

Curcumin Promotes Apoptosis, Increases Chemosensitivity, and Inhibits Nuclear Factor κ B in Esophageal Adenocarcinoma¹

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Abstract

The transcription factor, nuclear factor κ B (NF- κ B), plays a central role as a key mediator of cell survival and proliferation, and its activation may confer increased tumor chemoresistance. Curcumin, an orally available naturally occurring compound, has been shown to inhibit NF- κ B and has a potential role in cancer chemoprevention. We investigated the effects of curcumin on NF- κ B activity, on cell viability, and as a chemosensitizing agent with 5-fluorouracil (5-FU) or cisplatin (CDDP) in esophageal adenocarcinoma (EAC). Oligonucleotide microarray analysis of 46 cases, consisting of Barrett metaplasia, low-grade dysplasia, high-grade dysplasia and EAC, showed increased expression of NF- κ B and I κ B kinase subunits and decreased effector caspase expression in EAC compared with Barrett metaplasia. Stromal expression of both I κ B and phospho-I κ B was detected in several EAC samples by tissue microarray analysis. Curcumin alone inhibited NF- κ B activity and induced apoptosis in both Flo-1 and OE33 EAC cell lines as determined by Western blot analysis, NF- κ B reporter assays, and Caspase-Glo 3/7 assays. It also increased 5-FU- and CDDP-induced apoptosis in both cell lines. These data suggest that activation of NF- κ B and inhibition of apoptosis may play a role in the progression from Barrett metaplasia to EAC. In addition, curcumin, a well-known inhibitor of NF- κ B activity, was shown to increase apoptosis and enhance both 5-FU- and CDDP-mediated chemosensitivity, suggesting that it may have potential application in the therapy of patients with EAC.

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Introduction

The incidence of esophageal adenocarcinoma (EAC) has increased significantly, especially in western countries. Surveillance, Epidemiology, and End Results (SEER) registry data indicate a three- to four-fold increase in incidence during the past 30 years [1], with current estimates of approximately 7000 new cases per year in the United States alone. EAC is generally diagnosed at a late stage and has a poor prognosis, with a 5-year survival of less than 10%. Although the current treatment includes chemotherapy, radiation therapy, and, if possible, esophagogastric resection, many patients with EAC experience progression of disease despite such treatment, suggesting that such tumors are resistant to chemotherapy.

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Nuclear factor κ B (NF- κ B) is a transcription factor that is associated with tumorigenesis, and its increased activity has been associated with evasion of apoptosis, malignant transformation, sustained cell proliferation, metastasis, and angiogenesis [2]. NF- κ B is a protein complex composed of several subunits including p50, p52, RelA (p65), RelB, and c-Rel that dimerize, with the most common form being the p50/RelA heterodimer. Inactive NF- κ B is retained in the cytoplasm by its interaction with inhibitors of κ B (I κ B α , I κ B β , or I κ B ϵ) [3]. Activation of extrinsic pathway-mediated apoptosis is initiated by extracellular signaling such as that mediated by tumor necrosis factor- α (TNF α) [4]. Resultant phosphorylation of I κ B, its subsequent ubiquitination and proteasome-mediated degradation releases NF- κ B, which then translocates to the nucleus [2].

Activation of NF- κ B has been reported in several epithelial cancers, including breast [5–7], pancreas [8], oropharynx [9], lung [10], and esophagus [11]. Increased bile acid exposure and an acidic environment have been shown to induce NF- κ B in dysplastic Barrett esophagus, the precursor to EAC [12]. With its central role as a transcription factor in a number of malignancies, NF- κ B is a target for ongoing development of novel targeted pharmacotherapy.

Curcumin, a phytopolyphenolic pigment derived from turmeric (*Curcuma longa*), has been shown to have multiple anticancer effects, including inhibition of proliferation, induction of apoptosis, inhibition of angiogenesis, and inhibition of DNA topoisomerase II. A variety of mechanisms has been implicated as mediators of these effects. Specifically, inhibition of activator protein 1 (AP-1), c-Jun N-terminal kinase, Akt, and NF- κ B signaling pathways has been described [13,14]. Curcumin has been reported to inhibit NF- κ B activation by suppressing the I κ B kinases (IKKs), thereby resulting in decreased proliferation and increased apoptosis [15,16]. It seems to suppress constitutively active NF- κ B in a variety of cancers [17]. Although several clinical studies are in progress to evaluate the chemotherapeutic activity of curcumin in various cancers (www.cancer.gov/clinicaltrials, accessed November 21, 2009), none have investigated the effectiveness of curcumin in the treatment of EAC. Its pharmacology has been extensively studied, and data support the feasibility of evaluating the efficacy of its oral administration in further clinical trials for the treatment of gastrointestinal tract lesions [18].

We report in the present study that there is increased gene expression of NF- κ B and IKK subunits and decreased expression of apoptosis-effector genes in primary EAC samples compared with Barrett metaplasia. We demonstrate that curcumin inhibits NF- κ B activity and promotes apoptosis in EAC cell lines, as has been demonstrated in other types of epithelial malignancies [8,9,13,17]. We also show that curcumin can enhance the *in vitro* cytotoxicity of 5-fluorouracil (5-FU) and cisplatin (CDDP), two first-line chemotherapeutic agents used in the treatment of EAC.

Materials and Methods

Patients and Tissues

After obtaining informed consent, tissues were obtained from patients undergoing esophagectomy for adenocarcinoma at the University of Michigan Medical Center (Ann Arbor, MI) and transported to the laboratory in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) on ice. A portion of each sample was embedded in OCT compound (Miles, Inc, Elkhart, IN) and frozen in isopentane cooled in liquid nitrogen for cryostat sectioning. The remainder was

frozen in liquid nitrogen and stored at -80°C . Metaplastic or dysplastic mucosa and tumor samples with at least 70% cellularity were identified using hematoxylin and eosin-stained frozen sections, and 2-mm³ samples were obtained for RNA and protein isolation. The sections were then examined by two pathologists to confirm the histopathologic diagnosis of EAC, high-grade or low-grade dysplasia, Barrett metaplasia, or normal esophageal mucosa. None of the patients had received previous chemotherapy or radiotherapy.

Cell Lines

OE33 (European Collection of Cell Cultures, Sigma-Aldrich, St Louis, MO) and Flo-1 cell lines [19,20] were derived from EACs and grown in RPMI or Dulbecco's modified Eagle medium (Invitrogen), respectively, supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 1% penicillin/streptomycin/fungizone (Invitrogen) and incubated at 37°C in 5% CO₂/95% air.

Reagents

Curcumin was obtained from Sigma-Aldrich (catalog no. C7727), diluted in dimethyl sulfoxide (DMSO, catalog no. D4540; Sigma-Aldrich) to 100 mM and kept at -20°C . TNF α (catalog no. 2169; Cell Signaling, Danvers, MA) was used to stimulate NF- κ B. CDDP (catalog no. P4394; Sigma-Aldrich) and 5-FU (catalog no. F6627; Sigma-Aldrich) were used in chemosensitivity experiments.

Oligonucleotide Microarray

Oligonucleotide expression microarray analysis was performed as previously described [21]. Briefly, total RNA was isolated from 46 esophageal samples using TRIzol (Invitrogen) and purified with RNeasy spin columns (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA quality was assessed by 1% agarose gel electrophoresis and A_{260}/A_{280} spectrophotometer ratios. RNA quality was reassessed with Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA) at intermediate steps after double-stranded complementary DNA and RNA (cDNA and cRNA, respectively) syntheses. cDNA synthesis, cRNA amplification, hybridization, and washing of HG-U133A gene chips (Affymetrix, Santa Clara, CA) were performed by the University of Michigan Cancer Center Microarray Core according to the manufacturer's instructions.

To normalize microarray data, a summary statistic was calculated using 11 probe pairs for each gene and the robust multichip average method as implemented in the Affymetrix Library of Bioconductor (version 1.3, www.bioconductor.org), which provides adjustment, quantile normalization, and summarization. Expression values for each sample were then compared with the mean expression value for the seven Barrett metaplasia samples [22]. Hierarchical cluster analysis and gene expression "heat maps" were generated using the method of Eisen et al. [23].

Quantitative Reverse Transcription–Polymerase Chain Reaction

Total RNA from treated cells was isolated and column purified using the RNeasy Mini Kit (Qiagen) as per the manufacturer's instructions and was then reverse-transcribed with 250 μM dNTPs, 12.5 ng/ μl oligo(dT)_{12–18} primer, 75 ng/ μl random primers, 10 mM dithiothreitol, 1 U/ μl RNaseOUT, and 5 U/ μl SuperScript II reverse transcriptase (Invitrogen). Real-time polymerase chain reaction (PCR) amplification using 20 ng worth of total RNA, 1 \times Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), and 0.2 μM both forward

and reverse primers was performed on the Corbett Rotor-Gene 6000 (Qiagen). The following cycling parameters were used: 50°C hold for 2 minutes; 95°C hold for 2 minutes; 40 cycles of 95°C for 10 seconds, annealing for 15 seconds, and 72°C for 20 seconds. Significant differences of relative quantification were determined using the $2^{(-\Delta\Delta C(T))}$ method [24]. These data are represented as the fold increase in gene expression normalized to the endogenous reference gene, β_2 -microglobulin (*B2M*).

Primer sets (Invitrogen) were designed using DNASTAR software (Madison, WI).

Casp7: forward, 5'-TTCCGAAGCCTGGGTTTTGACG-3'; and reverse, 5'-GCCAAGCAGCGGCATTTGTA-3'

RelA: forward, 5'-GACCCCGGCCATGGACGAAC-3'; and reverse, 5'-CCGCTGCTTGGGCTGCTCA-3'.

B2M: forward, 5'-GCTGTGCTCGCGCTACTCTC-3'; and reverse, 5'-CAATGTCCGATGGATGAAACC-3'.

Immunohistochemistry and Tissue Microarray

The expression of IKK and Ser-32 phosphorylated IKK was determined using an esophageal tissue microarray (TMA) [25,26] from 73 patients including 64 tumor, 8 lymph node metastases, 8 dysplastic Barrett mucosa, and 11 nondysplastic Barrett metaplasia samples. Normal esophagus was also included. Immunohistochemical staining was performed on the DAKO Autostainer and the DAKO EnVision+ System, Peroxidase (DAKO, Carpinteria, CA). Microwave epitope retrieval in 1 mM EDTA (pH 8) was performed for 20 minutes. Dewaxed and rehydrated sections of the TMA at 4-mm thickness were labeled with I κ B (1:100, catalog no. 9242; Cell Signaling) or phosphorylated I κ B (Ser 32, 1:100, catalog no. 9241; Cell Signaling). Slides were lightly counterstained with hematoxylin.

TNF α -Mediated Stimulation of NF- κ B

To determine the optimal dosage for TNF α -mediated stimulation of NF- κ B, Flo-1 and OE33 cells were treated with TNF α at 0.1, 0.5, 1, 5, 7.5, and 10 ng/ml for 5, 15, 30, 60, 120, 240, and 480 minutes.

Cellular Proliferation and Chemosensitivity Assay

Cellular proliferation was determined by WST-1 assay (Roche Diagnostics, Indianapolis, IN), in accordance with the manufacturer's directions, with varying concentrations of curcumin, 5-FU, or CDDP.

Western Blot Analysis for Apoptosis and NF- κ B Activity

Cells were plated at a density of 1.4×10^6 cells/60-mm plate and allowed to adhere for 24 hours. Cells were treated with curcumin at 6.25, 12.5, 25, 50, or 100 μ M for 48 hours. Untreated and DMSO (100 μ M)-treated cells were used as controls. In addition, cells observed for NF- κ B activity were treated with curcumin for 48 hours at varying doses and then stimulated by TNF α at 10 ng/ml for 5 minutes. In separate experiments, Flo-1 and OE33 cell lines were treated with combinations of curcumin and either 5-FU or CDDP.

Total cellular protein was extracted in lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 2.5 mM Na₄P₂O₇, 1 mM β -glycerol phosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, and 1% Triton X-100) supplemented with additional protease inhibitor cocktail (catalog no. P8340; Sigma-Aldrich). Lysates (40 μ g) were separated by electrophoresis in an 8% to 16% gradient Tris-glycine gel (Invitrogen), then transferred to Immobilon-P nylon

membranes (Millipore, Bedford, MA). Membranes were blocked with 5% evaporated milk in 1 \times TBS/0.1% Tween 20 (TBST) for 1 hour at room temperature.

Primary antibodies against polyadenosine-5'-diphosphate-ribose polymerase 1 (PARP) (1:1000, catalog no. 9542; Cell Signaling), I κ B α (1:1000, catalog no. 4812; Cell Signaling), phospho-I κ B α (1:1000, catalog no. 9246; Cell Signaling), or β -actin (1:10,000, catalog no. ab6276; Abcam, Inc, Cambridge, MA) were incubated with membranes overnight at 4°C. Antirabbit HRP-conjugated secondary antibodies (1:5000, catalog no. PI-1000, Vector; Burlingame, CA) or antimouse HRP-conjugated secondary antibodies (1:5000, catalog no. 1010-05; Southern Biotech; Birmingham, AL) in 5% evaporated milk/TBST were incubated with membranes for 1 hour at room temperature. Membranes were washed five times for 5 minutes in TBST after each antibody incubation. Protein bands were visualized using the Pierce ECL kit (Thermo Fisher Scientific, Rockford, IL).

Apoptosis Assay

Cells were plated in white-walled 96-well tissue culture plates at a density of 5×10^3 cells per well in 100 μ l of medium and allowed to adhere for 24 hours. Cells were treated in triplicate for 8 to 48 hours with curcumin at 12.5, 25, and 50 μ M with DMSO as the vehicle control. In separate experiments, Flo-1 and OE33 cell lines were treated with a combination of curcumin and either 5-FU or CDDP. Caspase-3 and -7 activity was determined at 8, 24, and 48 hours using the Caspase-Glo 3/7 assay (catalog no. G8091; Promega, Madison, WI) as directed by the manufacturer. Briefly, Caspase-Glo 3/7 reagent was added to each well in a 1:1 ratio and incubated for 30 minutes before measuring luminescence as relative light units (RLUs) using a Xenogen IVIS 100 luminometer (Xenogen, Corp, Alameda, CA). Caspase activity was normalized to the cell number using the WST-1 reagent in side-by-side assays performed under the same plating density and conditions.

NF- κ B Luciferase Reporter Assay

Flo-1 and OE33 cells were plated at a density of 125,000 cells/well and transfected with NF- κ B-Luc reporter plasmid pNifty-Luc (InvivoGen, San Diego, CA) using FuGENE 6 (Roche Diagnostics) at a reagent-plasmid ratio of 16 μ l:4 μ g in accordance with the manufacturer's directions. Twenty-four hours after transfection, cells were treated as described above with curcumin 10 to 100 μ M for 48 hours and 10 ng/ml of TNF α for 5 minutes. At the end of treatment, 1 μ l of 40 mg/ml of D-luciferin (Promega) was added to cells, and bioluminescence was monitored using the Xenogen IVIS 100. A grayscale image was collected followed by luminescence acquisition, which was overlaid as a pseudocolor image representing the spatial distribution of the detected photons emitted from cells. This analysis was performed using Living Image software (Xenogen). Signal intensity was quantified as the sum of all detected photon counts within a uniform region of interest manually placed over the wells during data postprocessing. The total numbers of cells in each well were counted, and NF- κ B activity was normalized to cell count before the fold increase was determined.

Statistical Analysis

Data points are reported as experimental averages and error bars represent SD. Comparisons between experimental groups used the two-sided Student's *t* test, with *P* < .05 considered statistically significant.

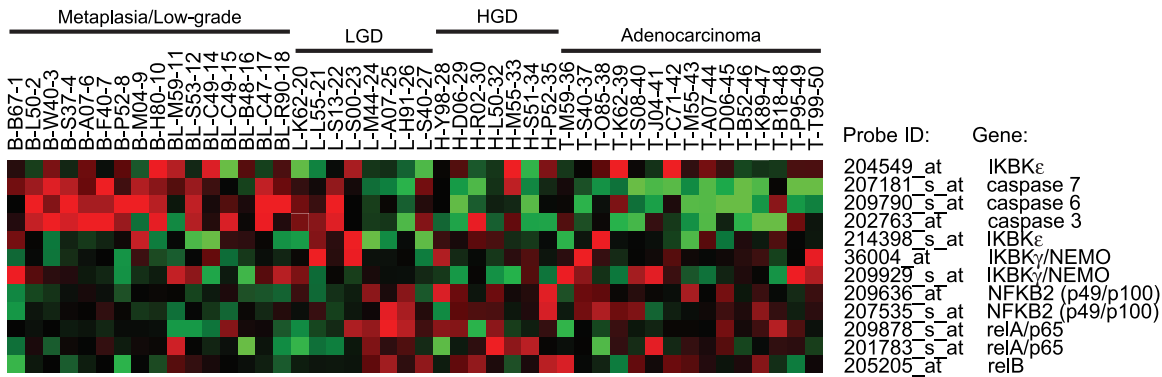
Results

NF-κB Subunit Gene Expression Increases and Apoptosis-Related Gene Expression Decreases in EAC Compared with Barrett Metaplasia

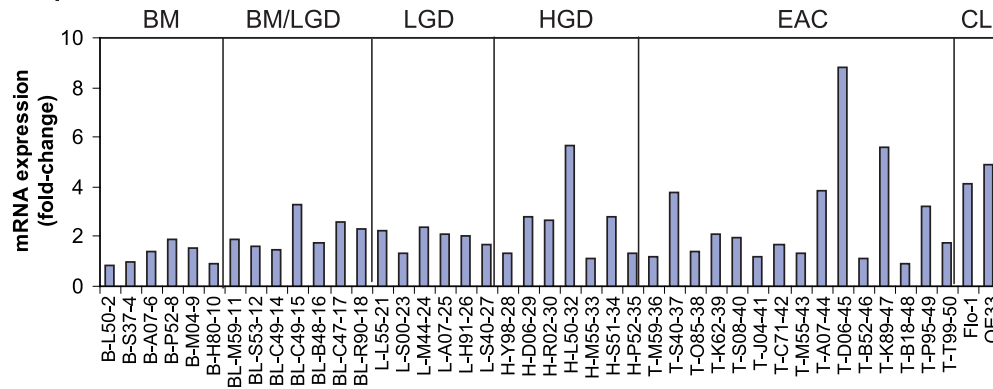
Oligonucleotide expression microarrays were used to evaluate a cohort of 46 esophageal tissue samples and revealed a 1.5-fold increase

or higher in *REL* (33%), *RELA* (40%), *RELB* (40%), and *NFκB2* (47%) messenger RNA (mRNA) expression in EAC compared with Barrett metaplasia ($P < .05$; Figure 1A). In addition, we observed over-expression of IKK β, γ, and ε subunits, components of the upstream NF-κB activation pathway [27,28], with 9% to 21% greater mean expression in adenocarcinoma compared with metaplastic Barrett esophagus ($P < .01$). The oligonucleotide microarray analysis also revealed a

A. Affymetrix HG-U133A oligonucleotide microarray



B. qRT-PCR *RELA*



C. qRT-PCR *CASP7*

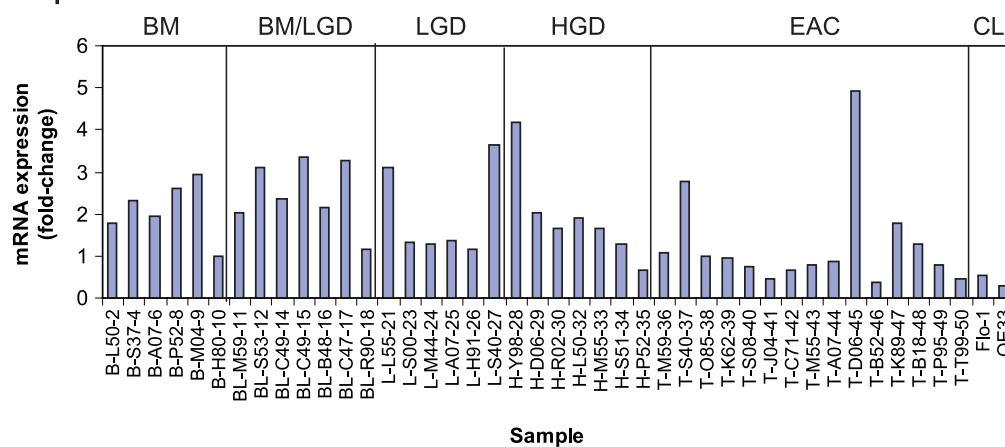


Figure 1. *NFκB* (*relA*, *relB*, and *NFκB2*), *IκB* kinase (γ , ϵ), and effector-caspase (3, 6, 7) mRNA expression in the progression from meta-plastic Barrett esophagus to dysplasia and EAC. (A) HG-U133A (Affymetrix) oligonucleotide microarray analysis of primary tissue samples from patients with Barrett metaplasia (Barrett, $n = 9$), low-grade dysplasia (LGD, $n = 15$), high-grade dysplasia (HGD, $n = 7$), and EAC (EAC, $n = 15$). Individual samples are labeled by tissue type (B, BL, L, H, or T), subject identifier code, and sample number for the purpose of cross-comparison. Gene expression is labeled as increased (red) or decreased (green). Quantitative real-time PCR analysis of *RELA* (B) and *CASP7* (C) expression in a panel of primary tissue samples from the oligonucleotide microarray analysis compared with normal intestinal epithelium. Significant differences of relative quantification were determined using the $2^{(-\Delta\Delta C(T))}$ method.

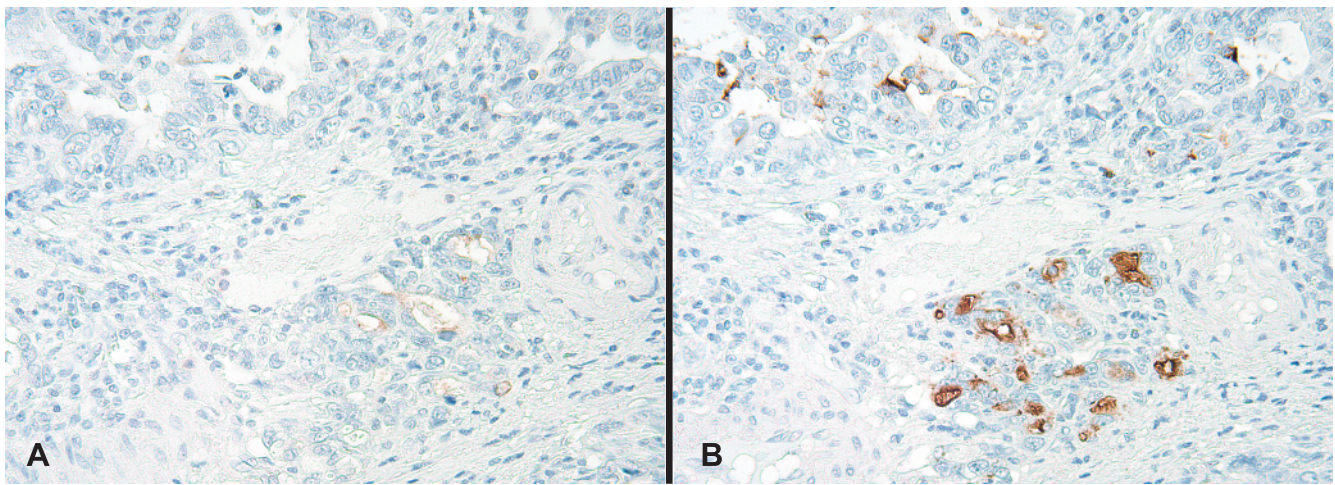


Figure 2. Immunohistochemical analysis of (A) I κ B and (B) phospho (Ser-32)-I κ B in EAC tissues. Paraffin-fixed tissue cores were prepared in a TMA. Representative sections from subject T-M59-36 (original magnification, $\times 200$) are displayed and indicate focal expression in tumor glands. This subject also had increased mRNA expression of *IKK γ* and *relB* (Figure 1A), consistent with increased NF- κ B signaling.

two-fold or more decrease in *CASP3* (30%), *CASP6* (47%), and *CASP7* (60%) mRNA expression in EAC compared with Barrett metaplasia ($P < .05$; Figure 1A). Reverse transcription–polymerase chain reaction analysis of *RELA* and *CASP7* confirmed the overall ex-

pression patterns observed in the oligonucleotide microarray data set. In addition, the EAC cell lines demonstrated similar expression patterns for *RELA* (Figure 1B) and *CASP7* (Figure 1C) when compared with Barrett metaplasia. To validate that NF κ B is activated in EAC, we

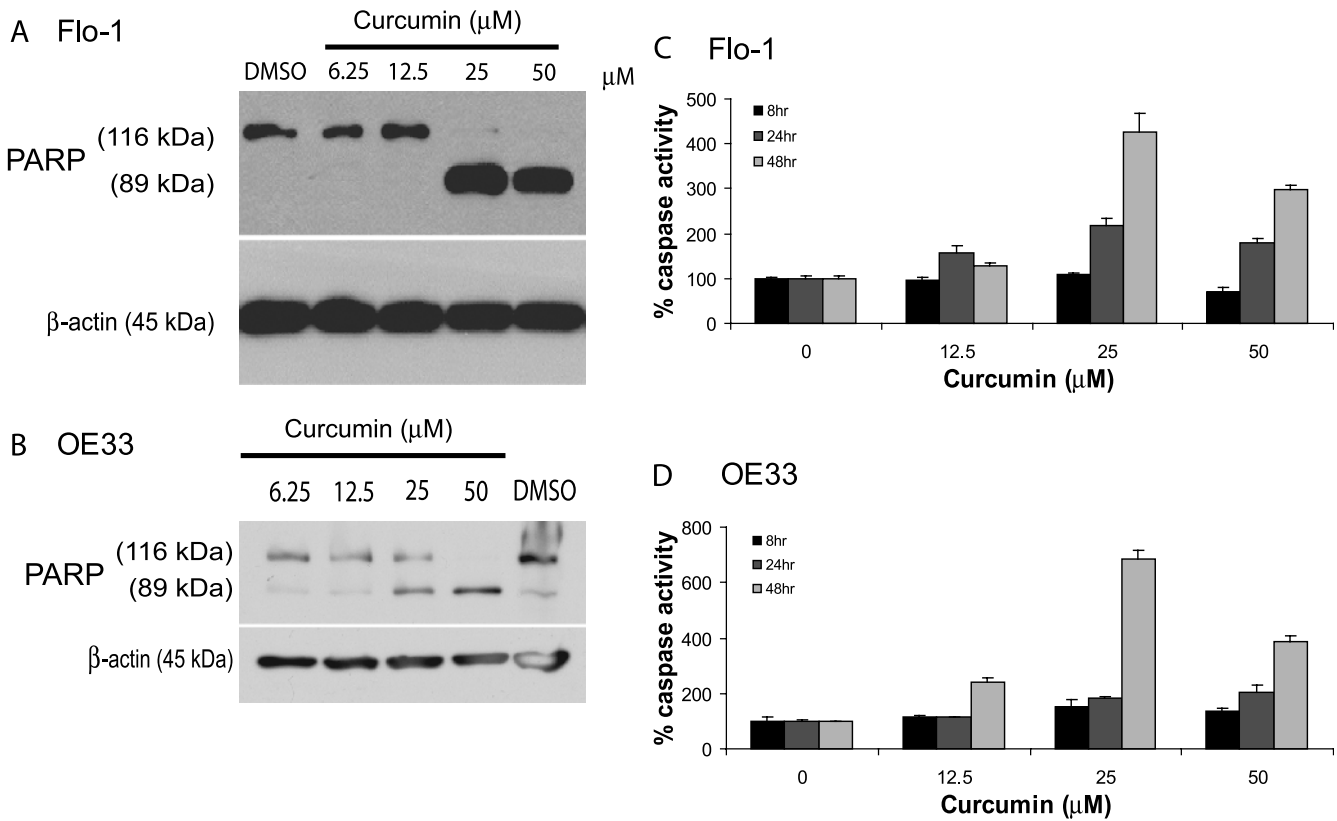


Figure 3. Curcumin dose–response and time course to determine the levels of apoptosis in the EAC cell lines Flo-1 and OE33. Western blot analysis of PARP cleavage (89-kDa subunit) after a 48-hour treatment with increasing doses of curcumin in both (A) Flo-1 and (B) OE33 cells. Levels of caspase 3/7 activity as determined by Caspase–Glo 3/7 luminescent-based assays after curcumin treatment at the indicated doses and with increasing duration of incubation in (C) Flo-1 and (D) OE33 cells. Caspase 3/7 activity was normalized to cell number as determined by WST-1 proliferation assay. These numbers were then compared with the level of vehicle control at each time point and expressed as percentage caspase activity (mean % \pm SD). Each data point represented three individual wells. Independently repeated experiments were performed to ensure reproducibility. DMSO was used as the vehicle control in all experiments.

performed immunohistochemical analysis of a TMA composed of tissue samples of normal, dysplastic, and EAC for the expression of I κ B and its phosphorylated product, P-I κ B. Stromal expression of I κ B was observed in 9 (12.3%) of 73 patients, and P-I κ B expression was observed in 5 (6.8%) of 73 patients. Representative photomicrographs from one subject (T-M59-36) indicate focal expression of both I κ B and P-I κ B in tumor glands (Figure 2), indicating activation of NF- κ B in this patient's adenocarcinoma tissue sample.

Curcumin Promotes Apoptosis in EAC Cell Lines

To determine whether caspase-dependent apoptotic activity could be modulated by curcumin, EAC cell lines Flo-1 and OE33 were treated with increasing doses of curcumin for 48 hours. Levels of apoptosis, as determined by immunoblot analysis of PARP expression and cleavage [29,30], were increased in a dose-dependent fashion up to 50 μ M curcumin for both Flo-1 (Figure 3A) and OE33 (Figure 3B). We confirmed this dose-dependent apoptotic response by luminescent-based caspase 3/7 assays, which showed more than a four-fold and six-fold increase in caspase 3/7 activity with a 48-hour treatment with 25 μ M curcumin in Flo-1 (Figure 3C) and OE33 (Figure 3D; $P < .05$) cells, respectively.

Curcumin Inhibits TNF α -Mediated NF- κ B Activation in EAC Cell Lines

To determine whether the inhibitory effects of curcumin could be mediated through the NF- κ B pathway, we examined I κ B α and phospho-I κ B α expression levels and NF- κ B luciferase reporter assay activity after curcumin treatment in the setting of TNF α stimulation. A 5-minute incubation with 10 ng/ml TNF α induced maximal NF- κ B activity as demonstrated by I κ B α phosphorylation (data not shown). Curcumin inhibited TNF α -mediated phosphorylation of I κ B at 25 μ M in Flo-1 and at 50 μ M in OE33 cell lines, respectively (Figure 4, A and B). Curcumin inhibited NF- κ B reporter gene activity in a dose-dependent fashion in both Flo-1 (90% at 25 μ M) and OE33 (50% at 50 μ M) cell lines ($P < .05$; Figure 4, C and D). In Flo-1 cells, maximal caspase 3/7 activity and PARP cleavage was detected with 25 μ M curcumin and correlated with almost complete inhibition of NF- κ B activity. The curcumin dose required for 50% inhibition (45 μ M) in OE33 cells was higher than in Flo-1 cells, and doses as high as 100 μ M were required for 90% inhibition. Curcumin at 25 μ M did not inhibit NF- κ B activity, yet it induced the maximum caspase 3/7 activity, suggesting that this induction of apoptosis was not through NF- κ B modulation.

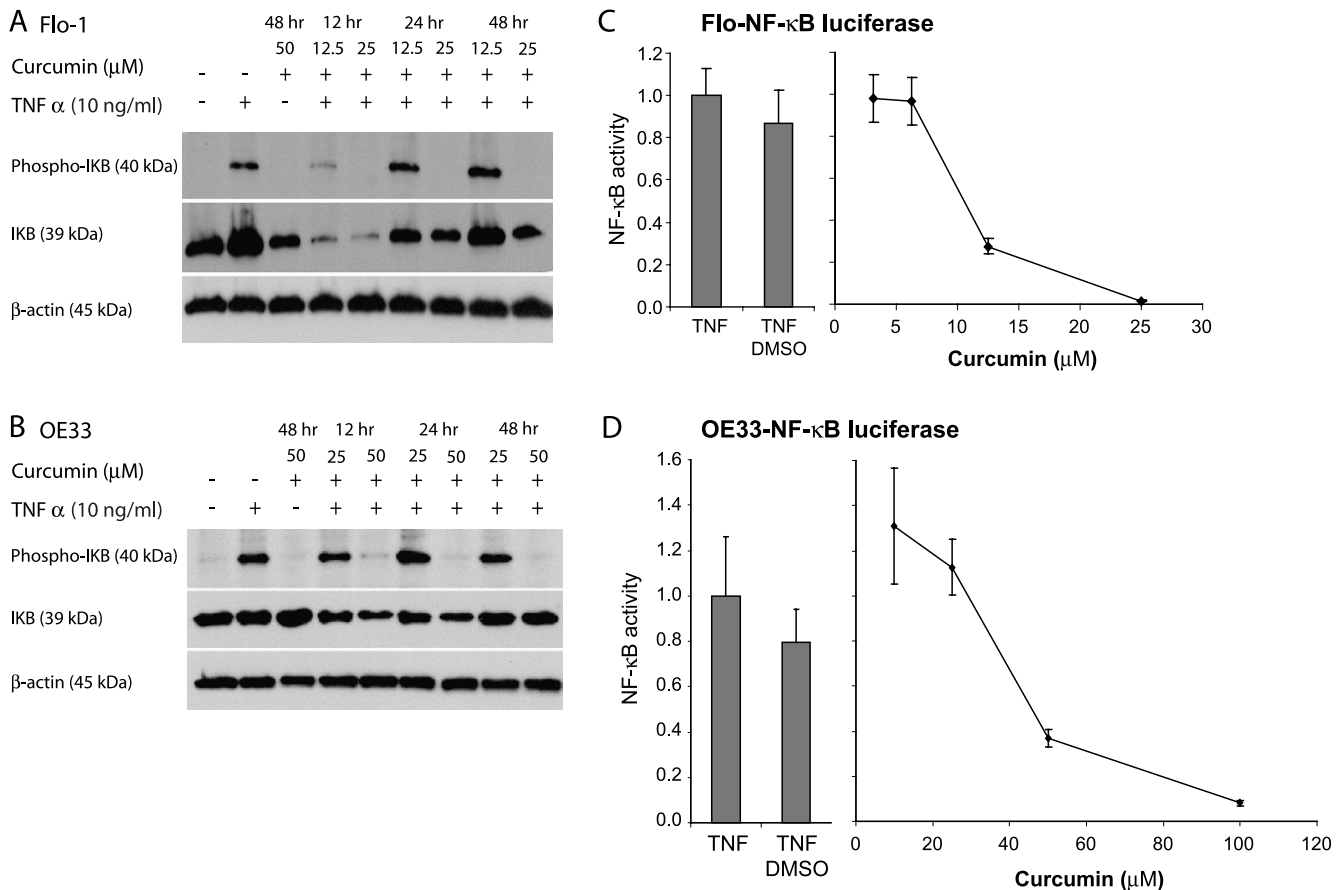


Figure 4. Inhibition of TNF α -induced activation (5 minutes) of NF- κ B by curcumin. Flo-1 and OE33 EAC cells were treated with increasing doses of curcumin that were administered for 12, 24, and 48 hours. TNF α was then added to the medium for 5 minutes followed by protein isolation for immunoblot analysis of I κ B and phospho-I κ B in both (A) Flo-1 and (B) OE33 cells. Actin was used as a loading control. A TNF α -inducible NF- κ B-luciferase reporter was transfected into (C) Flo-1 and (D) OE33 cell lines. After treatments with increasing doses of curcumin for 48 hours and TNF α for 5 minutes, D-luciferin was added, and bioluminescence was monitored. Luciferase reporter experiments were performed in triplicate and expressed as mean expression \pm SD relative to TNF α stimulation alone.

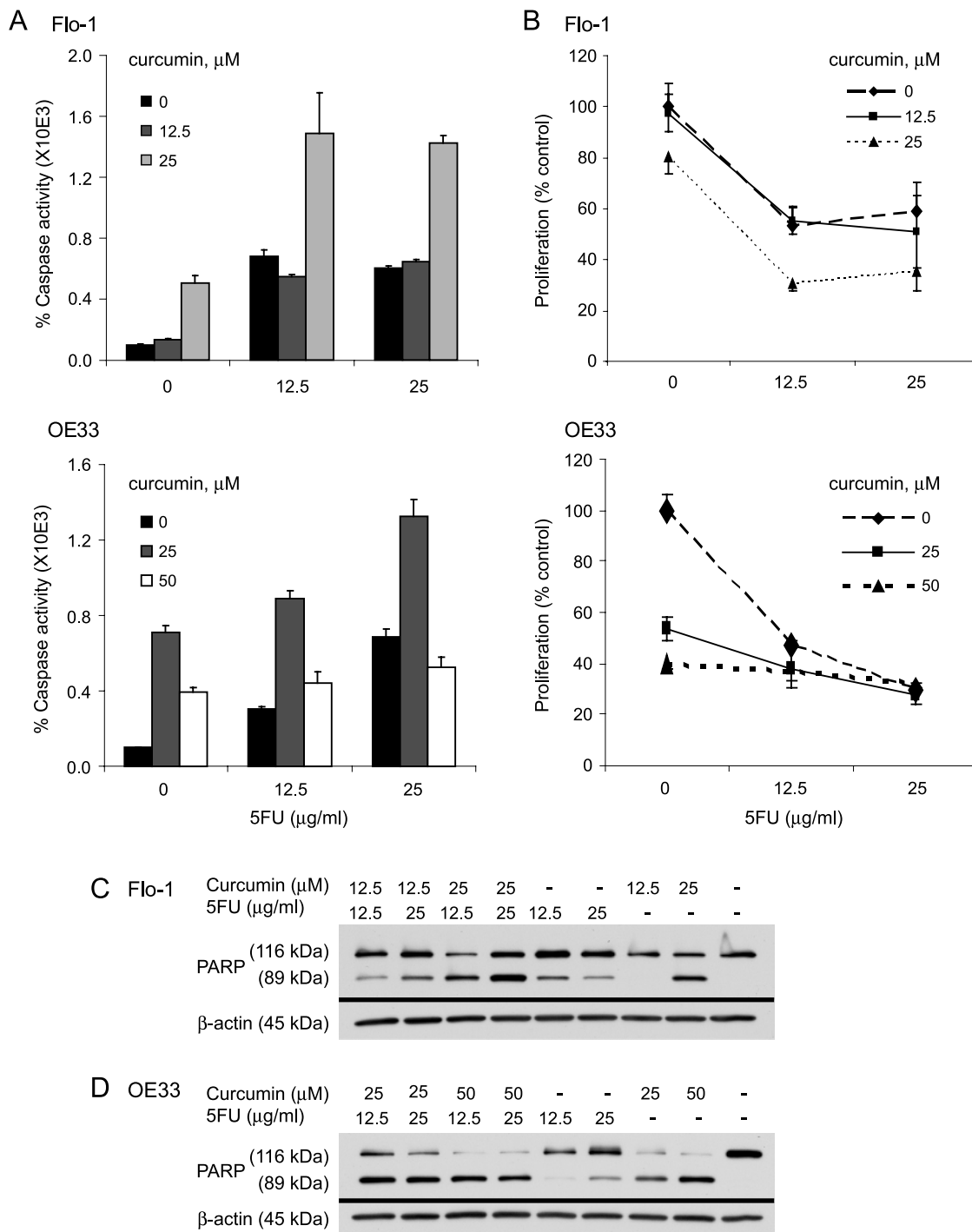


Figure 5. Effect of curcumin on 5-FU chemosensitivity in the EAC cell lines Flo-1 and OE33. (A) Levels of apoptosis induced by increasing doses of 5-FU and/or curcumin treatment for 48 hours in Flo-1 and OE33 cells were determined using the Caspase-Glo 3/7 activity assay. (B) Caspase 3/7 activity was normalized to cell number as determined by WST-1 proliferation assay. These numbers were then compared with the level of vehicle control and expressed as percentage caspase activity (mean % \pm SD). Each data point represented three individual wells. Immunoblot analyses of PARP cleavage were used to assess apoptosis in (C) Flo-1 cells and (D) OE33 cells that were treated with increasing doses of 5-FU and/or curcumin for 48 hours. β -Actin was used as a loading control. DMSO was used as the vehicle control in all experiments. Independently repeated experiments were performed to ensure reproducibility.

Chemosensitivity to 5-FU and CDDP Increases with Curcumin

The lethal dose, 50% (LD₅₀) values for Flo-1 and OE33 cell lines were 25 μ g/ml for 5-FU and 20 μ g/ml for CDDP (data not shown). Curcumin enhanced apoptosis in both Flo-1 and OE33 EAC cell lines

when administered in combination with 5-FU, as determined by both greater caspase-3/7 activity and PARP cleavage (Figure 5). 5-FU doses of 12.5 μ g/ml or greater seemed to have similar levels of cytotoxicity and caspase 3/7 activity in Flo-1 cells. Although a cytotoxic plateau effect was also noted in OE33 cells, a dose-dependent response to

5-FU was observed in both apoptotic assays. Both cell lines seemed to be sensitive to CDDP treatment in a dose-dependent fashion, with curcumin having an additive effect for both caspase activity and cellular proliferation (Figure 6). The decrease in caspase 3/7 activation observed in OE33 cells in response to 20 μg/ml CDDP/curcumin combinations and high doses of curcumin alone may be due to shifts toward nonapoptotic mechanisms of cell death such as mitotic catastrophe, as has been recently reported [31].

Discussion

Oligonucleotide expression microarray evaluation of a panel of tissue samples suggested that activation of NF-κB occurs in the neoplastic progression from Barrett metaplasia to preneoplastic high-grade dysplasia and adenocarcinoma. We also observed a concomitant decline in *caspase-3*, *-6*, and *-7* gene expression. Taken together, these findings suggest that NF-κB could be an important mediator of tumor cell survival in EAC, possibly through its inhibitory influence on

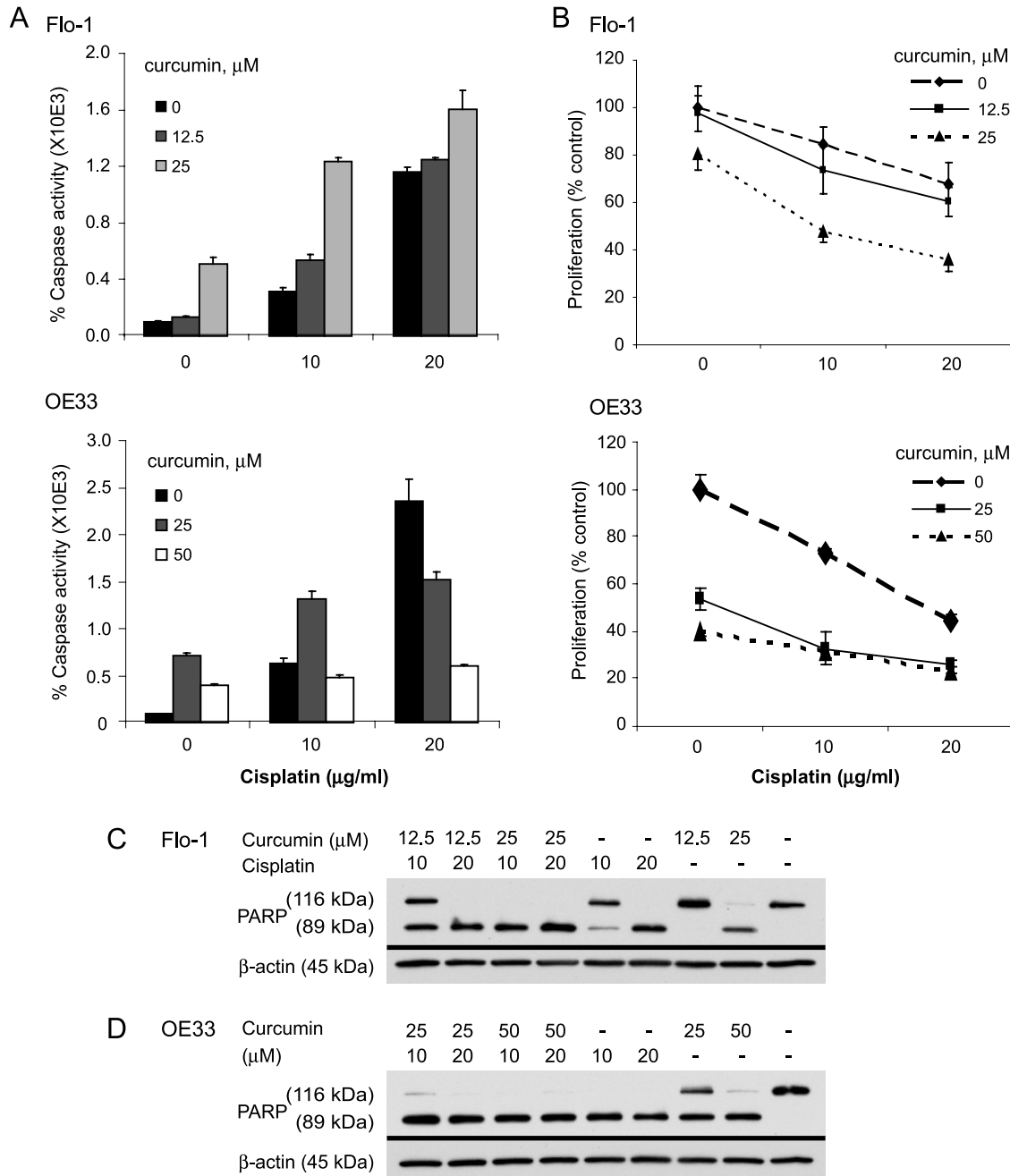


Figure 6. Effect of curcumin on CDDP chemosensitivity in the EAC cell lines Flo-1 and OE33. (A) Levels of apoptosis induced by increasing doses of CDDP (24-hour treatment) and/or curcumin (48-hour treatment) in Flo-1 and OE33 cells were determined using the Caspase-Glo 3/7 activity assay. (B) Caspase 3/7 activity was normalized to cell number as determined by WST-1 proliferation assay. These numbers were then compared with the level of vehicle control and expressed as percentage caspase activity (mean % ± SD). Each data point represented three individual wells. Immunoblot analyses of PARP cleavage were used to assess apoptosis in (C) Flo-1 cells and (D) OE33 cells that were treated with increasing doses of CDDP (24 hours) and/or curcumin (48 hours). β-Actin was used as a loading control. DMSO was used as the vehicle control in all experiments. Independently repeated experiments were performed to ensure reproducibility.

apoptosis. In addition, activation of NF- κ B might be an important mechanism of chemoresistance in the treatment of EAC.

The oligonucleotide expression data reported in this series of tissue samples are consistent with previous reports of increasing frequency of NF- κ B activation in the neoplastic progression from Barrett metaplasia to adenocarcinoma [32,33]. The impact of NF- κ B inhibition in EAC has not been widely reported. Our data demonstrated that activation of NF- κ B occurred not only in EAC but also in its preneoplastic lesion, Barrett esophagus with high-grade dysplasia (Figures 1 and 2). Recently, investigators have demonstrated that NF- κ B activation increases the resistance of Barrett epithelial cells to UV-B-mediated apoptotic signaling [34], suggesting one mechanism for increased survival of these precursor lesions as they progress to adenocarcinoma. There is additional indirect evidence regarding the role for NF- κ B activation as a mediator of cellular proliferation. Indeed, inhibitory RNA to the NF- κ B p50 subunit has been shown to reduce cyclooxygenase 2-dependent cellular proliferation in an EAC cell line [35]. NF- κ B activation may also have a role in epithelial tumor chemoresistance [36,37], possibly through its induction of cell survival genes including the inhibitors of apoptosis (IAPs) such as *survivin* and X-linked IAP (*XIAP*) [38].

Pretreatment NF- κ B status correlated significantly with overall survival, disease-free survival, and complete pathologic response, as reported in one retrospective study, in which pretreatment NF- κ B activation was detected in 78% of patients who failed to achieve complete pathologic response and 51% of patients who developed metastatic esophageal carcinoma [39]. Whether manipulation of NF- κ B activation will be of clinical significance remains to be determined.

A number of pharmacologic agents directed at inhibiting NF- κ B activation are under investigation including curcumin, a naturally occurring phytopolyphenol pigment that is considered the most active component of turmeric [14]. Curcumin has been shown to inhibit NF- κ B activation during tumor promotion [40] mediated by phorbol ester, TNF- α , hydrogen peroxide, and cigarette smoke. It also suppressed cellular proliferation and induced apoptosis through the inhibition of constitutively activated NF- κ B in pancreatic cancer [8]. In human melanoma cells, curcumin arrested cell cycle progression at the G₂/M phase and induced apoptosis by inhibiting NF- κ B activation [41].

Given these cell cycle effects, curcumin could potentially synergize with chemotherapeutic agents, inhibiting tumor growth and metastasis. In bladder cancer models, curcumin seemed to potentiate the apoptotic effects of the chemotherapeutic agents, gemcitabine and paclitaxel [42]. Furthermore, curcumin suppressed the paclitaxel-induced NF- κ B pathway in breast cancer cells and inhibited metastasis of human breast cancer to the lungs in nude mice [17]. Although curcumin has limited oral bioavailability as a result of hepatic clearance by glucuronidation and sulfation [43,44], it also has a low toxicity profile [14,45] and might be suitable for treatment of upper gastrointestinal malignancies, including esophageal cancers.

Curcumin dose-dependently inhibited NF- κ B activity, reduced cell viability and/or proliferation, and promoted apoptosis in EAC cell lines. We, therefore, hypothesized that curcumin might sensitize EAC cell lines to antiproliferative agents such as 5-FU or CDDP, two "first-line" chemotherapeutic agents for the treatment of EAC. We observed an enhanced apoptotic response to these chemotherapeutic agents when either Flo-1 or OE33 cells were concomitantly treated with curcumin. Curcumin may have enhanced the proapoptotic effects of these chemotherapeutic agents through the inhibition of NF- κ B

activation, or functioned in concert with CDDP, which has been shown to inhibit NF- κ B-dependent transcription, leading to the down-regulation of *XIAP* and, in turn, releasing caspase 3 inhibition [46]. In addition, it may attenuate CDDP-induced NF- κ B activation, as has been shown in several chemoresistant carcinoma cell lines [47–49]. Whereas curcumin likely has several modes of action, our data suggest that the combination of curcumin and "first-line" chemotherapeutic agents increased apoptosis, as demonstrated by increased caspase activity and PARP cleavage. Although we have shown that curcumin inhibits NF- κ B in EAC cells, other cell signaling pathways including Akt, AP-1, or c-Jun N-terminal kinase [14] might also be significant mediators of this curcumin-induced apoptotic activity. In fact, the lack of NF- κ B inhibition with the dose of curcumin that resulted in maximal caspase 3/7 activity in OE33 cells suggested that other signaling pathways were also involved.

It has been reported that curcumin can reduce cell survival in a p53- and caspase-independent manner, possibly through inhibition of both the NF- κ B and AP-1 signaling pathways. O'Sullivan-Coyne et al. recently showed that curcumin induced apoptosis-independent cell death in esophageal cancer cells [31]. Specifically, OE33 cells treated with 15 μ M curcumin for 24 hours resulted in 13.4% apoptosis and 22.2% mitotic catastrophe. Therefore, the decrease in caspase activity we observed with 50 μ M compared with 25 μ M curcumin may reflect a shift toward mitotic catastrophe at higher doses of curcumin in both OE33 and Flo-1 cells.

In summary, our findings provide further support for the investigation of curcuminoids in the treatment of patients with esophageal cancer or those with its neoplastic precursor, dysplastic Barrett esophagus. Although efforts to increase systemic bioavailability will likely be necessary for the treatment of more advanced esophageal cancer, proximal digestive tract lesions such as Barrett esophagus might be more amenable to oral-based chemoprevention with curcuminoids.

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