uicc
global cancer control

IJC
International Journal of Cancer

Curcumin decreases cholangiocarcinogenesis in hamsters by suppressing inflammation-mediated molecular events related to multistep carcinogenesis

Suksanti Prakobwong[1,2,3], Jarinya Khoontawad[1,2], Puangrat Yongvanit[2,4], Chawalit Pairojkul[2,5], Yusuke Hiraku[6], Paiboon Sithithaworn[1,2], Porntip Pinlaor[2,7], Bharat B. Aggarwal[3] and Somchai Pinlaor[1,2]

[1] Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
[2] Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
[3] Department of Experimental Therapeutics, The University of Texas, M.D. Anderson Cancer Center, Houston, TX
[4] Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
[5] Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
[6] Department of Environmental and Molecular Medicine, Mie University Graduate School of Medicine, Tsu, Mie, Japan
[7] Centre for Research and Development in Medical Diagnostic Laboratory, Faculty of Associated Medical Science, Khon Kaen University, Khon Kaen, Thailand

Cholangiocarcinoma (CCA) is a highly metastatic tumor linked to liver fluke infection and consumption of nitrosamine-contaminated foods and is a major health problem especially in South-Eastern Asia. In search for a suitable chemopreventive agents, we investigated the effect of curcumin, a traditional anti-inflammatory agent derived from turmeric (*Curcuma longa*), on CCA development in an animal model by infection with the liver fluke *Opisthorchis viverrini* and administration of *N*-nitrosodimethylamine and fed with curcumin-supplemented diet. The effect of curcumin-supplemented diet on histopathological changes and survival were assessed in relation to NF-κB activation, and the expression of NF-κB-related gene products involved in inflammation, DNA damage, apoptosis, cell proliferation, angiogenesis and metastasis. Our results showed that dietary administration of this nutraceutical significantly reduced the incidence of CCA and increased the survival of animals. This correlated with the suppression of the activation of transcription factors including NF-κB, AP-1 and STAT-3, and reduction in the expression of proinflammatory proteins such as COX-2 and iNOS. The formation of iNOS-dependent DNA lesions (8-nitroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine) was inhibited. Curcumin suppressed the expression of proteins related to cell survival (bcl-2 and bcl-xL), proliferation (cyclin D1 and c-myc), tumor invasion (MMP-9 and ICAM-1) and angiogenesis (VEGF), and microvessel density. Induction of apoptotic events as indicated by caspase activation and PARP cleavage was also noted. Our results suggest that curcumin exhibits an anticarcinogenic potential *via* suppression of various events involved in multiple steps of carcinogenesis, which is accounted for by its ability to suppress proinflammatory pathways.

Cholangiocarcinoma (CCA) is a bile duct cancer with high incidence rate in northeastern Thailand where liver fluke *Opisthorchis viverrini* is endemic.[1,2] The incidence rate of CCA is highest in Khon Kaen province with age standardized rate of 89.2/100,000 in males and 37.4/100,000 in females[3] with range from 93.8 to 317.6/100,000 person-years.[4] The risk factors for CCA in Thailand include not only parasite infection but also exposure to the carcinogen nitrosamine

Key words: *Opisthorchis viverrini*, cholangiocarcinoma, curcumin, DNA damage, apoptosis, cell proliferation, angiogenesis

Abbreviations: AP-1, activating protein-1; Bcl-2, B-cell lymphoma protein-2; Bcl-xL, B-cell leukemia-xL; COX-2, cyclooxygenase 2; EMSA, electrophoretic mobility shift assay; ICAM-1, intracellular adhesion molecule-1; iNOS, inducible nitric oxide synthase; JAK, janus kinase; MMP-9, matrix metalloproteinase-9; NF-κB, nuclear factor-kappaB; STAT-3, signal transducer and activator of transcription 3; TRAF, tumor necrosis factor receptor-associated factor; VEGF, vascular endothelial growth factor

Grant sponsors: Research Team Strengthening Grant, National Center for Genetic engineering and Biotechnology, National Science and Technology Development Agency, Thailand, Khon Kaen University Research Foundation, Thailand
DOI: 10.1002/ijc.25656
History: Received 13 Jun 2010; Accepted 23 Aug 2010; Online 7 Sep 2010

Correspondence to: Somchai Pinlaor, Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand, Tel.: +66-43-348-387, Fax: +66-43-202-475, E-mail: psomec@kku.ac.th; or Bharat B. Aggarwal, Department of Experimental Therapeutics, The University of Texas, M.D. Anderson Cancer Center, Houston, Texas 77030, Tel.: 713-794-1817, Fax: 713-794-1613, E-mail: aggarwal@mdanderson.org

contained in traditional fermented foods.[2,5] Both experimental and epidemiological evidences suggest that CCA proceeds through chronic inflammation-mediated DNA damage.[6–8] Currently, because of the ineffective diagnosis, there is no effective treatment available for CCA following surgery, radiation and chemotherapy for primary tumor.[9,10]

The activation of transcription factors is the key event in progression of human cancer and can be the target for cancer therapy.[11–13] Nuclear factor-kappaB (NF-κB) is a transcription factor, which acts as a modulator linked to inflammation and carcinogenesis.[12,13] Persistently activated NF-κB is implicated in the expression of various genes involved in inflammation [cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS)], apoptosis (caspases, bcl-2 and bcl-xL), proliferation (c-myc and cyclin D1) and metastasis [metalloproteinase (MMP)-9, and adhesion molecules such as ICAM-1].[14] Recently, we have shown that NF-κB-mediated iNOS expression leads to the formation of oxidative and nitrative DNA lesions [8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and 8-nitroguanine, respectively] in *O. viverrini*-infected hamsters[7] and in CCA patients.[8] These findings suggest that inflammation-mediated DNA damage plays a key role in the onset and development of cancers.[7,8,15] Moreover, NF-κB participates in tumor promotion and progression.[12,13] These evidences provide an avenue for chemopreventive strategies of multistep carcinogenesis in biliary systems.

Curcumin is a diferuloylmethane derived from turmeric (*Curcuma longa*) and a pharmacologically safe agent. Curcumin has been shown to suppress the activation of transcription factors, such as NF-κB, signal transducer and activator of transcription (STAT)-3 and activator protein (AP)-1.[16] We have recently reported that curcumin modulates the expression of NF-κB-relevant antioxidant genes and oxidative and nitrative DNA damage, inhibits cell proliferation, and reduces periductal fibrosis, resulting in the reduction of the pathogenesis of *O. viverrini*-infected hamsters.[17,18]

In this report, we determined whether curcumin prevents cholangiocarcinogenesis induced by the combination of *O. viverrini* infection and *N*-nitrosodimethylamine (NDMA) administration using a hamster model. We targeted on the anticancer potential of curcumin and its ability to suppress NF-κB activation and the expression of its gene products, resulting in inhibition of cell proliferation and tumor progression.[19] We found that curcumin inhibited CCA development through the suppression of NF-κB-related gene products involved in inflammation, DNA damage, apoptosis, cell proliferation, angiogenesis and metastasis and improved the survival rate of hamsters.

Material and Methods

Reagents

Curcumin, a mixture consisting of 97% curcumin was purchased from Merck-Schuchardt (Hohenbrunn, German). Polyclonal antibodies against ICAM-1, cyclin D1, c-jun, p50, p65, STAT-3, caspase-3, caspase-8 and caspase-9, and monoclonal antibodies against c-fos, COX-2, iNOS, phospho-STAT-3 (Tyr^{705}), c-myc, bcl-2 and bcl-xL were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibody against PCNA and monoclonal antibody against VEGF were obtained from Abcam (Cambridge, MA). Monoclonal antibody against phospho-p65 was purchased from Cell Signaling Technology (Beverly, MA).

Parasites

Opisthorchis viverrini metacercariae were isolated from naturally infected fish by pepsin digestion as described previously.[20] Cyprinid fish obtained from Khon Kaen province, Thailand, were digested in 0.25% pepsin-HCl and *O. viverrini* metacercariae were isolated and counted. Viable cysts were used for hamster infection.

Experimental animals

The Animal Ethics Committee of Khon Kaen University (AEKKU 17/2552) approved this study. Four- to six-week-old male golden hamsters were housed under conventional conditions and fed a stock diet and given water *ad libitum*. Animals were randomly divided into three groups: group I, animals that received conventional murine diet (CP-SWT, Thailand) supplemented with corn oil (normal); group II, those with *O. viverrini* infection and *N*-nitrosodimethylamine administration and fed with normal diet (OV + NDMA); group III, those with OV + NDMA and fed with 1% (w/w) curcumin (∼65 mg/day)-supplemented diet (OV + NDMA + Cur). Hamsters were treated with 50 *O. viverrini* metacercariae by oral inoculation and given with 12.5 ppm of NDMA in water *ad libitum* for 2 months and withdrawn thereafter. Curcumin-supplemented diet prepared weekly as described previously.[17] Curcumin supplement was started at day 0 and continued until animals were sacrificed at 21 days and 1, 2, 3, 4, 5 and 6 months post-treatment (each group, $n = 7$). Primary tumors in the livers were excised, and the final tumor volume was measured as length × width × depth. In addition, 45 hamsters of each group (OV + NDMA and OV + NDMA + Cur) were used for survival analysis, and the number of surviving hamsters was counted monthly.

Preparation of nuclear extract from liver samples

Liver tissues (75–100 mg) were minced and incubated on ice for 30 min in ice-cold lysis buffer [10 mM HEPES (pH 7.9), 1.5 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 2.0% NP-40, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. The minced tissue was homogenized using a Dounce homogenizer and centrifuged at 12,000*g* at 4°C for 10 min. The resulting nuclear pellet was suspended in nuclear extraction buffer [20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 2.5% NP-40, 0.5 mM PMSF, 2 μg/ml leupeptin] and incubated on ice for 2 hr with intermittent mixing. The suspension was then centrifuged at 12,000*g* at 4°C for 10 min. The supernatant

(nuclear extract) was collected and stored at −70°C until use. Protein concentration was measured by the Bradford assay with bovine serum albumin (BSA) as the standard.

Electrophoretic mobility shift assay

To assess NF-κB activation in nuclear proteins, we carried out electrophoretic mobility shift assay (EMSA) essentially as described previously.[21] Briefly, nuclear extracts were incubated with ^{32}P-end-labeled 45-mer double-stranded NF-κB oligonucleotide (4 μg of protein with 16 fmol of DNA) from the HIV long terminal repeat (5′-TTGTTACAA*GGGAC TTTC*CGCT*GGGGACTTTC*CAGGGGGAGGCGTGG-3′; italicized letters indicate NF-κB–binding sites) for 15 min at 37°C. The resulting DNA–protein complex was separated from free oligonucleotide on 6.6% native polyacrylamide gels. The dried gels were visualized, and radioactive bands were quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

To confirm the positive band for nuclear NF-κB activation, the Supershift assay was performed as described previously.[22] Nuclear extracts were incubated with specific antibodies against either p50 or p65 subunits of NF-κB for 30 min at room temperature before the DNA–protein complex was analyzed by EMSA. Preimmune serum was included as negative control.

Western blot analysis

To determine the levels of protein expression, liver tissues (75–100 mg) were minced and incubated on ice for 30 min in ice-cold lysis buffer (10% NP-40, 5 M NaCl, 1 M HEPES, 0.1 M EGTA, 0.5 M EDTA, 0.1 M PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin). Thirty micrograms of liver homogenates or the nuclear extracts was separated in 12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with the specific primary antibodies. Next, membranes were incubated with an appropriate secondary antibody and then detected by enhanced chemiluminescence (ECL kit, Amersham Bioscience, Piscataway, NJ). Relative band intensity was analyzed with a computer-assisted imaging densitometer system (Scion image, Scion Corporation, USA).

Gelatin zymography

Gelatinolytic activity of liver homogenates was examined by gelatin zymography as described previously.[20] The recombinant human MMP-2 (Calbiochem-Novabiochem Corporation, San Diego, CA) and MMP-9 (Sigma Chemical, St. Luis, MO) were included in each gel as standard protein. The intensity of MMP-2 and MMP-9 activity was analyzed by computer-assisted imaging densitometer as described above.

Immunohistochemical and histopathological study

Double immunofluorescent analysis was performed in formalin-fixed and paraffin-embedded tissue as described previously.[20] Deparaffinized sections (5 μm) were incubated with the specific primary antibodies against iNOS, p65, PCNA, 8-nitroguanine[15] or 8-oxodG (JAICA, Japan) for overnight at room temperature. Then, the sections were incubated with appropriate secondary antibodies, including Alexa Fluor® 488 goat anti-mouse IgG and Alexa Fluor® 594 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) for 3 hr at room temperature. The immunoreactivity was examined under a fluorescent microscope (Nikon, Japan).

To examine the localization of MMP-9, the sections were treated with monoclonal antibody against MMP-9 (R&D Systems, MN). The liquid DAB plus substrate chromogen system with horseradish peroxidase (DakoCytomation, Carpinteria, CA) was used followed by nuclear staining with hematoxylin. The positive staining was assessed by counting the number of MMP-9-positive cells in 10 randomly selected fields (×200) per section by two blinded observers from the authors and the percentage of positive cells in the livers was calculated as described previously.[23] Histopathological changes were evaluated by conventional H&E staining.

Microvessel density

Paraffin-embedded sections (5 μm) were stained with rat monoclonal anti-CD31 antibody (PharMingen, San Diego, CA). Vessel density was examined under higher magnification (×400) and a CD31-positive area was counted as a single vessel. A total of 20 high-power fields were examined from 5 animals at each time point of the experiment. Results were expressed as the mean number of vessels ±SD per field (×400).

Statistical analyses

Data are presented as mean ± SD. Student's *t*-test was used to compare between two groups, and one-way ANOVA was used to test for differences among two or more independent groups. Nonparametric Mann–Whitney *U* test was used to compare the intensity of bands of two independent groups in Western blot and gelatin zymography. The percentage of CCA development was compared using nonparametric χ^2 tests. Kaplan–Meier survival analysis was used to estimate the disease-specific survival and comparison between groups were done with a log-rank test. Statistical analyses were performed using SPSS version 11.5. p values less than 0.05 were considered statistically significant.

Results

Curcumin decreases CCA development and increases survival rate in hamsters

We have successfully established the CCA development in animal model by infection with the liver fluke *O. viverrini* and administration of NDMA.[24] Here, we demonstrated that 1% dietary curcumin, ∼65 mg/day, decreased CCA development and increased survival rate in hamsters. We examined the gross appearance in OV + NDMA group, and hepatomegaly, bile duct dilatation and abscesses were commonly found throughout the experiment. White granules and small tumors (average size < 0.5 cm) were observed at 2–3 months

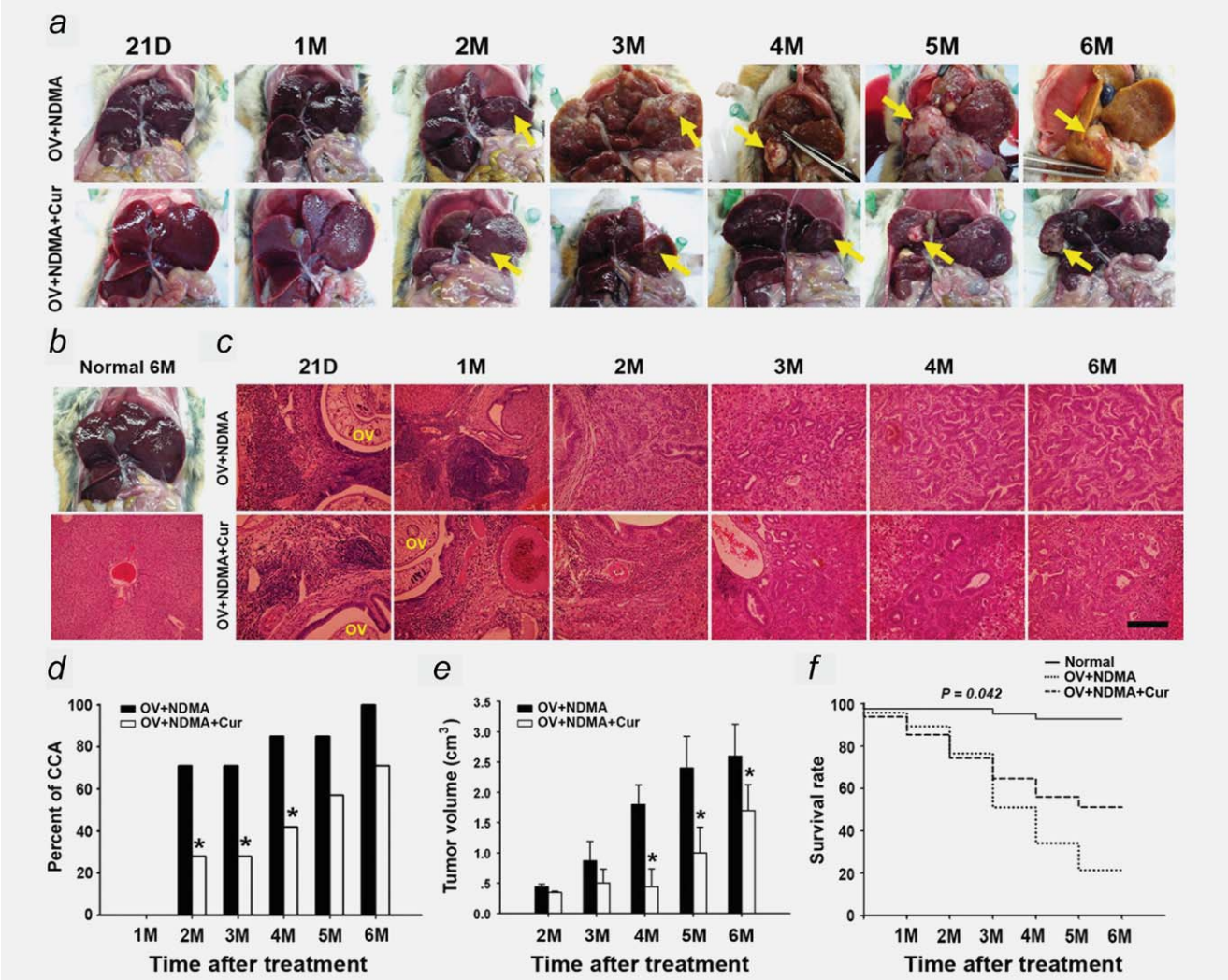

Figure 1. Effect of curcumin on cholangiocarcinogenesis and survival of hamsters with *O. viverrini* infection and NDMA administration. (*a*) Gross appearance of the hamster liver. Tumor tissues are indicated by arrows. (*b*) Gross appearance and histopathology of the liver of normal hamster at 6 months. (*c*) Histopathological changes in the liver examined by H&E staining. (*d*) Percentage of CCA in hamsters evaluated by H&E staining. (*e*) Effect of curcumin on tumor volume. Tumor volume was calculated as length × width × depth ($n = 7$). (*f*) Effect of curcumin on survival of hamsters ($n = 45$ in each group). OV + NDMA = hamsters with *O. viverrini* infection and NDMA administration. OV + NDMA + Cur = OV + NDMA with 1% (w/w) curcumin supplement. Bar = 100 μm, D = days, M = month(s). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Fig. 1*a*). Tumor mass in the liver was prominently found at 4–6 months (average size >2 cm), and attached to the neighboring organs at 5–6 months. In curcumin-treated group, the small granules firstly appeared at 3 months post-treatment. The size of tumor mass was 0.2–1.0 cm at 3–6 months, except two of seven hamsters bearing large tumors with the size of >1.0 cm at 6 months. At 4–6 months, curcumin treatment significantly reduced tumor volume compared with OV + NDMA group ($p < 0.05$) (Fig. 1*e*). Normal liver anatomy and histology are shown in Figure 1*b*.

We confirmed the CCA tissue using hematoxylin and eosin staining. Histopathological findings in OV + NDMA group revealed that inflammation and bile duct hyperplasia were observed at 21 days and 1 month (Fig. 1*c*). At 2 months, small bile ducts, lymphoid follicle and early stage of tumors were observed (71.42%, 5/7), and tumor progression occurred in all hamsters at 6 months (100%, 7/7). In contrast, in curcumin-treated group, inflammation was obviously diminished compared to OV + NDMA group, and CCA was firstly found at 3 months, followed by slow tumor growth until 6 months. The percentage of CCA in curcumin-treated group was significantly lower than that in OV + NDMA group throughout the experiment at 4–6 months (Fig. 1*d*). Notably, curcumin treatment was significantly increased the survival rate compared to OV + NDMA group at 4–6 months ($p = 0.042$, Fig. 1*f*).

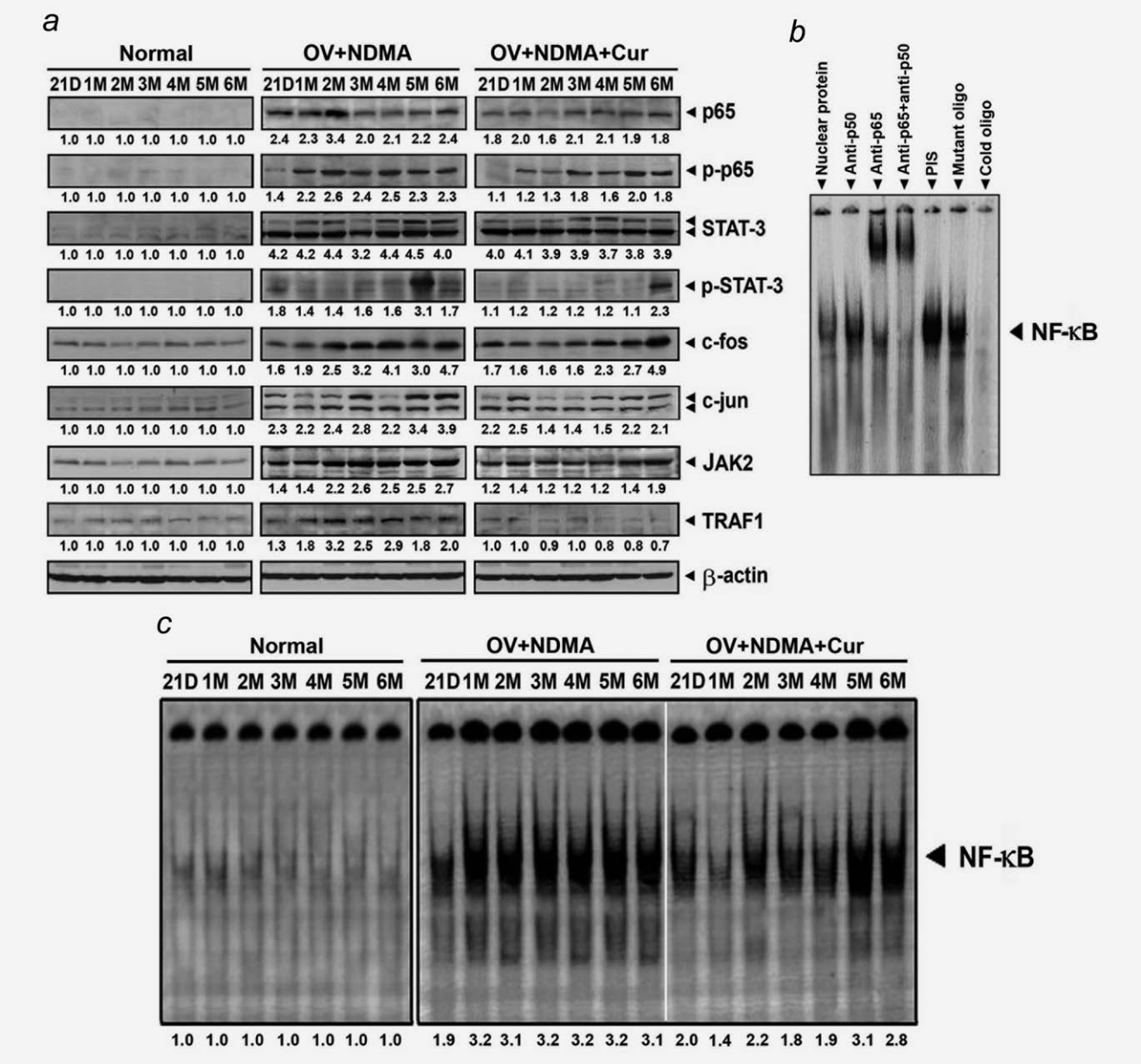

Figure 2. Effect of curcumin on the activation of transcriptional factors. (*a*) Expression of transcriptional factors. Nuclear extracts of the liver were used to examine the expression of phosphorylated NF-κB (p-p65), c-fos and c-jun, and liver homogenates were used to assess p65, STAT-3, p-STAT-3, JAK-2 and TRAF-1 by Western blot. (*b*) Supershift assay was performed to detect the specificity of NF-κB. (*c*) Effect of curcumin on NF-κB activation was assessed by electrophoretic mobility shift assay in nuclear extracts. The number below each photograph indicates fold increase in NF-κB activation relative to normal control in corresponding time point. Duplicate of experiment was performed. Representative data are shown from five animals. The experiment and abbreviations are the same as in Figure 1 legend. PIS = preimmune serum.

Curcumin inhibits the activation of transcription factors

We examined the effect of curcumin treatment on the activation of transcription factors by determination of the levels of nuclear translocated proteins, including NF-κB (p65), c-fos, c-jun and pSTAT-3 in liver homogenates. In OV + NDMA group, expression of phosphorylated (p)-p65 in nuclear extracts was observed in relation to CCA development (Fig. 2*a*), whereas curcumin treatment significantly inhibited p65 activation ($p < 0.05$). The nuclear proteins including c-fos, c-jun and p-STAT-3 were also upregulated, and in curcumin-treated group, the levels of these proteins were significantly lower than those in OV + NDMA group at 2–4 months ($p < 0.05$). Curcumin also significantly inhibited TRAF-1 and JAK2 expression ($p < 0.05$) involved in NF-κB and JAK2/STAT-3 pathway at 2–6 months. In addition, NF-κB activation in nuclear extract was assessed by EMSA. The

result revealed that p65 expression in OV + NDMA was prominently found and was significantly inhibited by curcumin treatment at 1–4 month(s) ($p < 0.05$), but there was no effect of curcumin on NF-κB activation at 5–6 months post-treatment (Fig. 2*c*).

Curcumin reduces inflammatory responses and related DNA damage

We determined the expression of proinflammatory biomarkers, iNOS and COX-2. Expression of these proteins was obviously increased in OV + NDMA group in association with NF-κB activation and cholangiocarcinogenesis (Fig. 3*a*). In curcumin-treated group, iNOS expression was almost completely and significantly inhibited at 1–6 month(s), whereas COX-2 was significantly downregulated at 1–3 months, but little or no inhibitory effect was observed at 5–6 months, suggesting that curcumin has a chemopreventive effect on tumor formation rather than a therapeutic effect on CCA.

NF-κB is known to regulate iNOS expression involved in NO production leading to DNA damage by oxidation and nitration. Double immunofluorescence revealed that coexpression of NF-κB in the nucleus and iNOS in the cytoplasm of epithelial bile duct and inflammatory cells was increased in parallel with the formation of 8-oxodG and 8-nitroguanine during CCA development (at inflamed tissue, 1 month; in early CCA, 2 months and in tumor tissue, 6 months) in OV + NDMA group (Figs. 3*b* and 3*c*). In curcumin-treated group, NF-κB and iNOS expression was suppressed, resulting in a significant reduction of oxidative ($p = 0.011$) and nitrative ($p = 0.035$) DNA damage compared with OV + NDMA group (Figs. 3*b* and 3*c*).

Curcumin induces apoptotic events

It has recently been demonstrated that curcumin exhibits chemopreventive activity by inhibiting cell growth and inducing apoptosis.[22] We investigated the effects of curcumin on protein expression involved in apoptosis (Fig. 4). In OV + NDMA group, the levels of antiapoptotic markers (bcl-2 and bcl-xL) were increased in a time-dependent manner and associated CCA development. The levels of proapoptotic proteins, caspase-8, caspase-9, caspase-3 and cleaved PARP, were higher than in normal hamsters. Curcumin treatment was significantly reduced the levels of bcl-2 and bcl-xL, and significant increases in the levels of proapoptotic caspases, and PARP cleavage were observed ($p < 0.05$).

Curcumin reduces cell proliferation

Inhibitory effects of curcumin on the expression of cyclin D1 and c-myc are shown in Figure 5. The expression of cyclin D1 and c-myc increased markedly in a time-dependent manner in OV + NDMA group with tumor progression (Fig. 5*a*). In curcumin-treated group, cyclin D1 expression was completely reduced to the level in normal hamsters with a significant difference from OV + NDMA group at 3–6 months ($p < 0.05$), whereas c-myc level was partially reduced by curcumin treatment and significantly different only at 6 months.

Colocalization of a proliferative marker, proliferating cell nuclear antigen (PCNA) and nitrative DNA damage (8-nitroguanine) is shown in Figure 5*b*. In OV + NDMA group, PCNA was expressed in the nucleus of proliferative bile duct epithelial cells, inflammatory cells and tumor cells at the inflamed tissues (1 month), early CCA (2 months) and tumor tissues (6 months), respectively, and a large part of the cells were positively stained with DNA damage. In curcumin-treated group, a significant decrease in PCNA expression was observed and 8-nitroguanine formation was partially reduced in hyperplastic tissues at the inflamed tissues (1 month), early CCA (2 months) and tumor tissues (6 months) ($p < 0.05$). These results are the first demonstration that curcumin reduces proliferation of cells with genetic damage, and curcumin shows a chemopreventive effect on tumor development.

Curcumin reduces the expression of molecules involved in angiogenesis and tumor invasion

The expression of VEGF, ICAM-1 and MMP-9 is known to be regulated by NF-κB, we therefore evaluated the expression of these molecules. Western blot analysis revealed that the expression of genes involved in tumor invasion (ICAM-1) and angiogenesis (VEGF) was prominently upregulated in relation to CCA development (Fig. 6*a*). Curcumin treatment significantly decreased the expression of these protein levels at 2–6 months ($p < 0.05$). The gelatinase activity in homogenated liver was assessed by zymography (Fig. 6*b*). In OV + NDMA group, the active form of MMP-2 and MMP-9 was increased after 3 and 4 months, respectively. In curcumin-treated group, only latent form of MMP-2 was observed, and suppressed MMP-9 activity was noted at 4 months and significantly higher than untreated group at 4–5 months ($p < 0.05$).

Immunohistochemistry revealed that MMP-9 expression was observed in the cytoplasm of fibroblasts, stroma cells, inflammatory cells and tumor cells located in invasive tissues in OV + NDMA group and increased with CCA development (Fig. 6*c*). The number of MMP-9 positive cells significantly increased throughout the experiment in OV + NDMA group. Curcumin treatment significantly decreased MMP-9-positive cells especially at 3–6 months with corresponding time points ($p < 0.05$, Fig. 6*d*).

To determine whether curcumin decreases angiogenesis, we examined the microvessel production using specific antibody against CD31. Curcumin significantly decreased CD31-positive cells (Fig. 6*e*), and led to a further reduction in microvessel density at 3–4 months compared with OV + NDMA group ($p < 0.05$) (Fig. 6*f*).

Discussion

CCA is a rare and fatal tumor leading to significant morbidity and mortality due to late diagnosis, with no effective

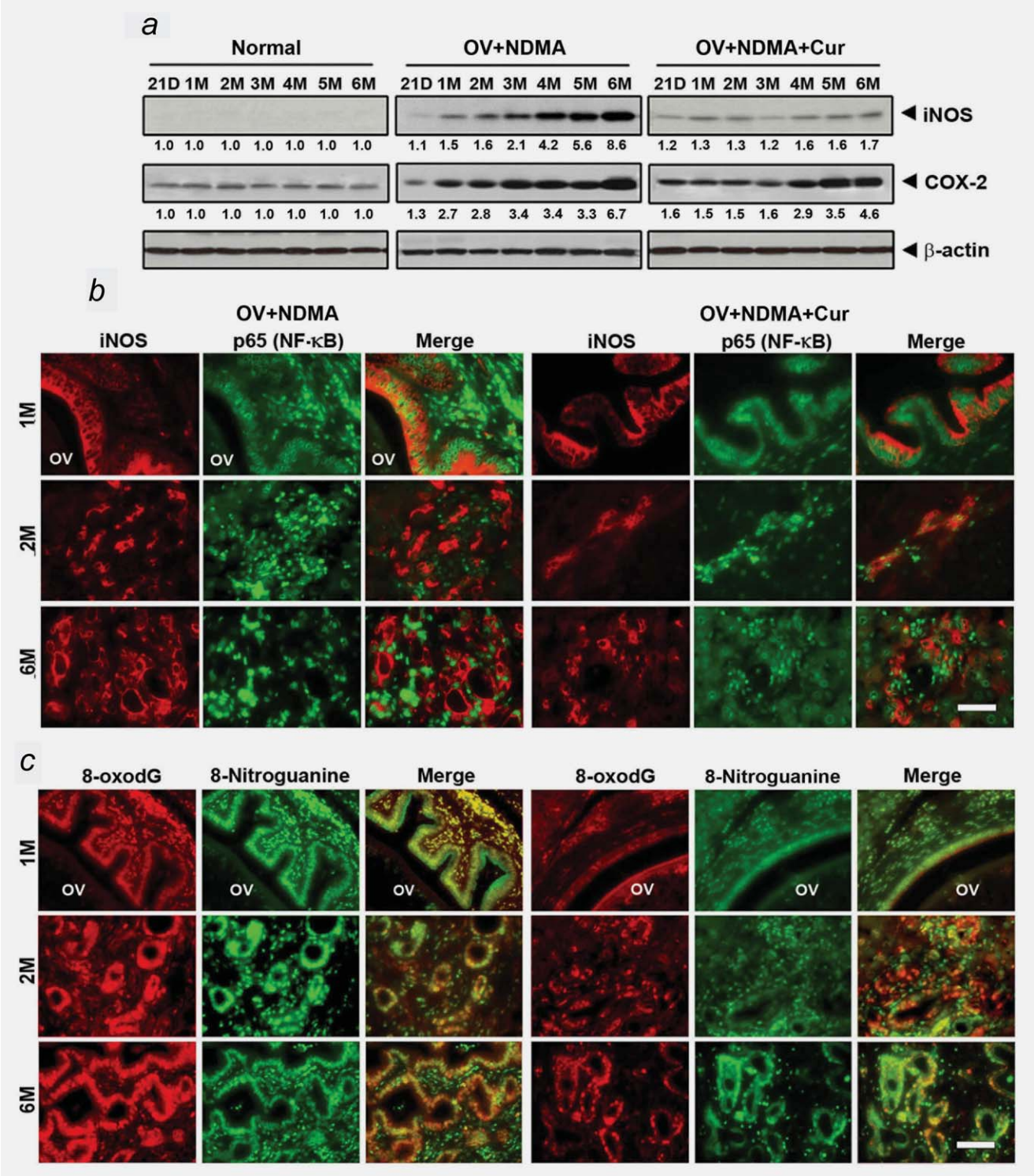

Figure 3. Effect of curcumin on the expression of inflammation-related molecules and resultant DNA damage. (*a*) Western blot analysis for the expression of iNOS and COX-2 in liver homogenates. Duplicate of experiment was performed. Representative data are shown from five animals. The number below each photograph indicates the fold increase in the levels of protein expression relative to normal control in corresponding time point. (*b*) Immunohistochemical analysis for the expression of iNOS and NF-κB. Colocalization of iNOS (red) and NF-κB (p65, green) is shown in merged pictures (yellow) of precancerous (1 month), early CCA (2 months) and malignant tissue (6 months). (*c*) Immunohistochemical analysis for the formation of 8-oxodG (red) and 8-nitroguanine (green). These DNA lesions are formed in the nucleus of bile duct epithelial cells, inflammatory cells and tumor cells and their colocalization is shown in merged pictures (yellow). The experiment and abbreviations are the same as in Figure 1 legend. Bar = 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

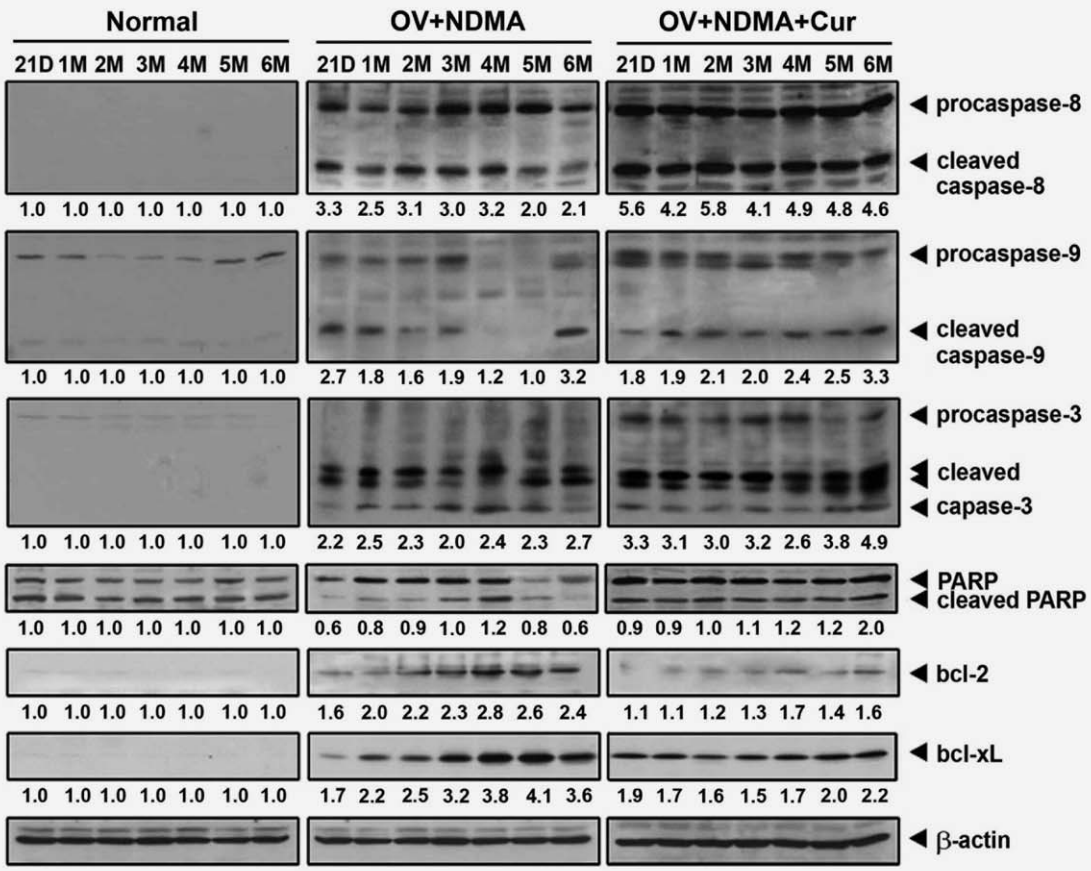

Figure 4. Effect of curcumin on the expression of proapoptotic and antiapoptotic molecules. Expression levels of proapoptotic proteins (caspase-8, caspase-9, caspase-3 and PARP cleavage), and antiapoptotic proteins (bcl-2 and bcl-xL) in liver homogenates were assessed by Western blot. Duplicate of experiment was performed. Representative data are shown from five animals. The number below each photograph indicates the fold increase in the levels of protein expression relative to normal control in corresponding time point. The experiment and abbreviations are the same as in Figure 1 legend.

treatment. The goal of the current study was to test whether curcumin has the preventive and therapeutic potential against CCA. We demonstrate that curcumin exhibited a chemopreventive and therapeutic effect on cholangiocarcinogenesis by affecting multiple molecular events. We found that dietary curcumin suppressed the activation of transcription factors involved in the process of inflammation-related carcinogenesis, including NF-κB, AP-1, JAK2/STAT-3 and TRAF-1, and inhibited the expression of NF-κB-regulated gene products, including iNOS, COX-2, cyclin D1, c-myc, MMP-9, ICAM-1, bcl-2, bcl-xL and VEGF. Suppression of iNOS expression by curcumin led to reduction in oxidative and nitrative DNA damage and inhibition of cell proliferation. In contrast, curcumin enhanced proapoptotic events such as the expression of caspase-8, -9 and -3 and PARP cleavage. We therefore hypothesized that curcumin prevents CCA development and increases the survival of animals by affecting molecular events involved in multiple steps of carcinogenesis, including tumor initiation, promotion and progression.

Our experimental cholangiocarcinogenesis model in this study is likely to mimic the multistep process of human CCA. Since 1978, Thamavit *et al.* first demonstrated the experimental cholangiocarcinogenesis induced by the combination of liver fluke infection and administration with a carcinogen, NDMA.[5] Up to date the molecular mechanism of CCA is not clear and there is lack of an effective drug for CCA treatment. The search for new chemopreventive and antitumor agents that are more effective and less toxic led us to our great interest in a phytochemicals agent, curcumin.[16] Our results provided the findings that curcumin decreased inflammation, the number and volume of tumor, and improved the survival of hamsters at the long-term treatment.

Several evidences have been provided for curcumin-induced reduction in the activation of NF-κB,[25,26] AP-1[27,28] and STAT-3[29,30] in various tumor types. This is the first study to show that curcumin downregulated these transcription factors in CCA model, although the effect of curcumin on the activation of CCA-specific transcription factors could

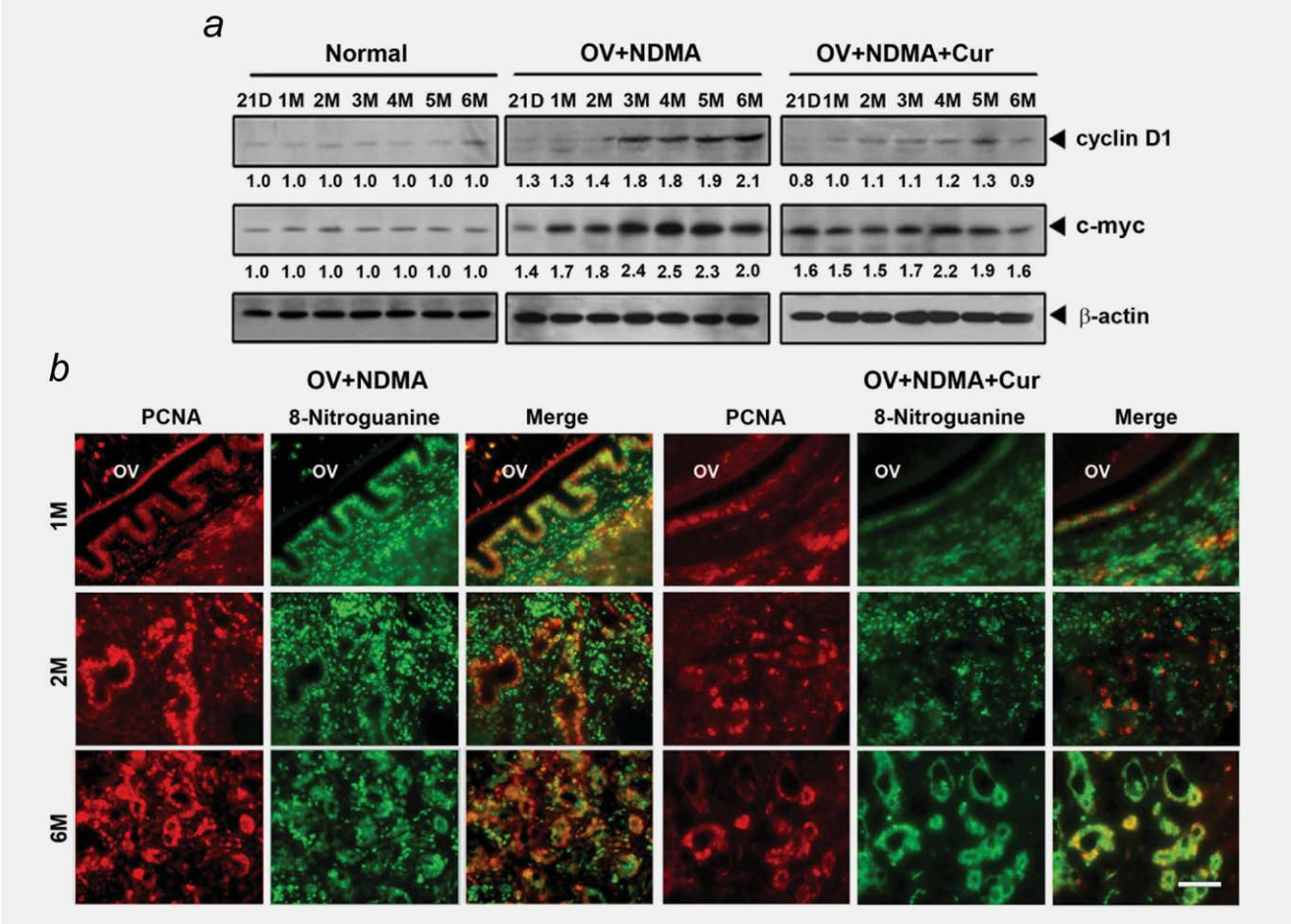

Figure 5. Effect of curcumin on the expression of molecules involved in cell proliferation. (*a*) Western blot analysis for the expression of cyclin D1 and c-myc in liver homogenates. Duplicate of experiment was performed. Representative data are shown from five animals. The number below each photograph indicates the fold increase in the levels of protein expression relative to normal control in corresponding time point. (*b*) Immunohistochemical analysis for PCNA expression and 8-nitroguanine formation. PCNA, a marker of cell proliferation (red) is partially colocalized with 8-nitroguanine (nitrative DNA damage marker; green) in precancerous (1 month), early CCA (2 months) and malignant tissue (6 months) as shown in merged picture (yellow). Bar = 50 μm. The experiment and abbreviations are the same as in Figure 1 legend. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

not be ruled out.[31] NF-κB mediates cellular transformation, proliferation, invasion, angiogenesis, and metastasis.[14] Thus, suppression of NF-κB activation should be effective in prevention and treatment of cancer. This hypothesis is supported by our findings that curcumin inhibited not only NF-κB expression and nuclear translocation (Fig. 2) but also the expression of its products as described above. Therefore, chemopreventive effect of curcumin on CCA may be largely accounted for by the suppression of NF-κB activation and the expression of its gene products involved in carcinogenesis.

Notably, we showed that curcumin inhibited not only NF-κB activation but also JAK2/STAT-3 activation resulting in a decrease in iNOS-dependent DNA damage. STAT-3 phosphorylation has recently been reported in CCA cell line.[32,33] JAK/STATs pathway is one of the key signaling cascades in CCA cell line, mediating the resistance to apoptosis.[34] *O. viverrini* infection increases IL-6 level in advanced periductal fibrosis[35] and upregulates iNOS expression,[6,15] resulting in DNA damage[7,15] leading to CCA. In this model, *O. viverrini* infection and administration with plus NDMA-induced cholangiocarcinogenesis may be explained by IL-6-induced activation of JAK2/STAT-3 and expression of iNOS.[36,37] Also, AP-1 is a transcription factor associated with NF-κB activation and closely linked to proliferation and transformation of tumor cells.[13] IL-6/STAT-3 signaling and enhanced expression of Mcl-1, a bcl-2 family, has been demonstrated in CCA cell line.[33] This pathway may be supported by our results that curcumin suppressed JAK-2/STAT-3 signaling and enhanced antiapoptotic events (bcl-2 and bcl-xL expression), suggesting that IL-6/STAT-3 signaling pathways may serve as new diagnostic and prognostic markers and targets for CCA therapy. We found that curcumin inhibited the expression of PCNA and proteins associated with cell proliferation (cyclin D1 and c-myc). This result is supported by

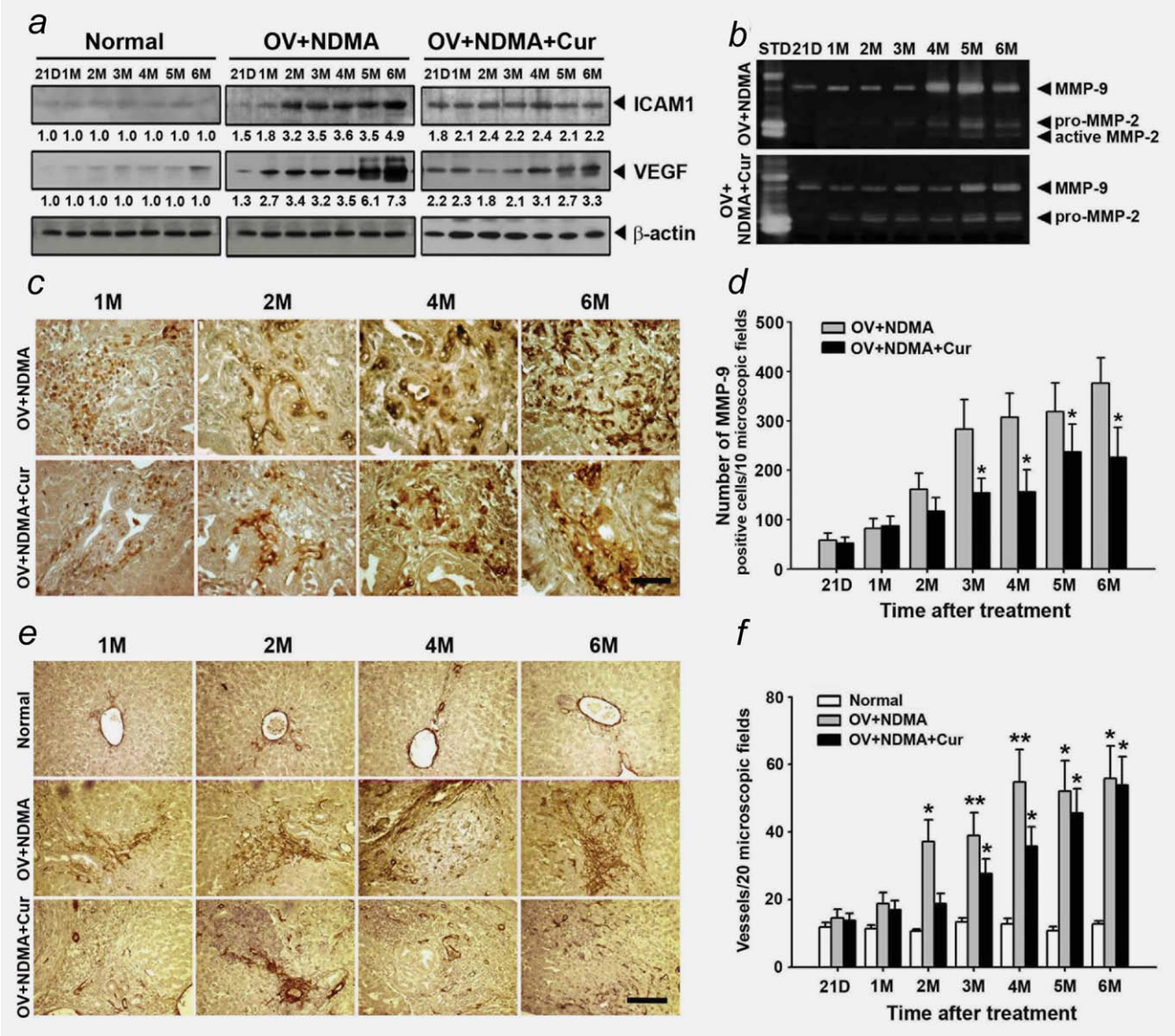

Figure 6. Effect of curcumin on the expression of molecules involved in tumor invasion and angiogenesis. (*a*) Western blot analysis for the expression of VEGF and ICAM1 in liver. Duplicate of experiment was performed. Representative data are shown from five animals. The number below each photograph indicates the fold increase in the levels of protein expression relative to normal control in corresponding time point. (*b*) Gelatin zymography for MMP-2 and -9 expression in the liver ($n = 7$). Gelatinase activity was performed individually. MMP-2, MMP-9 standard and normal control (data not shown) was included in each gel. (*c*) Immunohistochemical analysis for MMP-9 expression. MMP-9 expression in precancerous (1 month), early CCA (2 months) and malignant tissue (6 months) is shown. (*d*) MMP9-positive cells. (*e*) Immunohistochemical analysis for CD31, a marker of endothelial cells and angiogenesis. (*f*) Effect of curcumin on angiogenesis quantified by CD31-positive microvessel density. The values represent mean ± SEM ($n = 7$). * and **, statistically different ($p < 0.05$) by one-way ANOVA among three groups. The experiment and abbreviations are the same as in Figure 1 legend. STD = standard MMP-2 and -9, Bar; $c = 50$ μm, $e = 100$ μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the finding that curcumin mediated G2/M cell cycle arrest and apoptosis.[38] Taken together, JAK/STAT pathway is a therapeutic target of curcumin in not only CCA cell line[34] but also in CCA in animal model. STAT3-dependent iNOS expression occurs in patients with EB virus-induced nasopharyngeal carcinoma.[39] Therefore, our hypothesis can be applied to not only CCA but also other types of tumors as well as in nonendemic of liver fluke-associated CCA.[40]

JAK-2/STAT-3 pathway is not only involved in apoptosis but also participates in vessel formation.[41] Our results showed that the expression of ICAM-1, MMP-9 and VEGF, and activities of MMP-2 and MMP-9 time-dependently

increased in parallel to CCA development in OV + NDMA group. Curcumin supplement partially inhibited the expression and activities of these molecules, resulting in reduced microvessel density in hamsters (Figs. 6*a* and 6*e*). The decrease in these molecules is supported by the reduction in the activity of their transcription factors including NF-κB and JAK2/STAT-3. In addition, TRAF1 is a substrate of caspases activated during tumor necrosis factor receptor-alpha (TNFα)-induced apoptosis,[42] and regulates CD40-induced TRAF2-mediated NF-κB activation.[43] Carcinogen mediates TRAFs induction in many human tumor cells through a PKC/RAF/ERK/NF-κB-dependent pathway.[44] TNFα-induced p65 NF-κB binding as well as iNOS and TRAF1 expression has been demonstrated in liver cancer cell line.[45] We demonstrated here that curcumin completely inhibited TRAF1 in OV + NDMA-induced CCA in hamsters. It has recently been demonstrated that the chemopreventive activities of curcumin might be due to its ability to inhibit cell growth and induce apoptosis. In many studies, curcumin was proved to induce apoptosis of multiple cell lines and this feature might be an important mechanism for its antitumor effect in CCA. This hypothesis is supported by our results that curcumin suppressed the expression of proteins associated with cell survival (bcl-2 and bcl-xL) and cell proliferation (cyclin D1 and c-myc), and this correlated with apoptotic events including caspase expression and PARP cleavage. This study is the first report on the effect of curcumin on cholangiocarcinogenesis *via* induction of apoptosis.

In addition, the inhibitory effect of curcumin on CCA development may be induced by direct cytotoxic affect against *O. viverrini*. Likewise, in *in vitro* study, curcumin showed antihelminthic properties against *Schistosoma mansoni*.[46] We have previously reported that curcumin has no significant effect on worm burden in *O. viverrini*-infected hamsters as evaluated by egg count using formalin ether concentration technique.[18] In this study, curcumin slightly and nonsignificantly reduced the fecundity of adult worms in OV + NDMA-treated hamsters (data not shown). Thus, direct cytotoxic effect of curcumin against *O. viverrini* could not be ruled out and further study is required. The doses of curcumin used for chemoprevention of rat model of diethylnitrosamine-induced hepatic hyperplasia[47] and several murine tumor models range from 0.2 to 2% (w/w). Curcumin did not exert any toxicity at these doses.[48] Moreover, studies on the clinical trials against cancer have shown that curcumin can be orally administered safely at doses of 0.2–12 g/day.[48] In this study, we used 1% (w/w) curcumin, (equivalent to ~65 mg/day), a dose similar to that employed in human studies. For *O. viverrini*–infected subjects, a minimal dose within the range reported in human studies may be recommendable. Therefore, curcumin may serve as a safe and promising nutraceutical agent for practical use in chemoprevention against CCA development.

In conclusion, we showed that curcumin potentiates the antitumor and antimetastasis effects by inhibiting the expression of NF-κB-regulated gene products involved in DNA damage, cell proliferation, angiogenesis, tumor invasion and metastasis. Moreover, curcumin also inhibited CCA *via* suppression of JAK-2/STAT-3- and TRAF1-mediated pathways, and thus NF-κB-independent mechanisms may participate in cholangiocarcinogenesis. It is noteworthy that curcumin has the capacity of interaction with multiple molecular targets affecting the multistep process of cholangiocarcinogenesis. Our finding in animal model may be supported by a study in *in vitro* experiment indicating that curcumin inhibits CCA cell line.[49] The study may serve as powerful preclinical platforms for therapeutic and chemoprevention strategies for not only in human CCA but also in other types of tumors.

Acknowledgements

We thank Professor Shosuke Kawanishi and Profesor Ning Ma for providing us anti-8-nitroguanine antibody. We also thank the Office of the Higher Education Commission, Thailand for supporting by grant fund under the program Strategic Scholarships for Frontier Research Network for the Ph.D. Program Thai Doctoral degree for this research (to S.P.).

References

1. McLean L, Patel T. Racial and ethnic variations in the epidemiology of intrahepatic cholangiocarcinoma in the United States. *Liver Int* 2006;26:1047–53.
2. Sripa B, Pairojkul C. Cholangiocarcinoma: lessons from Thailand. *Curr Opin Gastroenterol* 2008;24:349–56.
3. Vatanasapt V, Martin N, Sriplung H, Chindavijak K, Sontipong S, Sriamporn H, Parkin DM, Ferlay J. Cancer incidence in Thailand, 1988–1991. *Cancer Epidemiol Biomarkers Prev* 1995;4:475–83.
4. Sriamporn S, Pisani P, Pipitgool V, Suwanrungruang K, Kamsa-ard S, Parkin DM. Prevalence of *Opisthorchis viverrini* infection and incidence of cholangiocarcinoma in Khon Kaen, Northeast Thailand. *Trop Med Int Health* 2004;9:588–94.
5. Thamavit W, Bhamarapravati N, Sahaphong S, Vajrasthira S, Angsubhakorn S. Effects of dimethylnitrosamine on induction of cholangiocarcinoma in *Opisthorchis viverrini*-infected Syrian golden hamsters. *Cancer Res* 1978;38:4634–9.
6. Ohshima H, Bandaletova TY, Brouet I, Bartsch H, Kirby G, Ogunbiyi F, Vatanasapt V, Pipitgool V. Increased nitrosamine and nitrate biosynthesis mediated by nitric oxide synthase induced in hamsters infected with liver fluke (*Opisthorchis viverrini*). *Carcinogenesis* 1994;15:271–5.
7. Pinlaor S, Ma N, Hiraku Y, Yongvanit P, Semba R, Oikawa S, Murata M, Sripa B, Sithithaworn P, Kawanishi S. Repeated infection with *Opisthorchis viverrini* induces accumulation of 8-nitroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanine in the bile duct of hamsters via inducible nitric oxide synthase. *Carcinogenesis* 2004;25:1535–42.
8. Pinlaor S, Sripa B, Ma N, Hiraku Y, Yongvanit P, Wongkham S, Pairojkul C, Bhudhisawasdi V, Oikawa S, Murata M, Semba R, Kawanishi S. Nitrative and oxidative DNA damage in intrahepatic cholangiocarcinoma patients in relation to tumor invasion. *World J Gastroenterol* 2005;11:4644–9.

9. Patel T, Singh P. Cholangiocarcinoma: emerging approaches to a challenging cancer. *Curr Opin Gastroenterol* 2007;23: 317–23.
10. Anderson C, Kim R. Adjuvant therapy for resected extrahepatic cholangiocarcinoma: a review of the literature and future directions. *Cancer Treat Rev* 2009;35:322–7.
11. Darnell JE, Jr. Transcription factors as targets for cancer therapy. *Nat Rev Cancer* 2002;2:740–9.
12. Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, Urieli-Shoval S, Galun E, Ben-Neriah Y. NF-κB functions as a tumour promoter in inflammation-associated cancer. *Nature* 2004;431:461–6.
13. Karin M. Nuclear factor-κB in cancer development and progression. *Nature* 2006; 441:431–6.
14. Aggarwal BB. Nuclear factor-κB: the enemy within. *Cancer Cell* 2004;6:203–8.
15. Pinlaor S, Hiraku Y, Ma N, Yongvanit P, Semba R, Oikawa S, Murata M, Sripa B, Sithithaworn P, Kawanishi S. Mechanism of NO-mediated oxidative and nitrative DNA damage in hamsters infected with *Opisthorchis viverrini*: a model of inflammation-mediated carcinogenesis. *Nitric Oxide* 2004;11:175–83.
16. Aggarwal BB, Shishodia S. Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem Pharmacol* 2006;71:1397–421.
17. Pinlaor S, Yongvanit P, Prakobwong S, Kaewsamut B, Khoontawad J, Pinlaor P, Hiraku Y. Curcumin reduces oxidative and nitrative DNA damage through balancing of oxidant-antioxidant status in hamsters infected with *Opisthorchis viverrini*. *Mol Nutr Food Res* 2009;53:1316–28.
18. Pinlaor S, Prakobwong S, Hiraku Y, Pinlaor P, Laothong U, Yongvanit P. Reduction of periductal fibrosis in liver fluke-infected hamsters after long-term curcumin treatment. *Eur J Pharmacol* 2010; 638:134–41.
19. Gescher AJ, Sharma RA, Steward WP. Cancer chemoprevention by dietary constituents: a tale of failure and promise. *Lancet Oncol* 2001;2:371–9.
20. Prakobwong S, Pinlaor S, Yongvanit P, Sithithaworn P, Pairojkul C, Hiraku Y. Time profiles of the expression of metalloproteinases, tissue inhibitors of metalloproteases, cytokines and collagens in hamsters infected with *Opisthorchis viverrini* with special reference to peribiliary fibrosis and liver injury. *Int J Parasitol* 2009;39:825–35.
21. Singh S, Aggarwal BB. Activation of transcription factor NF-κB is suppressed by curcumin (diferuloylmethane) [corrected]. *J Biol Chem* 1995;270: 24995–5000.
22. Aggarwal S, Ichikawa H, Takada Y, Sandur SK, Shishodia S, Aggarwal BB. Curcumin (diferuloylmethane) down-regulates expression of cell proliferation and antiapoptotic and metastatic gene products through suppression of IκBα kinase and Akt activation. *Mol Pharmacol* 2006;69: 195–206.
23. Duffield JS, Forbes SJ, Constandinou CM, Clay S, Partolina M, Vuthoori S, Wu S, Lang R, Iredale JP. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest* 2005;115:56–65.
24. Prakobwong S, Yongvanit P, Hiraku Y, Pairojkul C, Sithithaworn P, Pinlaor P, Pinlaor S. Involvement of MMP-9 in peribiliary fibrosis and cholangiocarcinogenesis via Rac1 dependent DNA damage in a hamster model. *Int J Cancer* 2010;127:2576–87.
25. Plummer SM, Holloway KA, Manson MM, Munks RJ, Kaptein A, Farrow S, Howells L. Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-κB activation via the NIK/IKK signalling complex. *Oncogene* 1999;18:6013–20.
26. Chun KS, Keum YS, Han SS, Song YS, Kim SH, Surh YJ. Curcumin inhibits phorbol ester-induced expression of cyclooxygenase-2 in mouse skin through suppression of extracellular signal-regulated kinase activity and NF-κB activation. *Carcinogenesis* 2003;24:1515–24.
27. Han SS, Keum YS, Seo HJ, Surh YJ. Curcumin suppresses activation of NF-κB and AP-1 induced by phorbol ester in cultured human promyelocytic leukemia cells. *J Biochem Mol Biol* 2002;35: 337–42.
28. Kunnumakkara AB, Diagaradjane P, Anand P, Harikumar KB, Deorukhkar A, Gelovani J, Guha S, Krishnan S, Aggarwal BB. Curcumin sensitizes human colorectal cancer to capecitabine by modulation of cyclin D1, COX-2, MMP-9, VEGF and CXCR4 expression in an orthotopic mouse model. *Int J Cancer* 2009;125:2187–97.
29. Bharti AC, Shishodia S, Reuben JM, Weber D, Alexanian R, Raj-Vadhan S, Estrov Z, Talpaz M, Aggarwal BB. Nuclear factor-κB and STAT3 are constitutively active in CD138+ cells derived from multiple myeloma patients, and suppression of these transcription factors leads to apoptosis. *Blood* 2004;103:3175–84.
30. Kim YS, Ahn Y, Hong MH, Joo SY, Kim KH, Sohn IS, Park HW, Hong YJ, Kim JH, Kim W, Jeong MH, Cho JG, et al. Curcumin attenuates inflammatory responses of TNF-α-stimulated human endothelial cells. *J Cardiovasc Pharmacol* 2007;50:41–9.
31. Chadalapaka G, Jutooru I, Chintharlapalli S, Papineni S, Smith R, III, Li X, Safe S. Curcumin decreases specificity protein expression in bladder cancer cells. *Cancer Res* 2008;68:5345–54.
32. Taniai M, Grambihler A, Higuchi H, Werneburg N, Bronk SF, Farrugia DJ, Kaufmann SH, Gores GJ. Mcl-1 mediates tumor necrosis factor-related apoptosis-inducing ligand resistance in human cholangiocarcinoma cells. *Cancer Res* 2004; 64:3517–24.
33. Isomoto H, Mott JL, Kobayashi S, Werneburg NW, Bronk SF, Haan S, Gores GJ. Sustained IL-6/STAT-3 signaling in cholangiocarcinoma cells due to SOCS-3 epigenetic silencing. *Gastroenterology* 2007; 132:384–96.
34. Blechacz BR, Smoot RL, Bronk SF, Werneburg NW, Sirica AE, Gores GJ. Sorafenib inhibits signal transducer and activator of transcription-3 signaling in cholangiocarcinoma cells by activating the phosphatase shatterproof 2. *Hepatology* 2009;50:1861–70.
35. Sripa B, Mairiang E, Thinkhamrop B, Laha T, Kaewkes S, Sithithaworn P, Tessana S, Loukas A, Brindley PJ, Bethony JM. Advanced periductal fibrosis from infection with the carcinogenic human liver fluke *Opisthorchis viverrini* correlates with elevated levels of interleukin-6. *Hepatology* 2009;50:1273–81.
36. Ishimura N, Bronk SF, Gores GJ. Inducible nitric oxide synthase up-regulates Notch-1 in mouse cholangiocytes: implications for carcinogenesis. *Gastroenterology* 2005;128: 1354–68.
37. Kobayashi S, Werneburg NW, Bronk SF, Kaufmann SH, Gores GJ. Interleukin-6 contributes to Mcl-1 up-regulation and TRAIL resistance via an Akt-signaling pathway in cholangiocarcinoma cells. *Gastroenterology* 2005;128:2054–65.
38. Sahu RP, Batra S, Srivastava SK. Activation of ATM/Chk1 by curcumin causes cell cycle arrest and apoptosis in human pancreatic cancer cells. *Br J Cancer* 2009; 100:1425–33.
39. Ma N, Kawanishi M, Hiraku Y, Murata M, Huang GW, Huang Y, Luo DZ, Mo WG, Fukui Y, Kawanishi S. Reactive nitrogen species-dependent DNA damage in EBV-associated nasopharyngeal carcinoma: the relation to STAT3 activation and EGFR expression. *Int J Cancer* 2008;122: 2517–25.
40. Jinawath N, Chamgramol Y, Furukawa Y, Obama K, Tsunoda T, Sripa B, Pairojkul C, Nakamura Y. Comparison of gene expression profiles between *Opisthorchis viverrini* and non-*Opisthorchis viverrini* associated human intrahepatic cholangiocarcinoma. *Hepatology* 2006;44: 1025–38.

41. Valdembri D, Serini G, Vacca A, Ribatti D, Bussolino F. In vivo activation of JAK2/STAT-3 pathway during angiogenesis induced by GM-CSF. *FASEB J* 2002;16:225–7.
42. Leo E, Deveraux QL, Buchholtz C, Welsh K, Matsuzawa S, Stennicke HR, Salvesen GS, Reed JC. TRAF1 is a substrate of caspases activated during tumor necrosis factor receptor-α-induced apoptosis. *J Biol Chem* 2001;276:8087–93.
43. Fotin-Mleczek M, Henkler F, Hausser A, Glauner H, Samel D, Graness A, Scheurich P, Mauri D, Wajant H. Tumor necrosis factor receptor-associated factor (TRAF) 1 regulates CD40-induced TRAF2-mediated NF-κB activation. *J Biol Chem* 2004;279:677–85.
44. Wang X, Wang Q, Hu W, Evers BM. Regulation of phorbol ester-mediated TRAF1 induction in human colon cancer cells through a PKC/RAF/ERK/NF-κB-dependent pathway. *Oncogene* 2004;23:1885–95.
45. Du Q, Zhang X, Cardinal J, Cao Z, Guo Z, Shao L, Geller DA. Wnt/beta-catenin signaling regulates cytokine-induced human inducible nitric oxide synthase expression by inhibiting nuclear factor-κB activation in cancer cells. *Cancer Res* 2009;69:3764–71.
46. Magalhaes LG, Machado CB, Morais ER, Moreira EB, Soares CS, da Silva SH, Da Silva Filho AA, Rodrigues V. In vitro schistosomicidal activity of curcumin against *Schistosoma mansoni* adult worms. *Parasitol Res* 2009;104:1197–201.
47. Chuang SE, Cheng AL, Lin JK, Kuo ML. Inhibition by curcumin of diethylnitrosamine-induced hepatic hyperplasia, inflammation, cellular gene products and cell-cycle-related proteins in rats. *Food Chem Toxicol* 2000;38:991–5.
48. Goel A, Kunnumakkara AB, Aggarwal BB. Curcumin as "Curecumin": from kitchen to clinic. *Biochem Pharmacol* 2008;75:787–809.
49. Suphim B, Prawan A, Kukongviriyapan U, Kongpetch S, Buranrat B, Kukongviriyapan V. Redox modulation and human bile duct cancer inhibition by curcumin. *Food Chem Toxicol* 2010;48:2265–72.