

Significance of TLR4, MyD88 and STAT3 expression in colorectal cancer.

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Abstract

Objective: The aim of this study was to investigate the expression status of toll-like receptor 4 (TLR4), myeloid differentiation primary response protein 88 (MyD88) and signal transducers and activators of transcription 3 (STAT3) in colorectal cancer (CRC) by immunohistochemistry (IHC), and explore the relationship between their expression and clinicopathological features.

Materials and methods: Expression of TLR4/MyD88/STAT3 was detected using IHC staining in 20 colorectal adenomas, 40 CRC samples and 40 matched non-tumor tissues, and the correlations between their immunoscores and clinicopathological variables were analysed.

Results: The IHC results showed that the tumor sites exhibited significantly higher levels of all these three proteins compared to the adenoma and adjacent non-tumor sites. Further data analysis showed the expression of TLR4, MyD88 and STAT3 was positively correlated with each other in CRC tissue samples. Among clinicopathological parameters, overexpression of TLR4 was significantly correlated with lymph node metastasis (LNM) ($P=0.023$), MyD88 expression was significantly associated with the degree of differentiation ($P=0.006$), LNM ($P=0.009$) and Tumor-Node-Metastasis (TNM) stage ($P=0.018$), whereas STAT3 expression was significantly associated with tumor size ($P=0.013$), LNM ($P=0.001$) and TNM stage ($P=0.002$).

Conclusion: The overexpression of TLR4/MyD88/STAT3 proteins may play an important role in the pathogenesis of CRC.

Keywords: Colorectal cancer, TLR4, MyD88, STAT3, Immunohistochemistry.

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Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females, with over 1.3 million new cancer cases and 693,900 deaths estimated to have occurred in 2012 [1]. Over the recent decades, the incidence of CRC has been increasing dramatically in several developing countries and has attracted wide attention [2-5]. The theory of adenoma-carcinoma sequence is now accepted as the mechanism of CRC [6]. While many of the oncogenes and tumor suppressor genes that are involved in the occurrence and progression of CRC have been identified, the molecular mechanisms of CRC has not been fully determined.

Toll-like receptors (TLRs) is a type I transmembrane receptor that was originally identified in *Drosophila* for its involvement in embryo development [7]. They act as pathogen recognition receptors and play key roles in both innate and adaptive immune responses [8]. There are 10 human isoforms of TLRs (TLR1 to 10) have been identified. Human TLRs are expressed

in various cell types, including immune cells, intestinal epithelial cells and tumor cells [9,10]. TLR4 is the best characterized pathogen recognition receptor. There is increasing evidence suggests that TLR4 plays important roles in cancers [11]. TLR4 specifically binds to an adaptor molecule, myeloid differentiation primary response protein 88 (MyD88), resulting in the production of proinflammatory cytokines, such as IL-6 or TNF. Current studies suggest MyD88-dependent signaling has been shown to be critical for oncogenesis and tumor progression, such as lymphoma and hepatocellular carcinoma [12,13].

Signal transducers and activators of transcription (STATs) are cytoplasmic transcription factors, and STATs are key mediators of cytokine and growth factor signalling pathways [14]. STAT3 is a member of the STATs family and is also a target gene of TLR4/MyD88 signaling [15,16]. In addition, prolonged activation of STAT3 plays a significant role in promoting development and progression of many cancers, such as lung cancer, hepatocellular carcinoma and breast cancer [17].

Currently, few studies have been published on the relations between TLR4/MyD88/STAT3 expression and clinicopathological characteristics in CRC patients. In this study, we investigated the expressions of TLR4, MyD88 and STAT3 in colorectal adenomas, CRC and peritumoral tissues by performing IHC and we evaluated the relationship between these proteins and the clinicopathologic factors.

Materials and Methods

Patients and tissue specimens

We obtained colorectal adenomas from 20 patients and tumors and peritumoral colorectal tissues from 40 CRC patients who had undergone endoscopic resection or surgery at the Central Hospital of Wuhan in 2016. None of the 40 CRC patients had received chemotherapy or radiotherapy prior to surgery. Tissue samples were collected immediately following endoscopic resection or surgery. The non-malignant colorectal tissues were at least 2 cm in distance from the tumor margin. The tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin for histopathological diagnosis and immunohistochemical staining. The diagnoses were confirmed by histopathological study. Detailed clinical and histopathological information was collected based on case records and pathologic reports. Table 1 shows the general clinicopathological features of these 40 patients with CRC. Tumors located proximal to the splenic flexure were considered proximal, whereas those located after the splenic flexure were distal. Tumor staging was determined by the TNM Classification (7th edition) of the American Joint Committee on Cancer (AJCC) [18]. The present study was performed according to the guidelines of the Ethics and Research Committee of the Central Hospital of Wuhan and approved in accordance with the ethical standards of the World Medical Association Declaration of Helsinki.

Immunohistochemical staining

This was performed as previously described by the same author [19]. We used the primary antibodies were as following: a rabbit anti-TLR4 polyclonal antibody (Wuhan Boster Bio-engineering Co., Ltd., China) at 1:100 dilution, a rabbit anti-MyD88 polyclonal antibody (Wuhan Boster Bio-engineering Co., Ltd.) at 1:200 dilution, and a rabbit anti-STAT3 polyclonal antibody (Wuhan Boster Bio-engineering Co., Ltd.) at 1:200 dilution. Immunohistochemical staining was performed using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Negative controls were performed by replacing the primary antibody with phosphate-buffered saline (PBS).

Table 1. Clinicopathological characteristics of the 40 CRC patients.

Characteristics	Results
Gender	
Male	24

Female	16
Age (years)	
<65	25
≥ 65	15
Tumor site	
Distal	25
Proximal	15
Tumor diameter (cm)	
<5	22
≥ 5	18
Pathological grade	
Well differentiated	9
Well-moderately differentiated	7
Moderately differentiated	15
Moderately-poorly differentiated	6
Poorly differentiated	3
Depth of invasion	
T1+T2	5
T3+T4	35
LNM	
N0	17
N1	11
N2	12
Distant metastasis	
M0	34
M1	6
TNM stage	
I+II	15
III+IV	25
Lymphovascular invasion	
Present	29
Absent	11

LNM: Lymph Node Metastasis; TNM: Tumor Node Metastasis

Evaluation of immunohistochemical findings

Each slide was evaluated independently by two observers who were blinded to clinical data. The immunostaining score of TLR4, MyD88, and STAT3 proteins was assessed by the percentage of positively stained tumor cells as described earlier: 0, no positives staining; 1, 1-25% cells positive; 2, 26-50% cells positive; 3, 51-75% cells positive; 4, 76-100% cells positive [19,20]. Specimens with scores ≥ 2 were labeled

as ‘positive (+)’. The staining areas were imaged with a BX51 model light microscope (at x200 and x400 magnification; OLYMPUS Corporation, Tokyo, Japan).

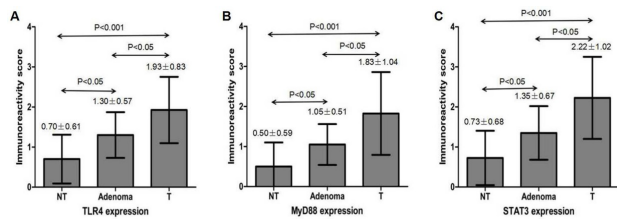


Figure 1. Comparison of TLR4, MyD88 and STAT3 expression levels in colorectal tissues. The immunostaining scores of TLR4 (A), MyD88 (B) and STAT3 (C) in T were significantly higher than in adenoma and NT. In addition, the immunostaining scores of TLR4 (A), MyD88 (B) and STAT3 (C) were significantly higher in adenoma compared to NT. Data are presented as mean ± standard deviation; T=CRC tissues; NT=adjacent non-tumor tissues.

Statistical analysis

Data were presented in terms of mean ± standard deviation, percentage and the number of cases. The analyses that compared the expressions of TLR4, MyD88 and STAT3 between adenoma, tumor and peritumoral tissues were performed using Kruskal-Wallis test. The relationships between the levels of three proteins expression and the various clinicopathological parameters in CRC were determined by Fisher's exact test. Correlations were calculated using the Spearman's rank correlation coefficient. P-value <0.05 was considered to indicate a statistically significant difference in all tests. All statistical analyses were performed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

The expressions of TLR4, MyD88 and STAT3 in colorectal adenoma, CRC and peritumoral colorectal tissues

TLR4 protein expression was located in the cytoplasm and membrane, and MyD88 and STAT3 protein expression was located in the cytoplasm, as brownish-yellow granules. The expression of TLR4, MyD88 and STAT3 was seen in 25% (5/20), 15% (3/20) and 45% (9/20) of the colorectal adenoma, respectively. The immunostaining score of TLR4, MyD88 and STAT3 was 1.30 ± 0.57, 1.05 ± 0.51 and 1.35 ± 0.67 in adenoma, respectively (Figure 1). Examples of adenoma tissue specimens of TLR4, MyD88 and STAT3 staining intensity are shown at (Figure 2). The expression of TLR4, MyD88 and STAT3 was seen in 77.5% (31/40), 60% (24/40) and 75% (30/40) of the CRC, respectively. The immunostaining score of TLR4, MyD88 and STAT3 was 1.93 ± 0.83, 1.83 ± 1.04 and 2.22 ± 1.02 in CRC, respectively (Figure 1). Examples of CRC tissue specimens of TLR4, MyD88 and STAT3 staining intensity are shown at (Figure 3).

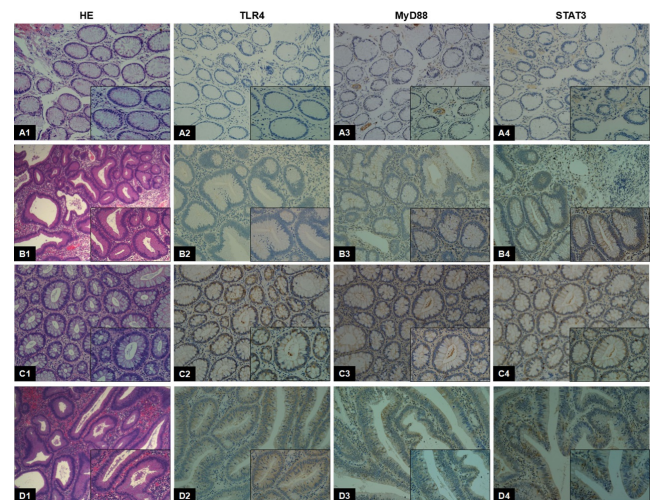


Figure 2. Detection of TLR4, MyD88 and STAT3 immunoreactivity in colorectal adenomas. (A) TLR4 (A2), MyD88 (A3) and STAT3 (A4) showed no immunoreactions in adenoma (A1). (B) No TLR4 (B2) and MyD88 (B3) immunostaining were present in adenoma (B1), but STAT3 (B4) had a positive staining (immunoreactivity score=1). (C) TLR4 (C2), MyD88 (C3) and STAT3 (C4) showed positive immunoreactions in adenoma (C1), the score was 2, 1 and 2, respectively. (D) TLR4 (D2), MyD88 (D3) and STAT3 (D4) showed positive immunoreactions in adenoma (D1), the score was 3, 2 and 1, respectively. Original magnification 200X and 400X.

The expression of TLR4, MyD88 and STAT3 was seen in 7.5% (3/40), 5% (2/40) and 12.5% (5/40) of the peritumoral colorectal tissues, respectively. The immunostaining score of TLR4, MyD88 and STAT3 was 0.70 ± 0.61, 0.50 ± 0.59 and 0.73 ± 0.68 in peritumoral tissues, respectively (Figure 1). Examples of peritumoral tissue specimens of TLR4, MyD88 and STAT3 staining intensity are shown at (Figure 3). The staining intensity of TLR4, MyD88 and STAT3 was significantly higher in the CRC as compared with that of the adenoma and adjacent non-tumor tissues respectively (P<0.05 and P<0.001, respectively) (Figure 1). The staining intensity of TLR4, MyD88 and STAT3 also tended to be significantly higher in the adenoma when compared with the adjacent non-tumor tissues respectively (P<0.05) (Figure 1).

Table 2. Correlation among TLR4, MyD88 and STAT3 in CRC tissues

		TLR4	MyD88	STAT3
TLR4	Spearman Correlation	-	0.328	0.346
	P-value	-	0.039	0.029
MyD88	Spearman Correlation	-	-	0.494
	P-value	-	-	0.001

Correlation among TLR4, MyD88 and STAT3 expression in CRC

In CRC, TLR4 and MyD88 protein expression was significantly positively correlated (r=0.328, P=0.039) as was MyD88 and STAT3 protein expression (r=0.494, P=0.001)

(Table 2). TLR4 and STAT3 protein expression was positively correlated ($r=0.346$, $P=0.029$) (Table 2).

Table 3. Relationship between clinicopathological features and expression of TLR4, MyD88 and STAT3 in CRC tissues.

Variables	TLR4 (+)			MyD88 (+)		STAT3 (+)	
	n	No. (%)	P	No. (%)	P	No. (%)	P
Gender							
Male	24	20 (83.3)	0.441	12 (50.0)	0.188	19 (79.2)	0.482
Female	16	11 (68.8)		12 (75.0)		11 (68.8)	
Age (years)							
<65	25	19 (76.0)	1	14 (56.0)	0.739	17 (68.0)	0.268
≥65	15	12 (80.0)		10 (66.7)		13 (86.6)	
Tumor site							
Distal	25	18 (72.0)	0.44	16 (64.0)	0.526	18 (72.0)	0.714
Proximal	15	13 (86.7)		8 (53.3)		12 (80.0)	
Tumor size (cm)							
<5	22	17 (77.2)	1	13 (59.1)	1	13 (59.1)	0.013
≥5	18	14 (77.7)		11 (61.1)		17 (94.4)	
Pathological grade*							
Group I	31	22 (70.9)	0.089	15 (48.4)	0.006	21 (67.7)	0.081
Group II	9	9 (100.0)		9 (100.0)		9 (100.0)	
LNM							
Negative	17	10 (58.8)	0.023	6 (35.3)	0.009	8 (47.1)	0.001
Positive	23	21 (91.3)		18 (78.3)		22 (95.6)	
TNM stage							
I+II	15	9 (60.0)	0.057	5 (33.3)	0.018	7 (46.7)	0.002
III+IV	25	22 (88.0)		19 (76.0)		23 (92.0)	
Lymphovascular invasion							
Absent	11	10 (90.9)	0.399	6 (54.5)	0.727	7 (63.6)	0.417
Present	29	21 (72.4)		18 (62.1)		23 (79.3)	

*CRC was subdivided into two groups according to differentiation degree; group I was defined as well, well-moderately and moderately differentiated and group II was defined as moderately-poorly and poorly differentiated. LNM: Lymph Node Metastasis; TNM: Tumor Node Metastasis

Relationship between clinicopathological features and expression of TLR4, MyD88 and STAT3 in CRC

When analyzing the relationship between protein expression and clinicopathological features, we found that the expression of TLR4 was significantly correlated with LNM ($P=0.023$) and potentially related to the pathological grade ($P=0.089$) and TNM stage ($P=0.057$) (Table 3). Expression of MyD88 was significantly associated with cancer histology ($P=0.006$), LNM ($P=0.009$) and TNM stage ($P=0.018$) (Table 3). Expression of

STAT3 was significantly associated with tumor size ($P=0.013$), LNM ($P=0.001$) and TNM stage ($P=0.002$) and potentially related to histologic type ($P=0.081$) (Table 3). The expression of each protein was not significantly different according to gender, age, tumor site and lymphovascular invasion.

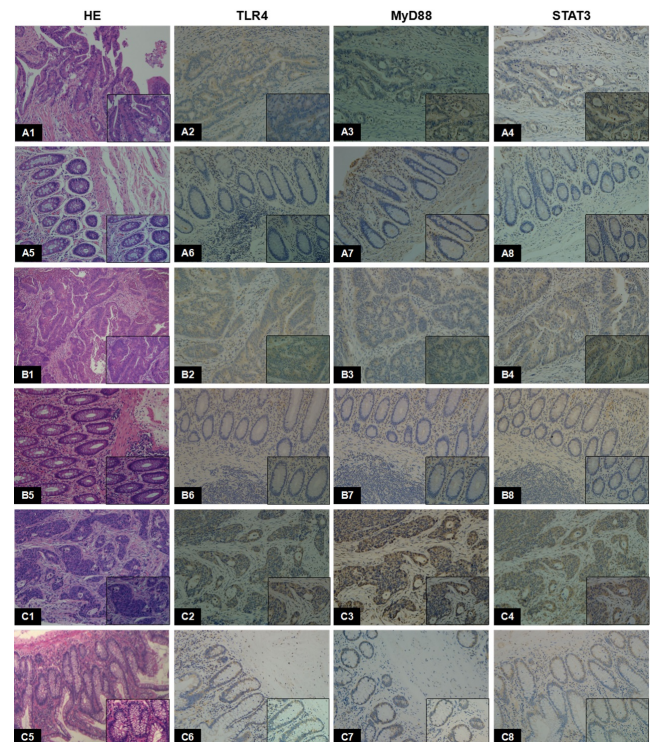


Figure 3. Detection of TLR4, MyD88 and STAT3 immunoreactivity in CRC and adjacent non-tumor tissues. (A) TLR4, MyD88 and STAT3 showed positive immunoreactions in well-moderately differentiated CRC (A1) (immunoreactivity score=2, 1 and 2, respectively), but no immunoreactions in matched non-tumor tissues (A5). (B) TLR4, MyD88 and STAT3 showed positive immunoreactions in moderately differentiated CRC (B1) (immunoreactivity score=4, 2 and 3, respectively), but no immunoreactions in matched non-tumor tissues (B5). (C) TLR4, MyD88 and STAT3 showed positive immunoreactions in poorly differentiated CRC (C1) (immunoreactivity score=3, 3 and 4, respectively) and matched non-tumor tissues (C5) (immunoreactivity score=2, 2 and 2, respectively). Original magnification 200X and 400X.

Discussion

In recent years, there is increasing evidence that TLR4 signaling pathway is involved in tumorigenesis [11]. MyD88 plays a critical role in TLR signaling. Some experimental studies have identified the contribution of TLR4/MyD88 signaling to tumor development and progression [21-23]. In this study, we observed that TLR4 and MyD88 signaling factors were highly expressed in CRC compared with adjacent non-tumor tissues and adenoma, and this result was near to Wang et al. who found that TLR4 and MyD88 immunostaining was absent or very weak in normal mucosa and adenoma, but a higher proportion of CRC cases showed positive expression of TLR4 and MyD88 [21]. Similar result had been reported in breast cancer by Chen et al. [22]. A correlation between TLR4 and MyD88 expression was detected. This agreed with Wang

et al and also agreed with Kim et al. who found that there was a positive correlation between the expression of TLR4 and MyD88 in CRC and ovarian epithelial cancer (OEC) through IHC, respectively [21,23]. Collectively, our study provided us with preliminary results on the possible involvement of TLR4/MyD88 signaling pathway in pathogenesis of CRC.

In the present study, there was a statistically significant association between TLR4 positivity and LNM. This result agreed with Ehsan et al. who found that the incidence of LNM was higher in patients with high TLR4 expression in the breast tumor cells [24]. Moreover, we noted that the expression level of TLR4 tended to be higher in poorly differentiated and advanced tumors, and this result agreed with Wang et al. who found that TLR4 expression was significantly associated with TNM stage in CRC [21]. Similar result had been reported in breast cancer by Chen et al. [22]. These findings indicated that increased TLR4 expression in CRC might be associated with tumor progression.

The current study showed that MyD88 positivity was significantly associated with LNM and TNM stage. This agreed with Chen et al, who found that the expression of MyD88 was significantly associated with axillary LNM and tumor staging in breast cancer [22]. Moreover, in the present study there was a statistically significant association between high expression of MyD88 and histological grade. This result was similar to Wang et al. who found that MyD88 expression had a significant association with differentiation grade in CRC [21]. Similar result had been reported by Kim et al. who found that there was a significant correlation between MyD88 expression and tumor stage and MyD88 was an independent prognostic predictor in OEC [23]. Our findings suggested that MyD88 may partially contribute to the progression of CRC.

STAT3, as an important downstream effector of the TLR4/MyD88 signaling has been identified [15,16]. Recently, many studies have demonstrated that the constitutively activated STAT3 protein contributes to malignant transformation and tumor progression [17]. In the present study, the expression of STAT3 was found to be more intense in CRC compared with adjacent non-tumor tissues and adenoma, and STAT3 protein expression was significantly associated with tumor size and potentially related to histologic type. These results were similar to the observation by Lee et al. conducted on 20 cases of colorectal adenomas and 39 cases of colorectal adenocarcinomas [20]. While they found the expression of STAT3 was increased in the adenocarcinomas as compared with the adenomas and was significantly higher in the tumors with a larger size. Artas et al. found that the expression of STAT3 was significantly higher in colon adenocarcinoma than in normal colon mucosa, and was significantly related to the tumor differentiation [25]. In addition, the current study showed that the STAT3 protein expression was significantly related to LNM and TNM stage. Jin et al. found that the 3- and 5-year survival rates of STAT3 protein-positive CRC patients were lower than the rates in STAT3 protein-negative patients [26]. Our findings might add evidence that STAT3 was participated with the development and progression of CRC.

Our results showed that the expressions of TLR4, MyD88 and STAT3 were increased in CRC as compared with the expressions in colorectal adenoma and adjacent normal mucosa, and the staining intensity was increased with the progression from adenoma to CRC. The results suggested that these proteins may play a driving role towards colorectal carcinogenesis with the progression from adenoma to carcinoma. Especially, our study found that the expression of TLR4, MyD88 and STAT3 was positively correlated with each other in CRC tissues. Further data analysis showed that all these three proteins had correlation with lymph node metastasis in CRC. Evidence from the literature and our findings indicated that the expression of TLR4, MyD88 and STAT3 may be interdependent, and that each has their own role and prognostic value in CRC; elevated these three proteins expression may partly contribute to the development and progression of CRC.

The limitations that we faced in the current study were the small number of CRC cases as they were 40 cases only, also the association between TLR4/MyD88/STAT3 signaling and cancer mortality has not been investigated in clinical samples.

Conclusion

Our study revealed that TLR4, MyD88 and STAT3 were overexpressed in the tumor cells of CRC using IHC and these proteins expression was significantly associated with lymph node metastasis. It is now more evident that TLR4/MyD88/STAT3 proteins may be one of the major signaling factors involved in the pathogenesis of CRC. However, further investigation is required to identify the detailed mechanisms of these proteins in colorectal carcinogenesis.

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