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Original Article

Expression of TLR4/MyD88 signaling pathway proteins and correlation with clinical and pathological features of colorectal cancer patients

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Abstract: Objective: Expression levels of TLR4/MyD88 signaling pathway-related proteins TLR4 and MyD88 in colorectal cancer tissues and correlation levels with clinicopathological features were explored. Methods: Cancer tissues and corresponding adjacent tissues of 122 colorectal cancer patients, retained after colorectal cancer resections, were selected. There were 62 patients with colon cancer and 60 patients with rectal cancer. Moreover, qRT-PCR and Western blotting were used to detect expression levels of TLR4 mRNA, MyD88 mRNA protein, TLR4, and MyD88 protein, respectively. Correlation between expression levels of TLR4 and MyD88 and clinicopathologic features and prognosis of patients was analyzed. Logistics were used to analyze risk factors affecting the prognosis of patients. Results: Expression levels of TLR4 mRNA, TLR4 protein, MyD88 mRNA, and MyD88 protein in cancer tissues of colon cancer and rectal cancer patients were higher than those in adjacent tissues ($P < 0.05$). High expression of TLR4 protein was closely related to clinical stage ($P < 0.001$), depth of invasion ($P < 0.001$), lymph node metastasis ($P=0.011$), and liver metastasis ($P < 0.001$) in patients with colorectal cancer. High expression of MyD88 protein was closely related to clinical stage ($P=0.002$), histological grade ($P < 0.001$), depth of invasion ($P < 0.001$), lymph node metastasis ($P < 0.001$), and liver metastasis ($P < 0.001$) in patients with colorectal cancer. A total of 122 patients with colorectal cancer had complete 5-year follow-up data. A total of 47 (38.52%) had died from tumors. According to univariate analysis, clinical stage ($P=0.002$), histological grade ($P=0.007$), immersion and serosal layer ($P=0.022$), lymph node metastasis ($P=0.012$), liver metastasis ($P=0.014$), peritoneal metastasis ($P=0.003$), TLR4 ($P=0.023$), and MyD88 ($P=0.020$) may be risk factors for survival prognosis. Further multivariate analysis found that clinical stage ($P=0.036$), histological grade ($P=0.024$), and MyD88 ($P=0.025$) were independent risk factors for prognosis of colorectal cancer patients. Five-year survival rates of patients with low expression of TLR4 protein were higher than those of patients with high expression of TLR4 protein ($P=0.027$). Five-year survival rates were higher in the MyD88 protein expression group than in the MyD88 protein expression group ($P=0.001$). Conclusion: TLR4 and MyD88 are highly expressed in tumor tissues of patients with colorectal cancer. Patients with high expression of TLR4 and MyD88 have shorter 5-year survival rates, closely related to clinical stage, depth of invasion, lymph node metastasis, and liver metastasis. MyD88 is also closely related to tumor histologic grades. Thus, MyD88 is an independent risk factor for patient prognosis.

Keywords: Colorectal cancer, TLR4, MyD88, clinicopathologic feature, prognosis

Introduction

Colorectal cancer is the most common malignancy in the digestive tract, occurring mainly in people over 40 years old. The ratio of male to female is about 2-3:1. It is one of the main causes of human death [1]. With improvements in living standards and dietary levels, incidence of colorectal cancer has increased year by year [2]. The clear pathogenesis and pathogenesis of colorectal cancer is the same as other malignant tumors. Yet, it has not been fully studied [3]. The biological behavior of colorectal cancer is complex. Early screening, timely detection, and timely treatment can effectively improve survival rates [4]. Therefore, it is of great significance to discover the pathogenesis of colorectal cancer and to search for new biological markers and prognostic indicators.

Toll-like receptor 4 (TLR4) is an antigen recognition receptor closely related to the natural

immune system, leading to the release of inflammatory mediators. TLR4 is highly expressed in colorectal cancer, breast cancer, liver cancer, and other tumors. It is involved in the immune escape of tumor cells [5-7]. Myeloid differentiation factor 88 (MyD88) is a key linker in the initiation of downstream signal transduction by TLR4 signaling pathways. It plays an important role in tumorigenesis, development, and immunosuppression [8, 9]. Occurrence and development of colorectal cancer are closely related to congenital immune function and tumorigenic pro-inflammatory response. Some studies have reported an increase in expression levels of TLR4 and MyD88 in colorectal cancer, with TLR4/MyD88 signaling pathways promoting the development of colorectal-associated colorectal cancer [10, 11]. However, few studies have reported the clinical significance of TLR4/MyD88 signaling pathway expression in patients with sporadic colorectal cancer.

Therefore, the current study examined expression levels of TLR4/MyD88 in tissues of patients with colorectal cancer, analyzing the relationship between TLR4/MyD88 and clinicopathological features and prognosis in patients with colorectal cancer.

Materials and methods

Objectives of the current study

Cancer tissues of 122 patients with colorectal cancer and corresponding adjacent tissues, after resections, were obtained. There were 62 patients with colon cancer and 60 patients with rectal cancer, aged 35-85 years. Inclusion criteria: Patients were pathologically diagnosed with colon or rectal cancer; Patients did not receive radiotherapy and chemotherapy and showed no dysfunction of the liver, kidneys, and other organs; Patients had no abnormal bleeding or coagulation function; Medical records were complete and follow-up data was complete; No carcinogenesis-related inflammatory bowel disease, familial adenomatous polyposis, or other diseases. Exclusion criteria: Patients with large lumps; Patients with other lung or chest wall diseases; Evidence of other benign or malignant tumors, with a history of tumors and malignant pleural effusion; Serious diseases in the heart, brain, liver, kidneys, and blood vessels; Severe infections, such as sepsis; Pregnant or lactating women. The current study consulted patients and their families, receiving informed consent. This study was approved by the Medical Ethics Committee of Jining No.1 People's Hospital.

Performance of qRT-PCR

After grinding and pulverizing 50 mg of cancer tissues or paracancerous tissues, 1 mL of TRIzol lysate was added to extract total RNA. The purity of RNA was determined using a micro-ultraviolet spectrophotometer. A260/A280 values were considered to meet the experimental requirements between 1.8 and 2.1. After RNA extraction, reverse transcription reaction was carried out. First strand cDNA was synthesized and then subjected to PCR amplification. The PCR amplification system was 1 μL of cDNA template, 10 μL of 2*Real-time PCR Master Mix (SYBR Green), 0.4 μL of upstream primer and downstream primer, double-distilled water to 20 μL, pre-denaturation at 95°C for 3 minutes, and denaturation at 95°C for 10 seconds. Annealing at 60°C, extending at 72°C for 20 seconds and 72°C for 5 minutes, for a total of 40 cycles, as well as dissolution curve analysis, was carried out after the end of the experiment. GAPDH was used for reaction parameters. All samples were repeated for 3 wells. Results were analyzed using the 2-ΔCt method. TRIzol Reagent was purchased from Xiamen Research Biotechnology Co., Ltd., Item No. 15596018, Real-time PCR Master Mix kit (SYBR Green) was purchased from Beijing Xinhua Luyuan Technology Co., Ltd., item number SS2110. The primer sequence was designed and synthesized by Shanghai Qiyin Biotechnology Co., Ltd., (**Table 1**) 50 mg 2*Real-time PCR Master Mix (SYBR Green) 10 μL.

Western blot

Proteins from cancer tissues and A549 cells were extracted using the repeated freeze-thaw method. Proteins were separated by polyacrylamide gel electrophoresis. Initial voltage was 90 V. The voltage was then increased to 120 V to move the sample to the appropriate position of the separation gel. After electrophoresis was completed and the membrane was transferred, 100 V constant pressure was applied for 100 minutes. The temperature was blocked at 37°C for 60 minutes. The transfer membrane was then placed in 5% skim milk for blocking and subjected to an immune response. The membrane was incubated with the primary antibody

Table 1. Primer sequences

	Upstream	Downstream
TLR4 mRNA	5'-GAATGAGGACTGGGTGAGAAAC-3'	5'-ACCAACGGCTCTGGATAAAGT-3'
MyD88 mRNA	5'-GCCTTGTTAGACCGTGAGGA-3'	5'-GGGACACTGCTTTCCACTCT-3'
GAPDH	5'-CGGAGTCAACGGATTTGGTCGTAT-3'	5'-AGCCTTCTCCATGGTGGTGAAGAC-3'

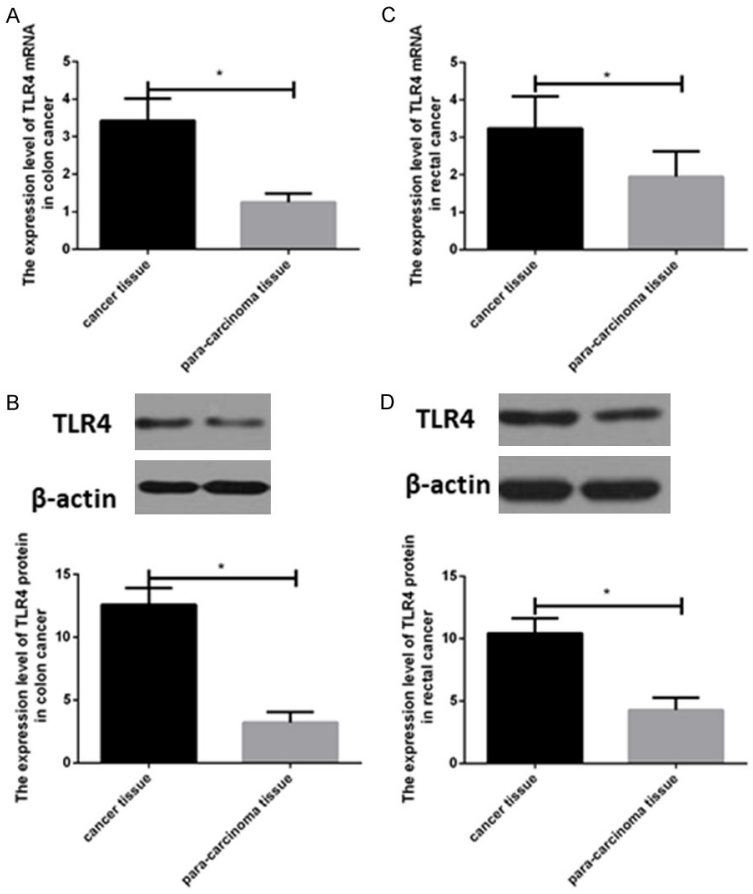

Figure 1. Expression levels of TLR4 in colorectal cancer. A: Expression level of TLR4 mRNA in colon cancer tissues. B: Expression level of TLR4 protein in colon cancer tissue. C: Expression level of TLR4 mRNA in rectal cancer tissues. D: Expression level of TLR4 protein in rectal cancer tissues. *P < 0.05.

overnight at 4°C. The next day, PBS washing was carried out three times for 5 minutes each time. They were then incubated with the secondary antibody for 1 hour at room temperature. After completion, the ECL luminescence reagent was developed and fixed. Quantity One software was used to perform statistical analysis on the strip after scanning the film. Relative protein expression level = band gray value/internal reference gray value. Western blot detection kit was purchased from Wuhan Boot Biotechnology Co., Ltd., goods number orb-342299. Rabbit anti-human TLR4, MyD88 polyclonal antibody was purchased from Mengcheng Technology (Shanghai) Co., Ltd, goods number P103461, P100350. Goat anti-rabbit IgG secondary antibody was purchased from Shanghai Xinyu Biotechnology Co., Ltd., goods number ZI225-1.

Statistical methods

SPSS19.0 (Asia Analytics power SPSS China) was used for analysis. Measurement data are represented by %, while the comparison of rate is presented by χ^2. Count data are expressed as mean ± standard deviation (mean ± sd). Student's t-test was used for comparisons between the two groups. Logistic analysis was used to analyze risk factors affecting the prognosis of patients. Kaplan-Meier survival curves were used to analyze the relationship between TLR4 and MyD88 and survival times of patients. P < 0.05 indicates statistical significance.

Results

Expression levels of TLR4 in colorectal cancer

Expression levels of TLR4 mRNA and TLR4 protein in cancer tissues of colon cancer and rectal cancer patients were higher than those in adjacent tissues (P < 0.05) (**Figure 1**).

Expression levels of MyD88 in colorectal cancer

Expression levels of MyD88 mRNA and MyD88 protein in cancer tissues of colon cancer and rectal cancer patients were higher than those in adjacent tissues (P < 0.05) (**Figure 2**).

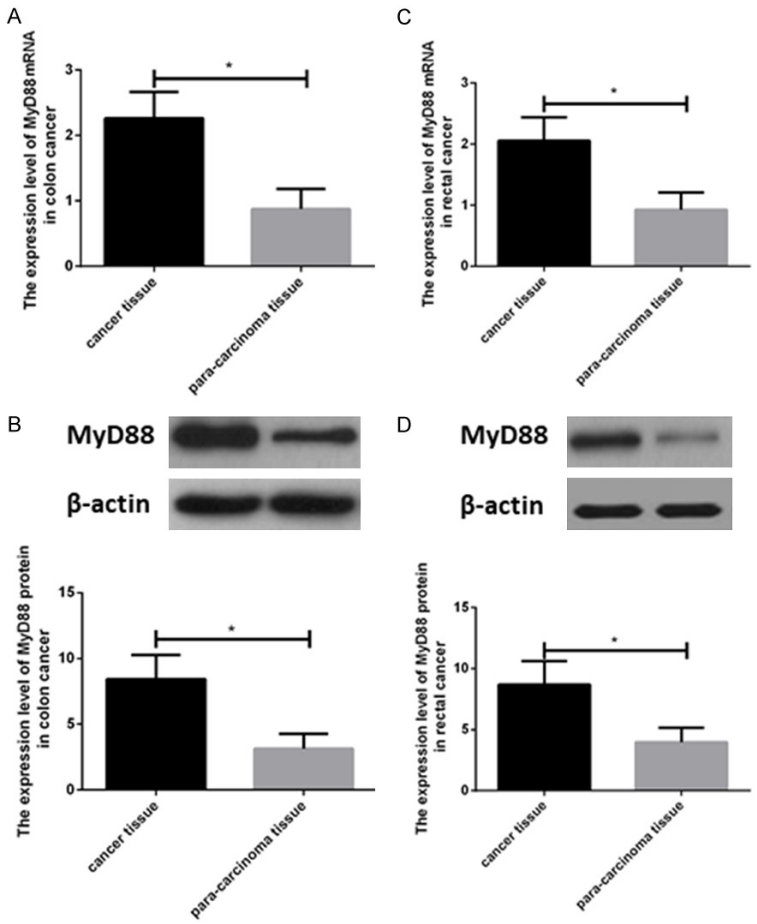

Figure 2. Expression levels of MyD88 in colorectal cancer. A: Expression level of MyD88 mRNA in colon cancer tissues. B: Expression level of MyD88 protein in colon cancer tissue. C: Expression level of MyD88 mRNA in rectal cancer tissues. D: Expression level of MyD88 protein in rectal cancer tissues. *P < 0.05.

Relationship between TLR4 and clinical case characteristics of colorectal cancer patients

According to relative expression levels of TLR4 protein in colorectal cancer tissues, the median of the relative expression level of TLR4 protein was 11.342. The two groups with the median as the critical value were divided into high (> 11.342) and low (less than or equal to 11.342) expression levels. High expression of TLR4 protein was closely related to clinical stage (P < 0.001), depth of invasion (P < 0.001), lymph node metastasis (P=0.011), and liver metastasis (P < 0.001) in patients with colorectal cancer. Differences were statistically significant. However, no significant correlation was found with other indicators, such as gender and age. Differences were not statistically significant (P > 0.05) (**Table 2**).

Relationship between MyD88 and clinical features of colorectal cancer patients

According to relative expression levels of MyD88 protein in colorectal cancer tissues, the median expression level of MyD88 protein was 8.135. The two groups with the median as the critical value were divided into high (> 8.135) and low (less than or equal to 8.135) expression levels. High expression of MyD88 protein was closely related to clinical stage (P=0.002), histological grade (P < 0.001), depth of invasion (P < 0.001), lymph node metastasis (P < 0.001), and liver metastasis (P < 0.001) in patients with colorectal cancer. Differences were statistically significant. Results suggest no significant correlation with other indicators, such as gender and age. Differences were not statistically significant (P > 0.05) (**Table 3**).

Analysis of prognostic factors in patients with ring colorectal disease

A total of 122 patients with colorectal cancer had complete 5-year follow-up data. Of these, 47 (38.52%) died tumor-related deaths. According to univariate analysis, clinical stage (P=0.002), histological grade (P=0.007), immersion and serosal layer (P=0.022), lymph node metastasis (P=0.012), liver metastasis (P=0.014), peritoneal metastasis (P=0.003), TLR4 (P=0.023), and MyD88 (P=0.020) may be risk factors for survival prognosis. Further multivariate analysis found that clinical stage (P=0.036), histological grade (P=0.024), and MyD88 (P=0.025) were independent risk factors for prognosis of colorectal cancer patients (**Tables 4** and **5**).

Relationship between TLR4 and MyD88 and survival time of patients

According to relative expression levels of TLR4 and MyD88 proteins in colorectal cancer tis-

Table 2. Relationship between TLR4 and clinical case characteristics of colorectal cancer patients

	High expression of group (n=61)	Low expression of group (n=61)	χ^2	P
Sex			0.300	0.574
Male	33 (54.10)	36 (59.02)		
Female	28 (45.90)	25 (40.98)		
Age			0.133	0.715
≥ 65	28 (45.90)	26 (42.62)		
< 65	33 (54.10)	35 (57.38)		
Clinical stages			19.305	< 0.001
I-II	14 (22.95)	38 (62.30)		
III-IV	47 (77.05)	23 (37.70)		
Tumor size			1.419	0.234
≥ 5 cm	46 (75.41)	40 (65.57)		
< 5 cm	15 (24.59)	21 (34.43)		
Tumor site			0.000	1.000
Colon cancer	31 (50.82)	31 (50.82)		
Carcinoma of the rectum	30 (49.18)	30 (49.18)		
Histological grade			0.137	0.711
Lower-level	36 (59.02)	38 (62.30)		
High-level	25 (40.98)	23 (37.70)		
Depth of invasion			14.219	< 0.001
Immersed serosa	49 (80.33)	29 (47.54)		
Unimmersed serosa	12 (19.67)	32 (52.46)		
Lymphatic metastasis			6.470	0.011
Yes	35 (57.38)	21 (34.43)		
No	26 (42.62)	40 (65.57)		
Vascular invasion			0.068	0.794
Yes	9 (14.75)	8 (13.11)		
No	52 (85.25)	53 (86.88)		
Hepatic metastases			19.938	< 0.001
Yes	31 (50.82)	8 (13.11)		
No	30 (49.18)	53 (86.89)		
Peritoneal metastasis			0.918	0.338
Yes	43 (70.49)	38 (62.30)		
No	18 (29.51)	23 (37.70)		

sues, the median of relative expression levels of TLR4 proteins was 11.342. The median was divided into high (> 11.342) and low (≤ 11.342) expression groups. The median level of MyD88 protein expression was 8.135. The median was also divided into high (> 8.135) and low (≤ 8.135) expression groups, with Kaplan-Meier survival analysis performed. Analysis showed that the 5-year survival rate was 72.13% (44 cases) in the TLR4 high expression group. The 5-year survival rate was 50.82% (31 cases) in the TLR4 low expression group. The 5-year survival rate of patients with low expression of TLR4 protein was higher than that of patients with high expression of TLR4 protein (P=0.027). The 5-year survival rate of patients with high expression of MyD88 protein was 47.54% (29 cases). The 5-year survival rate of patients with low expression of MyD88 protein was 75.41% (46 cases). The 5-year survival rate of patients with low expression of MyD88 protein was higher than that of patients with high expression of MyD88 protein (P=0.001) (**Figure 3**).

Discussion

Colorectal cancer is more common in middle-aged men. It is a kind of malignant tumor with

Table 3. Relationship between MyD88 and clinical case characteristics of colorectal cancer patients

	High expression of group (n=61)	Low expression of group (n=61)	χ^2	P
Sex			0.131	0.363
Male	30 (49.18)	28 (45.90)		
Female	31 (50.82)	33 (54.10)		
Age			0.293	0.585
≥ 60	26 (42.62)	29 (47.54)		
< 60	35 (57.38)	32 (52.46)		
Clinical stages			9.785	0.002
I-II	11 (18.03)	27 (44.25)		
III-IV	50 (81.97)	34 (55.74)		
Tumor size			2.682	0.102
≥ 5 cm	38 (62.30)	29 (47.54)		
< 5 cm	23 (37.70)	32 (55.74)		
Tumor site			0.131	0.717
Colon cancer	29(47.54)	31 (50.82)		
Carcinoma of the rectum	32(52.46)	30 (49.18)		
Histological grade			25.767	< 0.001
low-level	43 (70.49)	15 (24.59)		
High-level	18 (29.51)	46 (75.41)		
Depth of invasion			61.684	< 0.001
Immersed serosa	56 (91.80)	13 (21.31)		
Unimmersed serosa	5 (8.20)	48 (78.69)		
Lymphatic metastasis			72.504	< 0.001
Yes	55 (90.16)	8 (13.11)		
No	6 (9.84)	53 (86.89)		
Vascular invasion			3.301	1.817
Yes	38 (62.30)	28 (45.90)		
No	23 (37.70)	33 (54.10)		
Hepatic metastases			18.541	< 0.001
Yes	24 (37.34)	4 (6.56)		
No	37 (60.66)	57 (93.44)		
Peritoneal metastasis			1.479	0.224
Yes	8 (13.11)	4 (6.56)		
No	53 (86.89)	57 (93.44)		

high incidence. According to some studies, the proportion of patients with colorectal cancer that can be detected early is only about 2% of all colorectal cancer patients [12, 13]. The pathogenesis of colorectal cancer has not been studied thoroughly. Therefore, the search for molecular biological markers related to clinical features and prognosis of colorectal cancer patients is of great significance in elucidating the pathogenesis of colorectal cancer. In recent years, TLR4/MyD88 signaling pathways have become a popular direction in the study of tumor development. This pathway plays an important role in the development of tumors, mainly by mediating immunity and inflammatory response [14]. Previous studies have reported that TLR4/MyD88 signaling pathways play a role in promoting tumor development in liver cancer [15]. The current study explored expression levels in colorectal cancer, examining its relationship with clinical features and prognosis of colorectal cancer patients.

A total of 122 colorectal cancer patients with cancer tissues and corresponding para-cancerous tissues were selected. Carcinogenic-re-

Table 4. Single factor analysis of prognosis related to colorectal patients

	HR	95% CI	P
Sex (Male VS Female)	1.162	0.385-2.341	0.792
Age (≥ 60 vs < 60)	1.257	0.564-2.173	0.853
Part (Colon vs rectum)	1.493	0.485-6.241	0.125
Size (≥ 5 vs < 5 cm)	1.634	0.696-3.203	0.133
Stages (I-II vs III-IV)	8.035	3.594-20.248	0.002
Classification (low vs high)	2.623	1.024-5.157	0.007
Immersed serosa (Yes vs no)	2.141	0.283-43279	0.022
Lymphatic metastasis (Yes vs no)	2.341	1.2745.983	0.012
Vascular invasion (Yes vs no)	0.683	0.557-1.804	0.446
Hepatic metastases (Yes vs no)	2.225	1.368-3.801	0.014
Peritoneal metastasis (Yes vs no)	5.128	2.889-10.266	0.003
TLR4 (Low vs high)	1.523	1.775-5.253	0.023
MyD88 (Low vs high)	1.796	1.341-5.583	0.020

Table 5. Multivariate analysis of prognosis related to colorectal patients

	HR	95% CI	P
Stages (I-II vs III-IV)	1.625	0.423-7.025	0.036
Classification (Low vs high)	1.165	0.735-1.865	0.024
Immersed serosa (Yes vs no)	1.244	0.766-2.175	0.213
Lymphatic metastasis (Yes vs no)	0.716	0.257-2.428	0.316
Hepatic metastases (Yes vs no)	0.683	0.142-4.251	0.651
Peritoneal metastasis (Yes vs no)	0.341	0.027-3.328	0.213
TLR4 (Low vs high)	0.775	0.357-2.175	0.739
MyD88 (Low vs high)	1.164	0.538-2.446	0.025

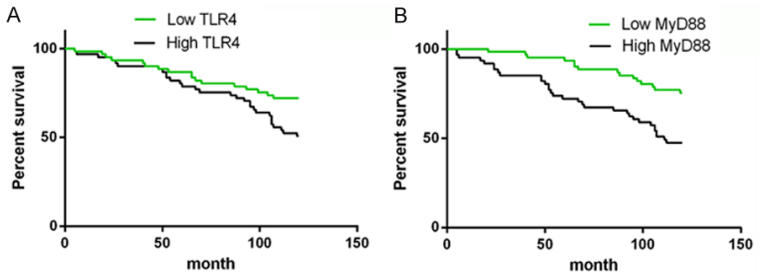

Figure 3. Relationship between prognosis of patients with colorectal cancer and TLR4/MyD88. A: Relationship between TLR4 protein expression and 5-year survival. B: Relationship between MyD88 protein expression and 5-year survival.

lated inflammatory bowel disease, familial adenomatous polyposis, or other diseases were not found in included patients. Results of this study showed that high expression of TLR4 and MyD88 were closely related to clinical stage, depth of invasion, lymph node metastasis, and liver metastasis of colorectal cancer patients. MyD88 was shown to be closely related to the histological grade of patients. Survival curves showed that the 5-year survival rate of patients with high expression of TLR4 and MyD88 was significantly lower than that of patients with low expression of TLR4 and MyD88. TLR4 is a potential risk factor for patient prognosis. MyD88 is an independent risk factor for prognosis of colorectal cancer patients. Present analysis showed that clinical stage and histologic grade were independent risk factors for prognosis.

In recent years, studies concerning TLR4/MyD88 in colorectal cancer have reported [16] that BMI-1 regulates NF-B signaling pathways mediated by the TLR4/md-2/MyD88 complex. It is involved in inflammatory induction of colorectal cancer cell invasion and epithelial mesenchymal transformation. Epithelial mesenchymal transition is the molecular basis for tumor cell infiltration and migration. There are also reports [17] indicating that lipopolysaccharide stimulates the TLR4/MD2 complex to activate PI3K/AKT signaling. This promotes the downstream β1 integrin function, thereby improving adhesion and metastatic abilities of colorectal cancer cells. It also inhibits lipopolysaccharide-induced TLR4 signal transduction. This can prevent tumor metastasis in the perioperative period of resections and improve therapeutic effects. Results indicated that TLR4/MyD88 plays an important role in the development of colorectal cancer. According to reports of TLR4/MyD88 and liver cancer [18], TLR4 is highly expressed in liver cancer tissues and closely related to tumor differentiation and TNM staging. TLR4/MyD88 signaling pathways are involved in the proliferation and metastasis of liver cancer cells by regulating IL-23/IL-17A axis. One study

also reported the clinical significance of TLR4/MyD88 in ovarian epithelial cancer [19]. Their results showed that TLR4 and MyD88 are related to histologic types of patients with ovarian epithelial cancer. TLR4/MyD88 signaling pathways are associated with survival in patients with ovarian epithelial cancer. MyD88 is an independent prognostic factor in patients with ovarian epithelial cancer. These previous studies have demonstrated the role of TLR4/MyD88 signaling pathways in tumors. They enhance the migration and invasion of tumor cells through multiple downstream signaling molecules, such as NF-κB, and promote further metastasis and deterioration of tumors. This also proves the accuracy of current results, at least to some extent.

In recent years, studies have reported the roles of TLR4 and MyD88 in tumor treatment. In a basic study [20], TLR4 agonists were shown to effectively improve the anti-tumor therapeutic effects of oxaliplatin or doxorubicin and improve long-term survival rates of mice. This is related to the fact that TLR4 agonist stimulates the production of CD4+ and CD8+ T-cells and amplifies the immune effects of chemotherapy. Moreover, in previous studies, TLR4 and MyD88 were found to be indispensable in the process of inhibiting growth of transplantable tumors with anthracyclines or radiotherapy immune-dependent pathways [21]. These two studies suggest that TLR4 can be a double-edged sword in tumors. Other studies have suggested that the TLR4/MyD88 pathway mediates activation of NF-κB and subsequent production of pro-inflammatory cytokines, including IL1β, IL-6, and TNF-α. These cytokines stimulate myeloid dendritic cells to secrete IL-23, which promotes Th17 cell differentiation, proliferation, and maintenance [22, 23]. It has been reported that the IL-23/IL-17A axis promotes the formation of lung metastases through tumor-endothelial transmigration [24]. This reinforces the need to continue to explore the roles of TLR4/MyD88 signaling pathway in tumors.

In summary, TLR4 and MyD88 are highly expressed in tumor tissues of patients with colorectal cancer. Five-year survival rates of patients with high expression of TLR4 and MyD88 are relatively short. Expression is closely related to clinical stage, depth of infiltration, lymph node metastasis, and liver metastasis of the patients. MyD88 is also closely related to tumor histologic grade. Thus, MyD88 is an independent risk factor for patient prognosis.

Disclosure of conflict of interest

None.

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