CYP24A1 Involvement in Inflammatory Factor Regulation Occurs via the Wnt Signaling Pathway*

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[**Abstract**] **Objective:** While the upregulation of cytochrome P450 family 24 subfamily A member 1 (*CYP24A1*) gene expression has been reported in colon cancer, its role in tumorigenesis remains largely unknown. In this study, we aimed to investigate the involvement of *CYP24A1* in Wnt pathway regulation *via* the nuclear factor kappa B (NF-κB) pathway. **Methods:** The human colon cancer cell lines HCT-116 and Caco-2 were subjected to stimulation with interleukin-6 (IL-6) as well as tumor necrosis factor alpha (TNF-α), with subsequent treatment using the NF-κB pathway-specific inhibitor ammonium pyrrolidinedithiocarbamate (PDTC). Furthermore, CYP24A1 expression was subjected to knockdown *via* the use of small interfering RNA (siRNA). Subsequently, NF-κB pathway activation was determined by an electrophoretic mobility shift assay, and the transcriptional activity of β-catenin was determined by a dual-luciferase reporter assay. A mouse ulcerative colitis (UC)-associated carcinogenesis model was established, wherein TNF-α and the NF-κB pathway were blocked by anti-TNF-α monoclonal antibody and NF-κB antisense oligonucleotides, respectively. Then the tumor size and protein level of CYP24A1 were determined. **Results:** IL-6 and TNF-α upregulated CYP24A1 expression and activated the NF-κB pathway in colon cancer cells. PDTC significantly inhibited this increase in CYP24A1 expression. Additionally, knockdown of *CYP24A1* expression by siRNA could partially antagonize Wnt pathway activation. Upregulated *CYP24A1* expression was observed in the colonic epithelial cells of UC-associated carcinoma mouse models. Anti-TNF-α monoclonal antibody and NF-κB antisense oligonucleotides decreased the tumor size and suppressed CYP24A1 expression. **Conclusion:** Taken together, this study suggests that inflammatory factors may increase CYP24A1 expression *via* NF-κB pathway activation, which in turn stimulates Wnt signaling.
Key words: CYP24A1; Wnt/β-catenin signaling pathway; colorectal neoplasms

The cytochrome P450 family 24 subfamily A member 1 (*CYP24A1*) gene encodes cytochrome P450

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oxidase, which is responsible for the catalysis of the hydroxylation reaction of 1,25-dihydroxy vitamin D_3 (1,25-D_3, calcitriol). The compound 1,25-D_3, the most physiologically active form of vitamin D, is converted to less active hydroxylated products following catalysis by CYP24A1 and is ultimately excreted after the conduction of various intermediate metabolic steps[1,2]. Vitamin D can suppress the development of colon cancer at multiple stages[1,3-5]. It has been reported that increasing dietary vitamin D supplementation in a colitis mouse model, chemically induced by azoxymethane (AOM)/dextran sodium sulfate (DSS), significantly reduces atypical

hyperplasia[6]. In addition, four single nucleotide polymorphisms distributed in the *CYP24A1* gene are associated with colonic polyps and colon cancer[7]. Moreover, the expression level of the *CYP24A1* gene is reportedly elevated in colonic polyps and colon cancer compared with adjacent normal tissues[8, 9] and correlates with a poor prognosis[8]. Furthermore, the expression level of CYP24A1 may be related to the 1,25-D$_3$ concentration. However, the relationship between *CYP24A1* and ulcerative colitis (UC)-related colorectal cancer has not been studied.

The cytokines interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) can upregulate the Wnt/β-catenin pathway activity *via* nuclear factor kappa B (NF-κB) signaling. However, whether the regulation of *CYP24A1* expression is implicated in these molecular cascades has not been investigated. It has been shown that IL-6 and TNF-α upregulate *CYP24A1* expression in COGA-1A colon cancer cells[10]. Additionally, in a cell model of chronic kidney disease, the NF-κB-specific inhibitor ammonium pyrrolidinedithiocarbamate (PDTC) antagonized the indophenol sulfate-induced upregulation of CYP24A1, indicating that *CYP24A1* expression may be regulated *via* the NF-κB pathway[11]. A further study revealed that the CYP24A1 enzyme determines the local concentration of 1,25-D$_3$, which can reduce the nuclear translocation of β-catenin, thereby downregulating the Wnt pathway activity[12]. Taken together, we speculate that *CYP24A1* expression may be involved in the IL-6- and TNF-α-mediated regulation of Wnt signaling *via* the NF-κB pathway.

In this study, we investigated the function of CYP24A1 expression in Wnt pathway regulation by the cytokines IL-6 and TNF-α *via* the NF-κB pathway at the cellular level. Furthermore, the significance of CYP24A1 in UC-associated carcinogenesis was assessed in an animal model to substantiate the relationship between inflammation, tumorigenesis and *CYP24A1*.

1 MATERIALS AND METHODS

1.1 Cell Culture

The human colon cancer cell lines HCT-116 and Caco-2 were acquired from the State Key Laboratory of Molecular Oncology, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences (the cell lines were not independently authenticated). The cells were cultured in Dulbecco's modified Eagle medium/Ham's nutrient mixture F-12 (F-12) medium (HyClone, USA) and minimum essential medium/Earle's balanced salt solution (HyClone, USA), respectively, supplemented with 10% (v/v) fetal bovine serum (HyClone, USA) and 1% penicillin/streptomycin (HyClone, USA). The cells were cultured at 37°C in a 5% carbon dioxide humidified incubator. One percent

of nonessential amino acids (HyClone, USA) were added to the culture medium for Caco-2 cell culture.

1.2 Oligonucleotides

The oligonucleotides for gene silencing were chemically synthesized by GenePharma (China) based on the sequence of *CYP24A1* and are as follows: *CYP24A1* small interfering RNA (siRNA) (si-CYP24A1), 5′-CAAACAGUCUAAUGUGGAUTT-3′ (sense), 5′-AUCCACAUUAGACUGUUUGTT-3′ (antisense); siRNA negative control (siNC), 5′-UUC-UCCGAACGUGUCACGUTT-3′ (sense), 5′-ACGUG-ACACGUUCGGAGAATT-3′ (antisense); *GAPDH* positive control, 5′-UGACCUCAACUACAUGGUU-TT-3′ (sense), 5′-AACCAUGUAGUUGAGGUCATT -3′ (antisense).

1.3 Luciferase Assay

To assay β-catenin/T-cell factor (TCF) activity, a luciferase reporter assay was performed using TOPflash and FOPflash TCF reporter constructs (Millipore, USA), which contained either the TCF/lymphoid enhancer factor consensus sequence (TOPflash) or the mutant (FOPflash) TCF-binding sites, as described previously[13]. The PRL-TK plasmid was from Promega (USA), and DH5α was from Invitrogen (USA). Cells were transfected with TOPflash or FOPflash (300 ng) and pRL-TK (30 ng) with siCYP24A1 or siNC using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cell lysates were harvested at 48 h after transfection. Luciferase activity of total cell lysates was assessed using a luminometer.

1.4 Electrophoretic Mobility Shift Assay for NF-κB

For the electrophoretic mobility shift assay (EMSA), nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction reagents (Pierce Biotechnology, USA). The biotin-labeled Bio-NF-κB probe (Bio-5′-TCGACAGAGGGACTTTCC-GAGAGGC-3′) and the Cold-NF-κB probe (cold-5′-TCGACAGAGGGACTTTCCGAGAGGC-3′) were synthesized by Sango Biotech (China). Lightshift Chemiluminescent EMSA kits were purchased from Thermo Scientific (Cat. No. 20148E). EMSAs were performed using established methods[13].

1.5 Western Blotting

Western blotting analysis was carried out as previously described[13]. Cells were subjected to lysis using 500 μL of RIPA lysis buffer (Applygen Technologies, USA). Primary anti-CYP24A1 rabbit monoclonal antibody (1:1000, Cat. No. ab175976; Abcam, USA), anti-CYP24A1 rabbit polyclonal antibody (1:1000, Cat. No. 21582-1-Ap; Proteintech, USA), anti-β-catenin rabbit monoclonal antibody (1:1000, Cat. No. ab32572; Abcam), anti-β-actin mouse monoclonal antibody [1:10 000, Cat. No. 3700, Cell Signaling Technology (CST), USA], anti-GAPDH mouse monoclonal antibody (1:100 000, Cat. No.

60004-1-Ig; Proteintech, USA), as well as secondary anti-mouse (1:10 000, Cat. No. 7076S; CST, USA) and anti-rabbit (1:10 000, Cat. No. 7074P2; CST, USA) horseradish peroxidase-conjugated antibodies were used for protein labeling.

1.6 RNA Extraction, cDNA Synthesis, and Quantitative PCR (qPCR)

Total RNA was extracted from cells using Trizol reagent (Invitrogen) and treated with RQ1 DNase (Promega) to remove DNA, according to the manufacturer's instructions. The quality and quantity of the purified RNA were determined by measuring the absorbance (A) value at wavelengths of 260 nm and 280 nm and then calculating their ratio (A_{260}/A_{280}) using a SmartSpec Plus Spectrophotometer (Bio-Rad Laboratories, USA). The RNA integrity was verified by 1.5% (w/v) agarose gel electrophoresis. All RNA samples were stored at –80°C until use. Reverse transcription from RNA to cDNA was carried out using a ReverTra Ace qPCR RT Kit (Toyobo Life Science, China), according to the manufacturer's instructions.

The expression levels of the *CYP24A1* gene were detected by qPCR. The actin gene of humans was used as a control. Specific primers were designed based on the cDNA sequences. The primer sequences were as follows: 5′-GACTACCGCAAAGAAGGCTACG-3′ (forward), 5′-ACACGAGGCAGATACTTTCAAACG-3′ (reverse). The qPCR was performed on a Bio-Rad S1000 instrument with Bestar SYBR Green RT-PCR Master Mix (Toyobo). The PCR conditions consisted of an initial denaturing step at 95°C for 1 min, and 40 cycles of denaturing at 95°C for 15 s followed by annealing and extension at 60°C for 30 s. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and then normalized to the endogenous reference gene actin. For each sample, amplifications were done in triplicate.

1.7 Mice

Eight-week-old male C57BL/6 mice were purchased from Beijing Vital River Laboratory Animal Technology (China) and used for the experiments, with each mouse weighing 22±2 g. The animals were housed in transparent plastic cages under specific pathogen-free conditions. They were maintained under controlled environmental conditions (22±1°C, 40%–60% humidity, and a 12-h light/dark cycle), with free access to water and a pellet-based diet at the animal facilities of National Cancer Center/Cancer Hospital. Initially, the mice (*n*=26) were housed together (no more than 5 animals/cage) for 1 week before they were randomly divided into one of the following four groups: blank control group (without UC-associated carcinogenesis, *n*=6), model control group (with UC-associated carcinogenesis, *n*=8), NF-κB antisense oligonucleotides enema group (with UC-associated carcinogenesis,

treated with NF-κB antisense oligonucleotides, *n*=6), and TNF-α monoclonal antibody-treated group (with UC-associated carcinogenesis, treated with TNF-α monoclonal antibody, *n*=6). The inflammation-related mouse colon carcinogenesis model was generated as previously described[13]. For the NF-κB antisense oligonucleotides enema group, the UC carcinogenesis model was established, and synthesized oligonucleotides (5′-GAAACAGATCGTCCATGGT-3′) at a concentration of 20 nmol in 300 µL of drinking water were administered to mice *via* enema every 2 weeks from week 0 until the end of week 12. In the TNF-α monoclonal antibody-treated group, the UC carcinogenesis model was established, and 75 mg/kg body weight TNF-α monoclonal antibody (infliximab injection, clotrimazole) was administered intraperitoneally every 2 weeks from week 0 until the end of week 12. During the experiment, data on body weight, clinical symptoms of colitis, and animal behavior were recorded weekly. After week 12, all mice were euthanized with a lethal dose of sodium pentobarbital (200 mg/kg body weight) *via* intraperitoneal injection. Colon tissues were collected after the mice were sacrificed.

1.8 Histology and Immunohistochemical Staining

Immunohistochemistry (IHC) was carried out and analyzed as previously described[13]. An anti-CYP24A1 rabbit monoclonal antibody (1:1000 dilution, Cat. No. ab175976; Abcam) and a secondary antibody (1:1 dilution, No. PV-9000; Zsbio Commerce, USA) were used. The staining intensity (absent, 0; weak, 1; moderate, 2; and strong, 3) and the percentage of immunoreactive cells (<25%, 1; 25%–50%, 2; >50% and <75%, 3; and >75%, 4) in each sample were determined and scored independently by two researchers. The average values were documented. The concordance rate between observers was >95%.

1.9 Ethics Statement

Experiments were performed in accordance with local laws and the Council of the European Communities Directive of 1986 (86/609/EEC) Guidelines for the Care and Use of Laboratory Animals. All animal experiments were conducted in accordance with procedures approved by the Animal Care Ethics and Use Committee of Peking Union Medical College (Approval No. XHDW-2015-0032, China). Minimal numbers of animals were used, and all efforts were made to minimize animal suffering.

1.10 Statistical Analysis

The results are presented as the mean±standard error of the mean. Statistical significance was determined by the Student's *t*-test (two-tailed) or one-way analysis of variance followed by Tukey's *post hoc* test. All statistical calculations were performed using SPSS 22.0 software (IBM, USA). *P*<0.05 was considered statistically significant.

2 RESULTS

2.1 Effects of IL-6 and TNF-α on CYP24A1 Protein Levels in Caco-2 and HCT-116 Cells

Caco-2 and HCT-116 cells were subjected to treatment with different concentrations of IL-6 (Peprotech, USA) and TNF-α (Peprotech) for different time periods, and the CYP24A1 protein levels were assessed by Western blotting (fig. 1A–1H). Both

cell lines exhibited upregulated CYP24A1 protein expression that correlated with the stimulation duration and inflammatory factor concentration. Based on the results, 100 ng/mL IL-6 and 50 ng/mL TNF-α were selected as optimal concentrations for Caco-2 cell treatment, while 50 ng/mL IL-6 and 100 ng/mL TNF-α were selected for the treatment of HCT-116 cells. All stimulation experiments were performed for a duration of 6 h.
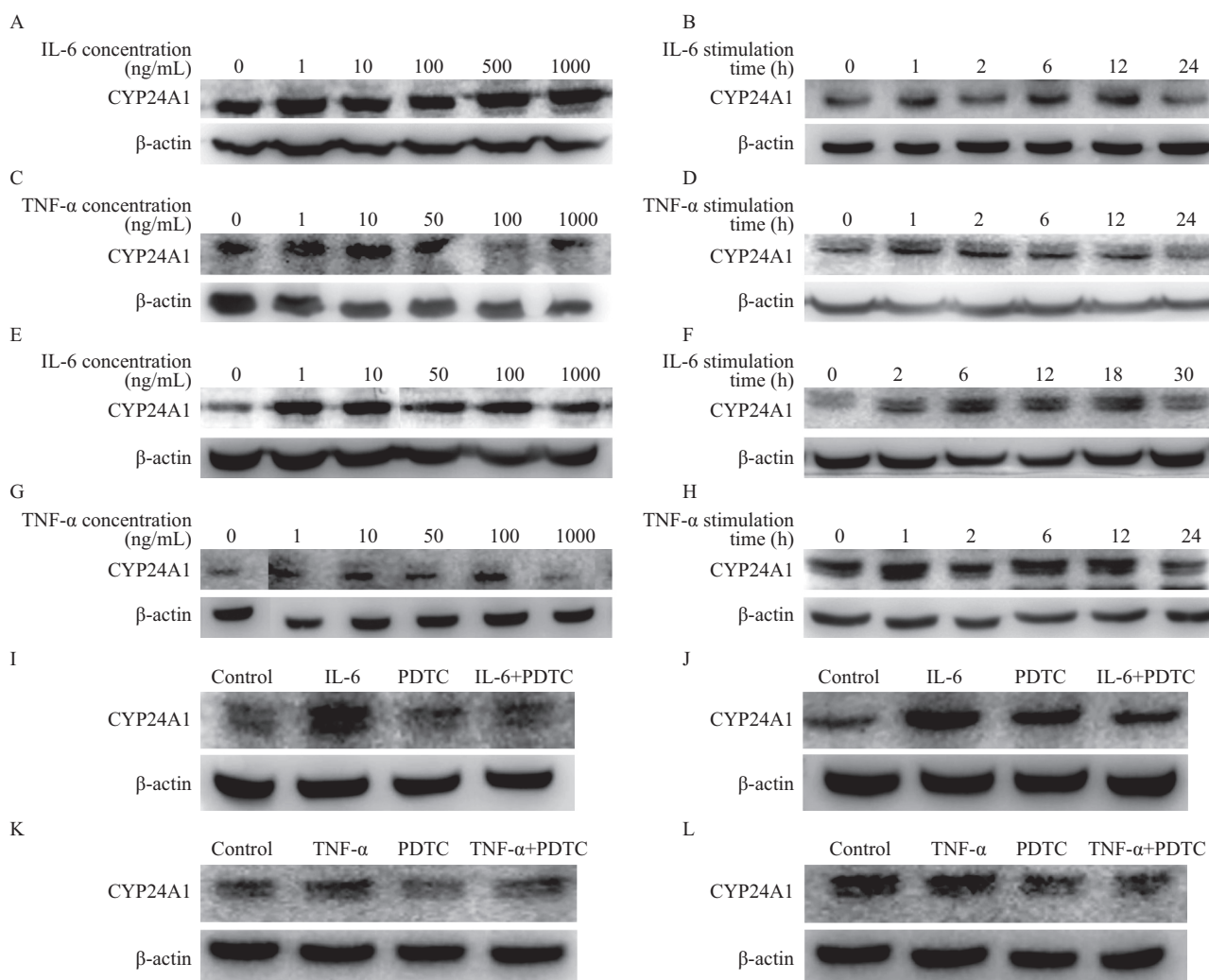
Fig. 1 Results of Western blotting analysis

A–D: *CYP24A1* expression in Caco-2 cells after stimulation with different (A) IL-6 concentrations, (B) IL-6 stimulation durations, (C) TNF-α concentrations, and (D) TNF-α stimulation durations. E–H: *CYP24A1* expression in HCT-116 cells after stimulation with different (E) IL-6 concentrations, (F) IL-6 stimulation durations, (G) TNF-α concentrations, and (H) TNF-α stimulation durations. I: *CYP24A1* expression in Caco-2 cells subjected to treatment with PDTC and IL-6. J: *CYP24A1* expression in Caco-2 cells subjected to treatment with PDTC and TNF-α. K: *CYP24A1* expression in HCT-116 cells subjected to treatment with PDTC and IL-6. L: *CYP24A1* expression in HCT-116 cells subjected to treatment by PDTC and TNF-α. Cells were subjected to treatment with 50 μmol/L PDTC for 1 h following 6 h of serum starvation and were then subjected to stimulation with different inflammatory factors for varying durations. Total protein extraction and analyses were performed, and β-actin was used as the loading control.

2.2 Effects of PDTC on the IL-6- and TNF-α-Induced Increase in CYP24A1 Expression

The NF-κB pathway-specific inhibitor PDTC was added prior to IL-6 and TNF-α treatment. PDTC significantly suppressed CYP24A1 upregulation (fig. 1I–1L) induced by the cytokines, indicating that

CYP24A1 expression might be regulated by the NF-κB pathway.

2.3 IL-6- and TNF-α-Induced Activation of the NF-κB Pathway

NF-κB activation is considered to be a major regulator of inflammation. To investigate whether

IL-6 and TNF-α can induce NF-κB translocation and DNA binding, cells were subjected to stimulation with inflammatory factors, and nuclear proteins were extracted and subjected to EMSA using an NF-κB-specific biotin-labeled probe (fig. 2A, representative experiment, *n*=3). Caco-2 and HCT-116 cells exposed to IL-6 showed induction of a nucleoprotein complex. The NF-κB DNA-protein complexes in TNF-α-treated cells did not change significantly.

2.4 IL-6- and TNF-α-Induced Wnt Pathway Activation

Wnt pathway activity was assessed *via* a dual-luciferase assay following inflammatory factor stimulation. IL-6 and TNF-α treatment activated Wnt signaling in both Caco-2 and HCT-116 cells (fig. 2B). Then *CYP24A1* expression was markedly silenced by siCYP24A1 (fig. 2C). As calcitriol is the primary inducer of CYP24A1 expression[14], cells were treated with calcitriol (10 nmol/L, Cat. No. 32222-06-3; Aladdin Reagent Factor, China) for 24 h and then with siCYP24A1. CYP24A1 expression increased with the treatment of calcitriol at both the mRNA and protein levels, and siCYP24A1 inhibited CYP24A1 expression in the presence of calcitriol, demonstrating

the specificity of the antibody and the efficacy of CYP24A1 siRNA.

Compared with the control group, the increased luciferase activity induced by IL-6 and TNF-α was attenuated after silencing *CYP24A1*, suggesting that *CYP24A1* knockdown could partially antagonize IL-6- and TNF-α-induced Wnt pathway activation (fig. 2E). To verify whether β-catenin was affected by CYP24A1 expression, *CYP24A1*-knockdown cells were subjected to treatment with inflammatory factors in the same manner. Proteins were extracted 48 h after transfection, and changes of β-catenin expression were assessed by Western blotting. The β-catenin levels did not change significantly after *CYP24A1* knockdown in both cell lines (fig. 2F).

2.5 The AOM/DSS-Induced UC Carcinoma Mouse Model

AOM/DSS-induced UC-associated carcinoma mouse models were established, and the TNF-α and NF-κB pathways were blocked using a TNF-α monoclonal antibody and NF-κB antisense oligonucleotides, respectively. The weight changes of the mice were recorded (fig. 3A). On the third day after DSS intake, the mice began to have irregular bloody stools and

E

F
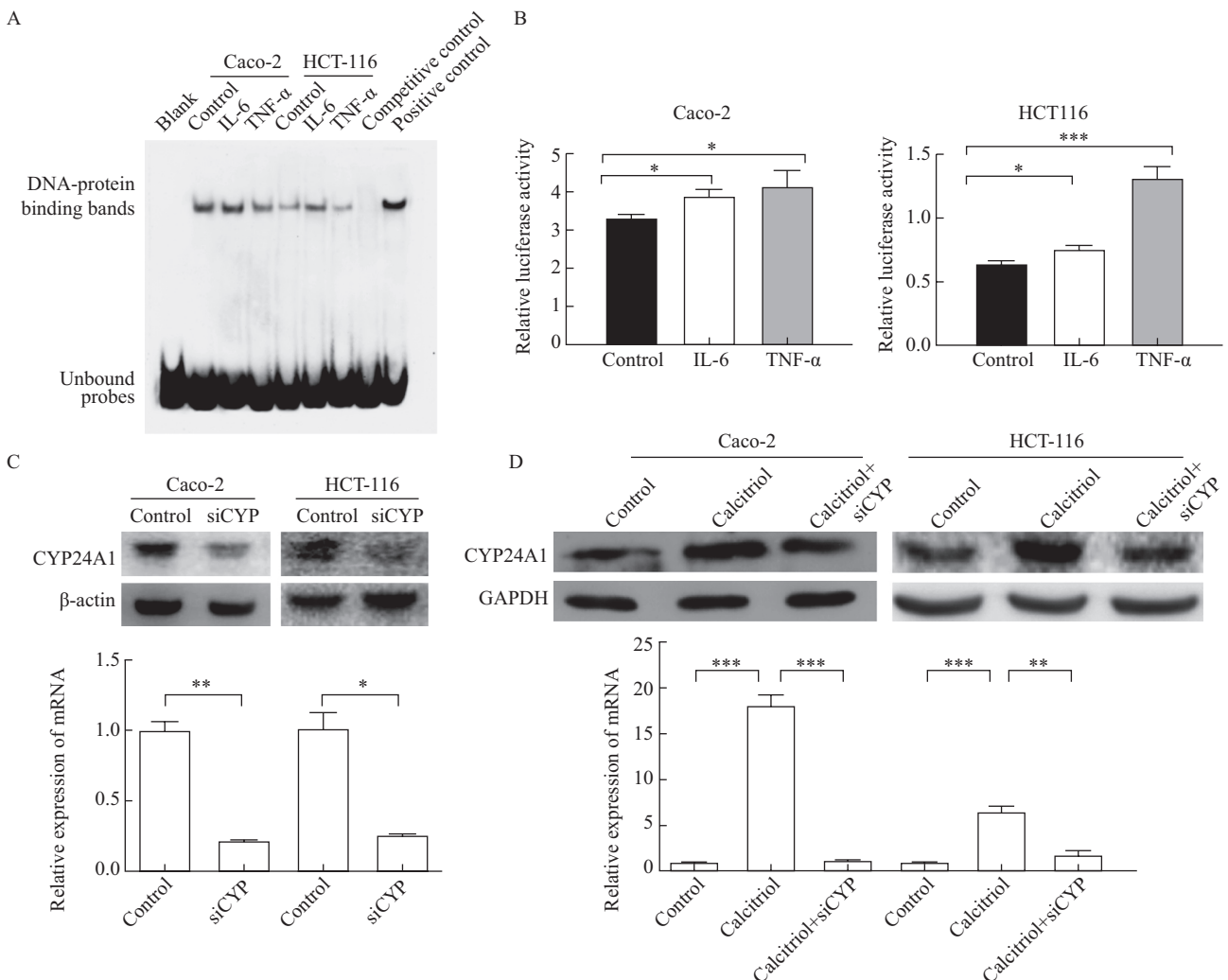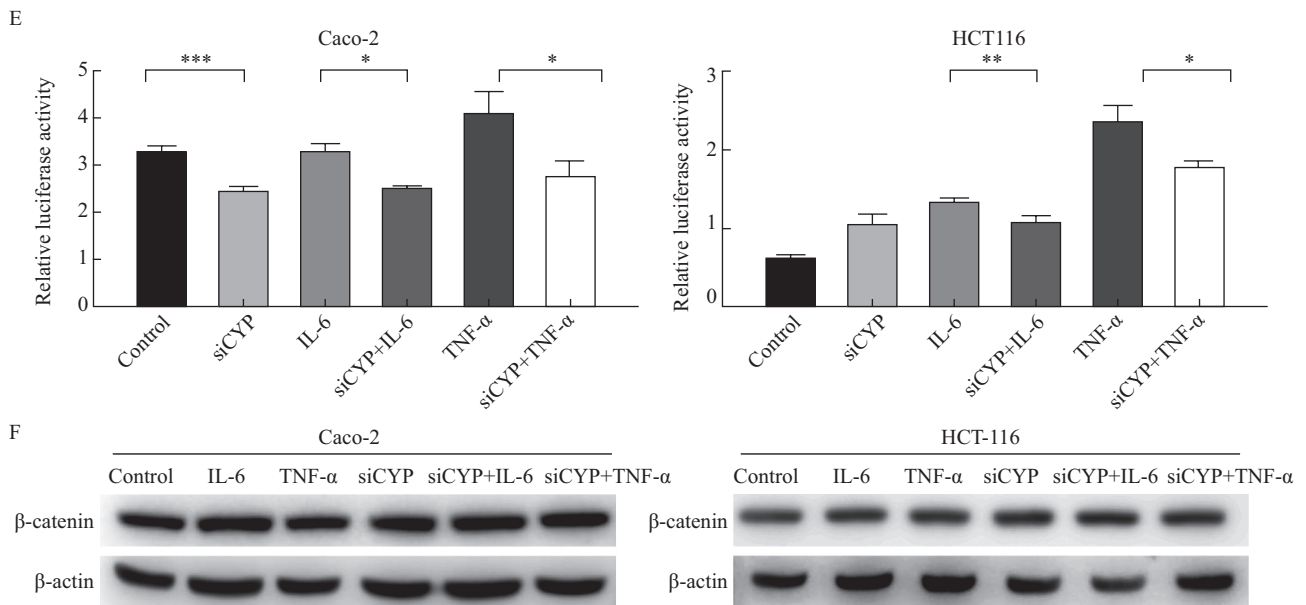
Fig. 2 Effects of inflammatory factors on the activity of different pathways

A: EMSA showing the binding of NF-kB to its promoter DNA consensus sequence. Blank lane, the background control group with only the probe and no nuclear extract. Control lane, the control group. Lane competitive control, excess cold probe blocking the specific band. Lane IL-6 and TNF-α, cells subjected to stimulation with IL-6 or TNF-α at previously determined optimal conditions. B: Stimulation with TNF-α and IL-6 increased β-catenin-mediated luciferase activity. A dual-luciferase reporter assay was performed to detect the WNT activity. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used ($^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$). C: *CYP24A1* was silenced by siRNA in different cells. CYP24A1 proteins were measured by Western blotting, and β-actin was used as the loading control. Primary anti-CYP24A1 antibody and anti-β-actin antibody were used. The expression of *CYP24A1* was also analyzed by qPCR. Data were normalized relative to actin and further normalized against the *CYP24A1* expression in the control groups. Three independent experiments were performed. Error bars represent the mean±standard deviation (SD) of triplicate experiments, and the data were analyzed by the *t*-test ($^*P<0.05$, $^{**}P<0.01$). D: Stimulation with calcitriol (10 nmol/L) for 24 h increased *CYP24A1* expression, and siCYP24A1 inhibited its expression in the presence of calcitriol at both the mRNA and protein levels. Primary anti-CYP24A1 antibody and anti-GAPDH antibody were used. The expression of *CYP24A1* was analyzed by qPCR, and the data were normalized relative to the gene actin and further normalized against the *CYP24A1* expression in the control groups of each cell line. Three independent experiments were performed. Error bars represent the mean±SD of triplicate experiments, and the data were analyzed by ANOVA ($^{**}P<0.01$, $^{***}P<0.001$). E: Transcriptional activity of β-catenin/TCF was detected by a dual-luciferase reporter gene assay. The relative luciferase activity of Caco-2 and HCT-116 cells after silencing of *CYP24A1* by siRNA decreased. Three independent experiments were performed. Error bars represent the mean±SD of triplicate experiments, and the data were analyzed by the *t*-test ($^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$). F: β-catenin proteins were measured by Western blotting in Caco-2 and HCT-116 cells. Experiments were independently repeated three times. Anti-β-catenin rabbit monoclonal antibody and anti-β-actin antibody were used. Secondary anti-mouse and anti-rabbit horseradish peroxidase-conjugated antibodies were also used for protein labeling.

diarrhea, both of which gradually worsened. After drinking plain water for 7–8 days, the mice gradually exhibited a normal fecal morphology. At week 12, no statistically significant differences in body weight were detected between the NF-κB antisense oligonucleotide group and the AOM/DSS control group, nor between the TNF-α monoclonal antibody group and the AOM/DSS control group ($P>0.05$). After the mice were sacrificed at the end of week 12, colon tumors were found in all of the AOM/DSS-treated mice, and the tumors were confined to the distal two-thirds of the colon. Several tumors of the terminal rectum could be seen fused and growing in the annulus of mice exhibiting anal prolapse (fig. 3B). The pathology of the colon tumors revealed intramucosal carcinoma or high-grade intraepithelial neoplasia. The colonic gland structure was disordered, with large nuclei, deep

staining, reduced ratio of nucleus to cytoplasm, as well as a distinctive size and morphology of tumor cells (fig. 3C). In contrast, no tumors formed in the blank control group.

The IHC results revealed that CYP24A1 expression in the AOM/DSS control group was significantly higher than that in the blank control group (fig. 4A). In addition, the tumor size in the NF-κB antisense oligonucleotide group (1.130±0.1678 cm) was significantly less than that in the AOM/DSS control group (2.294±0.3638 cm, $P=0.0354$). As expected, the expression of CYP24A1 in the NF-κB antisense oligonucleotide group was also significantly less than that in the model control group (fig. 4B). In the TNF-α monoclonal antibody group, the tumor size (1.780±0.5142 cm) was less than that in the model control group (2.294±0.3638 cm), but the difference was not statistically significant ($P=0.4195$).

A

- ■ NF-κB antisense oligonucleotide group
- ▼ TNF-α monoclonal antibody group
- ◆ AOM/DSS control group
- ● Blank control group

B

TNF-α monoclonal antibody group

NF-κB antisense oligonucleotide

AOM/DSS control group

Blank control group

C

Anti-NF-κB   Anti-TNF-α   AOM/DSS control   Blank control
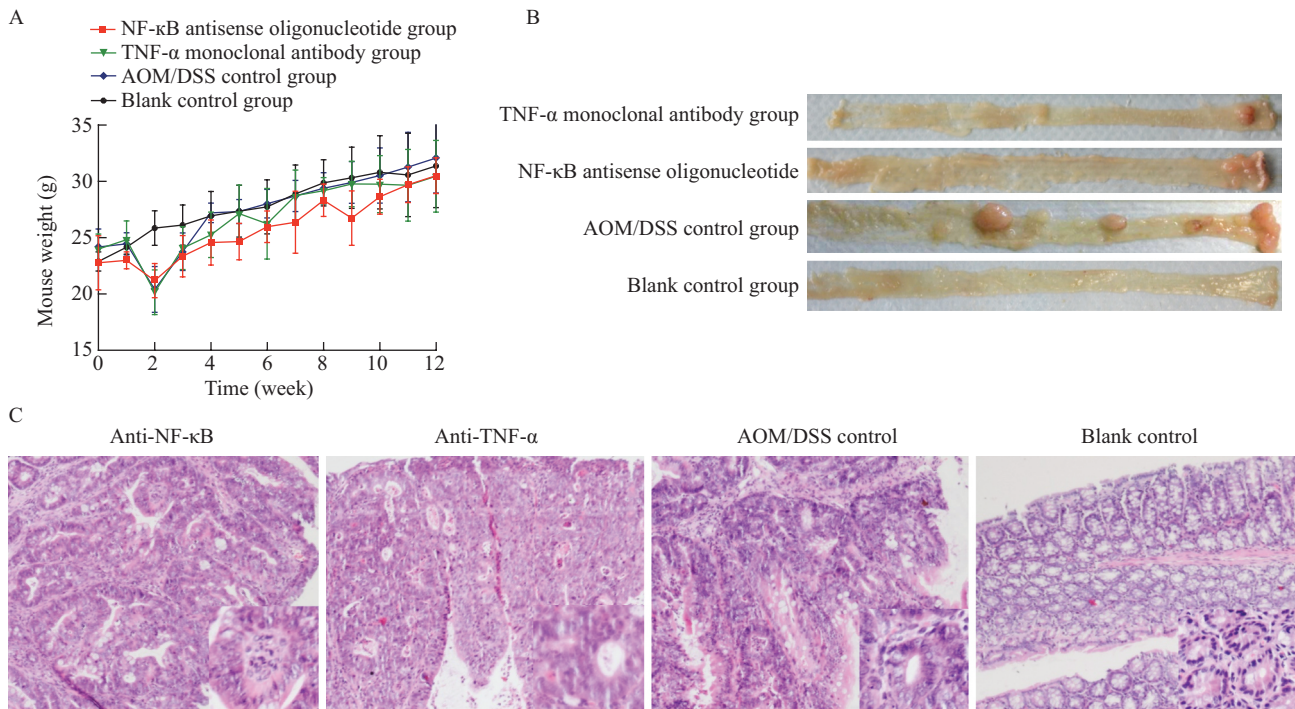
Fig. 3 Tumor size in each group
A: weight curves of mice in each group over 12 weeks; B: images of the colon and rectum of mice from each group; C: morphological examination of the colon and rectum of mice from each group

However, the TNF-α monoclonal antibody group had lower levels of CYP24A1 expression than the model control group (fig. 4C).

3 DISCUSSION

In recent years, elevated *CYP24A1* expression in colorectal tumors has received increasing attention, but the associated molecular mechanism remains unclear. Typically, *CYP24A1* is mainly expressed in the kidney and at low basal levels in other organs[3]. However, *CYP24A1* expression can be upregulated transiently in response to 1,25-D$_3$[14]. Extensive clinical studies have shown that the 1,25-D$_3$ concentration in the plasma of patients with colon polyps and colon cancer is lower than that in healthy people[4]. Considering the intrinsic low concentration of 1,25-D$_3$, *CYP24A1* expression should not be elevated in colon tissues. However, *CYP24A1* is abnormally overexpressed in colon polyps and colon cancers. Therefore, it is likely that the elevated expression of *CYP24A1* during tumorigenesis may result from other factors.

Unlike the "mutation-adenoma-cancer" carcin-ogenesis of sporadic colon cancers, the chronic inflammatory process in UC is considered to be a key factor in UC-associated carcinogenesis, but the causative mechanism is not fully understood. Inflammatory factors such as IL-6, TNF-α, IL-1β, and IFN-γ and the associated inflammatory signaling pathways, including the NF-κB, signal transducer and activator of transcription 3 (STAT3), and p38 pathways,

are critical for the transition from inflammation to cancer[15, 16]. For instance, elevated IL-6 and phosphorylated STAT3 expression levels have been reported in the intestinal epithelial cells of patients with active UC, UC intraepithelial neoplasia, UC-related colorectal cancer, and sporadic colon cancer[17], which may be instrumental for the development of colitis-associated cancer (CAC)[18–20]. In colon cancer SW480 cells, STAT3 pathway activation upregulates Wnt signaling, possibly by increasing nuclear β-catenin[21]. However, the specific mechanism underlying IL-6-induced CAC remains unknown. During inflammation, substantial amounts of TNF-α are secreted by myeloid cells in the lamina propria of the intestinal wall, thus activating the NF-κB pathway in intestinal epithelial cells. Therefore, NF-κB activation is a hallmark of the inflammatory response, and its activation can be detected in both the epithelial cells and macrophages of UC patients. Moreover, NF-κB upregulation is also considered important for inflammation-associated carcinogenesis, but whether its activation directly affects cell proliferation remains unclear[16, 22]. Taken together, the specific mechanisms of IL-6- and TNFα-induced CAC as well as the exact role of NF-κB in UC-associated carcinogenesis remain to be explored.

According to previous studies, IL-6 and TNF-α have been shown to induce an increased *CYP24A1* expression in COGA-1A colon cancer cells and human trophoblast cells[10, 23]. Furthermore, in a cell model of chronic kidney disease (proximal tubular epithelial cells subjected to treatment with indoxyl sulfate), the NF-κB-
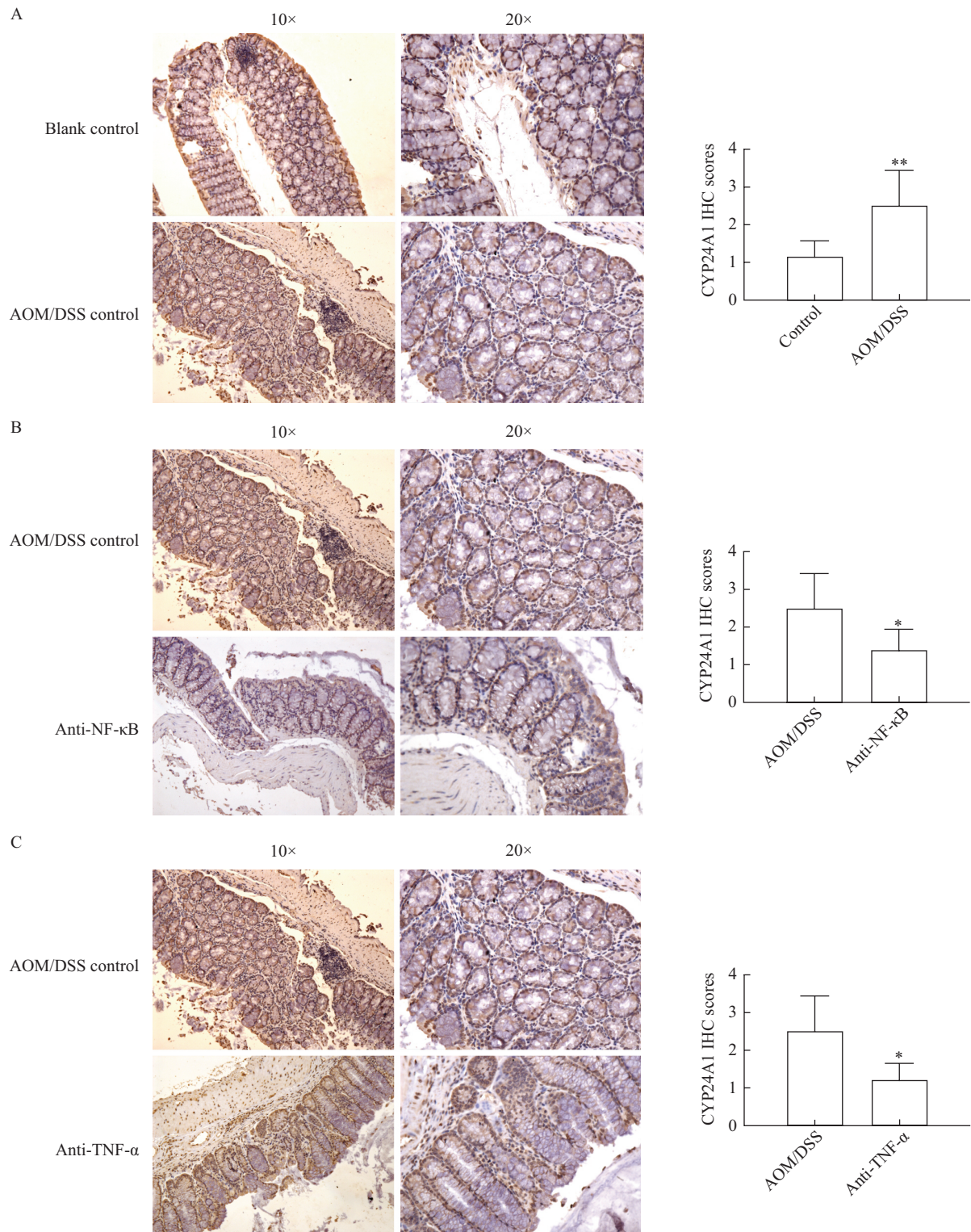
Fig. 4 CYP24A1 expression in each group

A: immunohistochemistry (IHC) of CYP24A1 and the corresponding CYP24A1 IHC scores of the AOM/DSS control group; B: IHC of CYP24A1 and the corresponding CYP24A1 IHC scores of the NF-κB antisense oligonucleotide group; C: IHC results of CYP24A1 and the corresponding CYP24A1 IHC scores of the TNF-α monoclonal antibody group. The Student's t-test with Bonferroni correction was used. $^*P<0.05$, $^{**}P<0.01$

specific inhibitor PDTC antagonized the indophenol sulfate-induced upregulation of CYP24A1, suggesting that *CYP24A1* gene expression may be regulated by the NF-κB pathway[24]. The present study revealed that IL-6 and TNF-α could upregulate *CYP24A1* expression

in HCT-116 and Caco-2 cells. Additionally, IL-6- and TNF-α-induced *CYP24A1* expression was almost suppressed upon NF-κB pathway inhibition caused by treatment with PDTC, suggesting that the process may depend on NF-κB pathway activation, in agreement

with previous research.

The EMSA results also suggested that IL-6 could induce NF-κB pathway activation, while TNF-α had no significant effect. In a previous study, NF-κB pathway activation in Caco-2 cells could be detected *via* EMSA at 1 h after stimulation with IL-6 (100 ng/mL), peaking at 2 h. Using dual-luciferase reporter genes, NF-κB pathway activation could be observed 6 h after IL-6 (100 ng/mL) and TNF-α (10 ng/mL) stimulation[25]. In another study, a dual-luciferase reporter gene assay revealed that TNF-α (10 ng/mL) stimulation led to activation of the NF-κB pathway at 0.5 h and peak of the NF-κB pathway activity at 6 h[26]. One difference between the dual-luciferase reporter gene assay and the EMSA assay is that the former may detect nontranslocated activated p65, resulting in increased signals. In contrast, only nuclear NF-κB bound to target genes was detected by EMSA in this study. Considering that NF-κB signaling upregulates the expression of its inhibitor IκBα, NF-κB pathway activation is reportedly short-lived compared to the half-life of CYP24A1. This study suggested that the level of CYP24A1 began to increase at 1–2 h after stimulation *via* cytokines and decreased at 12–24 h, consistent with downstream target gene expression changes. In the present study, NF-κB pathway activation could not be detected by EMSA at 6 h after TNF-α treatment, while the TNF-α-induced increase of CYP24A1 expression remained detectable.

The DSS-induced acute colitis mouse model is a commonly employed animal model for the recapitulation of human UC. Combining AOM and DSS allows for the rapid establishment of a UC-associated carcinoma mouse model with a high tumorigenesis rate[27]. In this study, the IHC results of the AOM/DSS control group samples suggested that CYP24A1 expression was higher than that in the blank control group, confirming the inflammation-induced localized elevation of CYP24A1 expression in colonic tissues.

In AOM/DSS mice, NF-κB antisense oligo-nucleotides administered by gavage can enter the intestinal mucosal epithelium and inhibit the activation of NF-κB, suppressing UC inflammation[24]. As the core pathway of various signaling cascades contributing to colonic inflammation, NF-κB activation can be detected in both the macrophages and epithelial cells of UC patients. While its function in UC has been elucidated, its role in UC-related colorectal cancer is not fully understood. Naugler *et al*[28] have suggested that NF-κB inhibits apoptosis by regulating the expression of B-cell lymphoma 2, B-cell lymphoma-extra large, and cellular FLICE-like inhibitory protein. Moreover, Greten[29] has found that IKKβ promotes tumorigenesis by regulating NF-κB expression and that inhibition of IKKβ activity can significantly suppress tumorigenesis in mice. In this study, the tumor size

in the NF-κB antisense oligonucleotide enema group was significantly less than that observed in the control group. In parallel, the expression level of *CYP24A1* in colon epithelial cells was also significantly lower, indicating that inhibition of the NF-κB pathway in the AOM/DSS model could lead to a decrease in both *CYP24A1* expression and tumor load. These observations suggest that the high expression of *CYP24A1* during inflammation may depend on NF-κB pathway activation and may contribute to UC carcinogenesis. As for the TNF-α monoclonal antibody group, the observed tumor size was less than that in the model control group, but the difference was not statistically significant. Additionally, the expression level of *CYP24A1* was significantly lower. Moreover, the colonic inflammation, tumor incidence, and tumor size of P55 mice lacking TNF-α receptors were significantly decreased compared to those of the wild-type mice. Tumors were also found to be significantly suppressed in wild-type mice after blockade *via* TNF-α antagonist administration[29]. In the present study, the targeted inhibition of TNF-α decreased the tumor size of mice, but the tumor size was not significantly different from that of the control group. This finding may be attributed to an insufficient number of mice in our experiments. Other inflammatory factors, such as IL-6 and IL-1β, also influence the development of UC carcinogenesis[26]; thus, inhibition of TNF-α alone cannot completely block inflammation.

Wnt/β-catenin is one of the key pathways in colon carcinogenesis, and dual-luciferase reporter gene assays can detect Wnt signaling pathway activation based on the relative luciferase activity. In this study, IL-6 and TNF-α induced an increase in Wnt pathway activation. After knockdown of *CYP24A1 via* siRNA, IL-6 and TNF-α treatment caused partially antagonistic effects in terms of Wnt pathway activation. siRNA could not completely reverse the above-described effect, and this result might be attributable to the function of various other target genes downstream of STAT3 and NF-κB.

In summary, this study revealed that IL-6 and TNF-α could upregulate the expression of *CYP24A1* in Caco-2 and HCT-116 cell lines, an effect that may be dependent on NF-κB pathway activation. Additionally, *CYP24A1* knockdown *via* siRNA partially antagonized cytokine-triggered Wnt pathway activation *via* NF-κB signaling. Moreover, *CYP24A1* was upregulated in the AOM/DSS-induced UC-associated carcinoma mouse model, and the inhibition of TNF-α and NF-κB partially reversed this effect. Nevertheless, the present study includes some limitations. For instance, due to the lack of specific IL-6 inhibitors, the effect of IL-6 on *CYP24A1* expression in AOM/DSS mouse models was not investigated. In the future, the specific function of *CYP24A1* in this context should be explored by using enemas to deliver CYP24A1 siRNA to specifically

reduce its expression in the colon, and then the inflammatory cancer transformation-related indexes can be observed.

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Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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