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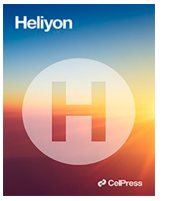

Research article

Toll-like receptor 6 inhibits colorectal cancer progression by suppressing NF-κB signaling

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ABSTRACT

Background: Toll-like receptors (TLRs) are implicated in the pathogenesis and progression of inflammation-associated cancers, except their role in regulating innate immunity. Specifically, a berrant expression of TLR6 has been observed in colorectal cancers (CRC). However, the effect of abnormal TLR6 expression on CRC remians unclear. Therefore, the present study evaluated TLR6 expression in CRC, its effect on CRC proliferation, and its underlying mechanism.

Methods: The expression of TLR6 in CRC was assessed using data from TCGA, GTEx, and HPA datasets and immunohistochemical assays of tumor tissues from patients with CRC. In human CRC cell lines, TLR6 signaling was activated using the TLR6 agonist Pam2CSK4 and was blocked using antiTLR6-IgG; subsequently, cell growth, migration, invasion, cell cycle, and apoptosis were compared in CRC cells. The levels of the anti-apoptotic protein Bcl-2 and the apoptotic protein Bax were identified using western blotting. In addition, the effect of TLR6 knockdown by shRNAs in CRC cells was observed both in vitro and in vivo. Nuclear factor κB (NF-κB) level was evaluated using immunofluorescence and western bolt.

Results: TLR6 expression was significantly downregulated in CRC tissues. The activation of TLR6 by Pam2CSK4 (100 pg/mL to 10 ng/mL) inhibited the proliferation of CRC cells. Compared with blocking TLR6 signaling using antiTLR6-IgG, activating TLR6 signaling significantly inhibited CRC cell growth, migration, and invasion as well as decreased the proportion of cells in the S and G2/M phases and promoted apoptosis. Furthermore, the knockdown of TLR6 by shRNA promoted the biological activity of CRC cells both in vitro and in vivo. Moreover, the activation of TLR6 signaling by Pam2CSK4 significantly downregulated NF-κB and Bcl-2 levels but upregulated Bax levels.

Conclusion: The findings of this study demonstrate that TLR6 may play a inhibitive role in CRC tumorigenesis by suppressing the activity of NF-κB signaling.

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1. Introduction

Colorectal cancer (CRC) is a common malignant tumor of the colon and is the second leading cause of cancer-related mortality globally. According to the GLOBOCAN project of the WHO center for cancer research, the number of new cases of CRC worldwide in 2020 is estimated to be approximately 1.93 million, and the number of deaths is estimated to be approximately 935,000 [1]. Owing to this high disease burden, CRC is considered a major global public health concern. Moreover, the prognosis for CRC is poor owing to metastasis, late detection, and limited availability of effective treatments [2].

Genetic and microenvironmental factors have been identified as risk factors for CRC development [3]. High-calorie, high-fat, low-fiber diets, particularly increased consumption of red/processed meat, as well as increasing rates of obesity and sedentary lifestyle are the major factors implicated in the initiation and progression of CRC [4]. Moreover, inflammatory diseases, such as inflammatory bowel disease, are strongly associated with the development of CRC [5–7]. Thus, owing to the intricate heterogeneity and unfavorable prognosis, new biomarkers and molecular targets for early diagnosis and effective treatment of CRC are urgently needed.

Evidence indicates that cancer development is associated with the dysregulation of innate immunity [8]. In humans, the innate immune system induces nonspecific immune responses that are activated by specific pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are cellular transmembrane receptors belonging to the interleukin-1 receptor superfamily and are the most studied type of pattern recognition receptors that play a crucial role in the recognition of PAMP and initiation of a pro-inflammatory response. Chronic inflammation has been implicated in the development of several tumors, including colon cancer [9]. Reportedly, TLRs are expressed in various normal epithelial and cancer cells. Moreover, previous studies have shown that TLRs are widely implicated in gut inflammation in colitis-associated cancers, as well as in the development of CRC [10–12].

To date, 10 functional TLRs have been identified in humans and these have been labeled TLR1 to TLR10 based on their subcellular localization. Specifically, TLR6 is expressed on the cell membrane and is an indispensable heterozygous partner of TLR2. The TLR2–TLR6 heterodimer recognizes a variety of ligands, including bacteria-derived diacyl lipoprotein, heat shock proteins, and HMGB1. In addition, the heterodimer can recognize the synthetic lipoprotein FSL-1 and Pam2CSK4 to regulate the nuclear factor κB

Table 1
Basal characteristics of 81 patients with colorectal cancer or adenomas.

Characteristics	Patients without tumor	Patients with tumor	χ^2	*p*
Number	33	48		
Age			3.098	0.078
< 65	21 (64%)	21 (44%)		
≥65	12 (36%)	27 (56%)		
Sex			0.053	0.819
Male	24 (73%)	36 (75%)		
female	9 (27%)	12 (25%)		
Localization				
Sigmoid	9	10		
Ascending colon	8	11		
Descending colon	3	6		
Transversal	5	8		
Recto	6	9		
Ileocecum	2	4		
Polyp size			3.313	0.069
	<1 cm 20 (60.6%)	<5 cm 38 (79.2%)		
	≥1 cm13 (39.4%)	≥5cm10 (20.8%)		
Histological grade				
Tubular adenoma II	27 (82%)			
Tubular adenoma III	3 (9%)			
Tubulovillous adenoma II	3 (9%)			
Grade of adenocarcinoma				
Low		7 (14.6%)		
Moderate		29 (60.4%)		
High		12 (25%)		
Type of morphology				
Uplift type		9 (19%)		
Ulcer type		36 (75%)		
Infiltration type		3 (6%)		
TNM staging				
T staging				
Tis + T1+T2		19 (39.6%)		
T3+T4		29(60.4%)		
N staging				
N0		24 (50%)		
N1+N2		24 (50%)		
M staging				
M0		24 (50%)		
M1		24 (50%)		

(NF-κB) signaling pathway. Studies indicate that TLR2 and TLR6 are widely expressed on human melanoma cells; are associated with increased risk of prostate cancer, pancreatic cancer, and non-Hodgkin lymphoma; and are negatively associated with ulcerative colitis and asthma [13–18]. Moreover, TLR6 has been reported to enhance the sensitivity of PAMP to TLR2 or expand its ligand range, enhance its signal transduction ability through heterodimerization, induce cytokine production, and promote tumor metastasis [19]. Studies have reported that TLR2 protein expression is upregulated in CRC and may be associated with poor prognosis [20]. In contrast, the expression of TLR6 is reduced in CRC tissues [21]; however, the mechanism of TLR6 and its function in CRC remains to be fully elucidated.

TLRs bind to ligands, activate intracellular molecules, release several cytokines, and participate in inflammatory and immune responses. Owing to their varied biological functions, the signaling pathways associated with each TLR may not be identical. Depending on the involvement of MyD88, the signal transduction pathways of TLRs are classified into MyD88-dependent and MyD88-independent pathways. TLR1, 2, 4, 5–7, and 9 are regulated by the MyD88-dependent signaling pathway. When stimulated, TLRs can interact via MyD88 to activate downstream signaling molecules, resulting in a series of responses. Particularly, NF-κB plays a central regulatory role in the cytokine network during TLR activation [22–24].

Accordingly, the present study evaluated the expression and effect of TLR6 on CRC progression. For this, data from Tumor genome Atlas (TCGA) and human Protein Atlas (HPA) databases were analyzed the identify the correlation between TLR6 expression and tumor. Then, expression profiles of TLR6 in CRC tissues and colon carcinoma cell lines were assessed using immunohistochemical and immunofluorescence analysis. In addition, CRC cell lines were subjected to treatment with the TLR2/TLR6 agonist Pam2CSK4 as well as antiTLR6-IgG or genetic silencing of TLR6, in order to elucidate the role of TLR6 signaling in carcinogenesis impact on cell proliferation, migration, and invasion. Furthermore, the role of the NF-κB signaling pathway in the CRC progression was evaluated.

2. Materials and methods

2.1. Dataset and data processing

Data on TLR6 expression in CRC samples were collected from TCGA, GTEx, and HPA datasets. For the TCGA dataset, the DiffExp module was used to investigate the differential expression of TLR6 in tumor and adjacent normal tissues. The distribution of *TLR6* expression was determined using box plots, and the statistical significance of differential expression was assessed using the Wilcoxon test.

2.2. Cells and CRC tissues

Tumor tissues, paired adjacent normal tissues, and colorectal adenoma tissues were collected from patients with CRC (60 males and 21 females; average age: 64.96 ± 11.90 years) who underwent surgery from 2018 to 2019 at the Yantai Yuhuangding Hospital. Information on patient sex, age, tumor anatomic site, histology, and TNM staging was obtained from clinical and pathological records (Table 1). These specimens were fixed with 10% formalin. The study was approved by the Ethics Committee of Binzhou Medical University, and all patients provided informed consent for participating in the study.

The human CRC cell lines SW480, HT29 and human normal colonic epithelial cell NCM460 were obtained from the American Type Culture Collection (Rockville, MD, USA) and authenticated using Short Tandem Repeat (STR) analysis as decribed in ANSI Standard (ASN-0002) by the ATCC Standards Development Organization (SDO). In brief, DNA was extracted by a DNA extraction Kits (CORNING, China). The twenty STRs including Amelogenin locus were amplified by six multiplex PCR, separated on Genetic Analyzer (ABI 3730XL, USA) and analyzed by the software GeneMapper. Then all cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (Gibco BRL) and 1% penicillin/streptomycin (Gibco BRL, Grand Island, NY, USA) at 37 °C under 5% CO2 and saturated humidity.

2.3. Immunohistochemistry analysis

For IHC analysis, CRC tissues, colorectal adenoma tissues, and paired adjacent normal tissues were embedded in paraffin wax, followed by sectioning them in 5-μm thick slices using a microtome (Leica Microsystems GmbH, Wetzlar, Germany). Tumor areas in the tissues were identified using hematoxylin and eosin staining. Subsequently, IHC staining was performed using the labeled streptavidin-biotin method, and the stained tissues were visualized using a Zeiss Axio Vert. A1 light microscope (Zeiss, Germany).

IHC staining was scored according to staining intensity and percentage of positive cells, and the calculation rules were as follows: staining color intensity (0, no color; 1, weak; 2, mild; 3, strong), percentage of positive cells (0, <5%; 1, 6–25%; 2, 26–50%; 3, 51–75%; 4, (>76%). The two scores were multiplied together to get the final scores. Then images (magnification, 400 times) were captured and integrated optical density (IOD) value was calculated by Image J software to verify and evaluate the expression level of TLR6. In both methods, 5 visual fields were selected randomly by blind method for evaluation and statistical analysis.

2.4. Detection of TLR6 protein expression by immunofluorescence

SW480 (50,000 cells/well) and HT29 (30,000 cells/well) cells were cultured on poly L-lysine-coated cover glass slides in 24-well culture plates. After 24 h of incubation, the slides were washed twice with phosphate-buffered saline (PBS) for 3 min each and then fixed with 4% paraformaldehyde for 30 min at 4 °C. Then, TLR6 expression in SW480 and HT29 cells was detected using

immunofluorescence staining. In brief, the cells were permeabilized, blocked, incubated with antibodies against TLR6 (InvivoGen, France) and the fluorescent secondary antibodies, and combined with the anti-fluorescence attenuation sealing solution (containing DAPI).

2.5. Determination of Pam2CSK4 levels

To screen optimal acting concentration, different concentrations of TLR6 agonist, pam2csk4, was added to activate the TLR6 of cells. Cell proliferation was detected with a cell counting kit (CCK-8) after 24h, 48h and 72h of culture. And the cell colony formation assay was determined by crystal violet staining after 10d culture.

2.6. Grouping and treatment

To dissect the impact of TLR6 on CRC cell growth, migration, invasion, and related signaling pathways, TLR6 was stimulated and inhibited in SW480 and HT29 cells. For TLR6 activation, cells were treated with the TLR2/6 agonist Pam2CSK4 at the screened concentration. For TLR6 inhibition, cells pretreated with anti-TLR6-IgG (100 ng/mL) for 1 h were subsequently treated with Pam2CSK4 (100 pg/mL). The control group received no treatment.

2.7. Cell proliferation assay

SW480 (5000 cells/well) and HT29 cells (3000 cells/well) were inoculated in 96-well plates. Then, the cells were treated with CCK-8 solution, and the absorbance was measured at 450 nm using a microplate reader to determine the cell proliferation.

For the colony formation assay, SW480 and HT29 cells were inoculated in 6-well plates at a density of 1000 cells/well. Then, colony formation was determined using crystal violet staining.

2.8. Cell migration and invasion assays

Wound-healing assay was used to determine cell migration. Briefly, cells were subjected to serum starvation overnight and were then bruised by scratching a line using a sterile pipette tip, followed by washing with PBS. Cells with different treatments were observed and imaged after 24 h of culture.

Furthermore, a cell invasion assay was conducted using 24-well Transwell chambers. Matrigel matrix 20 μg was prepared and polymerized at 37 °C for 1 h to reconstruct the substrate in the upper chamber of transwell. HT29 cells (10000/well) and SW480 cells (50000/well) were added to the upper chambers, and DMEM containing 10% FCS was added in the lower chamber. The SW480 cells were stimulated with pam2csk4 for 24 h or cultured with anti-Tlr6 for 1 h before the addition of pam2csk4. Meanwhile, HT29 cells were stimulated with pam2csk4 for 12 h or cultured with anti-TLR6-IgG for 1 h before pam2csk4 was added for 12 h. Then cells on the reverse side of the insert filter were detected by crystal violet staining.

2.9. Flow cytometry analysis for determination of cell apoptosis and cell cycle

SW480 (50,000 cells/well) and HT29 (50,000 cells/well) cells were inoculated in 6-well plates and treated with Pam2CSK4 for 24 h or with anti-TLR6-IgG for 1 h, stimulated with Pam2CSK4 for 24 h. Then cells were digested, collected, resuspends, mixed with 5 μl annexin V-FITC and 5 μl PI, incubated in dark for 15min, followed by mixed with 400 μl binding buffer, apoptosis was detected and completed within 1 h by flow cytometry.

SW480 and HT29 cells were digested, collected, resuspended, precipitated, resuspended with cooled 70% ethanol and fixed overnight at 4 °C, followed by resuspended, mixed with 0.5 mL staining working solution, incubated in dark at 37 °C for 30min, then kept in dark at 4 °C and cell cycle assay completed within 24 h by Flow cytometry.

2.10. shRNA and plasmid transfection

shRNAs targeting TLR6 were inserted into the lentiviral vector PLL3.7 to knockdown the expression of TLR6 in HT29 cells. The lentivirus used was constructed and infected as described previously [25,26]. The target sequences used were as follows:TLR6-1: 5′-CCTGCCCATCTGTAAGGAATT-3′and TLR6-2: 5′-CCCATTCCACAGAACAGCATT-3′. Then, the TLR6 shRNA plasmid was mixed with pSPAX2 and pMD2G packaging plasmids at a ratio of 4:3:1 and transfected into HEK-293T cells using the PolyJet reagent. After 48 and 72 h of transfection, the supernatant containing lentivirus was collected, filtered, and infected in HT29 cells. Subsequently, the infected cells were treated with puromycin. The effect of TLR6 knockdown was validated using quantitative polymerase chain reaction and western blotting. Then, proliferation, migration, and invasion of TLR6-knockdown HT29 cells stimulated with Pam2CSK4 were evaluated.

2.11. In vivo tumor xenograft

Ten, 4-week-old, male BALB/c-nu mice (Shanghai Model Organisms, China) were randomized (1:1) into two groups (shRNA2 group and shScr group). To establish the xenograft model, TLR6-knockdown HT29 cells and control cells were subcutaneously injected

into the back of the mice (5.0×10^6, 500 μL). The volume of tumors was measured at 1, 2, and 3 weeks. All mice were sacrificed at 3 weeks after model establishment to determine tumor weights. All animal experiments were performed according to the guidelines and were approved by the ethics committee of Binzhou Medical University.

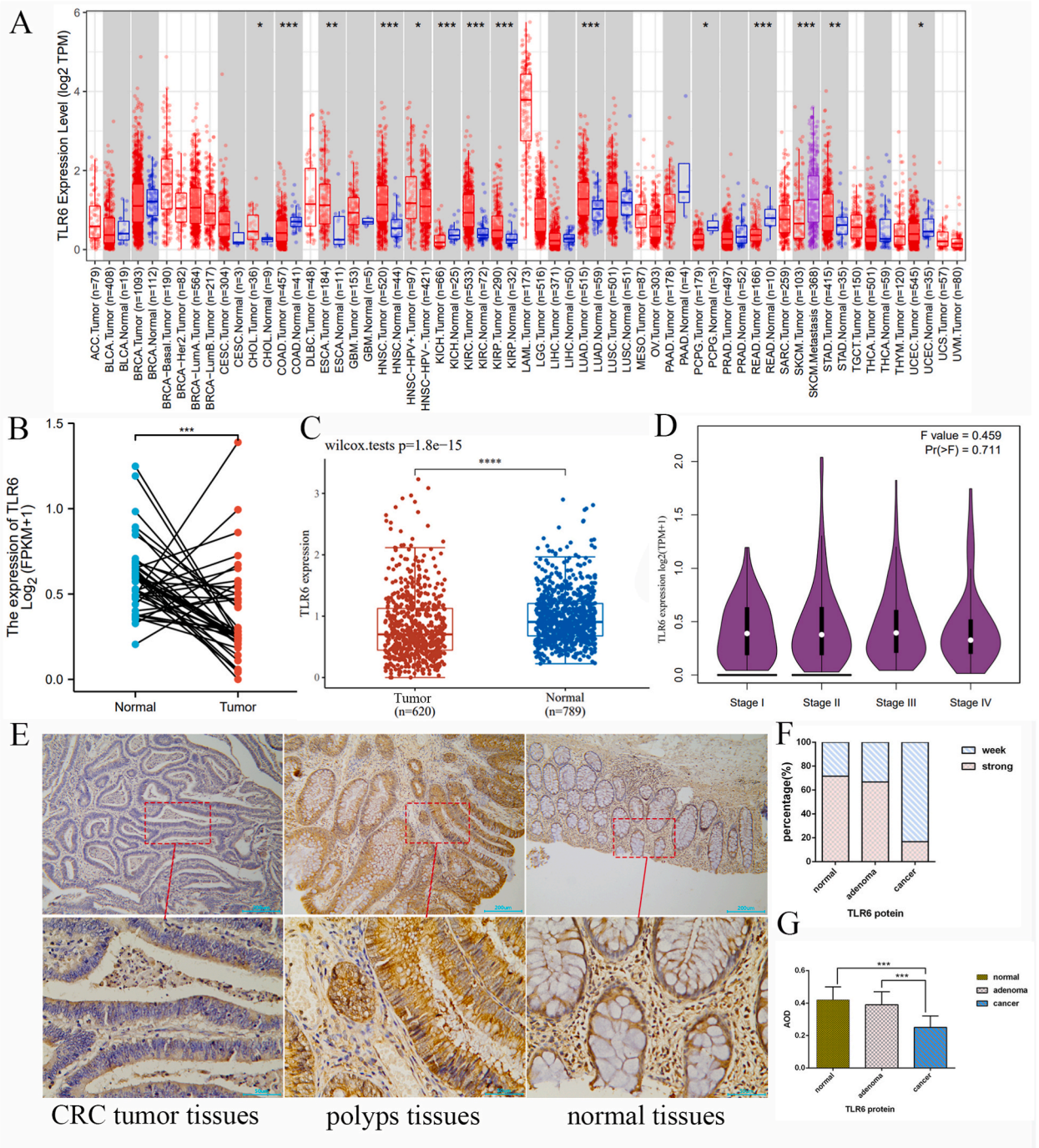

Fig. 1. Expression level of TLR6 is down-regulated in clinical CRC patients. (A) The mRNA expression of TLR6 in pan-cancer patients. (B) Paired comparison analysis showed the expression level of TLR6 genes in COAD and READ were significantly lower than that in normal tissue. (C) Expression distribution of TLR6 gene in tumor tissues lower than that in normal tissues. (D) Association between TLR6 expression and pathological stage analyzed showed there was no difference in the expression level of TLR6 in tumor with different stages. (E) Representative IHC for TLR6 in CRC tumor tissue, polyps tissue and adjacent normal tissue. (F) IHC staining scores analysis. (G) Average optical density value of IHC staining. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

2.12. Immunofluorescence to determine the effect of TLR6 signaling on NF-κB pathway

SW480 (50,000 cells/well) and HT29 (50,000 cells/well) cells were cultured on poly-L-lysine-coated cover glass slides in 24-well cell culture plates. After 24 h of incubation, the slides loaded with cells were taken out, washed twice with PBS solution for 3 min each time and fixed with 4% paraformaldehyde for 30min at 4 °C. Then, immunofluorescence staining was conducted to assess TLR6 levels in both cell types. In brief, the cells were permeabilized, blocked, incubated with antibodies against p–NF–κB and the fluorescent secondary antibodies, and combined with the anti-fluorescence attenuation sealing solution (containing DAPI).

2.13. The effect of TLR6 signal on NF-κB, bax, bcl-2 by Western blot

Apoptosis in SW480 and HT29 cells was determined using commercial kits, as per the manufacturer's instructions. The expression of NF-κB, p–NF–κB, bax, and bcl-2 in both cell types was detected using Western blot [26].

2.14. Statistical analysis

All data were analyzed by IBM SPSS Statistics 25.0 (IBM Co., Armonk, NY, USA). The data presented as the mean ± SEM ($\overline{X} \pm s$). Statistical comparisons were determined using student's t-test, one-way ANOVA test, and p value < 0.05 was considered statistically significant.

3. Results

3.1. Bioinformatics analysis demonstrated low expression of TLR6 in CRC tissues

The mRNA expression of TLR6 was evaluated in pan-cancer patients using the TCGA database. The expression of TLR6 genes in colorectal adenocarcinoma (COAD) or rectum adenocarcinoma (READ) was significantly downregulated compared with that in adjacent normal tissues ($p < 0.001$) (Fig. 1 A). Furthermore, paired comparison analysis showed that the expression of TLR6 genes in COAD and READ was significantly lower than that in normal tissues ($p < 0.001$) (Fig. 1 B). Moreover, the distribution of TLR6 expression in tumor tissues ($n = 620$) was significantly lower than that in healthy tissues ($n = 752$) obtained from TCGA and GTEx databases ($p < 0.0001$) (Fig. 1C). Moreover, no association was determined between TLR6 expression and the pathological stage of COAD and READ ($p > 0.05$) (Fig. 1 D).

Table 2
Relationship between TLR6 and clinicopathological features of colorectal cancer.

Characteristics	Patients with tumor	positive	negative	χ^2	p
Age				0.152	0.696
< 65	21	3 (14.3%)	18 (85.7%)		
≥65	27	5 (18.5%)	22 (81.5%)		
Sex				0.561	0.454
Male	36	5 (13.9%)	31 (86.1%)		
Female	12	3 (25%)	9 (75%)		
Localization of the polyp				0.246	0.620
Ascending colon + Ileocecum Descending colon + Sigmoid + Transversal	39	7 (17.9%)	32 (82.1%)		
Recto	9	1 (11.1%)	8 (88.9%)		
Polyp size				0.101	0.751
<5 cm	38	6 (15.8%)	32 (84.2%)		
≥5 cm	10	2 (20%)	8 (80%)		
Grade of adenocarcinoma				0.033	0.855
Low	7	1 (14.3%)	6 (85.7%)		
Moderate + High	41	7 (17.1%)	34 (82.9%)		
Type of morphology				0.246	0.62
Uplift type	9	2 (22.2%)	7 (77.8%)		
Ulcer type + Infiltration type	39	6 (15.4%)	33 (84.6%)		
TNM staging					
T staging				2.108	0.147
Tis + T1+T2	19	5 (26.3%)	14 (73.7%)		
T3+T4	29	3 (10.3%)	26 (89.7%)		
N staging					
N0	24	6 (25%)	18 (75%)	2.4	0.121
N1+N2	24	2 (8.3%)	22 (91.7%)		
M staging				0.600	0.439
M0	24	5 (20.8%)	19 (79.2%)		
M1	24	3 (12.5%)	21 (87.5%)		

The expression level of TLR6 in CRC samples showed no significant difference amongst the parameters of age, sex, tissue type, degree of differentiation, distant metastasis status, lymph node invasion, tumor site, tumor size, and tumor stages. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

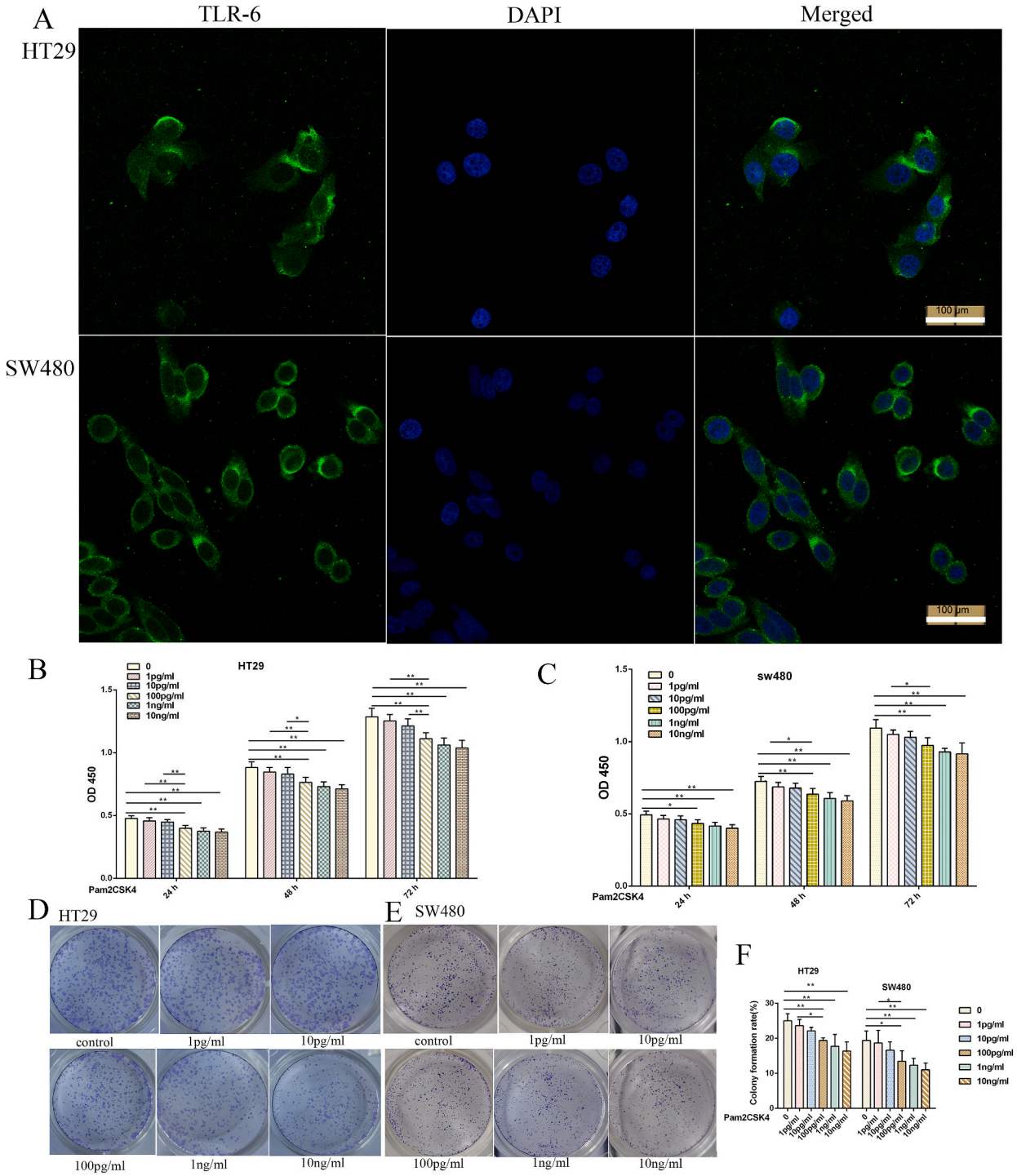

Fig. 2. Activation of TLR6 effect on CRC cell growth. (A) Representative immunofluorescence picture for TLR6 in the CRC cell lines HT29 and SW480. (B) HT29 cell proliferation was determined by CCK8 assay. (C) SW480 cell proliferation was determined by CCK8 assay. (D) Representative clone formation of HT29. (E) Representative clone formation of SW480. (F) The clone formation rate of cells treated by pam2csk4 in varied concentration. *$p < 0.05$, **$p < 0.01$, $n = 5$.

3.2. TLR6 expression in CRC tissues and matching normal colon tissues

The protein levels of TLR6 in paired CRC tissues and adjacent normal tissue were determined by IHC analysis. Approximately 16.7% (n = 48) of CRC tissues exhibited strong or moderate TLR6 staining, whereas 71.6% of matched adjacent normal tissue (n = 81) and 66.7% of polyps (n = 33) demonstrated TLR6 staining (Fig. 1 E, F). The comparison of the average optical density value was similar to the result of positive expression. The protein level of TLR6 in CRC tissues (0.25 ± 0.07) was significantly lower than that in adenoma tissues (0.39 ± 0.08) and normal tissues (0.42 ± 0.08; all $p < 0.001$) (Fig. 1 G). Furthermore, the level of TLR6 did not significantly differ among CRC tissues with regard to age, sex, tissue type, degree of differentiation, distant metastasis status, lymph node invasion, tumor site, tumor size, and tumor stages (Table 2).

3.3. Expression of TLR6 in CRC cell lines

The results of STR showed that the DNA of the cell lines matched HT29, SW480, and NCM460 in the cell line retrieval, respectively. Multiple alleles and cross-contamination of human cells were not observed in the cell lines. (Supplementary Table 1). Immunofluorescence analysis revealed the presence of TLR6 protein in the cytomembrane and cytoplasm. Moreover, TLR6 expression was higher in HT29 cells and lower in SW480 cells (Fig. 2 A). These results are consistent with the TLR6 expression observed in various CRC cell lines (https://www.proteinatlas.org/and https://portals. broadinstitute.org/ccle).

3.4. Activation of TLR6/TLR2 by Pam2CSK4 affects CRC cell growth

As shown in Fig. 3B and C, treatment of HT29 and SW480 cells with Pam2CSK4 suppressed the growth of both cell types, in a dose-

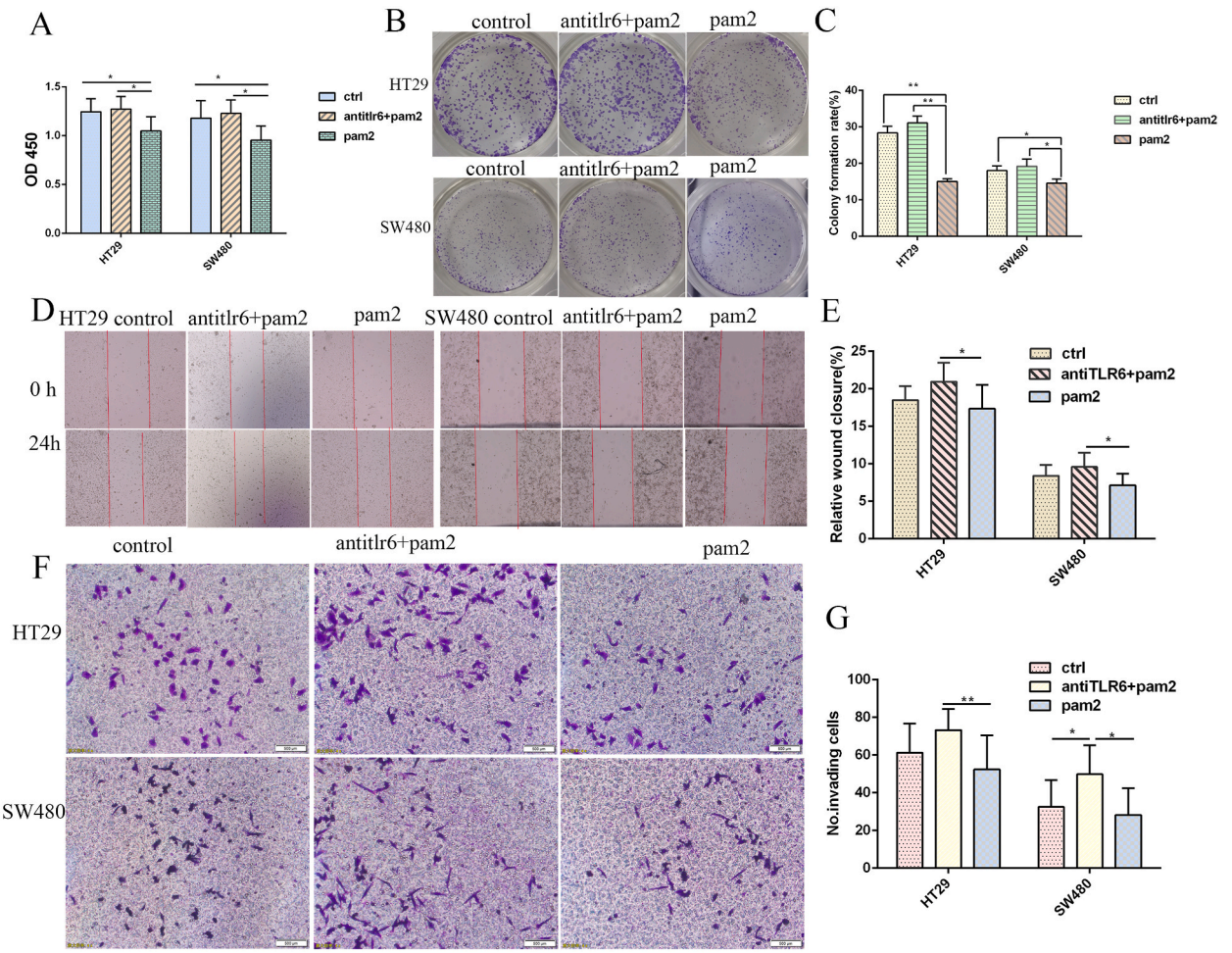

Fig. 3. TLR6 inhibits CRC cell proliferation, migration and invasion. (A) Proliferation of CRC cells treated with 100 pg/mL Pam2CSK4 or treated with antibodies against TLR6 before Pam2CSK4 stimulation respectively. (B and C) Cell colony formation. (D and E) Cell migration was detected by wound healing assay. (F and G) Cell invasion was measured using transwell assay. *$p < 0.05$, **$p < 0.01$, $n = 5$.

dependent manner (1 pg/mL–10 ng/mL) at 24 h, 48 h, and 72 h. Furthermore, the growth of HT29 cells was significantly lower after treatment with 100 pg/mL of Pam2CSK4 than after treatment with 1 pg/mL or 10 pg/mL of Pam2CSK4 ($p < 0.01$). However, cell growth did not significantly differ following treatment with 100 pg/mL, 1 ng/mL, and 10 ng/mL of Pam2CSK4 ($p > 0.05$) (Fig. 2B and

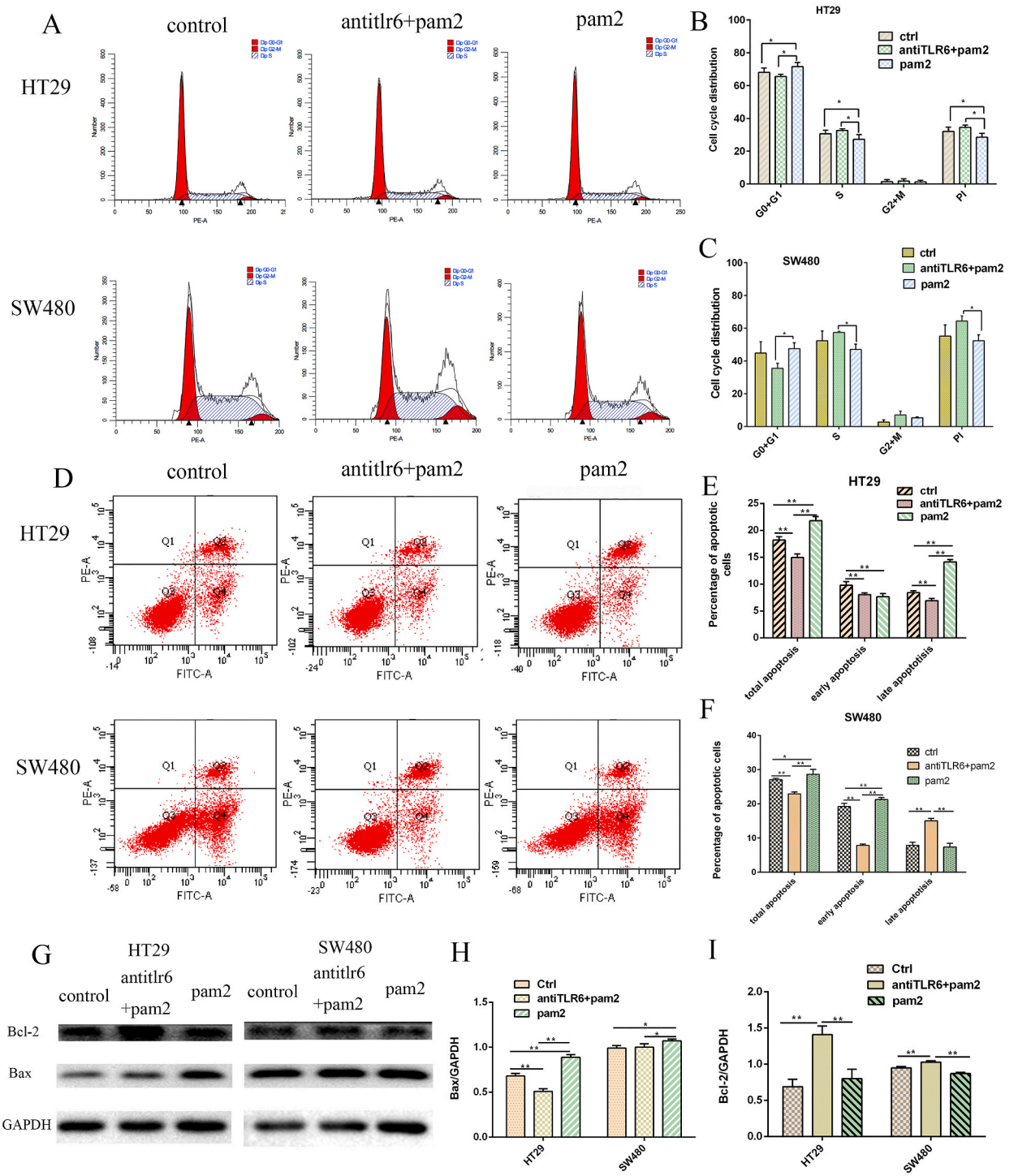

Fig. 4. *TLR6 influences on cell cycle progress and apoptosis of CRC cells.* (A and B) The effect of TLR6 on the progression of cell cycle in HT29 cells. (A and C) The effect of TLR6 on the progression of cell cycle in sw480 cells. (D, E and F) TLR6 induces CRC cell apoptosis. CRC cell lines with different treatments. (G) Representative Western blots with antibodies against Bax, Bcl-2, from HT29 and SW480 CRC cells with different treatments. (H) Analysis of Bax. (I) Analysis of Bcl-2. *$p < 0.05$. **$p < 0.01$, $n = 5$.

C). As in the cell colony formation assay, the clone formation rate of the two cell types decreased with increasing concentrations of Pam2CSK4 (1 pg/mL–10 ng/mL) on day 10. Moreover, the clone formation rate of cells was lower after treatment with 100 pg/mL of Pam2CSK4 than after treatment with 1 pg/mL ($p < 0.05$). However, the clone formation rate did not differ significantly between cells treated with 100 pg/mL, 1 ng/mL, and 10 ng/mL of Pam2CSK4 ($p > 0.05$) (Fig. 2D–F). These results indicate that Pam2CSK4 can inhibit CRC cell growth and colony formation in vitro. Moreover, simultaneous TLR6/TLR2 activation may play a role in inhibiting tumor cell growth. Accordingly, 100 pg/mL of Pam2CSK4 was chosen to stimulate TLR6/TLR2 in subsequent experiments.

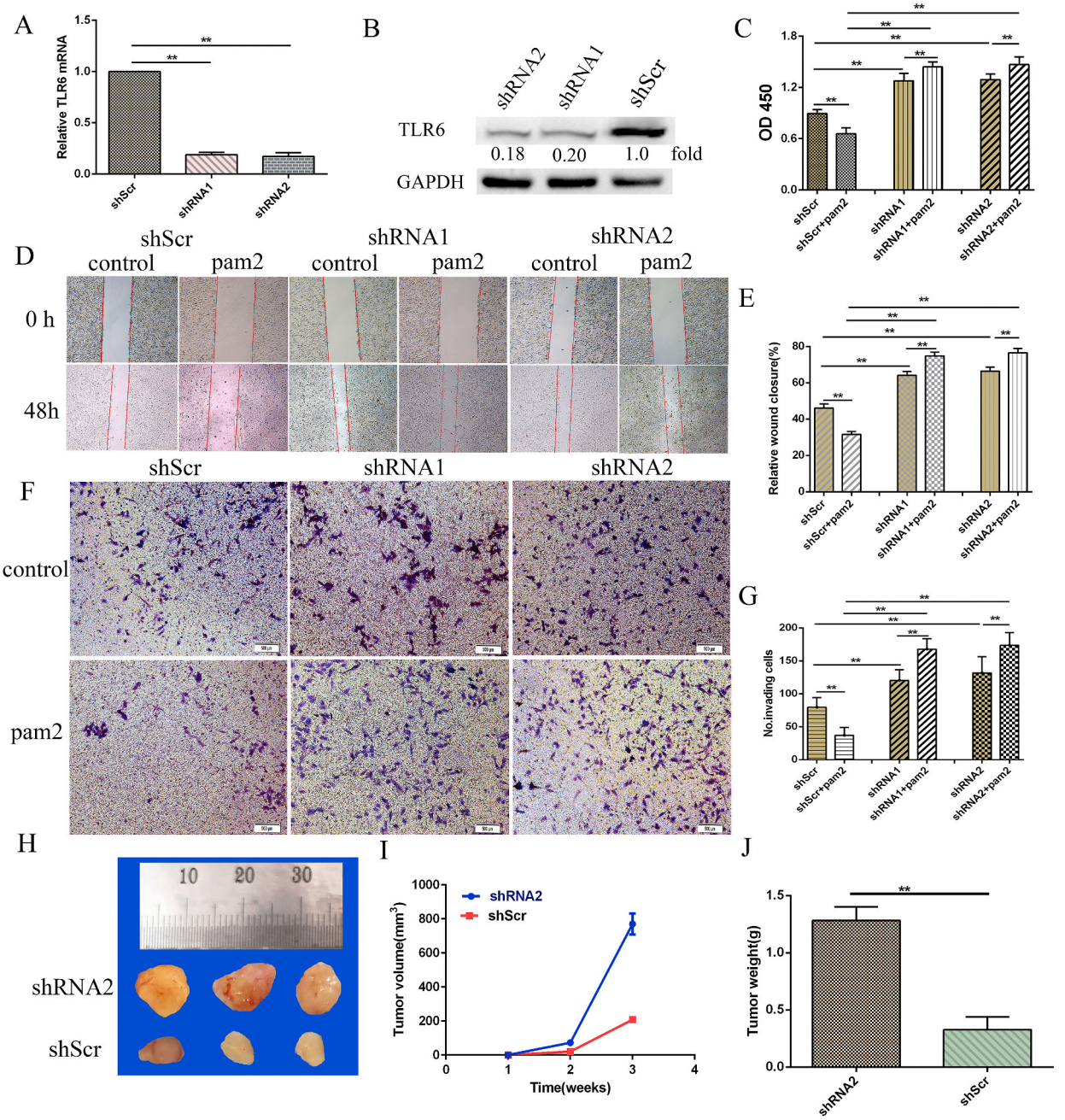

Fig. 5. Knockdown of TLR6 promotes CRC cell proliferation, migration, and invasion. (A, B) The efficiency of TLR6 silencing in HT29 cells was detected by qRT-PCR (A) and western blotting assay (B). (C) The viability of the cells was assessed using the CCK-8 assay. (D and E) Cell migration was detected by wound healing assay. (F and G) Cell invasion was measured using transwell assay. (H) Representative xenograft tumors collected from mice after 3 weeks of hypodermic injection (I) The volumes of tumors were measured at 1–3 weeks after hypodermic injection. (J) The weights of xenograft tumors after 3 weeks of hypodermic injection. *$p < 0.05$, **$p < 0.01$, $n = 5$.

3.5. TLR6 inhibits CRC cell proliferation, migration, and invasion

To further investigate the contribution of TLR6 in CRC growth, HT29, and SW480 cells were treated with anti-TLR6-IgG before Pam2CSK4 stimulation. Compared with Pam2CSK4 treatment, anti-TLR6 + Pam2CSK4 treatment significantly increased the proliferation of both CRC cell types ($P < 0.05$) (Fig. 3A). Moreover, the colony formation rate of both cell types was greater in the anti-TLR6 + Pam2CSK4 group than in the Pam2CSK4 group ($P < 0.05$) (Fig. 3B and C). In contrast to the individual treatment with Pam2CSK4, the migration of HT29 and SW480 cells before stimulation with the antibody against TLR6 was significantly increased (Fig. 3D and E). The findings of the Transwell assay demonstrated that the cellular invasion of both cell types was significantly elevated in the anti-TLR6 + Pam2CSK4 group (Fig. 3F and G). However, treatment with Pam2CSK4, there was no significant alteration in colon epithelial NCM 460 cells, and the growth rate had not any notable difference compared to the control group (Supplementary Fig. 1). Collectively, these results indicate that TLR6 signaling inhibits CRC cell growth, migration, and invasion.

3.6. TLR6 affects CRC cell cycle progress

To determine the effect of TLR6 on CRC cell cycle progression, HT29 and SW480 cells were stained by propidium iodide and detected using flow cytometry. After Pam2CSK4 stimulation, the proportion of both cell types in the S phase and G2/M phase decreased significantly ($p < 0.05$). However, compared with Pam2CSK4 treatment, anti-TLR6 + Pam2CSK4 treatment increased the proportion of cells in the S phase and G2/M phase ($p < 0.05$) (Fig. 4A, B, C). These data suggest that the activation of TLR6 signaling plays a role in inhibiting the progression of the CRC cell cycle.

3.7. TLR6 induces apoptosis in CRC cells

To determine whether TLR6 is involved in the apoptosis of CRC cells, the impact of TLR6 on apoptosis in CRC cells and the protein levels of Bax and Bcl-2 were detected. Compared with Pam2CSK4 treated HT29 cells, antiTLR6 + Pam2CSK4treated and untreated cells demonstrated significantly lower apoptosis, with the anti-TLR6 + Pam2CSK4treated group demonstrating the lowest rate of late-stage apoptosis and total apoptosis ($p < 0.01$). The early apoptosis rate and total apoptosis rate of SW480 cells in the anti-TLR6 + Pam2CSK4 group were the lowest, with statistically significant differences ($p < 0.01$) (Fig. 4 D, E, F). Furthermore, the protein levels of Bax in the anti-TLR6 + Pam2CSK4 group were lower than those in the untreated group or Pam2CSK4 group. The level of the anti-apoptotic protein Bcl-2 was higher in the anti-TLR6 + Pam2CSK4 group than in the untreated or Pam2CSK4 group (Fig. 4 G, H, I and Supplementary Fig. 2). These data demonstrate that Pam2CSK4 promotes apoptosis in CRC cells by activating the TLR6 signaling pathway while pre-antagonizing the TLR6 signal decreases the apoptosis rat.

3.8. Knockdown of TLR6 promotes CRC cell proliferation, migration, and invasion

To further evaluate the function of TLR6 in CRC, TLR6 expression in HT29 cells was suppressed using shRNA. And proliferation, migration, and invasion of TLR6-knockdown HT29 cells stimulated with Pam2CSK4 were evaluated. The results revealed that both shRNA-1 and shRNA-2 could considerably knockdown TLR6 expression at both mRNA and protein levels (Fig. 5 A, B and Supplementary Fig. 3); thus, these two were used for further assessments. The results of the CCK-8 assay revealed that the proliferation of HT29 cells was significantly higher in the TLR6-knockdown group than in the control group. The proliferation of TLR6-knockdown cells was significantly increased following Pam2CSK4 treatment, while a decrease in proliferation was observed in shScr-transfected cells ($P < 0.05$) (Fig. 5C). Furthermore, shTLR6-transfected HT29 showed a significantly faster wound healing rate than shScr-transfected cells. The wound healing rate of TLR6-knockdown cells was significantly enhanced upon stimulation with Pam2CSK4, whereas that of shScr-transfected cells was suppressed. ($P < 0.05$) (Fig. 5 D, E). Findings of the Transwell invasion assay demonstrated that the downregulation of TLR6 induced by shRNA1 and shRNA2 remarkably increased the invasiveness of HT29 cells. Meanwhile, the cellular invasion of TLR6-knockdown cells exhibited a significant increase demonstrated a substantial augmentation. (Fig. 5 F, G). Taken together, these findings suggest that TLR6 knockdown promotes the biological activity of HT29 cells, indicating a inhibitive role of TLR6 in CRC progression.

3.9. Knockdown of TLR6 promotes tumor growth in the xenograft model BALB/c-nu mice

To further investigate the effect of TLR6 on CRC cells in vivo, the tumorigenicity of HT29 cells with shRNA-2-induced TLR6 knockdown was evaluated in a xenograft model of nude mice. The results revealed that tumor volumes and weights were significantly increased in TLR6-knockdown mice (Fig. 5H, I, J). The collective findings suggest that the downregulation of TLR6 growth in the xenograft model BALB/c-nu mice, thereby the inhibitory effect of TLR on CRC was further confirmed.

3.10. TLR6 suppresses NF-κB activity in CRC cells

To identify the mechanism underlying the effect of TLR6 on CRC cells, the level of pNF-κB was determined using immunofluorescence staining. In addition, western blotting was used to determine the concentrations of protein extracted from HT29 and SW480 cells treated with either Pam2CSK4 or anti-TLR6 + Pam2CSK4. The results revealed that in the Pam2CSK4 group, pNF-κB was primarily located around the nuclear membrane of cytoplasm, whereas in the anti-TLR6 + Pam2CSK4 group, it was mainly located in the

cell nucleus (Fig. 6 A, B). Western blotting showed that in both CRC cell lines, pNF-κB levels increased in the anti-TLR6 + Pam2CSK4 group and were higher than those in the untreated group or Pam2CSK4 group (Fig. 6C, D and Supplementary Fig. 4). These results indicated that TLR6 inhibited the growth of CRC cells by suppressing the activity of NF-κB signaling.

4. Discussion

Studies have found that TLRs are not only involved in innate immunity but also expressed in tumor tissues and affect the genesis and development of tumors. In addition to epidemiological links, genetic links exist between TLRs and cancer [18]. TLR6, a transmembrane protein of the TLR family, forms heterodimers with TLR2 to enhance the PAMP sensitivity of TLR2 or to broaden its ligand range [27]. Reportedly, patients with CRC have altered expression of TLR6 [21]. A study by Kim et al. revealed that TLR6-deficiency mice had more CRC and a worse prognosis [28]. Despite these findings, this study elucidated the protective role and specific mechanism of TLR6 in colorectal cancer (CRC) pathogenesis.

The present study found that the expression of TLR6 was significantly downregulated in patients with CRC. However, TLR6 expression did not differ between tumors at different stages. In addition, the expression was independent of age, sex, tumor location, degree of differentiation, and metastasis. These findings are consistent with those of previous studies, suggesting that TLR6 plays a protective role against CRC [21]. In addition, CCK-8 and colony formation assays revealed dose-dependent inhibition of CRC cell growth in response to TLR6 stimulation with Pam2CSK4 at concentrations ranging from 100 pg/mL to 10 ng/mL. These findings provide additional support regarding the inhibiting effect of TLR6 on the growth of CRC.

Notably, the present study found that inhibiting TLR6 signaling by antibody or genetic silencing of TLR6 promoted the proliferation, migration, and invasion of CRC cells. These findings prove that TLR6 simulation inhibits CRC cell growth. Moreover, TLR6 stimulation significantly reduced the proportion of CRC cells in the S phase and G2/M phase, whereas inhibiting TLR6 significantly

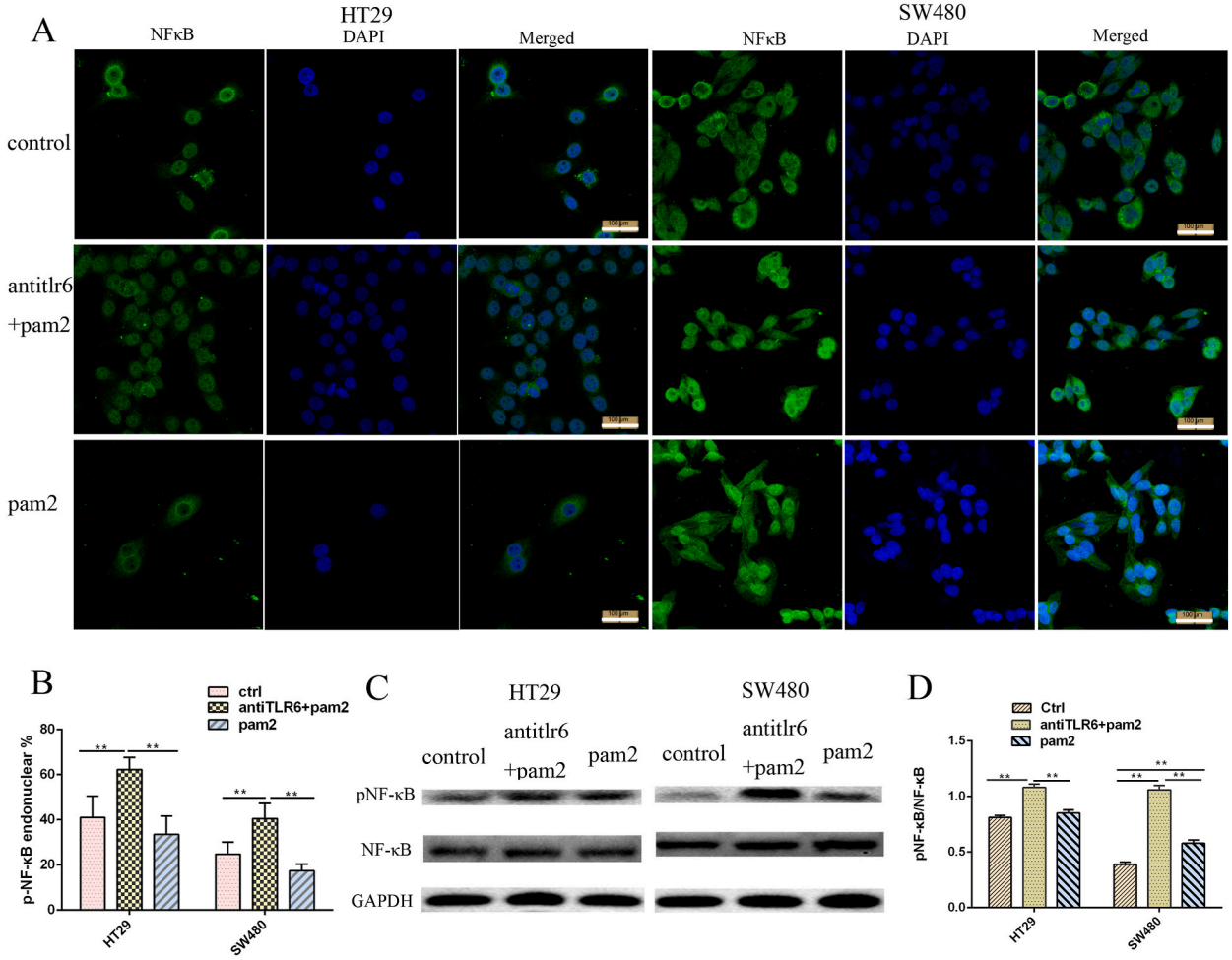

Fig. 6. Immunofluorescence picture for pNF-κB and the result of Western blot. (A) Representative immunofluorescence picture for pNF-κB in the CRC cell lines with different treatments. (B) Analysis of Immunofluorescence for pNF-κB. (C) Representative Western blots with antibodies against NF-κB, pNF-κB from HT29 and SW480 CRC cells with different treatments. (D) Analysis of activity of NF-κB. *p < 0.05, **p < 0.01, n = 5.

increased the proportion of CRC cells in these phases. In addition, the study demonstrated that the apoptosis of CRC cells activated by TLR6 was significantly higher than that of cells blocked by TLR6. In TLR6 knockdown or blocked cells, treatment with Pam2CSK4 could induce cell growth, migration, and invasion possibly due to the activation of TLR2 by Pam2CSK4. Meanwhile, the level of the apoptotic protein Bax increased and that of the anti-apoptotic protein Bcl-2 decreased significantly after the activation of TLR6 signaling. Conversely, inhibiting TLR6 signaling decreased Bax levels and significantly increased Bcl-2 levels in CRC cells. These findings further confirm that TLR6 promotes apoptosis in CRC. Thus, TLR6 may play a inhibitive role in the occurrence and development of colon cancer by inhibiting the proliferation, migration, and invasion of tumor cells and promoting cell apoptosis.

A number of examples of agonists of TLRs exhibiting antitumor activity [29–36]. Reportedly, the activation of TLR2 and TLR6 receptors by synthetic lipopeptide significantly reduces tumor growth in mice [37]. Moreover, intratumoral injection of a toll-like receptor 2/6 agonist has been reported to reduce pancreatic tumor proliferation and metastasis [12,38]. The present study revealed that TLR6 exerted a protective role in the pathogenesis and progression of colon cancer. However, the underlying mechanisms of these functions still remain unclear. The conventional mechanism of TLRs signal pathway transmission involves the engagement of various intracellular molecules, such as MyD88, thereby initiating the activation of NF-κB [39]. The NF-κB signaling pathway is involved in the regulation of a variety of biological responses. The activation of NF-κB is implicated in the development and progression of CRC through its enhancement of cell proliferation, angiogenesis, promotion of cell invasion and metastasis, as well as inhibition of apoptosis [40]. Furthermore, the crucial adaptor molecule involved in TLRs signaling, MyD88, has been identified as a mediator of CRC cell proliferation, migration, and invasion through the NF-κB signaling pathway [41].Subsequently, we examined whether TLR6 activation elicited alterations in NF-κB proteins. The activation of TLR6 by Pam2CSK4 treatment was observed to result in a decrease in pNF-κB levels and nuclear localization, while an increase was observed in TLR6 antibody-blocking CRC cells. Collectively, these findings suggest that TLR6 functions as a negative regulator of NF-κB in CRC cells and potentially exerts inhibitory effects on CRC tumorigenesis and development through the modulation of NF-κB signaling. The regulation was not validated by blocking of NF-κB in this study due to the intricate nature of NF-κB and its interactions with multiple cytokines within cells. The detailed mechanism by which TLR6 inhibits the development of CRC via NF-κB signaling will be further investigated in future studies.

In summary, this study demonstrated that the expression of TLR6 is downregulated in CRC tumor tissues. Moreover, TLR6 maybe inhibit CRC cell proliferation, migration, and invasion and promote CRC cell apoptosis by suppressing the activity of NF-κB signaling. Thus, TLR6 plays a inhibitive role against CRC tumorigenesis and development.

Ethics approval and consent to participate

This study was reviewed and approved by the Ethics Committees of Binzhou Medical University (Approval number: 2021-105).

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Data availability statement

Data include in article, supplementary material and referenced of the article. The original data supporting the conclusions of this article can be obtained from the corresponding author on reasonable request. And the data that support the findings of this study are openly available in TCGA at https://www.cancer.gov/ccg, GTEx at https://commonfund.nih.gov/GTEx and HPA at https://www.proteinatlas.org/.

CRediT authorship contribution statement

Lina Ma: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Yancun Yin:** Writing – review & editing, Writing – original draft, Investigation. **Zhenhai Yu:** Methodology, Investigation. **Ning Xu:** Methodology, Investigation, Funding acquisition. **Lianhuan Ma:** Software, Methodology. **Weiwei Qiao:** Software, Methodology. **Xiaowen Zhen:** Resources, Project administration, Data curation. **Fan Yang:** Data curation. **Naili Zhang:** Writing – review & editing, Writing – original draft, Visualization, Resources, Conceptualization. **Yue Yu:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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