

Bile Acid Exposure Up-regulates Tuberous Sclerosis Complex 1/Mammalian Target of Rapamycin Pathway in Barrett's-Associated Esophageal Adenocarcinoma

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Abstract

Barrett's esophagus, a columnar metaplasia of the lower esophagus epithelium related to gastroesophageal reflux disease, is the strongest known risk factor for the development of esophageal adenocarcinoma (EAC). Understanding the signal transduction events involved in esophageal epithelium carcinogenesis may provide insights into the origins of EAC and may suggest new therapies. To elucidate the molecular pathways of bile acid-induced tumorigenesis, the newly identified inflammation-associated signaling pathway involving I κ B kinases β (IKK β), tuberous sclerosis complex 1 (TSC1), and mammalian target of rapamycin (mTOR) downstream effector S6 kinase (S6K1) was confirmed to be activated in immortalized Barrett's CPC-A and CPC-C cells and esophageal cancer SEG-1 and BE3 cells. Phosphorylation of TSC1 and S6K1 was induced in response to bile acid stimulation. Treatment of these cells with the mTOR inhibitor rapamycin or the IKK β inhibitor Bay 11-7082 suppressed bile acid-induced cell proliferation and anchorage-independent growth. We next used an orthotopic rat model to evaluate the role of bile acid in the progression of Barrett's esophagus to EAC. Of interest, we found high expression of phosphorylated IKK β (pIKK β) and phosphorylated S6K1 (pS6K1) in tumor tissues and the Barrett's epithelium compared with normal epithelium. Furthermore, immunostaining of clinical EAC tissue specimens revealed that pIKK β expression was strongly correlated with pS6K1 level. Together, these results show that bile acid can deregulate TSC1/mTOR through IKK β signaling, which may play a critical role in EAC progression. In addition, Bay 11-7082 and rapamycin may potentially be chemopreventive drugs against Barrett's esophagus-associated EAC. [Cancer Res 2008;68(8):2632-9]

Introduction

Approximately 14,550 people were diagnosed with esophageal cancer in the United States in 2006; half of whom had esophageal adenocarcinoma (EAC; ref. 1). In fact, its incidence has risen faster

than that of any other major cancer, including lung cancer, breast cancer, and melanoma in the United States since 1975. The prognosis of EAC is extremely poor, with a 5-year survival rate of <20%, even among patients who undergo combined modality therapy (2, 3). The causes underlying the dramatic increase in EAC incidence are poorly understood. A history of gastroesophageal reflux disease (GERD), which consists of the reflux of gastric contents into the lower esophagus, has been closely associated with EAC development (4, 5). Chronic GERD leads to the development of Barrett's esophagus, a pathologic transformation of the normal squamous esophagus epithelium into metaplastic intestinal-like epithelium. Barrett's esophagus is a true premalignant condition with an estimated 43-fold increase in the relative risk of developing EAC compared with the general population (6, 7). People with Barrett's esophagus diagnosed with endoscopy are more likely to develop adenocarcinoma of the esophagus than the normal population (8-10). Bile acids are one of the major harmful components of refluxed gastric material, and intraesophageal bile salt concentrations are higher in patients with Barrett's esophagus than in those with uncomplicated GERD (11, 12). Bile acids are promising candidates as esophageal carcinogens, because repeated exposure to bile acid, particularly exposure to the unconjugated bile acids deoxycholic acid and chenodeoxycholic acid (CDCA), can enhance Barrett's esophagus transformation to EAC (13, 14). As bile salts can also stimulate the signaling effects of antiapoptotic pathways, such as c-myc, cyclooxygenase-2, and nuclear factor- κ B (NF- κ B), it is likely that continuous bile damage enhances dysregulated proliferation, ultimately contributing to genomic instability and progression to EAC (15-18). However, the molecular signaling switch for Barrett's esophagus progression from dysplasia to invasive carcinoma is still unknown, especially in the refluxed bile acid-related cancer development and progression.

It is recognized that chronic inflammation is functionally important for the developmental process of several types of neoplasia, including lung and liver cancers (19, 20). Similarly, the multistep progression from Barrett's esophagus metaplasia to invasive EAC evolves in a chronic inflammatory microenvironment (21-24). In Barrett's esophagus, damaged esophageal cells and infiltrating inflammatory cells, including T lymphocytes and neutrophils, release inflammatory cytokines and chemokines, such as tumor necrosis factor α (TNF α), interleukin-8 (IL-8), and IL-10 (25-27). Published evidence suggest that the TNF α /I κ B kinase β (IKK β) signaling pathway plays a role in inflammation-mediated tumorigenesis. TNF α may be a proinflammatory cytokine involved in human cancers. When cells are exposed to TNF α stimuli, IKK β

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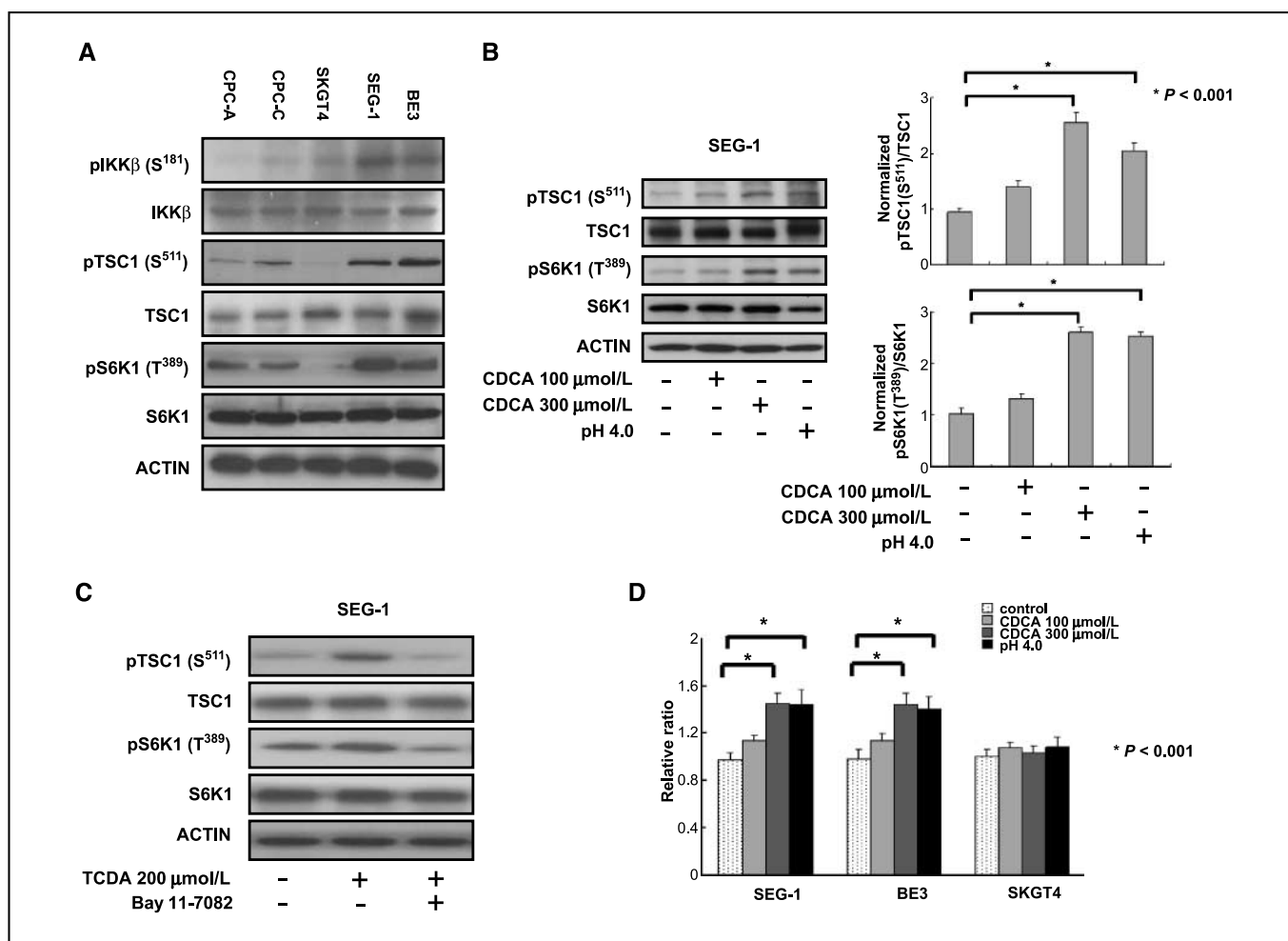


Figure 1. IKK β /TSC1/mTOR pathway was activated in Barrett's esophagus-associated EAC cancer cells. **A**, increased expression of pIKK β (S¹⁸¹) was correlated with higher level of pTSC1 (S⁵¹¹) and pS6K1 (T³⁸⁹) in SEG-1 and BE3 EAC cancer cell lines. Immortalized Barrett's esophagus cell lines CPC-A and CPC-C had lower expression level of pIKK β (S¹⁸¹) and expressed concurrently low levels of pTSC1 (S⁵¹¹) and pS6K1 (T³⁸⁹). The SKGT4 EAC cancer cell line also had lower expression of pIKK β (S¹⁸¹), pTSC1 (S⁵¹¹), and pS6K1 (T³⁸⁹). **B**, the phosphorylation of pTSC1 (S⁵¹¹) and pS6K1 (T³⁸⁹) increased two to three times in SEG-1 cancer cells treated with 300 μ mol/L CDCA ($P < 0.001$) and low-pH medium ($P < 0.001$) compared with those in untreated cells. **C**, conjugated bile acid TCDA increased the expressions of pTSC1 (S⁵¹¹) and pS6K1 (T³⁸⁹), and the IKK β inhibitor Bay 11-7082 blocked the TCDA stimulation effect in SEG-1 EAC cancer cells. **D**, cell proliferation was increased in SEG-1 and BE3 cancer cells compared with SKGT-4 cells, which had lower expression of the IKK β /TSC1/mTOR signaling pathway.

is activated, which triggers I κ B α degradation and enhances NF- κ B translocation to the nucleus, ultimately activating target genes (28, 29). NF- κ B has been found to be up-regulated in Barrett's epithelium (BE), and the nuclear translocation of NF- κ B in EAC is associated with poor prognosis and treatment response in patients (30).

Tuberous sclerosis complex 1 (TSC1), a well-known tumor suppressor involved in the development of tuberous sclerosis, negatively regulates the mammalian target of rapamycin (mTOR) pathway by inhibiting Ras homologue enriched brain (31, 32). Deregulation of the TSC/mTOR signaling pathway has been implicated in the development of cancer, and the activation of ribosomal protein S6 kinase (S6K1) by mTOR was found to lead to increased cell proliferation and angiogenesis (33).

Although the evidence suggests that inflammation is associated with human tumors, the underlying mechanism of tumorigenesis is not yet fully understood. In a recent study, we found that TNF α enhanced tumor angiogenesis through IKK β -mediated deregulation of the TSC/mTOR pathway in breast cancer (34, 35). In the present study, we found that the bile acid deregulated the TSC/mTOR

pathway through IKK β signaling. We also found that treatment of Barrett's esophagus cells and EAC cells with the IKK β inhibitor Bay 11-7082 or the mTOR inhibitor rapamycin inhibited cell proliferation and *in vitro* transformation measured by the anchorage-independent growth assay. In a rat animal model, the expressions of phosphorylated IKK β (pIKK β) and phosphorylated S6K1 (pS6K1) were higher in Barrett's esophagus and EAC compared with normal esophageal epithelium. We next evaluated normal esophageal epithelium, Barrett's esophagus lesions, and tumor samples from patients with Barrett's esophagus-associated EAC and found a concomitant sustained expression of both pIKK β and pS6K1 in Barrett's esophagus high-grade dysplasia and EAC. Together, our results indicate that bile acid may activate the mTOR pathway via IKK β signaling, leading to Barrett's esophagus and EAC.

Materials and Methods

Cell lines. We used immortalized human CPC-A and CPC-C cells which were derived from Barrett's esophagus premalignant epithelium (CPC-A from hyperplasia and CPC-C from high-grade dysplasia) and were

transfected with the human telomerase catalytic subunit to extend cell life span (kind gifts from Dr. P. Rabinovitch, University of Washington; ref. 36). These cells were maintained at 37°C in a 5% CO₂ incubator with MCDB153 (Sigma) plus 10% fetal bovine serum (FBS), 20 ng/mL epidermal growth factor, 20 mg/mL adenine, 140 µg/mL bovine pituitary extract (Invitrogen), 5 µg/mL insulin-transferrin-selenium (Sigma), 4 mmol/L L-glutamine, and 0.4 µg/mL hydrocortisone. Human esophageal cell lines SKGT4, SEG-1, and BE3, which were derived from Barrett's esophagus-associated EACs of the distal esophagus (kind gifts from Dr. D. Beer, University of Michigan), were maintained at 37°C in a 5% CO₂ incubator with DMEM/F12 plus 10% FBS.

Antibodies and reagents. The antibodies used in this study were anti-TSC1 (Zymed), anti-pS6K1 (T³⁸⁹; Cell Signaling Technology), anti-S6K1 (Cell Signaling Technology), anti-pIKKβ (S¹⁸¹; Cell Signaling Technology), anti-phosphorylated AKT (S⁴⁷³; Cell Signaling Technology), anti-AKT (Cell Signaling Technology), anti-actin (Sigma), and anti-IKKβ (Cell Signaling Technology). Antibodies against the phosphorylation sites of TSC1 S⁵¹¹ were produced using the synthetic phosphorylated peptides SPFYRDP_{SLPGSQ} as antigens and purified on a phosphopeptide column at Bethyl Laboratories, Inc. CDCA was obtained from Sigma Chemical Co. and dissolved in ethanol. Bay 11-7082 and rapamycin were purchased from Calbiochem. Recombinant human TNFα was obtained from Roche.

Chenodeoxycholate, taurochenodeoxycholate, Bay 11-7082, and rapamycin treatment. CDCA and taurochenodeoxycholate (TCDA), the major components of bile acid, were used to mimic the physiologic refluxed bile acid in Barrett's esophagus and EAC. In brief, EAC SEG-1 and BE3 cells

and Barrett's esophagus CPC-A and CPC-C cells were grown in the complete medium and exposed to 100 µmol/L, 300 µmol/L CDCA, or 200 µmol/L TCDA for 2 h with or without Bay 11-7082 (40 µmol/L, 45 min pretreatment) or rapamycin (50 or 100 nmol/L, 3 h pretreatment). Cell proliferation/viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and the anchorage-independent growth was measured by soft agar assay. Western blotting was performed to determine the expression of members of the IKKβ/TSC1/mTOR pathway (e.g., IKKβ, TSC1, and S6K1).

Small interfering RNAs. SEG-1 cells were transfected with IKKβ SMARTpool small interfering RNAs (siRNA; Upstate Biotechnology) or control SMARTpool siRNAs (Upstate Biotechnology) by Lipofectamine with plus reagent. After 72 h of siRNA transfection, the cells were treated with 300 µmol/L CDCA for 2 h and lysed for Western blotting.

MTT and anchorage-independent growth assays. Cell growth was measured using the MTT assay (37). In brief, 5,000 cells were seeded in 96-well plates and incubated in culture medium overnight. We then treated cells with 100 µmol/L CDCA, 300 µmol/L CDCA for 2 h, then washed, and cultured with media containing 10% FBS for 24 h. For acid treatment, cells were incubated for 24 h with serum-free medium that was adjusted with 0.1 mol/L HCl to pH 4.0, as monitored by pH meter. An equivalent volume of PBS was added to serum-free, normal pH medium to control the difference in osmolarity. At the end of the exposure period, 20 µL of MTT was added to the cell culture and the cells were incubated for 2 h. Subsequently, 100 µL of DMSO (Sigma) was added to each well and the plate

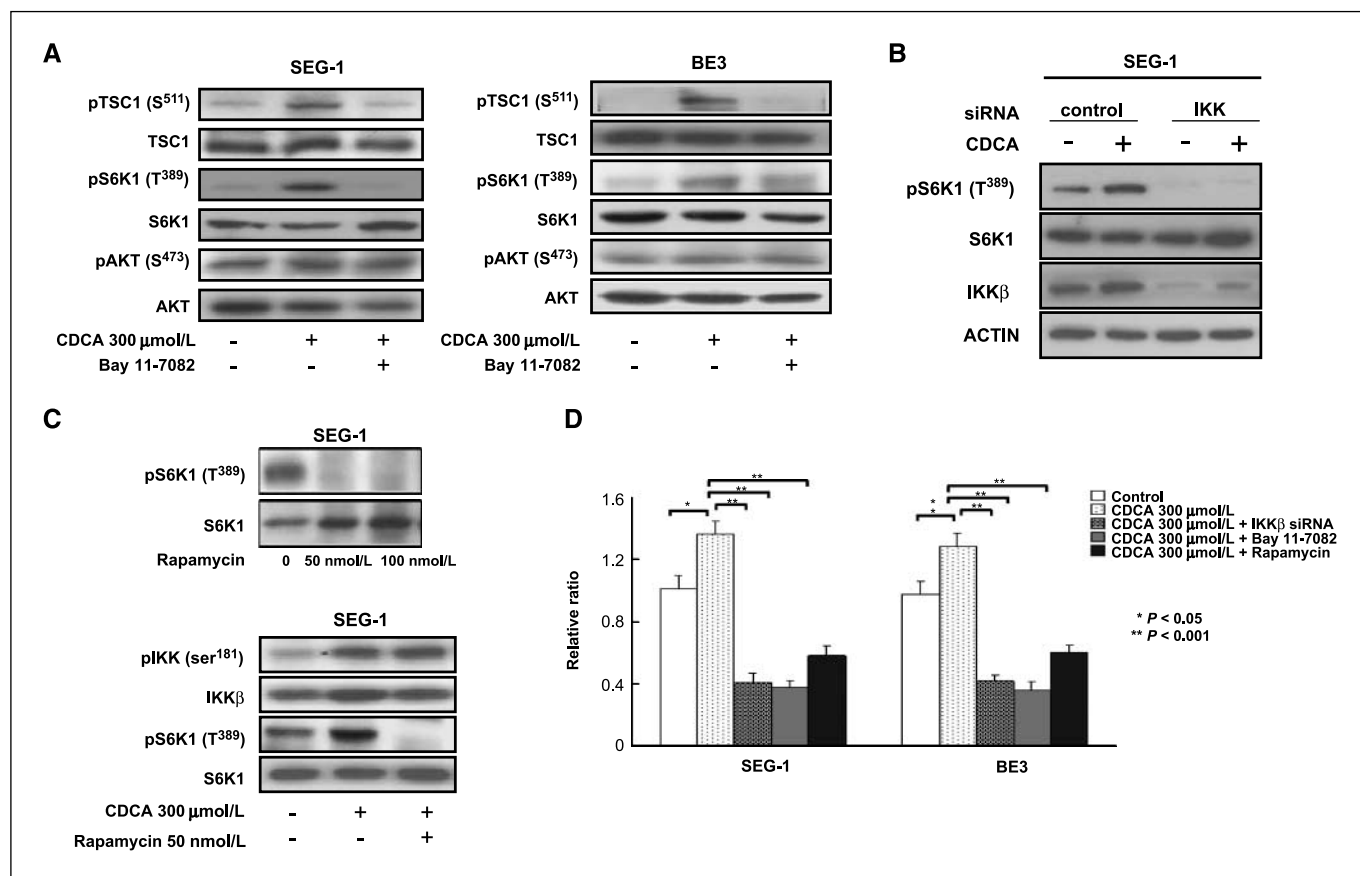


Figure 2. Bile acid stimulated the IKKβ/TSC1/mTOR pathway and increased cell proliferation, which was blocked by IKKβ and mTOR inhibitors. **A**, CDCA induced pTSC1 (S⁵¹¹) and pS6K1 (T³⁸⁹) in the SEG-1 and BE3 EAC cancer cell lines. The increased expression of pTSC1 (S⁵¹¹) and pS6K1 (T³⁸⁹) by CDCA were blocked by the IKKβ inhibitor Bay 11-7082 (40 µmol/L, 45 min pretreatment). CDCA did not activate Akt in SEG-1 and BE3 cancer cells. **B**, siRNA-mediated knockdown of IKKβ inhibited CDCA-induced pS6K1 (T³⁸⁹) in SEG-1 EAC cancer cells. **C**, CDCA exposure increased the expression of pS6K1 (T³⁸⁹) in SEG-1 EAC cancer cells, and rapamycin (50 nmol/L, 3 h pretreatment) blocked activation of the mTOR pathway. **D**, CDCA exposure enhanced SEG-1 and BE3 EAC cancer cell proliferation ($P < 0.05$). Knockdown of IKKβ by IKKβ siRNA inhibited CDCA stimulation effect ($P < 0.001$), and CDCA-induced cell proliferation was also blocked by the IKKβ inhibitor Bay 11-7082 and mTOR inhibitor rapamycin ($P < 0.001$).

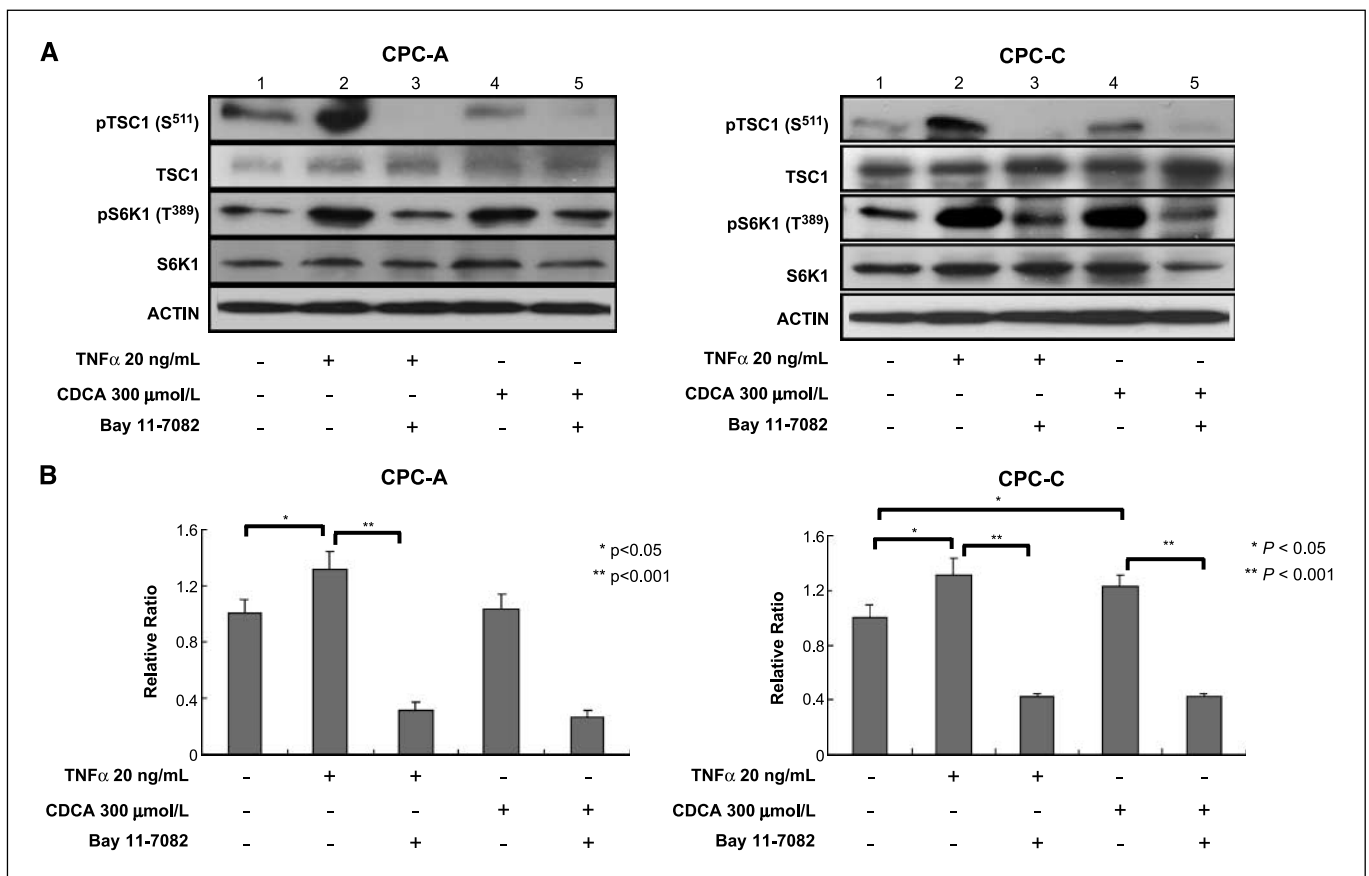


Figure 3. Bile acid and TNF α activated the IKK β /TSC1/mTOR pathway in Barrett's esophagus CPC-A and CPC-C cells. **A**, inflammatory cytokine TNF α induced expression of pTSC1 (S⁵¹¹) and pS6K1 (T³⁸⁹) in Barrett's esophagus CPC-A and CPC-C cells (lane 2). CDCA induced expression of pTSC1 (S⁵¹¹) and pS6K1 (T³⁸⁹) in CPC-C cells (lane 4). The stimulation effect of TNF α or CDCA was blocked by IKK β inhibitor Bay 11-7082 (lanes 3 and 5). Cells were serum-starved overnight and then treated with 20 ng/mL TNF α for 30 min or 40 μ mol/L Bay 11-7082 for 45 min. **B**, TNF α induced CPC-A and CPC-C cell proliferation ($P < 0.05$) and CDCA induced CPC-C cell proliferation ($P < 0.05$). Both TNF α -induced and CDCA-induced cell proliferation was blocked by IKK β inhibitor Bay 11-7082 ($P < 0.001$).

was incubated for 5 min to dissolve the formazan crystals. Light absorbance was measured at 570 nm using a multiwell scanner (Labsystems). To determine the number of colonies under anchorage-independent growth condition, we cultured EAC cells in soft agar medium after SEG-1 and BE3 EAC cells were treated with 300 μ mol/L CDCA for 16 h. In brief, aliquots of 2×10^4 cells were suspended in 1.5 mL of standard medium containing 0.3% agar at 43°C and layered on 1 mL of solidified 0.5% agar in the culture medium in 35-mm culture dishes. Dishes were incubated at 37°C in 5% CO₂ for 3 wk (38). Cells were fed with the culture medium, and viable colonies were stained with p-iodo-nitrotriazolium violet (Sigma). Each experiment was repeated thrice.

Rat model of Barrett's esophagus and adenocarcinoma. Six-week-old male Sprague-Dawley rats were purchased from Harlan and housed at two per cage under standard laboratory conditions. Rats were given commercial rat chow (Auto 5010 test diet; Purina Mills) before and up to 4 wk after surgical intervention. The rats were allowed to acclimate for 2 wk before surgery. Rat chow was withheld 24 h before surgical intervention, and liquids were stopped on the morning of surgery. The rats were anesthetized by isoflurane (99.9%, 0.5 mL/L volume), followed by an i.m. injection of xylazine hydrochloride (12 mg/kg) and ketamine (75 mg/kg) as maintenance anesthesia. Enteroesophageal reflux was induced by Levrat's esophagojejunostomy technique, as previously described (39).

After the reflux induction of esophageal cancer, the rats were euthanized by CO₂ narcosis. A midline incision was made from the laryngopharynx to the lower abdomen. The site of anastomosis was identified by the location of the polypropylene sutures, and the esophageal tissue was removed from the larynx to 2 mm above the anastomosis site. A longitudinal slice of

inflamed and tumorous esophageal tissue (<1-mm wide) was removed and stored at -70°C. The remaining esophageal tissue was fixed in 10% buffered formalin for 24 h and then transferred to 80% ethanol. The tissue was longitudinally divided into slices, with each slice representing the entire length and depth of the esophagus.

Protein expression. The expression of IKK β , pIKK β (S¹⁸¹), TSC1, pTSC1 (S⁵¹¹), S6K1, and pS6K1 (T³⁸⁹) was detected by Western blot analysis. The method of Western blot was described previously (40). In brief, Barrett's esophagus and EAC cells were plated in 10-cm dishes until they reached confluence. They were then harvested, washed in ice-cold PBS, and resuspended in whole-cell extraction solution [0.02 mol/L HEPES (pH 7.9), 0.005% NP40, 0.15% glycerol, 0.3 mol/L NaCl, 0.001 mol/L EDTA, and 0.01 mol/L NaF] with protease inhibitors. After 30-min incubation on ice, lysates were collected by centrifugation at 12,000 rpm for 10 min at 4°C. Protein concentrations were estimated using the Bradford method (Bio-Rad protein assay). Fifty micrograms of total protein were resolved by 6% to 10% SDS PAGE, transferred to polyvinylidene difluoride membranes (NEN Life Science), and probed with a specific antibody of the target protein. After binding with horseradish peroxidase coupled with secondary antibodies (Amersham Pharmacia), the immune complex was visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia) by exposure to X-ray films (Kodak).

Detection of pIKK β and pS6K1 protein expression by immunohistochemical staining in human EAC. Immunohistochemical staining for pIKK β and pS6K1 protein expression was performed on adjacent 4- μ m formalin-fixed paraffin-embedded tissue sections. One hundred twenty-three EAC, assembled in a tissue microarray, were examined. Adjacent BE

and normal esophageal epithelia were available in 20 of the 123 EAC cases and included in all 20 cases of BE metaplasia and 8 cases of BE high-grade dysplasia. Tissue specimens were obtained retrospectively from patients undergoing, as primary treatment, complete esophageal surgical resection at M. D. Anderson between January 1986 and December 1997. The Institutional Review Board approved this project. All specimens were reviewed by an experienced gastrointestinal pathologist (T.T. Wu). The tissue microarray contained BE premalignant (CPC-A, CPC-C) and EAC (SKGT-5, SKGT-5, BE-3, SEG-1, TT) cell lines as internal controls. Immunohistochemistry was performed as previously described using pIKK β (S¹⁸¹) or pS6K1 (T³⁸⁹) antibodies (41). Visualization was performed with aminomethylcarbazole chromogen. Only cytoplasmic immunoreactivity in cancer cells was considered positive for scoring purposes. Protein staining was evaluated on a semiquantitative scale combining staining intensity and percentage of positive cells in the cancer fields. Staining intensity was evaluated as follows: no/weak staining, negative; moderate or strong staining, positive. Percentage of positive cells was assessed on a four-point semiquantitative scale as follows: no positive cells, >0 to 10% positive cells, >10% to 25% positive cells, >25% to 50% positive cells, and >50% positive cells. Normal, BE, and cancer samples at $\geq 10\%$ of the scored cells were considered positive. Two investigators (J.G.I. and D.F.L.), independently, in a blind fashion, analyzed protein expression. Fisher's exact test and Spearman rank correlation tests were used for statistical analysis; $P < 0.05$ was considered statistically significant.

Results

IKK β /TSC1/mTOR pathway was activated in certain EAC cell lines but not in immortalized Barrett's esophagus cell lines. To determine whether the IKK β /TSC1/mTOR pathway may be involved in inflammation-associated Barrett's esophagus and EAC, immortalized Barrett's esophagus cell lines CPC-A and CPC-C and EAC cell lines SKGT-4, SEG-1, and BE3 were evaluated for the expression of pIKK β (S¹⁸¹), pTSC1 (S⁵¹¹), and mTOR downstream pS6K1 (T³⁸⁹). CPC-A and CPC-C cells had a lower expression level of pIKK β (S¹⁸¹) and expressed concurrently low levels of pTSC1 (S⁵¹¹) and pS6K1 (T³⁸⁹). The SKGT4 cancer cell line also had low expression of pIKK β (S¹⁸¹), pTSC1 (S⁵¹¹), and pS6K1 (T³⁸⁹). Expression of pIKK β (S¹⁸¹) was correlated with higher level of pTSC1 (S⁵¹¹) and pS6K1 (T³⁸⁹) in SEG-1 and BE3 EAC cancer cell lines. These phenomena were observed in two of three cancer cell lines, suggesting that the TSC1/mTOR pathway via IKK β signaling may be activated in certain inflammation-associated EAC (Fig. 1A).

Bile acid induced activation of IKK β /TSC1/mTOR pathway, leading to enhanced EAC cell proliferation. Because bile acid and a low pH environment have been thought to be important risk factors in the development of Barrett's esophagus and EAC, we determined whether CDCA or a low pH environment might activate the IKK β /TSC1/mTOR pathway in EAC cell lines and thus contribute to cell survival in the presence of bile acid. The relative phosphorylated TSC1 (S⁵¹¹) levels were increased 2.6-fold and concurrently pS6K1 (T³⁸⁹) levels were 2.8-fold higher under CDCA 300 μ mol/L stimulation in SEG-1 EAC cancer cells (Fig. 1B) and BE3 (data not shown) cells compared with those in CDCA untreated cells. CDCA is unconjugated bile acid, and to further prove that conjugated bile acid had the same stimulation effect in EAC cancer cells, we used the TCDA and treated the SEG-1 cancer cells. Interestingly, the TCDA induced the phosphorylation of TSC1 and S6K1 in SEG-1 cancer cells, and these inductions were suppressed by the IKK β inhibitor Bay 11-7082 (Fig. 1C). Accordingly, the cell growth rate was increased in both SEG-1 and BE3 cancer cells; however, the CDCA and acidic stimulation did not have an effect on the growth rate of SKGT4 cancer cells, in which the IKK β /TSC1/

mTOR pathway was not activated (Fig. 1D). Together, these results suggest that CDCA and acidic environment may stimulate the mTOR pathway and enhance cell growth in certain EAC cells.

Inhibition of IKK β and mTOR diminished bile acid-induced cell growth in EAC cells. To further evaluate whether a causal relationship exists between the induction of the IKK β /TSC1/mTOR pathway and an increased cell growth rate in response to bile acid, we used the IKK β inhibitor and IKK β siRNAs to block the IKK β kinase activity. The activation of pTSC1 (S⁵¹¹) and pS6K1 (T³⁸⁹) by bile acid was suppressed by the IKK β inhibitor Bay 11-7082

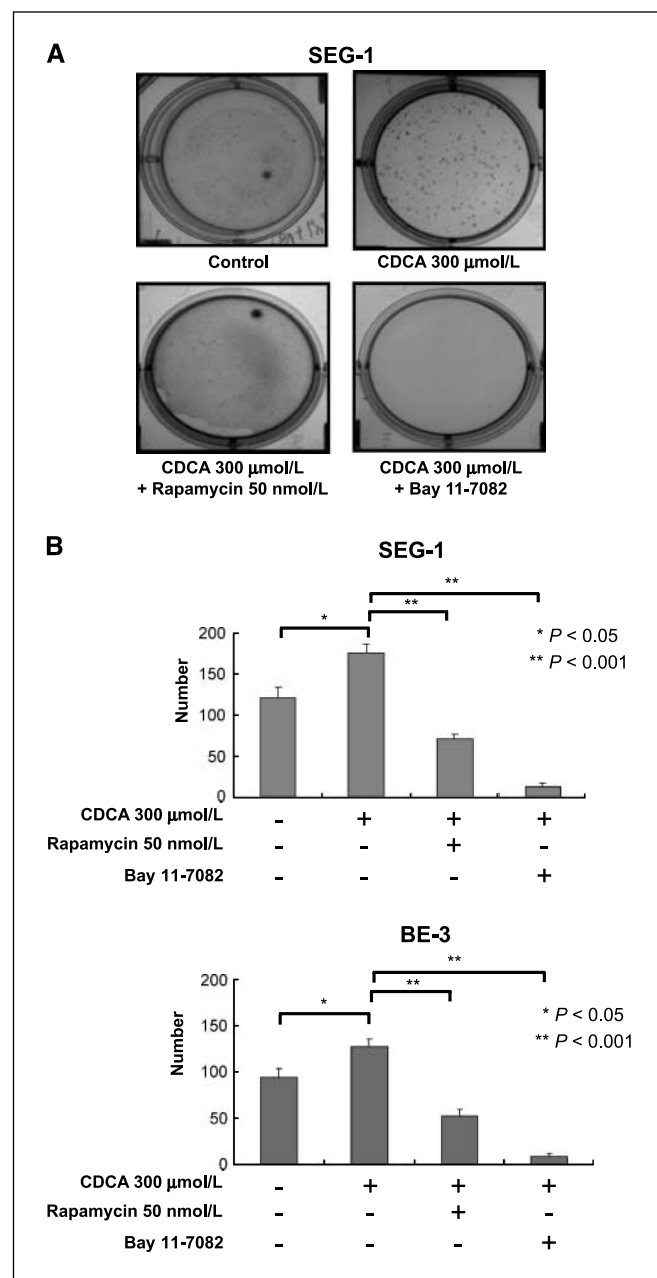


Figure 4. Bay 11-7082 and rapamycin suppressed bile acid-induced esophageal tumor anchorage-independent growth. **A**, CDCA enhanced the anchorage-independent growth abilities of SEG-1 EAC cancer cells in soft agar. **B**, quantitative result of **A**. CDCA-induced SEG-1 and BE3 EAC cancer cells' soft agar growth ability ($P < 0.05$). The enhanced anchorage-independent growth could be inhibited by Bay 11-7082 and rapamycin ($P < 0.001$).

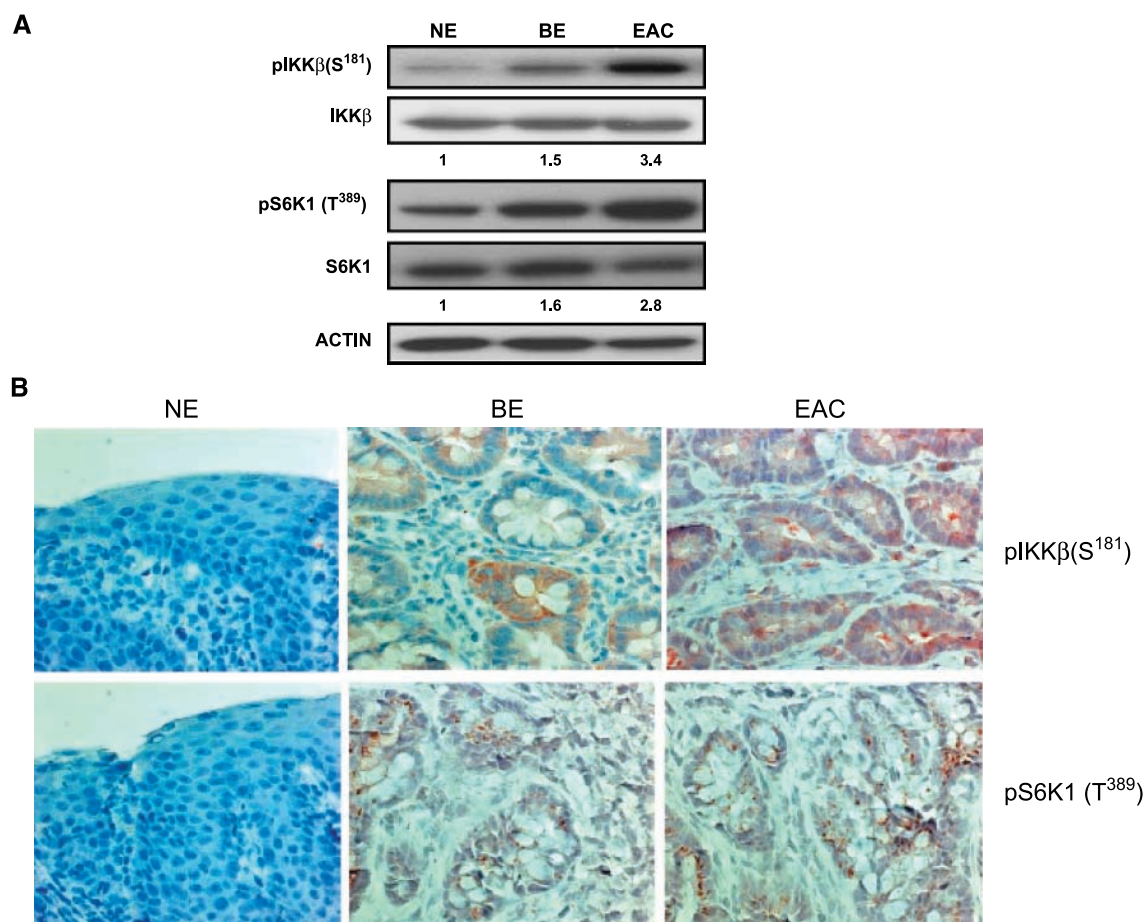


Figure 5. Activation of the IKK β /TSC1/mTOR pathway was observed in an orthotopic animal model. **A**, in rats, the relative expression density of pIKK β (S¹⁸¹) was 1.5 times higher in inflammatory BE and 3.4 times higher in EAC. The relative expression density of pS6K1 (T³⁸⁹) was 1.6 times higher in BE and 2.8 times higher in EAC compared with normal esophagus squamous epithelia (NE). **B**, immunohistochemical staining revealed the expression of pIKK β (S¹⁸¹) and pS6K1 (T³⁸⁹) in BE and EAC. pIKK β (S¹⁸¹) and pS6K1 (T³⁸⁹) staining in the adjacent NE was negative.

(40 μ mol/L, 45 min pretreatment) and IKK β siRNAs. CDCA did not activate AKT in SEG-1 and BE3 cancer cells (Fig. 2A and B). To ensure that the observed increase in S6K1 phosphorylation was caused by mTOR activity, we treated cells with rapamycin, a well-known mTOR inhibitor. CDCA exposure increased the level of pS6K1 (T³⁸⁹) in SEG-1 and BE3 cancer cells, and rapamycin (50 or 100 nmol/L, 3 h pretreatment) blocked activation of the mTOR pathway. Rapamycin inhibited both basal and CDCA-induced S6K1 phosphorylation (Fig. 2C). Consistently, both the IKK β and mTOR inhibitors (Bay 11-7082 and rapamycin), as well as IKK β siRNAs, suppressed the growth rates of the SEG-1 and BE3 cells (Fig. 2D). These results suggest that activation of the TSC1/mTOR pathway via IKK β signaling may be required for the proliferation of EAC cells in the presence of bile salts.

Bile acid and TNF α activated IKK β /TSC1/mTOR pathway in immortalized Barrett's esophagus CPC-A and CPC-C cells. Bile acid and cytokine TNF α , which exist in the inflammatory microenvironment, are major factors in the development of Barrett's esophagus. We hypothesized that CDCA and TNF α would activate the IKK β /TSC1/mTOR pathway in nontransformed Barrett's esophagus cell lines, resulting in enhanced cell proliferation. To test this hypothesis, we treated the Barrett's esophagus

cell lines CPC-A and CPC-C with CDCA or TNF α . We observed that TNF α increased phosphorylation of TSC1 and S6K1 in both CPC-A and CPC-C cells. The IKK β /TSC1/mTOR pathway can also be activated by bile acid CDCA in CPC-C cell line. Both CDCA-induced and TNF α -induced phosphorylation of TSC1 and S6K1 were blocked by the IKK β inhibitor Bay 11-7082 (Fig. 3A). Furthermore, the IKK β inhibitor Bay 11-7082 inhibited the TNF α -induced cell proliferation of CPC-A and CPC-C cells. The Bay 11-7082 also inhibited the CDCA-induced cell proliferation of CPC-C (Fig. 3B). These results show that bile acid and the inflammatory cytokine TNF α , both important risk factors for the progression of Barrett's esophagus, induce activation of the IKK β /TSC1/mTOR pathway and enhance the growth of epithelium cells in Barrett's esophagus.

Bile acid-induced esophageal anchorage-independent growth was suppressed by Bay 11-7082 and rapamycin. To evaluate the effect of bile acid on the transforming activity of EAC cells, we determined the anchorage-independent growth activity of SEG-1 and BE3 EAC cells, which is a transforming phenotype frequently associated with tumorigenic cancer cells. The activity level was increased in SEG-1 and BE3 EAC cancer cells after CDCA stimulation compared with that in control untreated cells ($P < 0.05$). This CDCA-enhanced SEG-1 and BE3 cancer cell

anchorage-independent growth was substantially blocked by treatment with Bay 11-7082 and rapamycin ($P < 0.001$; Fig. 4). These observations indicate that bile acid can increase the anchorage-independent growth activity of the esophageal cancer cells, and this stimulation effect may be due to activation of the IKK β /TSC1/mTOR pathway.

IKK β signaling led to activation of the TSC1/mTOR pathway in an orthotopic animal model. To further understand the contribution of the TSC1/mTOR pathway via IKK β signaling in the progression of Barrett's esophagus to EAC, we used an orthotopic animal model (39). Because intestinal bile acid reflux plays an important role in the development of Barrett's esophagus and EAC, we exposed the esophagus of the rat to intestinal bile acid by surgical anastomosis of the esophagus and intestines. After several months, esophageal inflammation and tumor developed, mimicking Barrett's esophagus and EAC in humans. Of interest, the relative expression density of pIKK β (S¹⁸¹) was 1.5 times higher in inflammatory BE and 3.4 times higher in EAC compared with normal epithelium (NE). The relative expression densities of pS6K1 (T³⁸⁹) was 1.6 times higher in BE and 2.8 times higher in EAC compared with NE (Fig. 5A). Immunohistochemical staining revealed the expression of pIKK β (S¹⁸¹) and pS6K1 (T³⁸⁹) in three of five rats with enteric bile acid reflux-related BE and EAC.

The adjacent normal esophagus squamous epithelia (NE) stained pIKK β (S¹⁸¹) and pS6K1 (T³⁸⁹) negative (Fig. 5B).

Thus, the animal model supported the role of the IKK β /mTOR pathway that was identified in cell line studies.

Immunohistochemical staining revealed a correlation between pIKK β and pS6K1 in Barrett's esophagus and EAC human tissue samples. To further determine the importance of the TSC1/mTOR pathway via IKK β signaling in Barrett's esophagus-associated EAC, we evaluated a tissue microarray of 123 EAC tissue specimens. Expression of both proteins was found in most EAC cases: 73 out of 98 scorable cases (74.5%) for pIKK β and 57 of the 96 scorable cases (59.3%) for pS6K1 were positive. As shown in Fig. 6B, the degree of positivity was heterogeneous between cases. In 90 cases, concomitant scoring of both proteins was possible and revealed a strong correlation between positive pIKK β pS6K1 expression [of the 90 specimens, 45 (50%) showed both pIKK β and pS6K1 positive, 15 (16.6%) showed both protein negative, 21 (23.3%) harbored only pIKK β positive, and 9 (10%) had only pS6K1 positive; $P = 0.016$; Fig. 6]. We also examined normal esophageal mucosa and BE lesions (20 metaplasia and 8 high-grade dysplasia) adjacent to resected EAC in 20 of the 90 cases for which both protein were scorable. We found that, whereas normal esophageal squamous epithelium did not show positivity for neither proteins, 3 (15%) BE

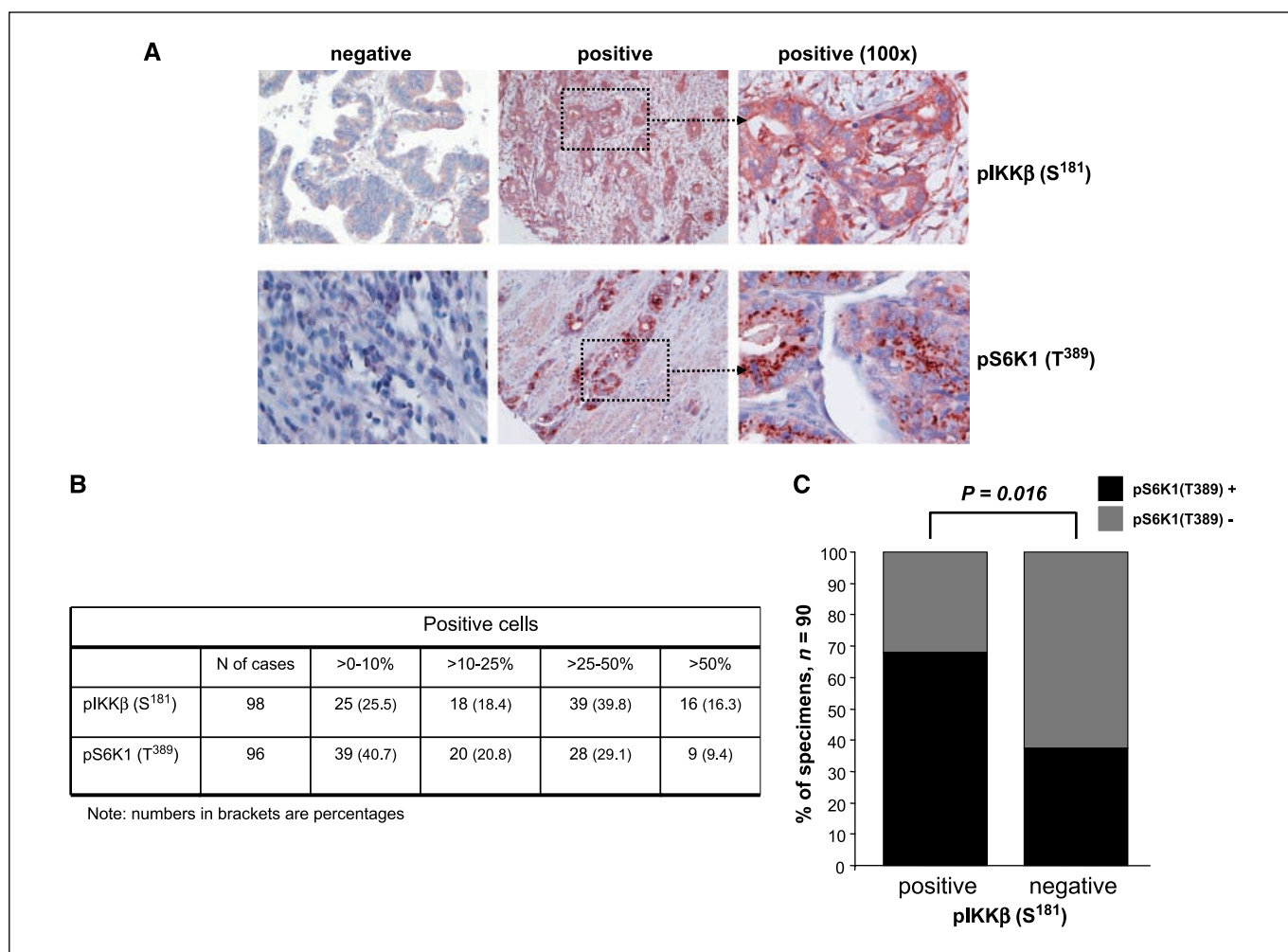


Figure 6. pIKK β (S¹⁸¹) and pS6K1 (T³⁸⁹) protein expression in 123 EAC human specimens. A, examples of immunohistochemical visualization of both proteins. B, degree of positivity for pIKK β (S¹⁸¹) and pS6K1 (T³⁸⁹) in EAC tissues. C, correlation between pIKK β (S¹⁸¹) and pS6K1 (T³⁸⁹) positive expression.

metaplasia and 5 (62.5%) BE high-grade dysplasia showed positivity for both proteins. Interestingly, in all BE-positive cases, the paired EAC showed also positivity for pIKK β and pS6K1. These results suggest that activation of IKK β /mTOR pathway plays a role in EAC development.

Discussion

Although the close relationship between Barrett's esophagus and EAC has been well established, the pathogenic mechanism responsible for the transformation of Barrett's esophagus to EAC is still far from understood, especially in view of the contribution of reflux substance, bile acid and gastric acid to cancer development and progression (42). Therefore, we undertook an investigation of the biological effects of bile acid and acidic condition on the Barrett's esophagus and EAC cells. We found that both bile acid (CDCA) and a low pH environment, the major content of gastro-esophageal reflux, triggered the activation of IKK β , leading to phosphorylation of TSC1 and S6K1 and activation of the mTOR pathway. The IKK β /TSC1/mTOR pathway may be a molecular switch, allowing Barrett's esophagus to progress to cancer. This is likely to be clinically relevant to pathogenesis as we found a statistically significant correlation between the phosphorylation of IKK β and phosphorylation of S6K1 in Barrett's esophagus-associated EAC.

Chronic exposure to bile acid and gastric acid can cause an inflammation process in the lower esophageal epithelium, resulting in Barrett's esophagus, which may subsequently evolve into EAC (23, 43). Bile acids are one of the major components of the gastro-esophageal refluxate and cause esophageal epithelium damage in Barrett's esophagus, which is highly associated with EAC (12, 44). However, the molecular mechanism by which bile acid exposure is involved in the esophageal epithelium inflammatory process and promotes malignant transformation in Barrett's esophagus is poorly understood. The results shown in this study from cell lines support the notion that CDCA may activate IKK β /mTOR signaling in certain types of EAC cancer cells, such as SEG-1 and BE3 cells, thereby increasing cell proliferation rate and transforming phenotype associated with tumorigenic potential. In Barrett's esophagus cell lines CPC-A and CPC-C, the inflammatory cytokine TNF α and CDCA also resulted in up-regulation of the IKK β /TSC1/mTOR pathway and in turn enhanced proliferation of Barrett's related cells; some of the surviving nontransformed Barrett's related cells may eventually develop into EAC. We validated our *in vitro* data using an *in vivo* bile salt reflux rat model, which promoted Barrett's esophagus and EAC. We showed expression of both pIKK β and pS6K1 in bile acid-induced Barrett's esophagus epithelial cells and EAC. Additionally, we further showed expression of both proteins in human tissues derived from Barrett's esophagus and EAC. These results suggest that deregulation of mTOR signaling in Barrett's esophagus cells may promote a stepwise progression from inflammatory epithelium to cancer development. It is worthwhile to

emphasize that both the animal model and human tumor tissues further supported the molecular mechanism, namely IKK β /mTOR pathway that was established from the cell line studies.

The mTOR signaling has been established in the formation of a number of cancers, including lung cancer (45), breast cancer (46), renal cell carcinoma (47), non-Hodgkin's lymphoma (48), and malignant melanoma (49), and activation of mTOR by IKK β has just recently been reported in breast cancer (34). The current study further links mTOR activation through the bile acid stimulation of the IKK β /TSC1 signaling in Barrett's esophagus-associated EAC. EAC is currently the cancer with the fastest rate of incidence in the United States (2). Currently available treatments for EAC patients, including chemotherapy and radiotherapy, are ineffective, resulting in a 5-year survival rate of <20% (2, 3). We investigated the mechanism by which bile acid stimulates the expression of IKK β , a downstream molecule of the TNF α signaling pathway, and further activates the TSC1/mTOR pathway. Activation of mTOR stimulates translation of ribosomal proteins via the activation of S6K1, which may be involved in the neoplastic process. Rapamycin can bind to FKBP12 and the complex blocks the mTOR activity very selectively. Bay 11-7082 has also been reported to effectively inhibit IKK β activity (50). Treatment of the esophageal cancer cells with rapamycin and Bay 11-7082 can effectively block the CDCA-induced cell proliferation and cell anchorage-independent growth ability. These findings suggest that incorporating targeted inhibition of the IKK β /TSC1/mTOR pathway into conventional chemoradiotherapy regimens might improve the EAC treatment outcome.

In conclusion, the results from this study clearly show that bile acid can activate mTOR activity, as measured by S6K1 through IKK β signaling, in Barrett's esophagus and EAC. Activation of IKK β /mTOR pathway enhances cell growth and may initiate the development of the EAC. Furthermore, blocking IKK β or mTOR signaling with Bay 11-7082 or rapamycin can interrupt the EAC growth and transformation. The results further suggest that Bay 11-7082 and rapamycin may be useful as chemopreventive drugs in the treatment of Barrett's esophagus-associated EAC.

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