Chloroquine inhibits MGC803 gastric cancer cell migration via the Toll-like receptor 9/nuclear factor kappa B signaling pathway

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Abstract. Stimulation of Toll-like receptor 9 (TLR9) has been associated with invasion in various types of cancer cell *in vitro*. The present study aimed to evaluate the expression of TLR9 in MGC803 gastric cancer cells and investigate the effect of a non-specific TLR9 inhibitor, chloroquine (CQ), on MGC803 cell migration via the TLR9/nuclear factor kappa B (NFκB) signaling pathway. The expression of TLR9 was investigated using reverse transcription polymerase chain reaction (RT-PCR), flow cytometry and western blot analysis. The effects of CQ on MGC803 cell proliferation were measured by MTT colorimetric assay. The mRNA expression levels of cyclooxygenase-2 (COX-2), matrix metalloproteinase (MMP)-2, MMP-7 and NFκB p65 were evaluated by RT-PCR in MGC803 cells stimulated by various concentrations of CQ. The migration of gastric cancer cells treated with CQ at 12, 24 and 36 h was measured by wound healing assay. The results indicated that MGC803 cells expressed TLR9 and that CQ had anti-proliferative effects on MGC803 cells and inhibited mRNA expression of COX-2, MMP-2, MMP-7 and NFκB p65 ($P<0.05$). Furthermore, CQ inhibited the bioactivity of NFκB p65 and prevented the migration of MGC803 cells in a dose-dependent manner ($P<0.05$). In conclusion, the results indicated that the TLR9/NFκB signaling pathway was involved in gastric cancer cell migration and that CQ had anti-tumor activity.

Introduction

According to the World Heath Organization, gastric cancer is the second most common cause of cancer mortality worldwide, with 870,000 novel cases occurring annually (1). The invasion and metastatic spread of gastric cancer is associated with patient survival and prognosis (2). Despite the optimization of surgery, radiotherapy and chemotherapy treatments, survival rates of patients with advanced gastric cancer have remained poor (3).

Toll-like receptors (TLRs) are transmembrane receptors, which are mainly expressed in immune and epithelial cells and have an important role in conferring innate immunity (4,5). Members of the TLR family comprise ≥13 associated genes, TLR1-TLR13 (6). TLR9 is one of the most important members of the TLR family (7). This previous study indicated that high expression of TLR9 occurred not only in immune cells, but also in numerous types of cancer cell, including breast, brain, ovarian, gastric, lung and prostate cancer cells (7). The role of TLR9 expressed by tumor cells in the evasion of immune surveillance was demonstrated in animal experiments and indicated that TLR9 stimulation may lead to tumor progression, inflammation and enhanced cell survival (8). TLR9 was demonstrated to be expressed by gastric epithelial cells in the human stomach and has critical roles in the development and progression of gastric cancer (9,10). TLR9-1486C polymorphism carriers have been found to be associated with an increased risk and poorer prognosis of patients with gastric carcinoma in the Chinese population (11). Ligands binding to TLR9 activate multiple signaling factors, including nuclear factor kappa B (NFκB) and result in increased production of inflammatory mediators which leads to a higher risk of developing chronic inflammatory diseases and cancer (12). NFκB is a transcription factor involved in conferring innate and adaptive immunity and has a crucial role in mediating inflammation against infectious molecules. The NFκB signaling pathway likely links chronic inflammation and tumor development, a hypothesis which was supported by the observation that constitutively active NFκB is detected in numerous human malignancies (13). A recent study directly demonstrated that NFκB had a pivotal role in TLR-induced tumorigenesis when TLRs were activated (14). In a previous study by our group, it was revealed that TLR9 was expressed in gastric cancer and associated with a high degree of tumor differentiation (15). These findings may be useful in identifying potential prognostic markers. However, at present, the mechanism of regulation of TLR9 expression and its specific role in gastric cancer cells remain to be elucidated.

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The purpose of the present study was therefore to investigate the expression of TLR9 in human gastric cancer cells and to analyze its potential association with gastric cancer cell proliferation and migration. The non-specific inhibitor of TLR9, chloroquine (CQ), was applied to gastric cancer MGC803 cells at various time-points to evaluate the involvment the of the TLR9/NFκB signaling pathway in gastric cancer cell migration and the proliferation of tumor cells using the wound healing assay and MTT analysis. The mRNA expression levels of tumor progression- and migration-associated factors, including matrix metalloproteinase-2 (MMP-2), MMP-7 and cyclooxygenase-2 (COX-2) were also examined by reverse transcription polymerase chain reaction (RT-PCR). The results of the present study may provide an experimental basis for the development of clinical immunology treatments for gastric cancer.

Materials and methods

Chemicals. The full phosphorothioated CpG-oligodeoxynucleotide (ODN)2006 and primers were synthesized by SBS Genetech Co., Ltd. (Beijing, China). Mouse monoclonal antibodies (mAbs) against TLR9, fluorescein isothiocyanate (FITC)-conjugated TLR9, GAPDH, NFκB and immunoglobulin G (IgG)2α isotype control were purchased from Abcam (Shanghai, China). The Annexin V-FITC/propidium iodide (PI) kit was obtained from Bender (Shenzen, China). TRIzol, RPMI-1640 and fetal bovine serum (FBS) were from Gibco-BRL (Invitrogen Life Technologies, Beijing, China). CQ was purchased from Sigma-Aldrich (St. Louis, MO, USA). The reverse transcription kit was purchased from TransGen Biotechnology Co., Ltd. (Beijing, China). Bicinchoninic acid (BCA) and enhanced chemiluminescence (ECL) kits were purchased from Pierce Biotechnology, Inc. (Rockford, IL, USA). A Nuclear Extract kit was obtained from Active Motif (Carlsbad, CA, USA). Protease inhibitor mixture was bought from Roche Diagnostics (Basel, Switzerland).

MGC803 cell line and cell culture. The gastric cancer cell line MGC803 (Cell Bank, Shanghai, China) was preserved in the Ningxia Key Laboratory of Cerebrocranial Diseases. The cells were cultivated in RPMI-1640 medium which contained 10% FBS (pH 7.2), 100 U/ml penicillin and 100 g/ml streptomycin (Invitrogen Life Technologies) in 5% CO_2 at 37˚C.

Flow cytometry. MGC803 cells were detached with 2.5 g/l trypsin (containing 0.02% EDTA; Invitrogen Life Technologies) and washed with cold phosphate-buffered saline (PBS). Mouse IgG2α anti-human TLR9 mAb (dilution, 1:50) or the appropriate isotypic control mAb were used at 0.5 mg/10^6 cells for 30 min on ice. Following washing with cold PBS, cells were stained with FITC-conjugated anti-mouse antibody (dilution, 1:50) and analyzed using a BD FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). Cells were gated using forward versus side scatter to exclude dead cells and debris. Fluorescence of 10^4 cells per sample was measured in logarithmic mode for visual inspection of the distributions and in linear mode for quantifying the expression of the relevant molecules by calculating the mean fluorescence intensity.

Western blot analysis. Nuclear extracts were prepared using a Nuclear Extract kit according to the manufacturer's instructions. Total cell extracts were prepared by lysing the cells in buffer containing 1% NP40, 150 mM NaCl, 50 mM Tris-HCl, a protease inhibitor mixture, 50 mM NaF and 1 mM Na_3VO_4 for phosphatase inhibition. The protein concentration in each sample was determined using a BCA Protein Assay kit. A total of 40 μg protein was loaded onto pre-casted 10% Bis-Tris Gels (Sigma, Beijing, China) and subjected to SDS-PAGE. Transferring to a nitrocellulose membrane was performed for 3 h at 4˚C and 60 V using a wet transfer system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal protein loading was confirmed with ponceau staining (Pierce Biotechnology, Inc.) (16). Membranes were blocked in 5% nonfat dry milk in 0.1% Tween-20-Tris buffered saline (TBST; pH 7.4) overnight at 4˚C. Anti-TLR9 (mouse monoclonal to TLR9; dilution, 1:100) and anti-GAPDH (mouse monoclonal to GAPDH; dilution, 1:1,000) antibodies were incubated overnight at 4˚C in 1% nonfat dry milk in TBST. Membranes were washed three times and incubated with appropriate goat anti-mouse secondary Abs (dilution, 1:1,000) for 1 h at room temperature. Following three washes in TBST, the membranes were developed with ECL detection reagents and exposed to Hyperfilm ECL.

RT-PCR. Total RNA was isolated using the RNeasy Mini kit (Qiagen, Beijing, China). DNA was removed from total RNA using the DNA-free kit (Turbo, Shanghai, China). A total of 1 μg RNA was used to synthesize cDNA using the Advantage RT-for-PCR kit (Clontech, Wuhan, China). A total of 50 ng cDNA was subsequently used for PCR. The TLR-9 and β-actin primers were as follows: TLR9 forward, 5'-GGACACTCCCAGCTCTGAAG-3' and reverse, 5'-TTGGCTGTGGATGTTGTTGT-3'; β-actin forward, 5'-TAGAGATTGGAGGTTGTTCCT-3' and reverse, 5'-TCCACCAACTAAGAACGGCC-3'. PCR was performed as follows: 94˚C for 5 min, 35 cycles of denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec, extension at 72˚C for 30 sec and a single extension at 72˚C for 5 min.

MGC803 cells were treated with CpG-ODN2006 (5'-TCGTCGTTTTGTCGTTTTGTCGTT-3') or CQ for 24 h, prior to total RNA extraction using TRIzol. Primers and annealing temperatures were as follows: MMP-2 forward, 5'-CTTCCAAGTCTGGAGCGATGT-3' and reverse, 5'-TACCGTCAAAGGGGTATCCAT-3' (annealing temperature, 65˚C); MMP-7 forward, 5'-CGGGGTACCATAATGTCCTGAATGATACC-3' and reverse, 5'-CCCAAGCTTTGCCGTCCAGAGACAATTG-3' (annealing temperature, 66˚C); COX-2 forward, 5'-GCCTGAATGTGCCATAAGACTGAC-3' and reverse, 5'-AAACCCACAGTGCTTGACACAGA-3' (annealing temperature, 62˚C); NFκB p65 forward, 5'-GTTCACAGACCTGGCATCCGT-3' and reverse, 5'-GAGAAGTCCATGTCCGCAATG-3' (annealing temperature, 57˚C); β-actin forward, 5'-TGGCACCCAGCACAATGAA-3' and reverse, 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3' (annealing temperature, 60˚C). PCR was performed as follows: 94˚C for 3 min, followed by 35 cycles of 94˚C for 30 sec, annealing at different temperatures for 30 sec and extension at 72˚C for 30 sec, followed by a final cycle of 72˚C for 10 min. Results were analyzed with BandScan 5.0 line image analysis software (Glyko, Novato, CA, USA) to calculate relative mRNA expression levels.

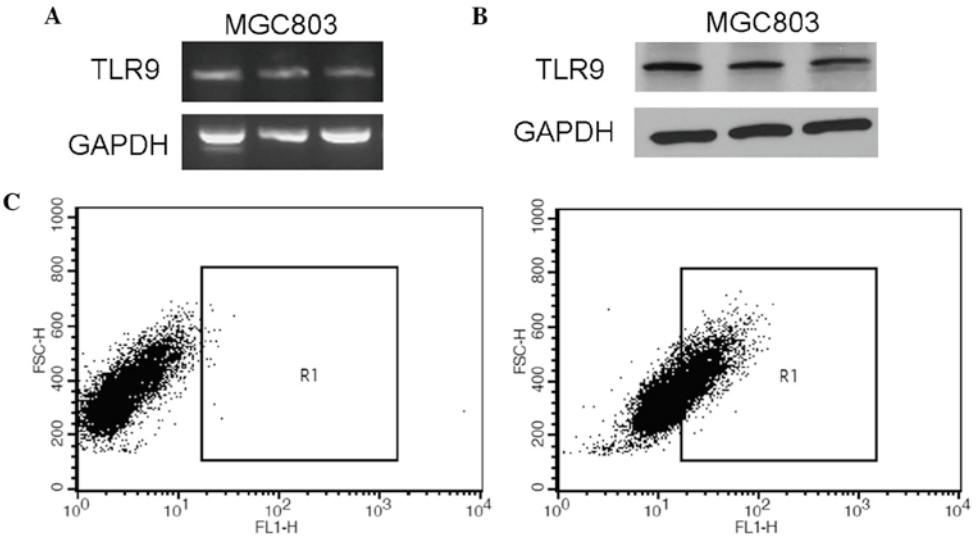

Figure 1. TLR9 expression in MGC803 cells detected by RT-PCR, western blot analysis and flow cytometry. (A) Results of RT-PCR analysis. (B) Results of western blot analysis. (C) Flow cytometric analysis. Left panel, immunoglobulin G2α control; right panel, TLR9. RT-PCR, reverse transcription polymerase chain reaction; TLR9, Toll-like receptor 9.

Cell proliferation analysis. The anti-proliferative effects of CQ on MGC803 cells were examined by MTT colorimetric assay. Cells were seeded in 96-well plates at a density of $5x10^4$ cells per well for 24 h, prior to exposure to the indicated concentrations of CQ for 24, 48 and 72 h, respectively. RPMI-1640 was used as a negative control. MTT was dissolved at a concentration of 5 mg/ml in sterile PBS at room temperature. Following removal of the medium, 20 μl was added to each well followed by 4 h of incubation. The MTT solution was aspirated and the purple formazan crystals produced by the mitochondrial dehydrogenase enzymes were dissolved in DMSO. The optical density (OD) of each well was measured at 570 nm on an ELISA reader (Bio-Rad, Beijing, China).

Wound healing assays. MGC803 cells were seeded into six-well culture plates ($1x10^5$ cells per well) and cultured for 24 h. The medium was subsequently removed and replaced with 20 μg/ml CpG-ODN 1816 RPMI-1640 medium or CQ at a concentration of 100 or 200 μg/ml once the cells had reached 80% confluence. The supernatant was discarded, scratches were produced in the confluent layer of cells using a sterile scraping cutter and the ability of cells to heal the scratch was analyzed at 12, 24 and 36 h. Images were captured of wound healing assays using an inverted microscope (CKX41SF; Olympus Corporation, Tokyo, Japan).

Statistics. Statistical differences were determined using Student's t-test for paired samples or by one-way analysis of variance followed by Student's t-test with the Bonferroni correction (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference between values.

Results

TLR9 mRNA and protein is expressed in MGC803 cells. The expression of TLR9 mRNA in the gastric cancer MGC803 cell line was analyzed by RT-PCR (Fig. 1A). The results revealed that TLR9 mRNA was expressed in MGC803 cells. To confirm the RT-PCR findings, the protein expression levels of TLR9 in MGC803 cells were examined by western blot analysis (Fig. 1B) and flow cytometric analysis (Fig. 1C). Analysis revealed that TLR9 protein was expressed in MGC803 cells.

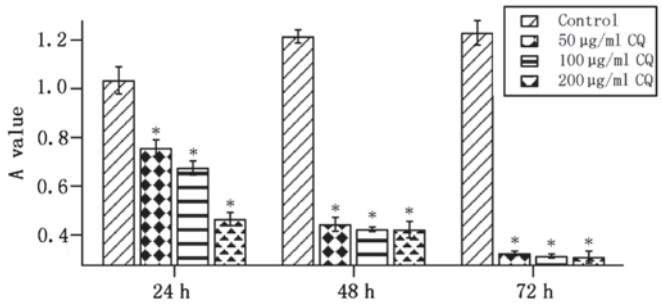

Figure 2. Effects of CQ on MGC803 cell viability, analyzed by MTT assay. Cells were treated with 50, 100 or 200 μg/ml CQ for 24, 48 or 72 h. Optical densities were measured in a microplate reader at a wavelength of 450 nm. Results were obtained from three independent experiments. *P<0.05 vs. control. CQ, chloroquine.

CQ inhibits MGC803 cell growth. When cells were treated with various concentrations of CQ (50, 100 or 150 μg/ml) for 24, 48 and 72 h, cell proliferation was significantly inhibited in all CQ-treated groups compared with that of the control group (P<0.05; Fig. 2).

CQ inhibits MMP-2, MMP-7, COX-2 and NFκB p65 mRNA expression. MGC803 cells were treated with various concentrations of CQ, in order to investigate the effects of TLR9 on MMP-2, MMP-7, COX-2 and NFκB p65 gene expression. CQ inhibited mRNA expression, while CpG-ODN enhanced mRNA expression of all four factors (P<0.05; Fig. 3).

CQ inhibits MGC803 cell migration. The wound healing assay results indicated that following CQ treatment, cell migration to

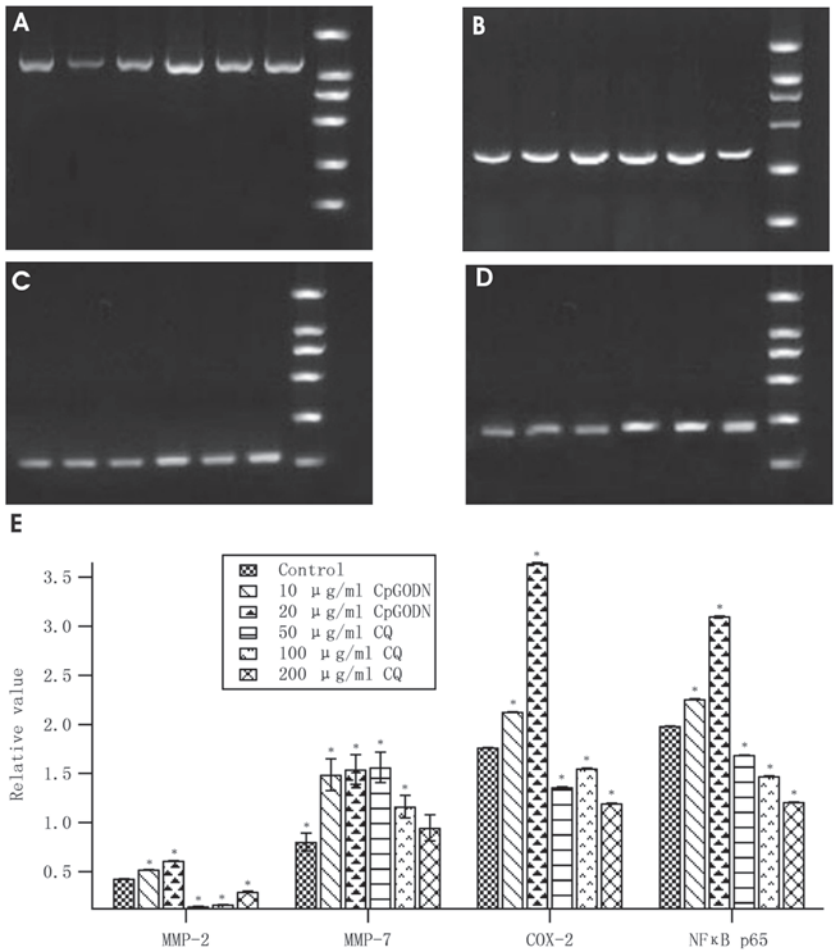

Figure 3. CQ inhibits mRNA expression levels of MMP-2, MMP-7, COX-2 and NFκB p65, detected by reverse transcription polymerase chain reaction. (A) MMP-2. (B) MMP-7. (C) COX-2. (D) NFκB p65. (E) Analysis of A, B C and D. Lanes, left to right: 1, marker; 2, 10 μg/ml CpG-ODN; 3, 20 μg/ml CpG-ODN; 4, 50 μg/ml CQ; 5, 100 μg/ml CQ; 6, 200 μg/ml CQ. *P<0.05 vs. control. CQ, chloroquine; MMP, matrix metalloproteinase; COX-2, cyclooxygenase-2; NFκB, nuclear factor-κB; CpG-ODN, CpG-oligodeoxynucleotide.

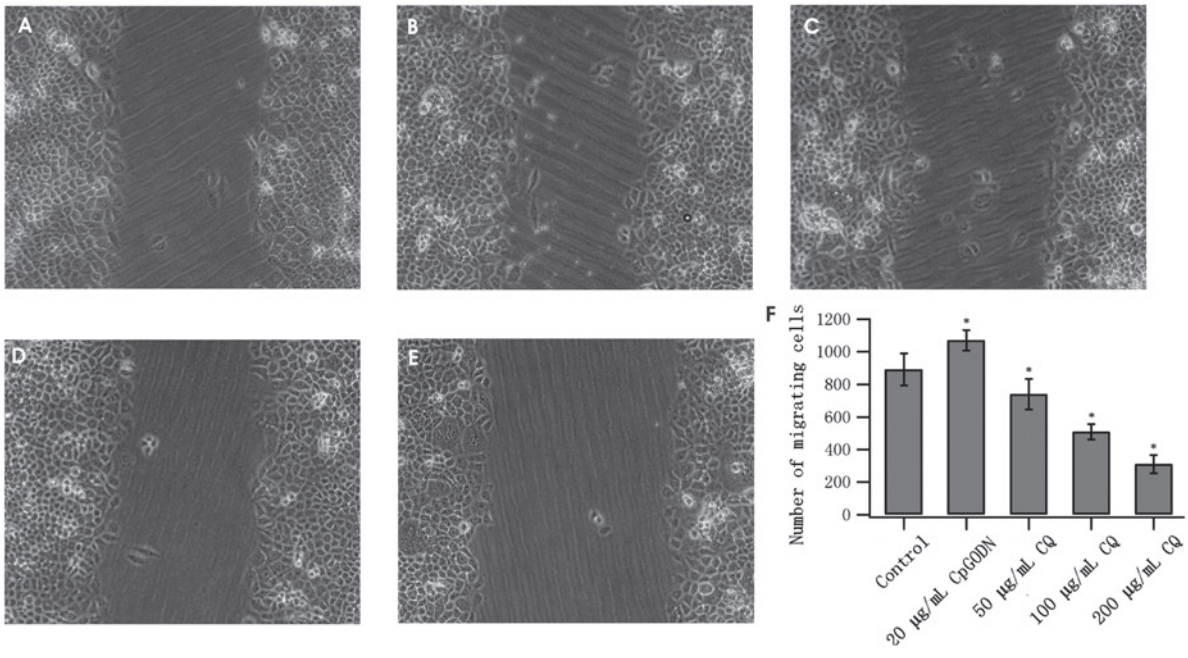

Figure 4. CQ inhibits MGC803 cell migration, indicated by wound healing assay. Cells were treated with 10 or 20 μg/ml CpG-ODN or 50, 100 or 200 μg/ml CQ for 12, 24 or 48 h. The supernatant was subsequently discarded, scratches were made in the confluent layer of cells using a sterile scraping cutter and the ability of cells to heal the scratch was analyzed. (A) Control. (B) 20 μg/ml CpG-ODN. (C) 50 μg/ml CQ. (D) 100 μg/ml CQ. (E) 200 μg/ml CQ. (Magnification, x40). (F) Analysis of A-E. *P<0.01 vs. control. CQ, chloroquine; CpG-ODN, CpG-oligodeoxynucleotide.

the damaged zone decreased and that the numbers of migrated cells following 36 h treatment were markedly decreased, except at a concentration of 50 μg/ml CQ. Conversely, CpG-ODN treatment promoted MGC803 cell migration (Fig. 4).

Discussion

Gastric cancer is one of the leading causes of mortality worldwide (17). Due to gastric cancer being prone to relapse and metastasis, the identification of prognostic factors is essential for improvement of the traditional risk classification system currently used in gastric cancer.

TLRs are important innate immunity regulators that may be activated upon recognition of bacterial and viral ligands, known as pathogen-associated molecular patterns (18). TLR9 is expressed in dendritic cells and various tissue types. TLR9 was found to be expressed in various cancer cell lines and human tumors, including non-small cell lung cancer, glioma and prostate cancer (19,20). TLR4 and TLR9 are known to be expressed by gastric epithelial cells in the human stomach (9,10). In the present study, TLR9 was demonstrated to be expressed and functional in gastric carcinoma cells. TLR9 recognizes unmethylated CpG-ODNs that are abundant in bacterial DNA, leading to NFκB activation (21,22). Participation of the NFκB signaling pathway in carcinogenesis differs between organs, cells and models. The crosstalk between an inflammatory cell and a neoplastic cell, which is instigated by the activation of NFκB, is critical for tumor organization (23). NFκB activation initiates the transcription of numerous cytokine genes involved in inflammation, evasion of apoptosis, tumor formation and transformation, including MMPs, COX and TNF-α (7,24,25). MMP-2 has been shown to have an important role in cancer metastasis (26,27). MMP-7 inhibits apoptosis of cancer cells, reduces cell adhesion and induces angiogenesis, making it easier for the cancer cells to invade small blood vessels and lymphatic tubes and metastasize (28,29). COX-2 has been detected in various tumor tissues, including pancreatic cancer, colorectal carcinoma and non-small cell lung cancer and is positively correlated with tumor invasion and lymphatic metastasis (30,31). In the present study, CQ, the non-specific inhibitor of TLR9, was demonstrated to decrease NFκB p65 mRNA expression levels and attenuate the bioactivity of NFκB p65. CQ also reduced mRNA expression of MMP-2, MMP-7 and COX-2, suggesting that the TLR9/NFκB signaling pathway may be involved in cancer occurrence and migration. However, elucidation of the specific roles of these three factors in gastric cancer requires further study.

In conclusion, in the present study, MGC803 gastric cancer cells were found to express TLR9 and the TLR9/NFκB signaling pathways were involved in cancer cell migration. The non-specific inhibitor of TLR9, CQ, inhibited cancer cell migration. Therefore, the TLR9/NFκB signaling pathway may be involved in gastric cell carcinogenesis and may represent an important therapeutic target in gastric cancer.

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