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Original Article

TLR4 promotes proliferation and predicts poor prognosis of colorectal cancer

Shuaixi Yang, Jinbo Liu, Quanbo Zhou, Fuqi Wang, Ke Shi, Junmin Song*, Weitang Yuan*

*Department of Anorectal Surgery, First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China. *Equal contributors.*

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Abstract: Objective: To investigate the relationship between TLR4 and colorectal cancer(CRC). Methods: 55 CRC patients were included in this study, we peformed a detailed analysis of TLR4 expression characteristic and function in CRC. Expression patterns were examined using Real-time quantitative polymerase chain reaction (RT-qPCR). Statistical analysis was performed to verify the relationship of TLR4 expression and clinical pathological factors, and its prognostic value. Results: TLR4 was high expressed in CRC and there was a positive link between TLR4 expression and TNM stage. The high TLR4 expression was closely related with TNM stage, lymphatic metastasis, distant metastases and vascular invasion (all $P<0.05$). Furthermore, the high expression of TLR4 was correlated with a poor survival. COX regression analysis demonstrated that Lymph node metastasis, TNM stage and TLR4 expression (HR, 6.321; p,0.013; 95% CI, 6.344-15.327) were independent risk factors of 5-year OS for patients with T1-stage CRC (both $p<0.05$). Moreover, knockdown of TLR4 using small interfering RNA (siRNA) significantly inhibited proliferation of colon cancer cells *in vitro*. Conclusion: We concluded that TLR4 might represent a potential role as a biomarker and therapeutic target for CRC diagnose and oncotherapy in the future.

Keywords: Colorectal cancer, TLR4, biomarker, prognosis

Introduction

CRC is the third most common malignant tumor worldwide [1]. Despite recent improvements in screening strategies and the development of more effective treatments for CRC, the prognosis of advanced CRC is still poor [2]. Furthermore, the newest line of molecularly targeted therapeutic agents, appear to only have activity in metastatic CRC and do not cure the patient, but they have exponentially increased treatment costs and the economic burden of CRC care. Therefore, robust diagnostic, prognostic and predictive biomarkers are clearly and urgently needed to detect advanced colon polyps and early stage CRC, which are most effectively treated with current therapies, and to identify the most effective treatments for specific CRC patients.

Chronic infection and inflammation are considered to be some of the most important epigenetic and environmental factors contributing to tumorigenesis and tumor progression [3, 4]. Although the adaptive immune system prevents tumor growth through immunosurveillance, the innate immune system is thought to promote tumor growth through inflammation-dependent mechanisms [5, 6]. The innate immune system recognizes the presence of bacterial pathogens through the expression of a family of membrane receptors known as Toll-like receptors (TLR) [7, 8]. Toll-like receptors (TLRs) are a family of receptors that are expressed on innate immune cells [9-11].

The aim of the present study was to improve our knowledge about the effect of genetic variation in immune/inflammation-related genes on different phenotypic features of CRC and survival. It has been suggested that an active TLR-4-MyD88 signaling pathway may be a risk factor for developing cancer and may be a biomarker and therapeutic target for ovarian cancer diagnose and therapy in the future [7]. However, the function of TLR4 in CRC remains still unknown. Here, the expression features of TLR4 were analyzed in CRC cancer tissues and its biologi-

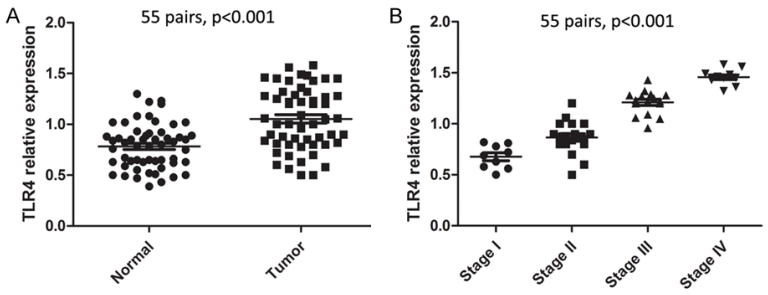

Figure 1. Expression of TLR4 detected in CRC specimens and normal colorectal mucosae. A. The expression of TLR4 was detected by qRT-PCR in normal and CRC tissues respectively of 55 pairs of CRC patients. B. TLR4 expression difference in TNM stages of 55 CRC tissues.

cal function was investigated in colon cancer cells to reveal a tumor biomarker and target therapeutic gene of CRC.

Materials and methods

Patients and samples

Tissue samples were collected from CRC patients. Tissues were cut in small aliquots and snap frozen in liquid nitrogen. All patients signed consent forms, and the use of patient samples was approved by the Human Investigations Committee of Zhengzhou University.

Real-time quantitative reverse transcription-PCR (RTqPCR) analysis

To analyze gene expression TLR4 by RT-qPCR, we extracted total cellular RNA using the RNeasy Minikit from Qiagen (Hilden, Germany). RNA extraction was performed according to the manufacture instructions. Reverse transcription was done on 1 ug of total RNA using the First Strand cDNA Synthesis kit (Amersham Biosciences, Buckinghamshire, United Kingdom) according to the manufacture instructions. The primers used for amplification of human TLR-4 were as follows: forward primer, TGGATACGTTTCCTTATAAG; reverse primer, GAAATGGAGGCACCCCTTC. Thirty cycles of PCR were done at 95jC for 15 seconds, 54jC for 45 seconds, and 72jC for 60 seconds. The size of the product was 449 bp. The relative expression of TLR4 and GAPDH were calculated through relative quantification ($2^{-\Delta\Delta Ct}$).

Cell culture

HCT116 cells were purchased from Chinese Academy of Sciences (Shanghai, China), HCT-116 cell line was cultured in RPMI 1640 (Gibco, Grand Island, NY, USA) medium with 10% fetal bovine serum (HyClone, Logan, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco) in humidified chamber at 37°C with 5% CO_2.

Cell transfection

Small interfering RNA (siRNA) against TLR4 (si-TLR4) and negative control were synthesized (Carlsbad, California, USA) and transfected into cells using Lipofectamine 2000 (Invitrogen, USA). The sequences of siRNAs targeting TLR4 were: Sense, 5'-CUUUAUCCAACCAGGUGCAUUUU-3'; Antisense, 5'-AAUGCACCUGGUUGGAUAAAGUU-3'. Control cells were transfected with negative control siRNA. The negative control siRNAs were: Sense, 5'-UUCUCCGAACGUGUCACGUTT-3'; Antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. Cells were harvested after 48 h for qRTPCR and western blot analyses.

Colony formation assays

800 transfected cells per well were seeded in six-well plate and cultured for 14 days. The cells were then fixed with methyl alcohol. for 15 min and stained with crystal violet solution for 15 min. Then, colonies were counted and the plates were photographed. The experiment was run in triplicate.

Cell viability assay

Cell viability assay was carried out using the MTT kit according to the manufacture instruction (Roche, Basel, Switzerland). Briefly, after transfection for 48 h, 3,000 cells per well were allowed to grow in 96-well plates with five replicate wells. After 6 h of culture, as well as at 24, 48, 72 and 96 h after starting the culture, the cells were treated with 100 μM TT by adding it to the medium. The cells were incubated at 37°C for another 4 h, then the medium was removed, and DMSO was added for 10 min to lyse the cells. Finally, the absorbance at a wave length of 490 nm was determined using a microplate reader.

Table 1. Univariate analysis between TLR4 expression and clinicopathologic features in CRC patients (n = 55 patients)

	n	Toll-like receptor 4		x^2	P
		Low (27)	High (28)		
Age at diagnosis (year)				0.150	0.698
≤60	32	17 (53.1%)	15 (46.9%)		
>60	23	11 (47.8%)	12 (52.2%)		
Gender				1.743	0.187
Male	36	20 (55.6%)	16 (44.4%)		
Female	19	7 (36.8%)	12 (63.2%)		
Tumor family history				0.682	0.409
No	40	21 (52.5%)	19 (47.5%)		
Yes	15	6 (40.0%)	9 (60.0%)		
Tumor size (cm)				0.908	0.341
≤5 cm	29	16 (55.2%)	13 (44.8%)		
>5 cm	26	11 (42.3%)	15 (57.7%)		
Localisation				1.547	0.214
Rectum	37	16 (43.2%)	21 (56.8%)		
Colon	18	11 (61.1%)	7 (38.9%)		
TNM stage				5.498	0.019
Stage I/II	20	14 (70.0%)	6 (30.0%)		
Stage III/IV	35	13 (37.1%)	22 (63.9%)		
Tissue type				0.154	0.695
Adenocarcinoma	42	20 (47.6%)	22 (52.4%)		
Nonadenocarcinoma	13	7 (53.8%)	6 (46.2%)		
Pathological lymph nodes (N)				8.054	0.005
N-	24	17 (70.8%)	7 (29.2%)		
N+	31	10 (32.3%)	21 (67.7%)		
Pathological metastases (M)				8.311	0.004
M-	39	24 (61.5%)	15 (38.5%)		
M+	16	3 (18.8%)	13 (81.2%)		
Vascular invasion				5.723	0.017
No	34	21 (61.8%)	13 (38.2%)		
Yes	21	6 (28.6%)	15 (71.4%)		

Statistical analyses

All statistical analyses were performed using SPSS version 18.0 (SPSS, Chicago, IL, USA). All measurements were in triplicate. Significant differences between TLR4 expression levels were analyzed using two-sided Student t-test. The clinic-pathological features of CRC patients were evaluated by Chi-square tests. The survival rates were calculated using the Kaplan-Meier method, the log-rank test was used to compare the survival curves. Multivariate survival analysis was performed by the Cox regression model to determine relative risk (RR) and 95% confidence intervals (CI). Statistical significance was defined as P< 0.05. Results were considered statistically significant at $p<0.05$.

Results

High TLR4 expressed in CRC tissues

To explore the expression of TLR4 in CRC tissues, qRT-PCR was performed in 55 pairs samples of CRC patients, containing 9 cases in stage I, 18 cases in stage II, 16 cases in stage III and 12 cases in stage IV. The results showed that TLR4 was significantly higher expressed in CRC tissues, compared with adjacent normal tissues ($p< 0.05$, **Figure 1A**). TLR4 expression level gradually increased along with the progression of TNM stage ($p<0.05$, **Figure 1B**).

Univariate analysis of correlation between clinicopathological features and TLR4 expression in 55 cases of CRC

The patients were divided into low and high expression groups based on the average expression value. Univariate analysis showed that TLR4 expression levels were associated with TNM stage, lymphatic metastasis, distant metastases and vascular invasion (all $p<0.05$). TLR4 expression levels were not associated with gender, age, tumor family history, tumor size, tumor location and tissue type. (all $p>0.05$) (**Table 1**).

Multivariate analysis of the correlation between clinicopathological features and TLR4 expression in 55 cases of CRC

Multivariate analysis showed that TLR4 expression levels were associated with TNM stage, lymphatic metastasis, distant metastases and vascular invasion (all $p<0.05$; **Table 2**). However,

Table 2. Multivariate analysis between TLR4 expression and clinicopathologic features in CRC patients (n = 55 patients)

		B value	OR value	95% confidence interval	*P* value
Age	≤60 vs >60 (year)	-0.324	0.698	0.515~1.184	0.216
Gender	Male vs female	-0.152	0.836	0.357~3.486	0.632
Tumor family history	Yes vs no	0.257	2.146	0647~2.578	0.307
Tumor size (cm)	≤5 vs >5	0.324	1.236	0.608~2.014	0.216
Tumor location	Rectal vs colon	0.129	1.846	0.618~1.392	0.481
TNM stage	I/II vs III/IV	1.536	2.835	2.114~4.815	0.003
Tissue type	Adenocarcinoma or nonadenocarcinoma	0.022	1.246	0.708~1.692	0.792
Lymphatic metastasis	Yes vs no	1.084	2.928	0.803~6.638	0.001
Distant metastasis	Yes vs no	-0.318	0.616	0.416~0.914	0.003
Vascular invasion	Yes vs no	1.187	2.916	0.786~3.216	0.001

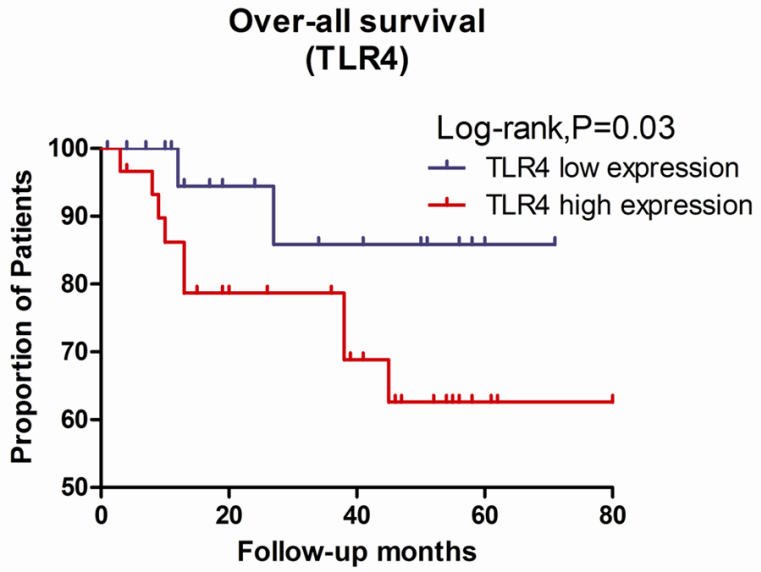

Figure 2. Prognostic effect analysis of TLR4 in patients with CRC. Survival analysis of TLR4 was carried out in 55 cases by Log-rank test and Kaplan-Meier curve (p<0.05).

they were not associated with gender, age, tumor family history, tissue type, tumor size and tumor location (all p>0.05).

Prognostic effect of TLR4 in patients with CRC

To estimate the prognosis level of TLR4 in CRC, we divided 55 CRC patients in two subgroups, low expression group (27 patients) and high expression group (28 patients) respectively. The results showed that the cases of 5-years OS between low TLR4 expression and high TLR4 expression was statistically significant (p<0.05, **Figure 2**).

Cox regress analysis of the correlation between clinicopathological parameters and 5-year OS

Multivariate survival analysis showed that TLR4 expression, Lymph node metastasis, and TNM stage were all closely associated with OS (all P<0.05). Meanwhile, gender, age, tumor family history, tumor size, tissue type, vascular invasion, localisation, and pathological lymph nodes (N) were not associated with 5-year OS (all P>0.05, **Table 3**).

Silencing TLR4 cellular proliferation in colon cancer cells

To explore the function of TLR4, si-TLR4 or negative control were transfected into HCT116 cells. As shown in **Figure 3A**, the downregulation of TLR4 significantly inhibited cell growth and cancer cell clonogenicity compared to cells transfected with control-shRNA. Then the cell proliferation was evaluated by MTT assay, the MTT assay showed that TLR4 knockdown signifcantly weakened the proliferation vitality of HCT116 (**Figure 3B**).

Discussion

Several studies focused on the effects of different TLRs in the context of tumor development and progression [12-14]. For example, F.A.

Table 3. COX regress analysis of the correlation between clinicopathological parameters and 5-year OS

	RR	*P* value	95% confidence interval	
			Lower bound	Upper bound
Gender	1.365	0.378	0.384	8.936
Male *vs* Female				
Age	4.316	0.086	0.869	9.635
<60 *vs* > = 60				
Tumor family history	3.654	0.176	2.667	4.286
No *vs* Yes				
Tumor size	0.647	0.532	0.328	11.235
<3 cm *vs* > = 3 cm				
Vascular invasion	0.486	0.483	0.986	6.589
No *vs* Yes				
Localisation	0.568	0.346	0.568	8.964
Rectum *vs* Colon				
TNM stage	0.684	0.023	4.569	14.278
Stage I/II vs Stage III/IV				
Pathological lymph nodes (N)	0.128	0.264	3.365	7.268
N- *vs* N+				
Tumor tissue type	0.957	0.436	0.056	2.543
Non-adenocarcinoma *vs* Adenocarcinoma				
Lymph node metastasis	5.489	0.042	2.358	12.612
No *vs* Yes				
TLR4 expression	6.321	0.013	6.344	15.327
Low *vs* High				

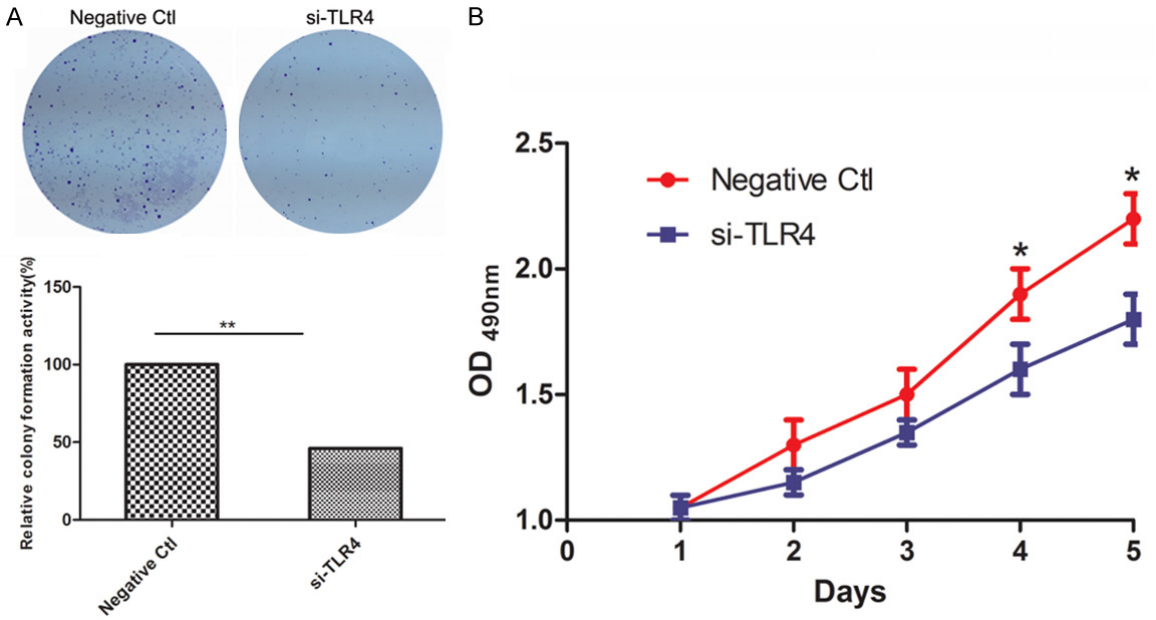

Figure 3. Downregulation of TLR4 inhibits CRC cells proliferation. A. Plate colony formation assays evaluated the impact of TLR4 on cell growth. Colony formation ability of si-TLR4 group was relative to Negative Ctl group. Asterisk indicates a significant change ($p<0.05$). B. MTT assay showing TLR4 knockdown inhibited cell vitality of HCT116 cells.

Castro et. reported that TLR-3 gene is an independent prognostic marker for disease specific survival in patients with stage II CRC [15]. Moreover, in ovarian cancer, TLR4 expression was associated with tumor progression and chemoresistance. Paclitaxel was identified as a ligand for TLR4 and the activation of the adapter protein MyD88 was linked to reduction of apoptosis in cancer cells [16]. In our study, we focused on the role of TLR4 in CRC.

To begin with, we observed significantly overexpressed in CRC tissues compared with adjacent normal tissues, and TLR4 expression level gradually increased along with the progression of TNM stage. In addition, we demonstrated that up-regulated TLR4 is associated with poor prognosis of CRC. In agreement with previous studies [17-19], we found that up-regulated TLR4 expression may be a risk factor for poor prognosis of CRC. Interestingly, multivariate analysis showed that TLR4 expression levels were associated with lymphatic metastasis and distant metastases, which indicated that high expression of TLR4 strongly correlates with increase of the metastatic potential of the tumor cells.

To further investigate the functions of TLR4 in CRC, we utilized siRNA-mediated knockdown of TLR4 and assessed the resultant effects on cell proliferation in HCT116 cells. The results implied that TLR4 can promote cell proliferation *in vitro*. Our results is in agreement with other studies which shown that chronic activation of TLRs expressed by tumor cells from CRC and pluripotent CD133+ colon cancer initiating cells may sustain inflammation responses, mediate resistance to apoptosis and promote further tumor progression [20, 21].

In conclusion, our studies demonstrated that TLR4 plays an important role in tumor development and metastasis of CRC. TLR4 will potentially become a potential diagnostic and prognostic biomarker for CRC patient.

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Disclosure of conflict of interest

None.

Address correspondence to: Junmin Song, Department of Anorectal Surgery, First Affiliated Hospital of Zhengzhou University, No.1, Jianshe Road, Zhengzhou 450052, China. Tel: +8615093351120; E-mail: songjunmin001@163.com; Weitang Yuan, Department of Anorectal Surgery, First Affiliated Hospital of Zhengzhou University, No.1, Jianshe Road, Zhengzhou 450052, China. Tel: +8615909910632; E-mail: yuanweitang@zzu.edu.cn

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