

Inhibition of nuclear factor- κ B activity by small interfering RNA in esophageal squamous cell carcinoma cell lines

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Abstract. Chemotherapy with 5-fluorouracil (5-FU) is commonly used in combination therapy for esophageal squamous cell carcinoma (ESCC), but its efficacy is limited in certain patients. Recent studies suggest that constitutive activation of nuclear factor- κ B (NF- κ B) has a critical role in tumorigenesis and is associated with poor prognosis and resistance to chemoradiation therapy in many types of human cancers. In the present study, we evaluated the effect of small interfering RNA targeting NF- κ B (NF- κ B siRNA) combined with 5-FU on the proliferation of two cell lines of cultured ESCCs. Immunofluorescence and immunoblot analyses revealed that the NF- κ B protein was localized mostly in the cytoplasm of ESCCs. When cultured ESCCs were exposed to tumor necrosis factor- α , NF- κ B was transferred to the nucleus and activated. ESCCs with activated NF- κ B had poor sensitivity to 5-FU. When cells were transfected with NF- κ B siRNA, the levels of NF- κ B protein were significantly decreased in the cytoplasm and the nucleus. Transcriptional activity of NF- κ B was significantly suppressed in cells treated with 5-FU and NF- κ B siRNA compared to cells treated with 5-FU alone. 5-FU consistently suppressed proliferation of ESCCs in a dose-dependent manner, and this effect was significantly enhanced when combined with NF- κ B siRNA. These results suggest that combination therapy of 5-FU with

NF- κ B siRNA may provide a new therapeutic option for ESCC.

Introduction

Esophageal squamous cell carcinoma (ESCC) remains a disease with a poor prognosis. The overall 5-year survival rate is approximately 50% (1), a figure that has recently been improved by surgical developments and perioperative management, chemotherapy and radiation therapy. 5-Fluorouracil (5-FU) is commonly used in combination therapy for esophageal cancer. It is converted to the active metabolite 5-fluorouridine monophosphate (FUMP); FUMP incorporates into RNA and inhibits RNA processing, thereby inhibiting cell growth. 5-FU is also metabolized to 5-fluoro-2'-deoxyuridine-5'-O-monophosphate (FdUMP); FdUMP inhibits thymidylate synthase, resulting in thymidine triphosphate depletion. This causes DNA damage during DNA synthesis, resulting in the induction of apoptosis (2,3). However, the sensitivity to 5-FU is different in each patient, and many esophageal cancers are inherently or developmentally resistant to chemotherapy with 5-FU. Thus, effective methods to suppress this resistance are being explored.

Nuclear factor- κ B (NF- κ B) belongs to a family of inducible transcription factors that regulate the gene expression involved in immune and inflammatory responses. NF- κ B is also involved in cell proliferation and anti-apoptotic pathways (4-6). NF- κ B is expressed in the cytoplasm of unstimulated cells and is comprised of a heterotrimer of p65, p50 and an inhibitor protein, I κ B. When cells are treated with certain stimuli, such as cytokines, NF- κ B is activated by phosphorylation and ubiquitination of I κ B. The degradation of I κ B results in the translocation of the NF- κ B heterodimer (p65-p50) from the cytoplasm to the nucleus, where it activates the expression of specific genes (4,6,7). Several lines of evidence show that the activation of NF- κ B is also related to inducible chemoresistance (8). Therefore, regulation of NF- κ B expression is thought to be extremely important for enhancing the efficacy of chemotherapeutic agents in the treatment of cancer patients.

Previously, we reported that NF- κ B siRNA with chemotherapy successfully prolongs survival in a xenograft model with peritoneal metastasis of gastric cancer (9). This result prompted us to test whether NF- κ B siRNA administration is

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Abbreviations: ESCC, esophageal squamous cell carcinoma; 5-FU, 5-fluorouracil; NF- κ B, nuclear factor- κ B; siRNA, small interfering RNA; TNF α , tumor necrosis factor α

Key words: esophageal squamous cell carcinoma, 5-fluorouracil, nuclear factor- κ B, siRNA

also effective for ESCC. In this study, we investigated the efficacy of 5-FU and NF- κ B siRNA on two ESCC cell lines and the effect of tumor necrosis factor- α (TNF α) on NF- κ B.

Materials and methods

Cell culture. The human esophageal cancer cell lines, TE4 and TE8, were obtained from Riken BioResource Center (National Bio-Resource Project of the MEXT, Tsukuba, Japan). They were routinely cultured in RPMI-1640 (Wako, Tokyo, Japan) supplemented with 10% FBS (Hyclone Lab, Waltham, MA, USA) at 37°C in a 5% CO₂ atmosphere. Histology of TE4 was that of a highly differentiated squamous cell carcinoma, and that of TE8 was a moderately differentiated squamous cell carcinoma. 5-FU (Wako) treatment was carried out by replacing the culture medium with medium containing indicated concentrations of 5-FU, and cells were incubated for 24 h. For TNF α treatment (Wako), cells were cultured in the medium with TNF α (0.05 μ g/ml) for 12 h.

Antibodies and siRNA. Rabbit polyclonal antibodies against NF- κ B p65 (sc-109) and β -tubulin (sc-9104) and NF- κ B siRNA (sc-29410) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibody to lamin A/C was purchased from Cell Signaling Technology (Danvers, MA, USA).

siRNA transfection. Transfection of siRNA was performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, cells were plated in a 35-mm dish to achieve a confluency of 30–50% in media containing 10% FBS and a mixture of NF- κ B p65 siRNA (sc-29410, Santa Cruz) and Lipofectamine RNAiMAX. After incubation for 24 h, cells were analyzed or collected for the following assays.

Immunofluorescence staining. All procedures were carried out at room temperature. Cells on coverslips were fixed with 3.7% formalin in phosphate-buffered saline (PBS) for 30 min, permeabilized with 0.1% Triton X-100 in PBS for 15 min and blocked with 1% bovine serum albumin (BSA) in PBS for 1 h. Cells were then incubated with the anti-NF- κ B antibody (diluted to 1:100 in 0.1% BSA in PBS) for 1 h, followed by incubation with Alexa Fluor 488-conjugated anti-rabbit IgG (Invitrogen) for 1 h. Fluorescence images were obtained using confocal microscopy (Leica TCS-SP2, Wetzlar, Germany).

Western blot analysis. Cytoplasmic and the nuclear lysates were separately extracted using a ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, Darmstadt, Germany), and protein concentrations were determined with a Protein Assay Rapid Kit (Wako). Cell lysates (20 μ g of cytoplasmic and 40 μ g of nucleic extracts) were separated by SDS-PAGE using 10% gels and transferred onto PVDF membranes. The membranes were blocked overnight at 4°C in Tris-buffered saline (TBS) containing 0.1% Tween-20 and skimmed-milk and then incubated for 1 h with primary antibodies at the following dilutions: rabbit-anti-NF- κ B (1:1000; Santa Cruz), rabbit-anti-tubulin (1:1000; Santa Cruz), mouse anti-lamin A/C (1:2000; Cell Signaling Technology, Japan). Membranes

were then incubated with HRP-conjugated anti-rabbit or anti-mouse IgG (1:5000; GE Healthcare, UK) for 1 h. The antibody-antigen complex was detected using Amersham ECL plus reagents (GE Healthcare, UK), and images were obtained with an LAS-4000 IR image analyzer (Fujifilm, Japan).

BrdU assay. Cell proliferation was measured using a BrdU cell proliferation assay, an immunoassay for the quantitation of bromodeoxyuridine incorporation (Calbiochem). Briefly, cells in 96-well plates were incubated with BrdU for 5 h. Cells that had incorporated BrdU were detected by incubation with anti-BrdU antibody and HRP-conjugated anti-mouse IgG. Absorbance was measured with a spectrophotometric plate reader Sunrise (Tecan Japan, Tokyo, Japan) at dual wavelengths of 450 and 540 nm.

Apoptosis assay. Apoptosis of cultured cells was measured by detecting caspase activity using the carboxyfluorescein FLICA apoptosis detection kit (Immunochemistry Technologies, Bloomington, MN, USA). Cells in 24-well culture plates were treated with or without 5-FU or NF- κ B siRNA overnight. After labeling with FLICA, cells were measured using a fluorescence plate reader Infinite F500 (Tecan Japan). Fluorescence of apoptotic cells induced by staurosporine was used as a positive control.

Luciferase assay. Transcriptional activity of NF- κ B was measured with a Ready-To-Glow Secreted Luciferase Reporter Assay Kit (Clontech, Mountain View, CA, USA). Briefly, cells in 6-well culture plates were transfected with the pNF- κ B-MetLuc2 reporter plasmid, which contains the NF- κ B binding site in the promoter region of the Metridia luciferase reporter gene. After incubation for 24 h, the activity of luciferase in the cell culture medium was measured as a representation of NF- κ B activity using a luminometer Infinite F500 (Tecan Japan) and was expressed in arbitrary units. pEGFP-N1 (Clontech) was used as an internal transfection control for each experiment.

Statistical analysis. Statistical analysis was performed using Student's t-test to compare control and test groups (Statview J version 5.0; SAS Institute, Cary, NC, USA). The results are expressed as mean \pm SD except as otherwise stated. $P < 0.05$ was considered statistically significant.

Results

NF- κ B siRNA efficiently suppresses NF- κ B expression in ESCC cells. We first examined the cellular localization of NF- κ B protein in TE4 and TE8 cells by immunofluorescence staining. Under normal culture conditions, NF- κ B was detected mostly in the cytoplasm with low levels in the nucleus in both cell lines. When cells were transiently transfected with NF- κ B siRNA, the level of NF- κ B in the cytoplasm was significantly decreased (Fig. 1A). These results were confirmed by Western blot analysis (Fig. 1B and C).

Effect of 5-FU and NF- κ B siRNA on ESCC cells. To assay the effect of 5-FU and NF- κ B siRNA on the proliferation of TE4

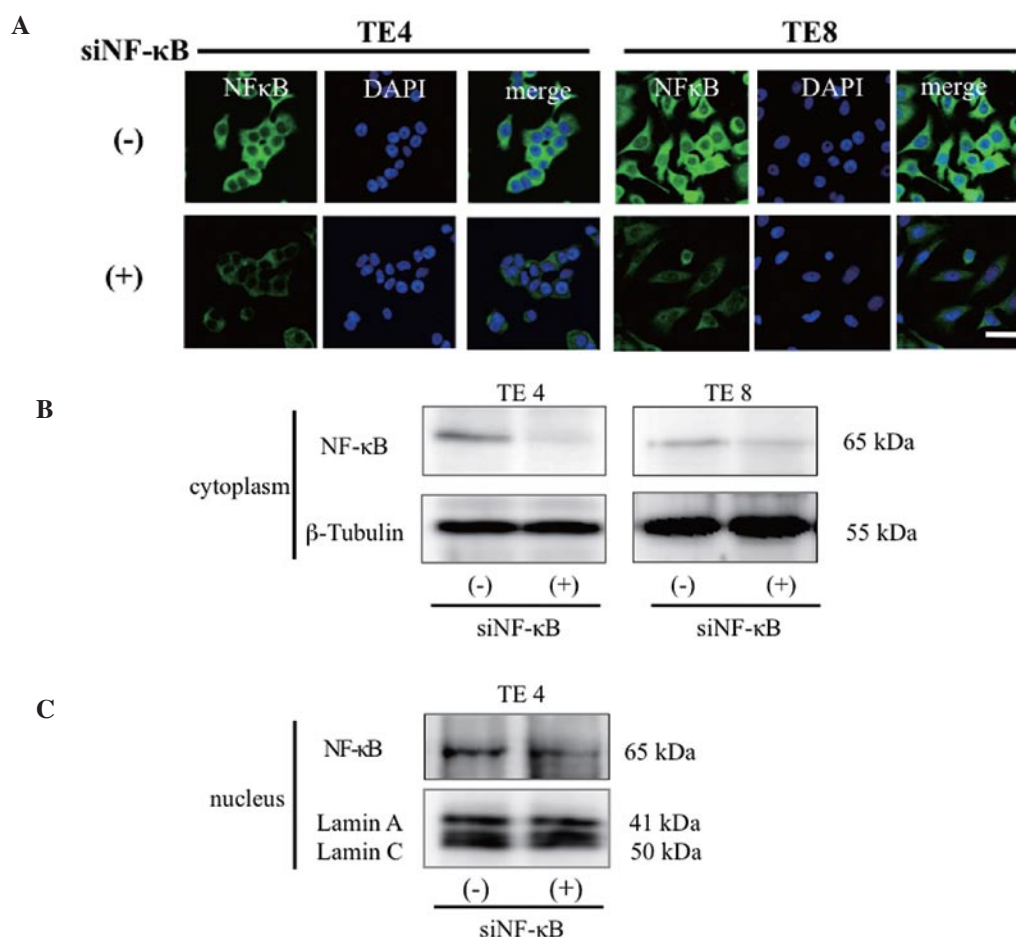


Figure 1. (A) NF-κB immunofluorescence staining of TE4 and TE8 cells treated with/without NF-κB siRNA (siNF-κB). Levels of NF-κB were decreased in TE4 and TE8 cells by NF-κB siRNA. (B) Western blot analysis of cytoplasmic NF-κB in TE4 and TE8 cells treated with NF-κB siRNA. Tubulin was used as the loading control. (C) Western blot analysis of nuclear NF-κB in TE4 and TE8 cells treated with NF-κB siRNA. Lamin A and C were used as the loading control. Nuclear NF-κB was not detected in the TE8 cells.

and TE8 cells, we performed the BrdU incorporation assay. Treatment with 5-FU caused a dose-dependent suppression of BrdU incorporation in the two ESCC lines. At the same concentration of 5-FU, TE4 and TE8 cells transfected with NF-κB siRNA had less BrdU incorporation compared to cells transfected with control siRNA (Fig. 2A).

We measured caspase levels to evaluate the effect of 5-FU and NF-κB siRNA on apoptosis of TE4 and TE8 cells. 5-FU induced apoptosis of TE4 and TE8 cells and NF-κB siRNA strongly induced apoptosis (Fig. 2B).

Effect of 5-FU and NF-κB siRNA on NF-κB transcriptional activity. We further examined the effects of 5-FU with NF-κB siRNA on the transcriptional activity of NF-κB in ESCC cells. To assess the transcriptional activity of NF-κB, we transfected ESCC cells with pNF-κB-MetLuc2 and then measured the luciferase activity in the culture medium, with reference to the luciferase activity of untreated cells. 5-FU reduced NF-κB activity in TE4 and TE8 cells, and the combination of 5-FU and NF-κB siRNA strongly suppressed NF-κB activity in both cell lines compared with 5-FU treatment only. Also the combination of 5-FU and NF-κB siRNA produced a difference in activity between TE4 and TE8 cells (Fig. 3).

Effect of TNFα on TE4 and TE8 cells. TE4 and TE8 cells were treated with TNFα. TNFα induced the accumulation of NF-κB in the nucleus. 5-FU slightly decreased the levels of NF-κB in cells treated with TNFα. A combination of 5-FU and NF-κB siRNA significantly decreased the levels of NF-κB in both the cytoplasm and nucleus of TNFα-treated cells (Fig. 4A).

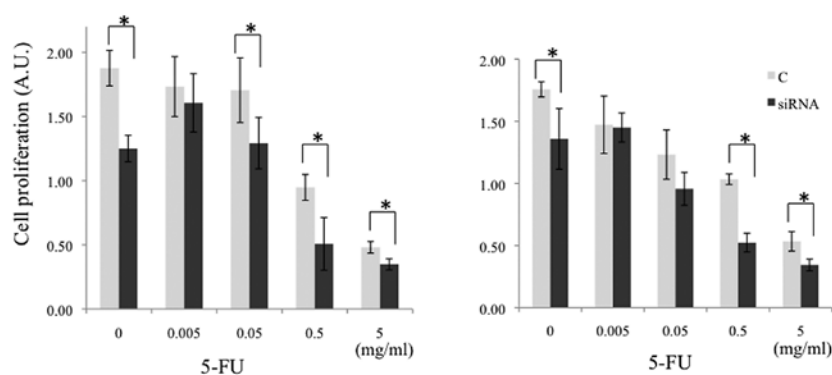
We measured the activity of NF-κB. TNFα strongly increased the activity of NF-κB. 5-FU only slightly suppressed NF-κB activity, but a combination of 5-FU and NF-κB siRNA strongly suppressed it. The level of response to TNFα in TE4 and TE8 cells was different (Fig. 4B).

TNFα decreased the effect of 5-FU on TE4 and TE8 cells. Adding NF-κB siRNA to TNFα- and 5-FU-treated TE4 and TE8 cells decreased the level of proliferation (Fig. 4C).

Discussion

Despite recent advances in surgical techniques and the combination of radiotherapy and chemotherapy treatments, the prognosis of many types of cancers remains poor due to advanced disease, metastasis and resistance to radiotherapy and chemotherapy (10). 5-FU is a commonly used treatment for ESCC and inhibits cell growth and induces apoptosis in

A



B

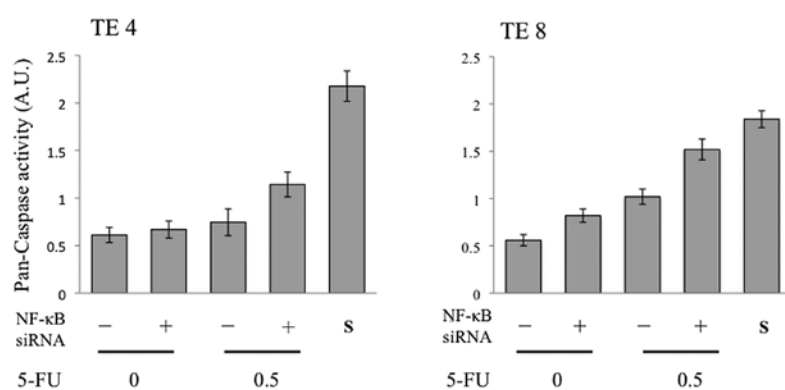


Figure 2. (A) Effect of NF- κ B siRNA with 5-FU on growth inhibition in TE4 and TE8 cells. Gray bar, untransfected cells; black bar, NF- κ B siRNA transfected cells. * $P < 0.05$ indicates that the viability of the transfected cells was significantly elevated compared to that of the untransfected cells. (B) Apoptosis assay showed that 5-FU and NF- κ B siRNA induced apoptosis of TE4 and TE8 cells. S, staurosporine was used as a positive control.

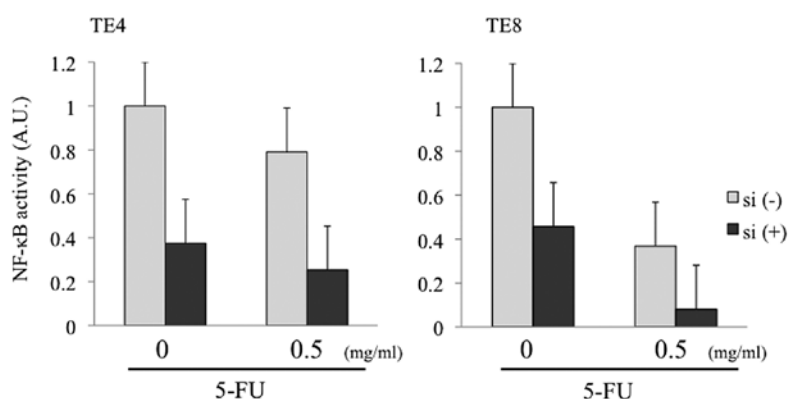


Figure 3. Luciferase assays were performed on TE4 and TE8 cells treated with 5-FU and NF- κ B siRNA. Activity of NF- κ B was inhibited by 5-FU and NF- κ B siRNA. Relative activities of NF- κ B are shown, with no treatment set at 1.0.

cancer cells (2). However, similar to other anticancer drugs, resistance is often observed (11). Moreover, the molecular mechanisms and the signaling pathways involved in ESCC chemoresistance are not yet clear.

Activation of NF- κ B signaling has a critical role in the development and progression of several types of cancer, including esophageal cancer (4). In our hospital, expression of NF- κ B was detected in 61.5% of patients with ESCC, whose prognosis was relatively poor compared with patients

who were negative for NF- κ B expression (date not shown). Therefore, we consider NF- κ B to be a key mediator of increased tumor chemoresistance. NF- κ B is a protein complex composed of multiple subunits, including p50, p52, RelA (p65), RelB and c-Rel. It is retained in an inactive form in the cytoplasm by its interaction with I κ B (4,12). Extrinsic stimuli such as TNF α cause phosphorylation of I κ B and its subsequent ubiquitination and degradation by the proteasome. This releases NF- κ B, which translocates to the nucleus (7).

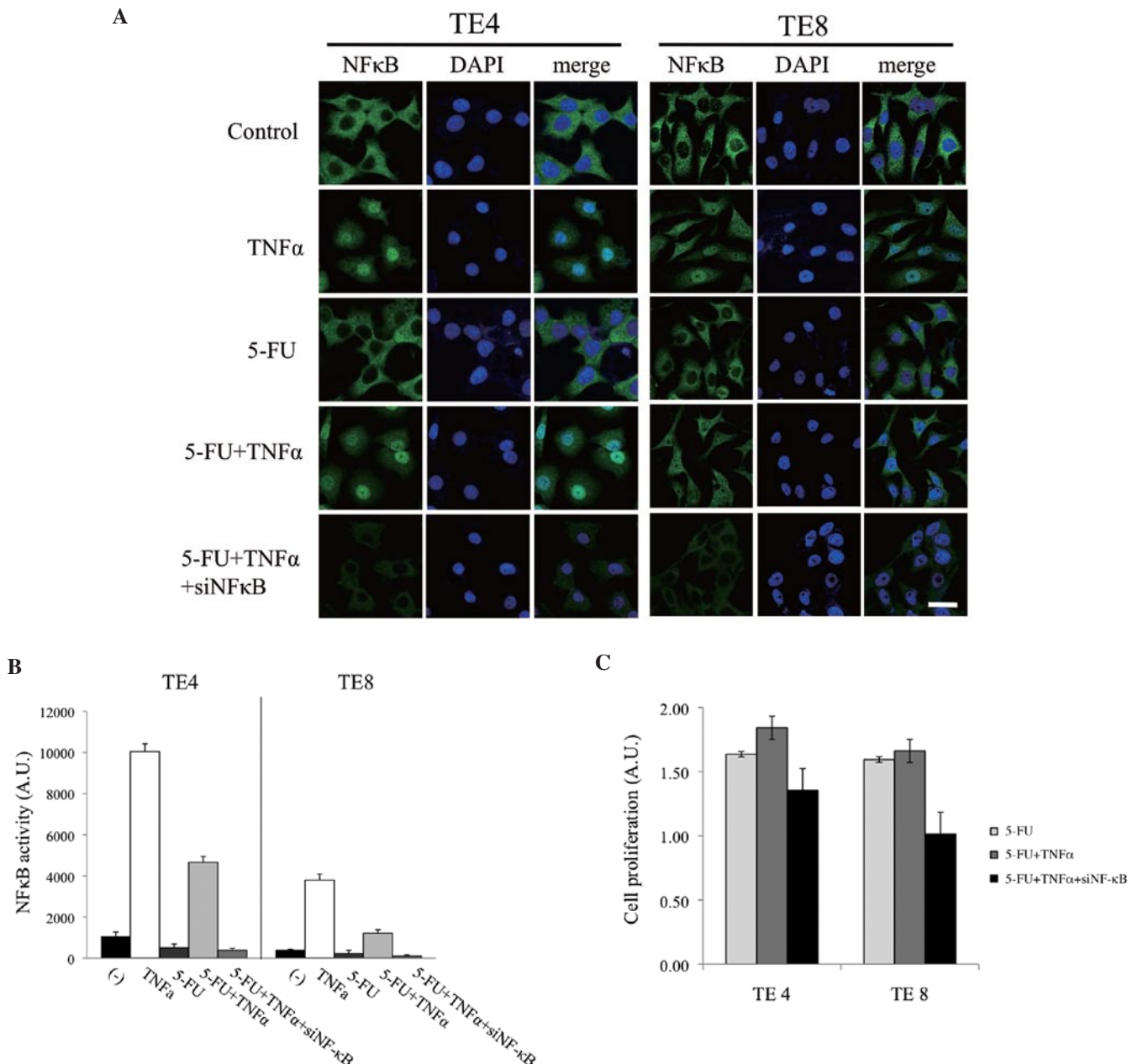


Figure 4. (A) Cells treated with 5-FU, TNF α and NF- κ B siRNA. TNF α induced NF- κ B to translocate to the nucleus. 5-FU slightly decreased the level of NF- κ B in cells treated with TNF α . A combination of 5-FU and TNF- α significantly decreased the levels of NF- κ B in the cytoplasm and nucleus. (B) NF- κ B activity in TE4 and TE8 cells was measured by luciferase assays. (C) Proliferation of TE4 and TE8 cells treated with 5-FU, TNF α and NF- κ B siRNA.

Several lines of evidence show that constitutive activation of NF- κ B induces expression of anti-apoptotic genes and drug resistance in cancer cells (7,12). Therefore, many strategies and compounds have recently been developed to target this pathway, including I κ B kinase inhibitors, proteasome inhibitors, acetylation inhibitors, gene transfer of antisense RNA and siRNA to inhibit proteins, anti-inflammatory agents and chemoprevention agents (9-11,13-18). However, whether the manipulation of NF- κ B activation is clinically significant remains unclear.

In this study, we showed that siRNA-mediated suppression of NF- κ B protein expression in cultured ESCCs resulted in an enhancement of the anti-proliferative effect of 5-FU. Li *et al* demonstrated that 5-FU or docetaxel induced NF- κ B

activation in human esophageal cancer cells, and pretreatment with an adenoviral-delivered I κ B suppressor followed by 5-FU or docetaxel significantly increased apoptosis (11).

Our present findings do not contradict these results, as high concentrations of 5-FU certainly inhibit both cell proliferation and NF- κ B activation, and inhibition of NF- κ B augments chemotherapeutic effects.

TNF α is a pro-inflammatory cytokine that regulates cell proliferation, differentiation and apoptosis. One of the most important downstream signaling targets activated by TNF α is NF- κ B (19-21). TNF α was found to be strongly expressed in an esophageal carcinoma cell line (22), and TNF α may have a critical role in ESCC. Immunostaining showed expression of NF- κ B in cultured ESCCs to be almost entirely located in

the cytoplasm, whereas that in resected ESCC specimens was in the nucleus (data not shown). When cultured ESCCs were exposed to TNF α , NF- κ B translocated to the nucleus, and the activity of NF- κ B increased. Esophageal cancer cells may be affected by cytokines such as TNF α or interleukin-6 from inflammatory cells in the body. 5-FU had a smaller effect on ESCCs that had elevated levels of TNF α -activated NF- κ B. Combination therapy of 5-FU and NF- κ B siRNA was effective in ESCCs, providing further evidence that NF- κ B is associated with the sensitivity of 5-FU.

A gene expression profiling study previously identified high expression levels of key regulatory genes in the NF- κ B pathway, including TRAF2, an inhibitor of NF- κ B kinase, and RalB in chemoresistant cancer (12). Moreover, curcumin inhibited NF- κ B activity and sensitized cancer cells to anti-proliferative agents, such as 5-FU or cisplatin (23). Our findings support further investigation of anti-NF- κ B strategies for the treatment of ESCC. Indeed, NF- κ B siRNA therapy may act cooperatively with chemotherapeutic agents. Moreover, NF- κ B inhibition may lower the effective therapeutic dose of 5-FU or of other antitumor agents, such that combination therapy may be a more effective treatment option for ESCCs than chemotherapy alone. The effect of NF- κ B siRNA may be more direct than that of inhibiting TNF α . Furthermore, since NF- κ B is overexpressed in many types of human cancer but present at only very low levels in normal tissues (24), inhibition of NF- κ B should have minimal side effects on normal tissues.

In conclusion, we demonstrated that NF- κ B activity was strongly inhibited by NF- κ B siRNA, resulting in suppression of ESCC proliferation. The effect of 5-FU on ESCCs was decreased by TNF α treatment, while the effect of 5-FU was significantly enhanced when combined with NF- κ B siRNA. Combination therapy of 5-FU and NF- κ B siRNA may provide a new therapeutic option for ESCCs.

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