

Original Article

High-level expression of MYH9 predicts poor prognosis in patients with colon cancer

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Abstract: MYH9 (myosin, heavy chain 9, non-muscle) as a gene encodes NM II-A (non-muscle II-A) protein, exists primarily in the cytoplasm. MYH9 consists of 2 myosin heavy chains with a relative molecular mass of 230 kDa. Previous studies have shown that MYH9 is involved in a number of diseases, including cancer. However, the exact role of MYH9 in colon cancer is still unclear. Therefore, we used a tissue microarray of 90 colon cancer cases using immunohistochemistry and western blotting on four cases of paired fresh tissues. After analyzing both the immunohistochemical scores and clinical data, the results suggest that colon cancer tissues had higher expression of MYH9 than normal colon tissues. Higher expression of MYH9 in colon cancer patients predicted a worse N classification and clinical stage. Additionally, colon cancer patients with high expression of MYH9 had a shorter survival time than those with low MYH9 expression. Further, subgroup analysis revealed that the expression level of MYH9 was associated with colon cancer patients' survival time in the T₃₋₄ stage, the N₀₋₁ stage, and the clinical stages I-II. Univariate and multivariate analyses of regression showed that MYH9 is an independent prognostic factor for colon cancer patients. These findings suggested that MYH9 is an oncogene and may be used as an indicator for clinical diagnosis in colon cancer patients. We also demonstrated that MYH9 could serve as an independent prognostic factor for colon cancer patients.

Keywords: MYH9, immunohistochemistry, colon cancer, prognosis

Introduction

Colon cancer has a high prevalence worldwide. In the USA, the American Cancer Society (ACS) estimated that 134,490 patients will be diagnosed with colon cancer and 49,190 patients with colon cancer will die during 2016 [1]. The Chinese Center for Disease Control and Prevention (CDC) has estimated that 376,300 new colon cancer cases would be diagnosed, and 191,000 patients with colon cancer would die during 2015 [2]. Thus, early diagnosis and targeted therapy are extremely important for colon cancer patients. In previous studies, MYH9 (myosin, heavy chain 9, non-muscle) was reported to be involved in cancer metastasis.

MYH9 as a gene encodes NM II-A protein which exists primarily in the cytoplasm. MYH9 consists of 2 myosin heavy chains, with relative

molecular mass of 230 kDa. MYH9 is related to a number of diseases, including focal segmental glomerulosclerosis [3], HIV-associated collapsing glomerulopathy [3], human non-syndromic hereditary deafness [4], macrothrombocytopenia [5], cataracts [5], anaplastic large cell lymphoma [6], and cancers. A study showed that MYH9 plays a protective role for patients with squamous carcinoma migration in head-neck squamous cell carcinoma [7]. However, other studies have reported that MYH9 is harmful to patients with adenocarcinomas through promotion of adenocarcinoma migration in various adenocarcinomas [8-12].

Studies have revealed that MYH9 promoted breast cancer metastasis [9]. Additionally, high-level expression of MYH9 in lung cancer patients was associated with a poor prognosis,

MYH9 expression in colon cancer

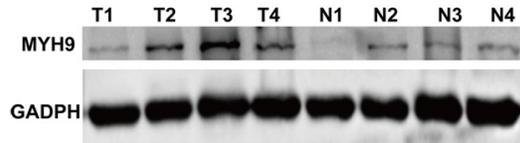


Figure 1. MYH9 is highly expressed in colon cancer tissues compared to normal colon tissues. T stands for colon cancer tissues, N stands for normal colon tissues. Results are from paired tissues from 4 cases of colon cancer patients.

indicating worse pathological stages and increased tumor invasion into the vessels [8]. Moreover, invasion and metastasis of gastric cancer was prevented through the use of microRNA Let-7f, which interfered with MYH9 expression, thus demonstrating that high-level MYH9 expression led to a poor prognosis in gastric cancer [10, 11]. Based upon the work in other cancers, MYH9 is likely to be a poor prognostic indicator for adenocarcinomas. Moreover, it has been shown that the expression of MYH9 in colon cancer is higher than that in normal colon tissues [13]. Although several studies on MYH9 and cancer have been done, the relationship between MYH9 expression and colon cancer and the prognosis of the colon cancer patients is still unclear.

In this study, we examined the expression MYH9 in colon cancer tissues using immunohistochemistry and Western blotting. We also investigated correlations between the MYH9 expression and the clinicopathologic features and the survival period of the colon cancer patients.

Materials and methods

Sample collection

A tissue microarray containing 90 paired colon cancer and normal colon tissues with clinical data was purchased from Shanghai Outdo Biotech Co., Ltd. (Shanghai, China). This tissue microarray included tissues from 45 males and 45 females between 47 and 90 years of age, with a duration of clinical follow-up of 84 months. All specimens were pathologically-confirmed and were classified according to the 7th edition of the American Joint Committee on Cancer (AJCC) criteria. Four cases of paired fresh tissues from colon cancer patients were collected from the Traditional Chinese Medicine-Integrated Hospital of Southern Medical University (Guangzhou, China).

Immunohistochemistry (IHC) and evaluation of staining

The tissue microarray was deparaffinized in 100% xylene and rehydrated in a descending ethanol series and water according to standard protocols. Heat-induced antigen retrieval was performed in 10 mM citrate buffer for 2 min at 100°C [14]. Endogenous peroxidase activity and nonspecific antigens were blocked with a peroxidase-blocking reagent containing 3% hydrogen peroxide and serum, followed by incubation with rabbit anti-human MYH9 antibody (1:200) overnight at 4°C. After washing, the sections were incubated with a biotin-labeled goat anti-rabbit antibody for 20 min at room temperature, and were subsequently incubated with a streptavidin-conjugated horseradish peroxidase (Maixin, Fuzhou, China). A 3, 3'-diaminobenzidine chromogen solution in with DAB buffer substrate was used to add color to the sections. The sections were visualized with DAB and counterstained with hematoxylin, mounted in neutral gum, and analyzed using a bright field microscope.

Two independent pathologists who were blinded to the origination of the samples and subject outcomes assessed the IHC results. The cell staining was semi-quantitatively expressed as an immunohistochemical score in combination with the percentage of based upon the percentage of positive cells (<10%, 0; 10%-25%, 1; 26%-75%, 2; and >76%, 3), and staining intensity (none, 0; weak yellow, 1; brown-yellow, 2; and brown-auburn, 3). The staining intensity and average percentage of positive tumor cells were assayed for 5 independent high magnification (×400) fields. The points for the percentage of positive cells and staining intensity were multiplied. Samples were then assigned to 1 of 2 groups according to the overall score (0-4 points, low expression or 5-6 points, high expression) [15, 16].

Western blot analysis

For protein analysis, 40 mg of tissues were used. The tissues were then cut into pieces and milled using a bead mill homogenizer. Finally, the tissues were lysed in RIPA buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0], 5 mM DTT, and 2% SDS) at 4°C. All protein concentrations were determined using the BCA assay (Beyotime, Beijing, China). Total protein (30 µg) was resolved using 10% SDS-polyacrylamide

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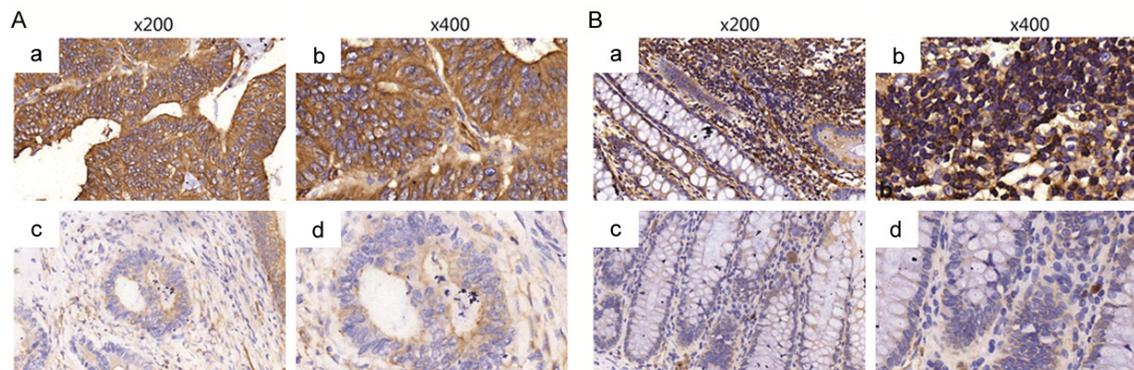


Figure 2. A. It has been observed that MYH9 expression primarily occurs in the cytoplasm of colon cancer. Aa and Ab (high power field) show high MYH9 expression. Ac and Ad (high power field) show low MYH9 levels. B. MYH9 expression occurs in the cytoplasm of normal colon tissue. Ba and Bb (high power field) show high MYH9 expression. Bc and Bd (high power field) show low MYH9 expression.

Table 1. Protein levels of MYH9 between colon cancer tissues and normal colon tissues

Group	Protein expression			P value
	Case	High expression	Low expression	
Cancer	90	43 (47.8%)	47 (52.2%)	
Normal	90	5 (5.6%)	85 (94.4%)	<0.001

gel electrophoresis and bands were electro-transferred to polyvinylidene fluoride membranes (Invitrogen, Los Angeles, CA, USA). The membranes were blocked with 5% BSA in Tris-buffered saline (pH 7.5), followed by immunoblotting overnight at 4°C with MYH9 (1:1000; Proteintect Group, Chicago, IL, USA) or GADPH (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG antibodies were used as secondary antibodies (Zhongshan, Beijing, China). Signals were detected using enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA).

Statistical analysis

All data were analyzed by SPSS (version 19.0). A χ^2 test was used to analyze MYH9 expression between colon cancer and normal colon tissues. The relationship between clinicopathologic characteristics and the MYH9 expression in individuals with colon cancer was also analyzed using a χ^2 test. Survival curves were plotted using the Kaplan-Meier method and compared using a log-rank test. Multivariate survival analysis was performed for all parameters that were significant based upon univariate

analyses using a Cox regression model. A $P < 0.05$ was considered statistically significant.

Results

High-level expression of MYH9 in colon cancer tissues

In order to evaluate the role of MYH9 in colon cancer, we used western blot analysis on four cases of paired fresh colon cancer and normal colon tissues. Results showed that MYH9 expression in colon cancer tissues is higher than that in the normal colon tissues (See **Figure 1**), suggesting that MYH9 is an oncogene in colon cancer.

IHC analyzes of MYH9 in colon cancer and normal colon tissues

We measured the subcellular localization of MYH9 and MYH9 protein levels in the tissue microarray of colon cancer tissues and normal colon tissues using immunohistochemical staining. Highly specific levels of MYH9 appeared in the cytoplasm of colon cancer tissues and normal colon tissues (**Figure 2**). The level of MYH9 in colon cancer tissues (47.8%) was significantly higher than that of normal colon tissues (5.6%, $P < 0.001$; **Table 1**).

The relationship between the clinicopathologic characteristics of colon cancer patients and the cytoplasmic expression of MYH9 in colon cancer tissues

Table 2 provides a summary of results on the relationship between the clinicopathologic

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Table 2. Relationship between clinicopathological characteristics and MYH9 expression levels in individuals with colon cancer (n = 90)

Characteristics	n	MYH9 (%)		P
		High expression	Low expression	
Gender				
Male	45	23 (51.1%)	22 (48.9%)	0.527
Female	45	20 (44.4%)	25 (55.6%)	
Age (years)				
≥65	57	31 (54.4%)	26 (45.6%)	0.099
<65	33	12 (36.4%)	21 (63.6%)	
T classification				
T ₁ + T ₂	6	3 (50.0%)	3 (50.0%)	1.000
T ₃ + T ₄	84	40 (47.8%)	44 (52.2%)	
N classification				
N ₀ + N ₁	78	33 (42.3%)	45 (57.7%)	0.008
N ₂	12	10 (83.3%)	2 (16.7%)	
Distant metastasis				
M ₀	89	43 (48.3%)	46 (51.7%)	1.000
M ₁	1	0 (0%)	1 (100%)	
Clinical stage				
I-III B	80	35 (43.8%)	45 (56.3%)	0.030
III C-IV	10	8 (80.0%)	2 (20.0%)	

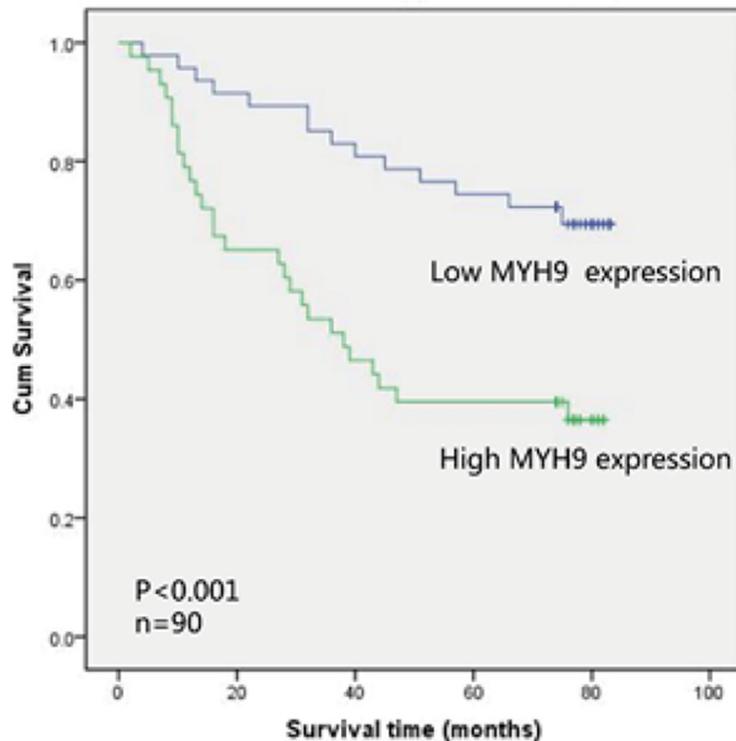


Figure 3. A Kaplan-Meier survival curve with data from 90 patients with colon cancer. Patients with colon cancer expressing low MYH9 levels had achieved better survival times when compared to those with high MYH9 expression ($P < 0.001$, log-rank test).

characteristics of colon cancer patients and MYH9 expression in colon cancer tissues. The expression level of MYH9 was not correlated with the gender, age, T classification, or M classification of the 90 patients with colon cancer. However, we did observe a positive correlation between the expression level of MYH9 and the N classification (N₀-N₁ vs. N₂; $P = 0.008$) and clinical stage (I-III B vs. III C-IV; $P = 0.030$; **Table 2**).

MYH9 expression is associated with the survival time of colon cancer patients

To investigate the prognostic value of MYH9 expression in colon cancer patients, we analyzed the relationship between MYH9 expression and survival time of the colon cancer patients, using a Kaplan-Meier analysis with a log-rank test. MYH9 expression was significantly correlated with the 90 patients' overall survival. Moreover, tumors with high MYH9 expression had a worse prognosis than those with low MYH9 expression ($P < 0.001$; **Figure 3**).

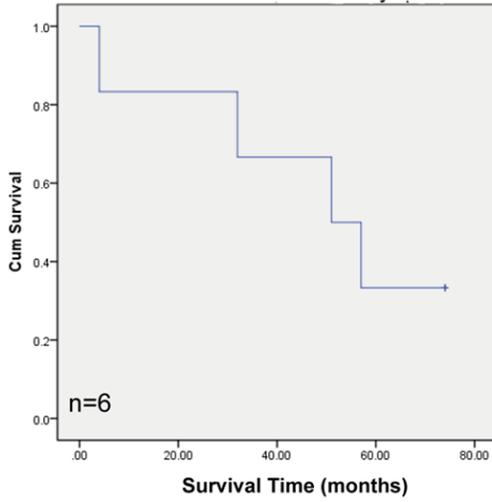
Subgroup analysis suggested that MYH9 expression was inversely associated with survival time for colon cancer patients with T₃₋₄ and N₀₋₁ tumors and those in clinical stage I and II

We analyzed the correlation between the cytoplasmic expression of MYH9 and the prognosis of colon cancer patients according to T classification, N classification, and clinical stage (**Figure 4**). High MYH9 expression was related to a shorter survival time in colon cancer patients with T₃₋₄ ($P < 0.001$) and N₀₋₁ stage

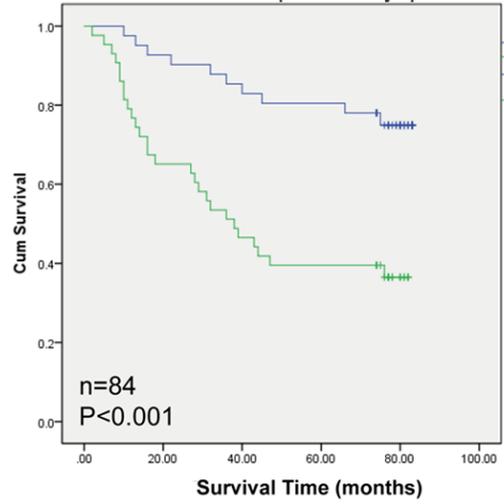
MYH9 expression in colon cancer

— Low MYH9 Expression
— High MYH9 Expression

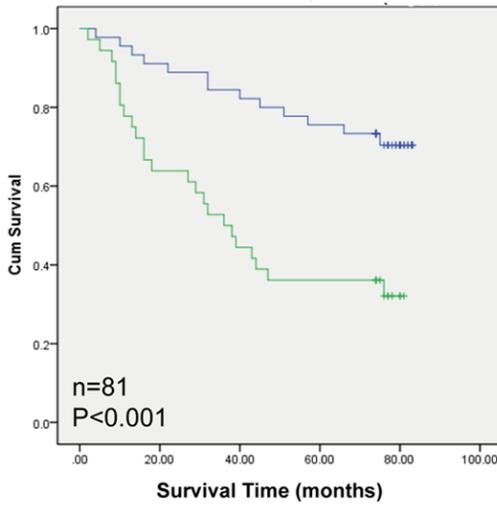
T1-T2



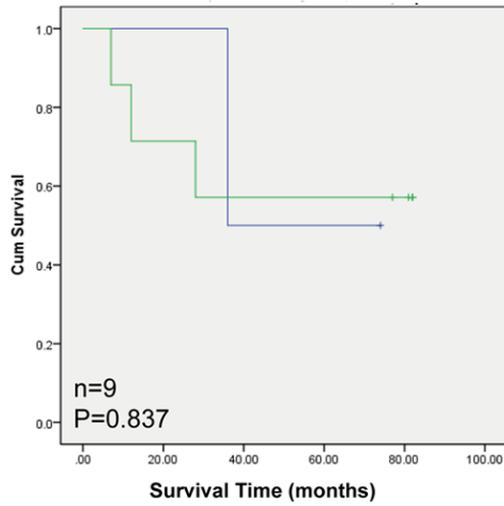
T3-T4



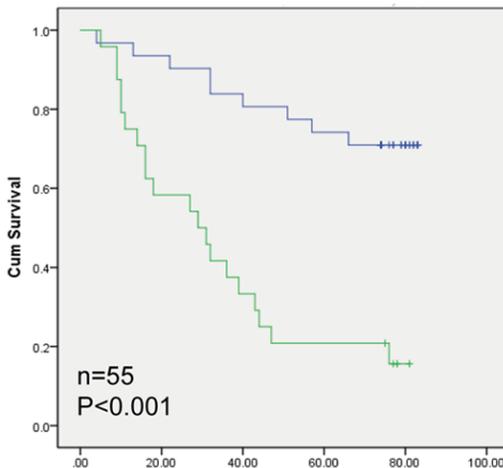
N0-N1



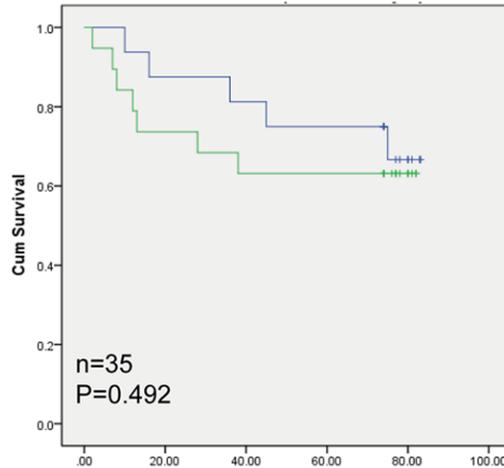
N2



Clinical Stage I-II



Clinical Stage III-IV



MYH9 expression in colon cancer

Figure 4. Correlation of MYH9 expression with colon cancer patients' survival time, stratified by T classification, N classification and clinical stage. MYH9 protein expression was associated significantly with a shorter survival for patients in stages T_{3,4}, stages N_{0,1}, and clinical stages I-II, but did not correlate with the survival of those in stages T_{1,2}, stage N₂, or clinical stages I-II.

Table 3. Summary of univariate and multivariate Cox regression analysis of overall survival duration (n = 90)

Parameters	Univariate analysis			Multivariate analysis		
	P	HR	95% CI	P	HR	95% CI
Age						
≥65 vs. <65	0.124	1.766	0.855-3.649			
Gender						
Male vs. Female	0.082	1.783	0.930-3.421			
T classification						
T ₁₋₂ vs. T ₃₋₄	0.979	505492.552	0.000-			
N classification						
N ₀₋₁ vs. N ₂	0.932	1.045	0.381-2.864			
M classification						
M ₀ vs. M ₁	0.926	1.114	0.113-10.929			
Clinical Stage						
I-II vs. III-IV	0.009	2.528	1.267-5.047	0.002	0.374	0.201-0.695
MYH9 expression						
High vs. Low	0.002	0.335	0.167-0.671	0.001	0.322	0.168-0.617

(P<0.001) tumors and those in clinical stage I-II (P<0.001).

High expression of MYH9 is an independent prognostic factor for colon cancer patients

Univariate analysis showed that clinical stage (P = 0.009) and cytoplasmic expression of MYH9 (P = 0.002) were significantly correlated with the survival time of colon cancer patients (**Table 3**). In order to identify whether MYH9 is an independent prognostic factor for colon cancer, we carried out a multivariate analysis on the variation of MYH9 expression in the clinical stages. The results showed that the expression level of MYH9 was indeed an independent prognostic factor (P = 0.001).

Discussion

MYH9 promotes cancer metastasis by lamellipodia formation at the leading edge of the cell [17]. Lamellipodia formation is controlled by Rac1, the WAVE complex, and the Arp2/3 complex [18]. Previous studies have reported that high MYH9 expression is a poor prognosis indicator in non-small cell lung cancer [8], gastric cancer [10, 11], bladder cancer [12], and breast

cancer [9]. Moreover, MYH9 promoted cancer metastasis. In contrast, others have shown that high MYH9 expression plays a protective role in squamous cell carcinoma, potentially due to its ability maintain the post-transcriptional stability of p53 [7] or maintain the mitotic stability of the chromosomes during karyomitosis [19]. The explanation of the role of MYH9 in squamous cell carcinoma versus other tumors is not clear. Additionally, the role of MYH9 expression in colon cancer and its influence on the survival period of colon cancer patients has not been examined.

In this study, we have showed that MYH9 expression in colon cancer tissues is higher than that in normal colon tissues using western blotting. These results agree with previous research in lung cancer [8], breast cancer [9], and gastric cancer [10].

Further, we evaluated the expression of MYH9 protein in colon cancer tissues immunohistochemical staining. MYH9 expression was mainly located in the cytoplasm, which matches similar findings in lung cancer [8], gastric cancer [10, 11], and breast cancer [9]. Since MYH9

can promote cell migration by forming lamellipodia at the edge of the cell, we analyzed the correlation between MYH9 expression and the clinical features of the colon cancer patients. We found that although MYH9 expression was not related to patient age, gender, T classification, or distant metastasis, it had a positive correlation with N classification and clinical stage, indicating that MYH9 is a potential oncogene.

Moreover, a negative correlation between MYH9 expression in colon cancer tissues and the survival period of colon cancer patients was noted, suggesting that high expression of MYH9 may act as an important clinical biomarker for colon cancer patients with poor prognosis. Additionally, the relationship with prognosis and the MYH9 levels were assessed further by the stratification of T and N classification and clinical stage. The results showed that MYH9 expression had a reverse correlation with the survival period of colon cancer patient in T_{3,4} stage, N_{0,1} stage, and clinical stage I-II, indicating that high MYH9 expression may promote colon cancer cell proliferation in advanced stages and promote cell migration in the early stages of colon cancer.

Finally, we assessed whether MYH9 is as an independent prognostic factor for patients with colon cancer. According to univariate analysis, the survival period of patients was inversely correlated to clinical stage and MYH9 expression. Moreover, multivariate analyzes showed that high MYH9 expression is a significant indicator of poor prognosis for colon cancer patients, which agrees with observations in lung [8], breast [8], gastric [10], bladder [11], and esophageal cancers [20].

In summary, we have confirmed that high MYH9 expression is associated with the clinical progression of colon cancer patients. MYH9 suggests a poor prognosis for colon cancer patients. Furthermore, data indicate that MYH9 may serve as an independent prognostic indicator for colon cancer patients.

Disclosure of conflict of interest

None.

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