells and elevated in IL-10-overexpressing TL4 cells (Figure 3A). The doubling time was significantly elevated in IL-10-knockdown TL1 cells and reduced in IL-10-overexpressing TL4 cells (23.4 ± 0.2, 26.1 ± 0.5, and 35.1 ± 1.0 for IL-10-knockdown TL1; 30.1 ± 0.3, 25.8 ± 0.3, and 20.6 ± 1.5 for IL-10-overexpressing TL4, Supplementary Figure 1). The representative soft-agar growth colony sizes decreased markedly in IL-10-knockdown TL1 cells and increased in IL-10 overexpression TL4 cells when compared with non-specific shRNA control (NC) and vector control (VC) cells (Figure 3B). The capability for soft-agar growth
and invasiveness was significantly reduced in IL-10-knockdown TL1 and elevated in IL-10-overexpressing TL4 cells in a dose-dependent manner when compared with NC and VC cells (Figure 3B). We further established a stable clone of IL-10-knockdown TL1 cells, in which IL-10 expression had almost disappeared (Figure 3C). We then injected nude mice with these clonal cells via the tail vein to determine whether a lower number of lung tumor nodules would form after 4 months, compared to injection with NC cells. The number of tumor nodules was significantly lower in nude mice injected with the IL-10-knockdown stable clone than in mice injected with NC cells (10.5 ± 7.6 vs. 26.5 ± 6.7, P = 0.003, Figure 3C). Therefore, IL-10 expression induced by E6 oncoprotein may be responsible for soft-agar growth, invasion, and xenograft tumor nodule formation.

IL-10 promotes tumor aggressiveness via upregulation of CIP2A

We explored the underlying mechanism of tumor aggressiveness induced by IL-10, using a PCR-array to examine which molecule might involve in IL-10-induced tumor progression. PCR-array analysis showed a marked decrease in c-Myc expression in IL-10 knockdown TL1 cells compared to expression in NC cells among the 94 gene examined. c-Myc expression has been shown to be regulated by a CIP2A-PP2A axis (20). Therefore, we expected that the IL-10 induced by E6 might upregulate CIP2A,
thereby contributing to c-Myc expression and consequently promoting tumor aggressiveness. Western blotting analysis showed that c-Myc and CIP2A expressions were concomitantly decreased in IL-10 knockdown TL1 cells and increased in E6-overexpressing TL4 cells (Figure 4A). The elevated expression of c-Myc and CIP2A by E6 was restored by IL-10 knockdown in E6-transfected TL4 cells (Figure 4A). Interestingly, CIP2A mRNA expression was consistent in its protein expression in IL-10 knockdown TL1, E6-transfected TL4, and IL-10-knockdown E6-transfected TL4 cells, suggesting that IL-10 could transactivate CIP2A transcription in E6-positive lung cancer cells (Figure 4A).

*Phosphorylation of CREB via PI3K/AKT pathway is responsible for IL-10-mediated CIP2A transcription*

We next examined which signaling pathway might be linked with the upregulation of CIP2A transcription induced by IL-10. Three promoters of the CIP2A gene (-972~+1, -452~+1, and -162~+1) were constructed: the putative transcriptional factors on these promoter regions are shown in Figure 4B (upper panel). These promoters were transfected into TL1 cells to verify which promoter region might be more important for CIP2A transcription. The luciferase reporter activity assays indicated that the -452~+1 promoter had 71% of the reporter activity of the -972~+1 promoter, whereas
the -162~+1 promoter had only 11% of the reporter activity of -972~+1 promoter (Figure 4B, middle panel). This finding suggests that CREB, NF-kB, and AP-1 might be involved in CIP2A transcription.

The reporter activity of the -452~+1 promoter in TL1 cells was markedly suppressed by E6-knockdown, LY294002, and wortmannin, but not by PD153035. Therefore, we expected that the PI3K/AKT pathway might be involved in IL-10 mediated CIP2A transcription via phosphorylation of CREB. Western blotting analysis indicated that the total CREB protein expression was not changed by E6-knockdown or by treatment of different inhibitors, but expression of phosphorylated CREB protein almost disappeared after treatment with LY294002 and wortmannin followed by E6- and IL-10-knockdown, and PD98059. ChIP analysis further confirmed that phosphorylated CREB was bound to the CIP2A promoter (Figure 4C). Phosphorylation of CREB therefore clearly played a crucial role in IL-10 mediated CIP2A transcription. We further verified whether IL-10-mediated CIP2A could be responsible for IL-10-induced cell invasion. TL4 cells were transfected with two doses of IL-10. Western blotting showed that CIP2A and c-Myc expression was concomitantly increased by IL-10 transfection in a dose-dependent manner (Fig. 4D upper panel). The invasion capability of TL4 cells was significantly elevated by IL-10 transfection (Fig. 4D lower panel). However, the invasion capability of
IL-10-transfected TL4 cells was restored by CIP2A-knockdown (Fig. 4D lower panel).
This result clearly indicates that CIP2A is responsible for IL-10-mediated cell invasion.

IL-10 mRNA expression levels are positively correlated with HPV16/18 E6 oncoprotein and CIP2A mRNA expression in tumors of lung adenocarcinoma patients

We verified whether IL-10 expression could be associated with HPV16/18 E6 oncoprotein expression by evaluating IL-10 mRNA expression levels and E6 oncoprotein levels in lung tumors from 98 lung adenocarcinoma patients using real-time RT-PCR and immunohistochemistry. The distribution and prognostic value of parameters of patients were summarized in Supplementary Table 2. Univariate analysis showed that patients with advanced stage (II, III), higher T value (T3, T4), and advanced nodal involvement (N1, N2) had shorter overall survival periods than with early stage (I), lower T value (T1, T2) and non-nodal involvement (N0) (P = 0.004 for stage, P = 0.001 for T, P = 0.002 for N; Supplementary Table 2). As shown in Supplementary Table 3, IL-10 mRNA expression levels were higher in E6-positive tumors than in E6-negative tumors (168.1 ± 40.5 vs. 64.7 ± 24.5, P = 0.032; Supplementary Table 3). We next examined whether IL-10 expression could be related to CIP2A expression in lung tumors. Real-time RT-PCR analysis showed that
CIP2A mRNA expression was significantly higher in tumors expressing high levels of IL-10 than in tumors expressing low levels (424.4 ± 85.5 vs. 188.5 ± 50.9, P = 0.020). These *in vivo* observations in lung tumors from lung adenocarcinoma patients were consistent with the earlier *in vitro* findings in lung cancer cell cultures.

*IL-10 mRNA and CIP2A mRNA expression may independently predict survival in lung adenocarcinoma patients*

Kaplan Meier analysis showed that patients with high IL-10 mRNA and CIP2A mRNA tumors had shorter overall survival than those with low IL-10 mRNA and CIP2A mRNA tumors (Supplementary Figure 2A). The prognostic significance of IL-10 and CIP2A mRNA levels was observed in patients with E6 positive tumors (Supplementary Figure 2B), not in patients with E6 negative tumors (Supplementary Figure 2C). Multivariate Cox regression analysis was used to estimate whether IL-10 and CIP2A mRNA expression level could independently predict survival in patients with lung adenocarcinoma. As expected, patients with stage II+III tumors had shorter median survival and lower 5-year survival percentage than did patients with stage I tumors (19.2 vs. 79.3 months, 18.3 vs. 57.4%; HR, 2.498, 95% CI, 1.373-4.546, P = 0.003; Table 1). Patients with high IL-10 mRNA levels had poorer survival than those with low IL-10 mRNA levels (HR, 2.083, 95% CI, 1.241-3.495, P = 0.005, Table 1,
Supplementary Figure 2). In addition, poorer survival was found in patients with high CIP2A mRNA levels than in patients with low CIP2A mRNA levels (HR, 1.809, 95% CI, 1.063-3.079, P = 0.029, Table 1, Supplementary Figure 2). Therefore, shorter median survival and lower 5-year survival rate were observed in patients with high IL-10 and CIP2A mRNA levels in tumor than in those with low IL-10 and CIP2A mRNA levels in tumor. Moreover, the prognostic significance of IL-10 mRNA and CIP2A mRNA expression levels was seen only in patients with E6-positive tumors and not in patients with E6-negative tumors (Table 2, Supplementary Figure 1). We further confirm the finding by the presence or absence of HPV 16/18 DNA in this study population. The prognostic significance of IL-10 mRNA and CIP2A mRNA levels were also observed in patients with HPV 16/18 DNA positive tumors and not in patients with HPV 16/18 DNA negative tumors (Supplementary Table 4). These clinical observations for lung adenocarcinoma patients were consistent with the findings from the mechanistic studies in cell models. Therefore, we suggest that IL-10 and CIP2A mRNA may independently predict survival in lung adenocarcinoma patients.
Discussion

Early studies reported that IL-10 is commonly expressed in human lung tumors and suggest that it may play an active immunoregulatory role in the lung tumor microenvironment (2, 21-25). IL-10 is considered to be an autocrine growth factor of immune cells, and it participates notably in increases of tumor cell proliferation of melanoma, gastric, and thyroid cancers (26, 27). The majority of reports indicate that IL-10 produced from immune cells may promote lung cancer growth via suppressing immune surveillance (28, 29). This suppression is due to defected function of both T cells and antigen presenting cells (2). No evidence has yet shown that IL-10 expressed in tumor cells could promote tumor progression. Therefore, the nature of IL-10-promoted tumor malignancy of lung cancer cells, i.e., whether it occurs via an autocrine or paracrine pathway, is still unclear. In the present study, we provided the molecular evidence to show that IL-10 is induced by HPV E6 oncoprotein and acts as an autocrine growth factor that not only promotes lung cancer growth, but also promotes anchorage-independent soft-agar growth and invasiveness (Figure 3). We also tested the effect of exogenous IL-10 on migration capability of lung cancer cells. As expected, the capability was decreased by IL-10 neutralized antibody in TL1 cells, but the capability was increased by IL-10 recombinant protein in TL4 cells (Supplementary Figure 3). The autocrine regulation of cell growth by IL-10 is
mediated through the IL-10 receptor (IL-10R). The results presented here for TL1
cells consistently showed that the capability for migration and invasion promoted by
IL-10 could be diminished by IL-10R-knockdown (Supplementary Figure 4). This
strongly suggests that IL-10 induced by E6 can directly promote lung cancer cell
invasiveness and soft-agar growth via the autocrine loop of IL-10/IL-10R.

IL-10 may play a dual role in the development and progression of human cancers (30).
A recent report demonstrated that IL-10 deficiency increases chemical-induced tumor
incidence, growth, and foci formation in IL-10 knockout C57BL/6 mice compared
with wild-type mice in a colitis-associated colon cancer model (31). The authors
further indicated that IL-10 deficiency increases the numbers of myeloid-derived
suppressor cells in which high levels of IL-1β was expressed to block tumor growth
(31). In melanoma, IL-10 has been shown to suppress tumor growth and metastasis
via inhibition of angiogenesis, indicating an anti-tumor action of IL-10 (32). However,
in animal models and in human tumors, IL-10 was shown to promote metastatic
potential in lung tumor cells in vivo by promoting angiogenesis and resistance to
apoptosis (22, 33). IL-10 is not only expressed by tumor cells but also expressed by
different types of immune cells (4, 5). The limitation of this study is to quantify the
IL-10 expression from tumor cells, and to exclude IL-10 expression from the
surrounding non-tumor and immune cells. In the present study, tumor tissues from
lung cancer patients were obtained from the frozen section of surgically resected lung
tumor parts according to the pathology examination. Therefore, we considered that
IL-10 was largely expressed from tumor cells. Elevation of serum or tumor-expressed
IL-10 may independently predict poor prognosis in advanced lung cancer patients (8,
34). Moreover, after chemotherapy, patients whose serum IL-10 levels were stable or
elevated showed a greater risk of tumor recurrence and distant metastasis, and of
chemoresistance, when compared with patients with lower serum IL-10 expression (8).
Our results appear to support this observation as we found that tumor-derived and
exogenous IL-10 may promote tumor aggressiveness and poor outcome in lung
adenocarcinoma patients who had HPV 16/18 E6-positive tumors.

Upregulation of IL-10 production by phosphorylation of CREB via the PI3K/AKT
pathway has been shown in immune cells such as monocytes and macrophages (35,
36). Notably, HPV E6 upregulates cIAP2 via the EGFR/PI3K/AKT cascades, and in
turn contributes to cisplatin resistance in HPV associated lung cancer. These results
give the clue that HPV E6 might regulate IL-10 expression in lung cancer via the
PI3K/AKT signaling pathway (37). In the present study, IL-10 production in
E6-positive lung cancer cells was predominantly regulated by phosphorylation of
CREB via the PI3K/AKT pathway (Figure 2). Unexpectedly, IL-10 was able to
activate CIP2A transcription via the phosphorylation of CREB induced by the PI3K
signaling pathway and also promoted tumor malignancy (Figure 4). CIP2A has been shown to have an oncogenic role in human malignancies, operating via inactivation of PP2A and stabilization of c-Myc protein (20). CIP2A over-expression has been associated with poor prognosis in various human carcinomas, including lung cancer (38-40). A prognostic value of CIP2A and IL-10 mRNA levels was also indicated in the present study population (Table 1). Moreover, the correlation of CIP2A with IL-10 expression in lung tumors was also supported by our mechanistic studies from lung cancer cell models (Figure 4).

Recently, ectopic CIP2A expression in hepatocellular carcinoma and head and neck squamous cell carcinoma cells has been suggested to enhance PI3K/AKT activation (41, 42). Therefore, it is conceivable that a feedback loop of IL-10-CIP2A-phosphorylated-CREB may be involved in the progression of E6-mediated IL-10 lung adenocarcinoma. Previous studies indicated that a proteasome inhibitor, bortezomib, significantly reduced CIP2A expression and increased apoptosis in hepatocellular carcinoma and head and neck carcinoma cells (41-45). Therefore, we expected that bortezomib or a PI3K inhibitor could be used to suppress tumor invasiveness and to improve the outcome in HPV-associated lung adenocarcinoma patients who had high IL-10 expression. In summary, we provide evidence that IL-10-mediated CIP2A may play a crucial role in the tumor Research.
aggressiveness of lung adenocarcinoma, particularly in patients with HPV 16/18 E6-positive tumors.

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Author contributions

Conception and design: WWS, HL; Analysis and interpretation: WWS, YCW, PLL, YWC, TCW, HL; Drafting the manuscript for important intellectual content: WWS, YCW, HL

Conflicts of interest

The authors declare no conflict of interest.
References


Legends

Figure 1. High IL-10 mRNA and protein expression in HPV16 E6-positive cell lines compared with HPV negative cell lines in a panel of lung and cervical cancer cell lines. (A) Relative IL-10 mRNA and protein expression levels of cancer cell lines were determined by real-time RT-PCR and western blot. The IL-10 mRNA expression of CL3 cell line was used as reference (mRNA expression level = 1). The E6(+) and E6(-) indicated the HPV E6 expression of cell lines. (B) TL1 cells were transfected with HPV16 E6 shRNA (left panel) and TL4 cells were transfected with HPV16 E6 cDNA plasmid (right panel) as indicated. HPV16 E6, p53, IL-10 protein expressions were determined by western blot. The changes of IL-10 mRNA expression levels were evaluated by real-time RT-PCR. Experiments in this figure were repeated at least three times and similar results were obtained.

Figure 2. CREB and C/EBP β may be responsible for IL-10 transcription via PI3K pathway in TL1 and TL4 cell lines. (A) TL1 cells were treated with MEK (PD98059, 20μM and U0126, 10μM), EGFR (PD153035, 0.5μM), NF-κB (BAY11-7082, 20μM) and PI3K inhibitors (LY294002, 30μM and wortmannin, 10μM) and protein expression of IL-10, AKT, p-AKT and β-actin was measured by western blot. TL1 cells were treated with PI3K inhibitors at various concentrations as indicated. TL4
cells were transfected with 3 μg HPV16 E6 cDNA plasmid or vector control plasmid for 48 hr. The medium was renewed and then those cells were treated with PI3K inhibitors at various concentrations as indicated. Protein expression was measured by western blot. (B) Diagram summarized the positions of the putative binding sites of transcriptional factors on IL-10 promoter constructs (-855→+1) predicted by software analysis. Luciferase reporter assay was performed to evaluate the promoter activity of these three constructs including -855→+1, -458→+1, and -349→+1. TL1 cells were transfected with these three promoter constructs separately and β−gal was served as an internal control. The luciferase reporter activity of these three constructs was determined and the reporter activity of IL-10 (-349→+1) construct was served as control (activity = 1) for presentation. Luciferase reporter assay was performed to measure the promoter activity of IL-10 (-458→+1) construct in TL1 cells which were transfected with HPV E6 shRNA or treated with EGFR or PI3K inhibitors as indicated. TL1 cells were transfected with IL-10 (-458→+1) construct, TL1 cells transfected with IL-10 (-458→+1) construct were treated with E6 shRNA, EGFR (PD153035, 0.5μM) and PI3K inhibitors (LY294002, 30μM and wortmannin, 10μM) for 48 hr, and then to determine the reporter activity by luciferase reporter assay. β−gal was served as an internal control. (C) TL1 cells were treated with EGFR (PD153035, 0.5μM) and PI3K inhibitors (LY294002, 30μM and wortmannin, 10μM).
Phosphorylated CREB (p-CREB), total CREB, p-CEBP/β, and total C/EBPβ expression levels were evaluated by western blot, and β-actin was used as a protein loading control. ChIP assay was performed to evaluate the DNA bonding ability of p-CREB and p-CEBP/β on the putative binding site of IL-10 promoter region. TL1 cells were treated with EGFR (PD153035, 0.5 μM) and PI3K inhibitors (LY294002, 30 μM and wortmannin, 10 μM) and fixed for ChIP assay. The products were amplified by PCR and the result was presented by gel-electrophoresis. Experiments in this figure were repeated at least three times and similar results were obtained.

Figure 3. IL-10 promotes the capability of soft-agar growth, invasiveness and lung tumor nodule formation in nude mice in TL1 and TL4 cells. (A) shRNA and cDNA plasmid of IL-10 were respectively transfected into TL1 and TL4 cells, and then IL-10 protein expression was determined by western blot and β-actin was as a protein loading control. (B) Matrigel invasion and soft-agar colony formation assay were used to evaluate the invasiveness and soft-agar growth capability after TL1 cells transfected with IL-10 shRNA and TL4 cells transfected with IL-10 cDNA plasmid as compared with their control cells. The results were show as representative pictures in left panel and the quantitative graph in the right panel. (C) IL-10 and β-actin protein expression of stable clones was shown as upper panel. NC and IL-10 knockdown TL1 stable cells were injected into nude mice via tail vein (1*10⁶). The mice were
sacrificed after four months (N = 5 for each group). The gross pictures of the tumor nodules formed in mice lung shown in right panel and the quantitative graph shown in left panel (p = 0.003). Experiments of figure 2A and 2B were repeated at least three times and similar results were obtained.

Figure 4. IL-10 upregulates CIP2A transcription by CREB phosphorylation via PI3K/AKT pathway in TL1 and E6-positive TL4 cells. (A) shRNA and cDNA plasmid of IL-10 were respectively transfected into TL1 and TL4 cells and various concentrations of IL-10 shRNA transfection in both cells were as indicated. Protein expression of HPV E6, IL-10, CIP2A, and c-Myc was determined by western blot and β-actin was used as a protein loading control. The change of CIP2A mRNA expression levels was evaluated by real time RT-PCR in IL-10 knockdown TL1 cells and E6-overexpressed TL4 cells and then transfected with IL-10 shRNA. (B) Diagram summarized the positions of the putative binding sites of transcriptional factors on CIP2A promoter constructs (-972→+1) predicted by software analysis. Luciferase reporter assay was performed to evaluate the promoter activity of these three constructs including -972→+1, -452→+1, and -162→+1. TL1 cells were transfected with these three promoter constructs separately and β-gal was served as an internal control. The luciferase reporter activity of these three constructs was determined and the reporter activity of CIP2A (-452→+1) construct was served as control (activity = 1) for
presentation. Luciferase reporter assay was performed to measure the promoter activity of CIP2A (-452~+1) construct in TL1 cells which were transfected with HPV E6 or IL-10 shRNA or treated with EGFR or PI3K inhibitors as indicated. TL1 cells were transfected with CIP2A (-452~+1) construct, TL1 cells transfected with CIP2A (-452~+1) construct were treated with E6 shRNA, EGFR (PD153035, 0.5μM) and PI3K inhibitors (LY294002, 30μM and wortmannin, 10μM) for 48 hr, and then to determine the reporter activity by luciferase reporter assay. β-gal was served as an internal control. (C) TL1 cells were transfected with 5μg HPV E6, IL-10 or NC shRNA; treated with EGFR (PD153035, 0.5μM) or PI3K inhibitors (LY294002, 30μM and wortmannin, 10μM). Protein expression of p-CREB, total CREB, and β-actin was measured by western blot. ChIP assay was performed to evaluate the DNA bonding ability of CREB on CIP2A promoter -478 to -346. Transfected or inhibitor-treated TL1 cells were fixed for ChIP assay. The products were amplified by PCR and the result was presented by gel-electrophoresis. (D) shRNA plasmid of CIP2A were respectively transfected into TL4 cells and various concentrations of IL-10 shRNA transfection in both cells were as indicated. Protein expression of IL-10, CIP2A, and c-Myc was determined by western blot and β-actin was used as a protein loading control. Matrigel invasion assay were used to evaluate the invasiveness after TL4 cells transfected with IL-10 cDNA and CIP2A shRNA plasmid as compared with
their control cells. Experiments in this figure were repeated at least three times and similar results were obtained.
Figure 1

(A) IL-10 mRNA expression levels in various cell lines: SiHa, HeLa, C33A, TL1, TL2, TL4, A549, CL3, H23, H1299.

(B) IL-10 mRNA level in TL1 and TL4 cells treated with HPV16 E6 at different concentrations (0, 1, 5 μg).
Figure 2

(A) Table showing the effects of different treatments on IL-10, Akt, p-Akt, and β-actin levels.

<table>
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<th>p-Akt</th>
<th>β-actin</th>
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<td>PD153035 (MEK)</td>
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(B) Figure showing the activation folds of IL-10 and other proteins.

(C) Figure showing the ChIP results for CREB and CEBP/β.
Figure 3

(A) TL1 shIL-10 TL4 IL-10
0 1 5 (μg)
IL-10
β-actin

(B) TL1
0 1 5 (μg)
shIL-10
IL-10

IL-10
β-actin

IL-10
β-actin

IL-10
β-actin

IL-10
β-actin

(C) Tumor nodules
NC shIL-10

Soft agar colony
Invasion ability

Relative to NC

shIL-10 (μg)

IL-10 (μg)

0 1 5

0 1 5

Relative to NC
Figure 4

(A) TL1 and TL4 E6 expression with varying IL-10 levels. IL-10, CIP2A, c-Myc, and β-actin expression levels are shown.

(B) Bar graphs showing CIP2A mRNA levels.

(C) Western blot analysis of p-CREB, CREB, and β-actin expression.

(D) Bar graphs representing activation folds for CIP2A, IL-10, c-Myc, and β-actin with shCIP2A and IL-10 treatments.

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Table 1. Multivariate analysis of the influence of IL-10 and CIP2A on overall survival in lung adenocarcinoma patients.

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Table 2. Multivariate analysis of the influence of IL-10 and CIP2A mRNA on overall survival in HPV E6 positive lung adenocarcinoma patients.

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IL-10 Promotes Tumor Aggressiveness via Upregulation of CIP2A Transcription in Lung Adenocarcinoma


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