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ORIGINAL ARTICLE

Retrospective Study

Evaluation of clinical significance of claudin 7 and construction of prognostic grading system for stage II colorectal cancer

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Author contributions: Quan JC wrote the manuscript; Wang HY, Zou SM and Wang XS conceived of and designed the study and contributed equally; Guan X, Zhuang M, Wang S, Chen HP, Liu Z and Sun P collected the data; Quan JC, Peng J and Jiang Z analyzed the data; all authors made critical revisions for the manuscript and approved the final version.

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Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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Abstract

BACKGROUND

Claudin 7 is often abnormally expressed in cancers and promotes the progression of some malignancies. However, the role of claudin 7 in stage II colorectal cancer (CRC) has not been studied.

AIM

To assess the expression and prognostic value of claudin 7 in stage II CRC.

METHODS

We retrospectively studied 231 stage II CRC patients who underwent radical surgery at our hospital from 2013 to 2014. The protein expression level of claudin 7 was assessed and its relationship with clinicopathological features and prognosis was statistically analyzed. The independent prognostic factors were identified by Cox proportional hazards models. A prognostic grading system was constructed to stratify the survival of CRC patients.

RESULTS

The expression of claudin 7 was significantly reduced in cancer tissues compared

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with normal tissues ($P < 0.001$), and its low expression was closely related to recurrence of the disease ($P = 0.017$). Multivariate analysis confirmed that claudin 7 low expression (claudin 7-low) ($P = 0.028$) and perineural invasion positivity (PNI+) ($P = 0.026$) were independent predictors of poor disease-free survival (DFS). A prognostic grading system based on the status of claudin 7 and PNI classified the patients into three prognostic grades: grade A (claudin 7-high and PNI-), grade B (claudin 7-low and PNI-, claudin 7-high and PNI+), and grade C (claudin 7-low and PNI+). The DFS was significantly different among the three grades (grade B *vs* grade A, $P = 0.032$; grade C *vs* grade A, $P < 0.001$; grade C *vs* grade B, $P = 0.040$).

CONCLUSION
Claudin 7 can be used as a new prognostic marker to predict the DFS of patients with stage II CRC. The prognostic grading system with the addition of claudin 7 can further improve prognosis stratification of patients.

Key words: Colorectal cancer; Stage II; Claudin 7; Perineural invasion; Prognosis; Prognostic grading system

Core tip: The clinical significance of claudin 7 in stage II colorectal cancer has not been studied. This is believed to be the first study to assess the expression and prognosis of claudin 7 in stage II colorectal cancer. We found that claudin 7 and perineural invasion were independent prognostic factors associated with disease-free survival. A prognostic grading system was constructed based on the status of claudin 7 and perineural invasion, which can better stratify disease-free survival.

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INTRODUCTION

Colorectal cancer (CRC) is a common malignancy, and its incidence and mortality rank fourth and second, respectively[1]. Furthermore, the incidence and mortality of CRC is still on the rise in many countries[2], which seriously threatens human health; thus, it is crucial to identify the factors influencing prognosis. At present, the International Union Against Cancer tumor node metastasis (TNM) staging system is widely used to predict prognosis and guide treatment. For patients with the same stage, they should have similar outcomes according to the TNM staging system. However, there are often different outcomes among these patients in clinical practice, including stage II CRC patients.

Although the overall prognosis of stage II patients is relatively good, some patients will develop local recurrence or distant metastasis within 5 years after radical surgery, which is the leading reason for poor prognosis and cancer-associated death. At present, although some conventional clinicopathologic factors have been used to distinguish these high risk patients, use of only these conventional factors can not better classify such patients. Therefore, the identification of risk factors and precise classification of these patients are still challenging. In addition to conventional clinicopathological factors, more and more attention has been paid to the study of molecular markers, as the application of molecular markers may be helpful in further stratifying prognosis.

Claudins, the main constituents of intercellular tight junctions, play a crucial role in maintaining the paracellular barrier[3]. However, the deregulation of claudins can lead to the development of many diseases[4,5]. Currently, the claudin family is known to contain 27 members[6], and as one of the important claudin family members, claudin 7 has received a remarkable amount of attention. It has been reported that claudin 7 is

abnormally expressed in many types of tumors[7-9], and its deregulation promotes the invasion and metastasis of cancer[10,11]. In addition, the aberrant expression of claudin 7 was also found to be related to prognosis of breast carcinoma[12], lung cancer[13], nasopharyngeal carcinoma[14,15], oral and oropharyngeal squamous cell cancer[16], hepatocellular cancer[17], ovarian cancer[18], laryngeal carcinoma[19], gastric carcinoma[20], and clear cell renal cell carcinoma[21]. However, only limited studies have focused on CRC, and, as far as we know, there have been no studies on the clinical significance of claudin 7 in stage II CRC to date. Therefore, it is necessary to clarify the potential value of claudin 7 in stage II CRC.

In our study, the purpose was to assess the protein expression of claudin 7 in stage II CRC and its relationship with clinicopathological features and prognosis. In addition, we also comprehensively analyzed the prognostic factors in stage II CRC and developed a prognostic grading system to accurately classify the survival difference among patients.

MATERIALS AND METHODS

Patients

This retrospective study included 231 stage II CRC patients who underwent radical surgery at the Cancer Hospital, Chinese Academy of Medical Sciences from 2013 to 2014. All patients had histopathology-confirmed stage II CRC. The patients' clinicopathological data, including age, gender, tumor location (colon or rectum), preoperative carcinoembryonic antigen (CEA) level (negative or positive), T stage (T3 or T4), tumor differentiation (well/moderate or poor), perineural invasion (PNI, negative or positive), lymphovascular invasion (negative or positive) and postoperative adjuvant chemotherapy, were obtained. This study was approved by the Institutional Review Board of the Cancer Hospital, Chinese Academy of Medical Sciences.

Tissue microarray and immunohistochemistry

Paraffin-embedded cancer tissue blocks were acquired from all the patients. An additional 72 paraffin-embedded adjacent normal tissue blocks were also prepared. After assessing the hematoxylin and eosin-stained slides, the donor paraffin blocks were punched (1.0 mm diameter of the core), and the tissue cores were placed into recipient paraffin blocks to construct the tissue microarray (TMA) blocks. Following construction of the TMA blocks, 5 μm-thick tissue sections were cut for subsequent immunohistochemical study.

The protein expression level of claudin 7 was investigated by immunohistochemistry staining of the above TMAs. The immunohistochemical procedures were as follows: TMA sections were deparaffinized and rehydrated in xylene and gradient ethanol, respectively. After deparaffination and rehydration, the sections were heated following immersion in ethylenediaminetetraacetic acid buffer and using a microwave oven for antigen retrieval, and then washed with distilled water. Afterwards, the sections were incubated in 3% hydrogen peroxide for 30 min, and then washed in phosphate buffered saline (referred to as PBS) before blocking of nonspecific binding. Rabbit polyclonal antibody against claudin 7 (ab27487, 1:200; Abcam, Cambridge, United Kingdom) was used as the primary antibody and the sections were incubated overnight at 4 °C. After washing with PBS, the sections were incubated with the corresponding secondary antibody for 30 min at 37 °C, and then washed with PBS again. Subsequently, diaminobenzidine and hematoxylin were used for developing the color and counterstaining, respectively.

Scoring of immunostaining results

The immunoreactivity of claudin 7 was evaluated by two independent investigators, based on the staining intensity and percentage of positively stained cells. The immunostaining intensity was divided into four categories: 3, strong intensity; 2, moderate; 1, weak; 0, negative. The percent of positive cells was classified as 0, less than 5%; 1, 6%-25%; 2, 26%-50%; 3, 51%-75%; 4, > 75%. The results of staining intensity and staining percentage were multiplied to obtain the final comprehensive score (range: 0-12), and 0 score was defined as negative expression, scores of 1-5 as weak expression, scores of 6-8 as moderate expression, scores of 9-12 as strong expression. According to the comprehensive score, the immunoreactivity of claudin 7 was further classified as high expression (scores of 6-12) and low expression (scores < 6) for subsequent statistical analysis.

Statistical analysis

Clinicopathological variables are expressed in the form of median with corresponding

range (continuous variables) and number with percent (categorical variables). The χ^2 test was used to evaluate the relationship between claudin 7 expression and clinicopathological features. The Mann-Whitney *U* test was applied to examine the difference between groups. The prognostic factors were identified by Cox proportional hazards models. Disease-free survival (DFS) rates were assessed by the Kaplan-Meier method, and the differences were compared with the log-rank test. In further analysis, we performed a stratified analysis of prognosis using a combination of independent prognostic factors, and divided patients into different prognostic grades. The DFS among different grades was compared by Cox analysis, and the corresponding hazard ratio (HR) and 95% confidence interval (CI) were estimated. All statistical analyses were performed using SPSS version 20.0 (IBM Corp., Armonk, NY, United States), and the difference was considered statistically significant when the *P* value was less than 0.05.

RESULTS

Protein expression level of claudin 7 and its relationship with clinicopathological features

The claudin 7 expression status in normal tissues and cancer tissues was evaluated, respectively. The immunohistochemical results showed that 87.50% of normal tissue samples had strong expression and the remaining 12.50% had moderate expression, while the proportion of strong expression, moderate expression and weak/negative expression in cancer tissue samples was 23.81%, 50.65% and 25.54%, respectively. The representative images are shown in Figure 1. Compared with normal tissues, the claudin 7 protein expression in cancer tissues was statistically reduced ($P < 0.001$; Figure 2). Table 1 shows the detailed patient characteristics. Table 2 presents the analysis of relationships between claudin 7 expression and clinicopathological features. The results revealed that claudin 7 expression was only related to disease recurrence, and the incidence of disease recurrence was higher in the claudin 7 low expression group than in the high expression group ($P = 0.017$). However, no correlations were found regarding age, gender, CEA level, tumor location, T stage, tumor differentiation, PNI, and lymphovascular invasion.

Prognostic factors of stage II CRC

Univariate and multivariate prognostic analyses were performed to identify the factors that affected patient survival (Table 3). In univariate analysis, it was found that T stage, PNI, and claudin 7 expression levels were the most important factors influencing DFS; whereas, no statistical differences were observed for gender, age, CEA level, tumor location, postoperative adjuvant chemotherapy, tumor differentiation, and lymphovascular invasion. With regard to T stage, claudin 7 expression levels and PNI were included in the multivariate analysis. The results revealed that only PNI (HR = 2.586; 95%CI: 1.121-5.966; $P = 0.026$) and claudin 7 (HR = 2.366; 95%CI: 1.100-5.091; $P = 0.028$) were independent prognostic factors associated with DFS. Figure 3A shows the DFS curves of different claudin 7 subgroups, and DFS was significantly worse in the claudin 7 low expression group than in the high expression group ($P = 0.011$, log-rank test). Similarly, compared to patients with PNI, those without PNI had a better DFS ($P = 0.002$, log-rank test), the survival curves are shown in Figure 3B.

Construction of the prognostic grading system

In order to determine whether the combined assessment of claudin 7 and PNI can better classify patient survival, we further developed a prognostic grading system based on the above two independent prognostic factors (Table 4), and the patients were classified into three prognostic grades: Grade A, grade B, and grade C. Grade A was defined as claudin 7 high expression (claudin 7-high) and PNI negative (PNI-), grade B was defined as claudin 7 low expression (claudin 7-low) and PNI negative or claudin 7 high expression and PNI positive (PNI+), and grade C was defined as claudin 7 low expression and PNI positive. Cox analysis showed that grade B (HR = 2.512; 95%CI: 1.084-5.817; $P = 0.032$) and grade C patients (HR = 7.963; 95%CI: 2.704-23.452; $P < 0.001$) had a statistically poorer DFS compared with grade A patients. Similarly, the DFS in grade C patients was also significantly worse than in grade B patients (HR = 2.987; 95%CI: 1.051-8.489; $P = 0.040$). Figure 3C shows the survival curves of the three grades ($P < 0.001$, log-rank test).

Table 1 Clinicopathological features of the patients with stage II colorectal cancer, *n* (%)

Variable	No. of patients
Age in yr	
Median (range)	60 (30-79)
≤ 60	124 (53.7)
> 60	107 (46.3)
Gender	
Male	140 (60.6)
Female	91 (39.4)
CEA level	
Negative	119 (51.5)
Positive	54 (23.4)
Unknown	58 (25.1)
Tumor location	
Colon	130 (56.3)
Rectum	101 (43.7)
Tumor differentiation	
Well/moderate	209 (90.5)
Poor	22 (9.5)
T stage	
T3	189 (81.8)
T4	42 (18.2)
Lymphovascular invasion	
Negative	200 (86.6)
Positive	31 (13.4)
Perineural invasion	
Negative	191 (82.7)
Positive	40 (17.3)
Postoperative chemotherapy	
No	147 (63.6)
Yes	84 (36.4)
Recurrence	
No	204 (88.3)
Yes	27 (11.7)

CEA: Carcinoembryonic antigen.

DISCUSSION

More and more studies have reported the effects of claudin 7 on various cancers[11,18,21]. However, the role of claudin 7 in CRC has not been fully elucidated. Therefore, further studies are necessary to examine the potential value of claudin 7 in CRC. In the present study, we analyzed claudin 7 expression level in stage II CRC, and evaluated its relationship with clinicopathological features and prognosis. As far as we know, we are the first group to investigate the value of claudin 7 in stage II CRC.

Claudin 7 expression differs in different types of cancers. For example, in gastric carcinoma, Jun *et al*[20] reported that claudin 7 expression was up-regulated, while Zhou *et al*[19] showed that claudin 7 expression was down-regulated in laryngeal carcinoma. For CRC, Tang *et al*[22] revealed that claudin 7 had low expression in cancer tissues; similarly, Bornholdt *et al*[23] reported the same conclusion at the mRNA level, based on PCR analysis. In the present study, we analyzed the protein expression levels of claudin 7 using immunohistochemistry and found that claudin 7 expression was decreased in CRC tissues compared to that in adjacent normal tissues, which further confirmed the findings of Tang *et al*[22] and Bornholdt *et al*[23].

At present, only a limited number of articles have reported the relationship between claudin 7 and clinicopathological features in CRC. Moreover, the conclusions vary among different studies. Wang *et al*[24] assessed claudin 7 expression in 80 CRC patients and showed that there was a significant association between claudin 7

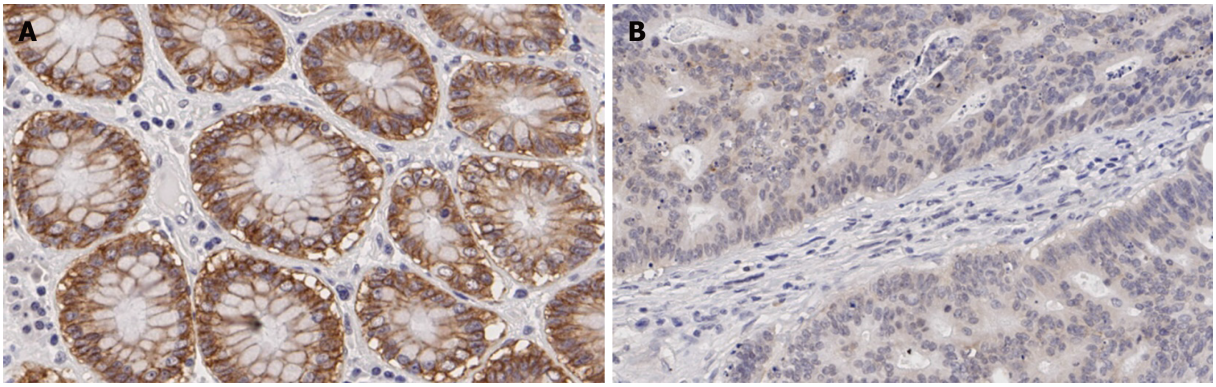

Figure 1 Immunochemical staining of claudin 7. A: Immunochemical staining of claudin 7 in normal colorectal tissue; B: Immunochemical staining of claudin 7 in cancer tissue.

expression and degree of differentiation and liver metastasis. In addition, Oshima *et al*[25] analyzed 205 CRC patients, and revealed that claudin 7 expression was correlated with liver metastasis and venous invasion but not with the degree of differentiation. However, unlike the above studies, our study specifically focused on stage II CRC and found that low expression of claudin 7 was only related to disease recurrence, and no correlations were found with other clinicopathological features, including tumor differentiation, T stage, PNI, and lymphovascular invasion. The differences in variables and sample sizes in different studies may be the reason for the above contradictions. Therefore, more and larger studies are needed to obtain definitive conclusions in the future.

In addition to its involvement in the development of cancer, claudin 7 is also an important prognostic factor. Its prognostic value has been previously reported in different cancers, such as gastric cancer, ovarian cancer, hepatocellular cancer and so on. In the study by Li *et al*[21], a significant correlation was found between low expression of claudin 7 and poor prognosis in clear cell renal cell carcinoma. Additionally, Kim *et al*[18] also revealed that claudin 7 expression was related to progression-free survival of epithelial ovarian carcinoma patients. Jun *et al*[20] analyzed the survival of patients with gastric cancer, and reached the same conclusion that claudin 7 was a significant factor affecting the prognosis of patients. Similarly, Bouchagier *et al*[17] and Yamamoto *et al*[13] also emphasized the prognostic value of claudin 7 in hepatocellular carcinoma and lung cancer. However, little is known about the relationship of claudin 7 with survival outcomes in CRC due to a limited number of cases and lack of survival information in previous studies.

In order to understand whether claudin 7 has prognostic significance in CRC, we evaluated the effects of claudin 7 on DFS in stage II CRC patients and confirmed that claudin 7 was an independent predictor of DFS and that the DFS in the claudin 7 high expression group was significantly better than in the low-expression group. Our study showed that claudin 7 was a novel marker for predicting DFS in stage II CRC patients. Moreover, the protein expression level of claudin 7 can be detected using routine immunohistochemistry; therefore, claudin 7 detection can be easily applied in clinical practice to provide guidance for better prediction of DFS in stage II CRC.

In addition to claudin 7, PNI was another important predictive factor for DFS. In our study, the DFS of patients with PNI was significantly worse than those without PNI, which was consistent with the findings of Huh *et al*[26]. In order to assess whether claudin 7 combined with PNI could more accurately classify DFS in patients with stage II CRC, we constructed a prognostic grading system and divided the patients into three subgroups based on the above two independent predictors and found that DFS was worse in patients with low expression of claudin 7 and the presence of PNI; in addition, patients with high expression of claudin 7 and absence of PNI had the best DFS. By prognostic stratification analysis, statistical differences in DFS were found among the three subgroups, and claudin 7 combined with PNI can better stratify DFS. Therefore, in clinical practice, we can make individualized risk assessments based on this prognostic grading system.

In conclusion, the present study revealed the clinical value of claudin 7 in stage II CRC. Moreover, we also constructed a prognostic grading system using the combination of claudin 7 and PNI, which can more accurately distinguish the survival difference among different patients.

Table 2 Relationship between the expression of claudin 7 and clinicopathological features, *n* (%)

Variable	No. of patients	Claudin 7 expression		*P* value
		High	Low	
Age in yr				
≤ 60	124	97 (78.2)	27 (21.8)	0.158
> 60	107	75 (70.1)	32 (29.9)	
Gender				
Male	140	99 (70.7)	41 (29.3)	0.106
Female	91	73 (80.2)	18 (19.8)	
CEA level				
Negative	119	91 (76.5)	28 (23.5)	0.708
Positive	54	40 (74.1)	14 (25.9)	
Unknown	58	41 (70.7)	17 (29.3)	
Tumor location				
Colon	130	96 (73.8)	34 (26.2)	0.809
Rectum	101	76 (75.2)	25 (24.8)	
Tumor differentiation				
Well/moderate	209	159 (76.1)	50 (23.9)	0.082
Poor	22	13 (59.1)	9 (40.9)	
T stage				
T3	189	143 (75.7)	46 (24.3)	0.374
T4	42	29 (69.0)	13 (31.0)	
Lymphovascular invasion				
Negative	200	150 (75.0)	50 (25.0)	0.632
Positive	31	22 (71.0)	9 (29.0)	
Perineural invasion				
Negative	191	144 (75.4)	47 (24.6)	0.477
Positive	40	28 (70.0)	12 (30.0)	
Recurrence				
No	204	157 (77.0)	47 (23.0)	0.017
Yes	27	15 (55.6)	12 (44.4)	

CEA: Carcinoembryonic antigen.

Table 3 Univariate and multivariate prognostic analyses of patients with stage II colorectal cancer

Variable	Univariable analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age in yr				
≤ 60	Reference			
> 60	1.310 (0.616-2.788)	0.483		
Gender				
Male	Reference			
Female	0.903 (0.413-1.971)	0.797		
CEA level				
Negative	Reference			
Positive	1.224 (0.488-3.067)	0.667		
Unknown	1.133 (0.452-2.842)	0.789		
Tumor location				
Colon	Reference			
Rectum	1.384 (0.651-2.945)	0.399		
Tumor differentiation				
Well/moderate	Reference			

Poor	1.228 (0.370-4.080)	0.737		
T stage				
T3	Reference		Reference	
T4	2.558 (1.147-5.701)	0.022	1.817 (0.771-4.285)	0.172
Lymphovascular invasion				
Negative	Reference			
Positive	1.984 (0.800-4.919)	0.139		
Perineural invasion				
Negative	Reference		Reference	
Positive	3.190 (1.457-6.985)	0.004	2.586 (1.121-5.966)	0.026
Postoperative chemotherapy				
No	Reference			
Yes	0.859 (0.386-1.913)	0.710		
Claudin 7 expression				
High	Reference		Reference	
Low	2.578 (1.206-5.509)	0.015	2.366 (1.100-5.091)	0.028

CEA: Carcinoembryonic antigen; CI: Confidence interval; HR: Hazard ratio.

Table 4 Prognostic stratification analysis based on different grades

Variable	HR	95%CI	*P* value
Grade A (claudin 7-high and PNI-)	1	-	-
Grade B (claudin 7-high and PNI+, claudin 7-low and PNI-)	2.512	1.084-5.817	0.032
Grade C (claudin 7-low and PNI+)	7.963	2.704-23.452	< 0.001

Grade C *vs* Grade B, $P = 0.040$ (HR = 2.987; 95%CI: 1.051-8.489). HR: Hazard ratio. Claudin 7-high: Claudin 7 high expression; claudin 7-low: Claudin 7 low expression; PNI-: Perineural invasion negative; PNI+: Perineural invasion positive. CI: Confidence interval; HR: Hazard ratio.

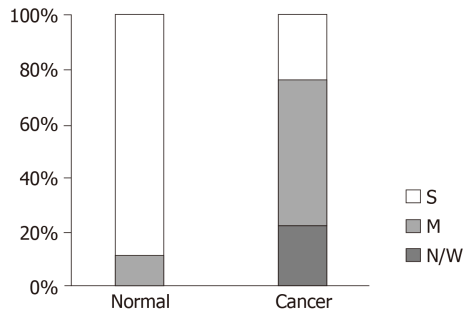

Figure 2 Comparison of claudin 7 expression in colorectal cancer tissues and normal tissues (*P* < 0.001). N/W: Negative/weak expression; M: Moderate expression; S: Strong expression.

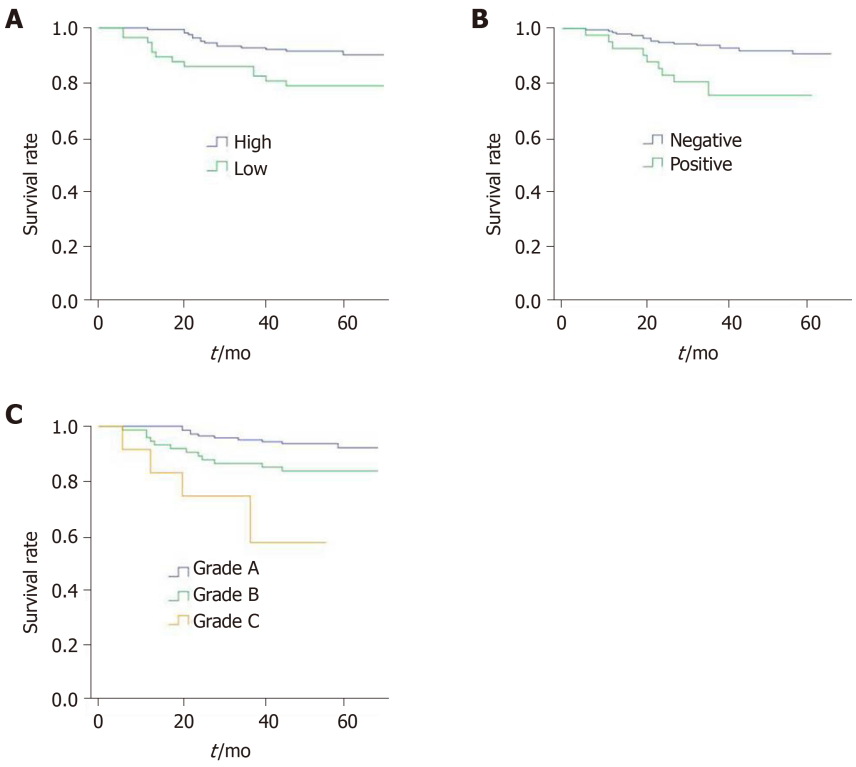

Figure 3 Kaplan-Meier curves for disease-free survival. A: Kaplan-Meier survival curves for patients with high and low claudin 7 expression (P = 0.011, log-rank test); B: Kaplan-Meier survival curves for patients with or without perineural invasion (P = 0.002, log-rank test); C: Kaplan-Meier survival curves for patients with different prognostic grades (P < 0.001, log-rank test).

ARTICLE HIGHLIGHTS

Research background

Claudin 7 has been found to be abnormally expressed in cancers and plays a critical role in the progression of some malignancies. However, there have been no studies on the clinical significance of claudin 7 in stage II colorectal cancer (CRC) to date. Therefore, it is necessary to clarify the potential value of claudin 7 in stage II CRC.

Research motivation

We evaluated the expression and prognostic value of claudin 7 in stage II CRC, and further constructed a prognostic grading system to accurately classify the survival difference among patients.

Research objectives

To assess the clinical significance of claudin 7 and construct a prognostic grading system for stage II CRC.

Research methods

This retrospective study included 231 stage II CRC patients who underwent radical surgery at our hospital from 2013 to 2014. The protein expression level of claudin 7 was assessed and its relationship with clinicopathological features and prognosis was statistically analyzed. The Kaplan-Meier method was used to assess the disease-free survival, and Cox proportional hazards models were used to determine the independent prognostic factors. A prognostic grading system was constructed to stratify the patients into different subgroups, and the survival differences among different subgroups were compared.

Research results

The expression of claudin 7 was decreased in cancer tissues compared to that in normal tissues, and its low expression was related to disease recurrence. Prognostic analysis showed that claudin 7 low expression (claudin 7-low) and perineural invasion positivity (PNI+) were independent prognostic factors associated with disease-free survival. A prognostic grading system based on the above two independent prognostic factors divided the patients into three prognostic grades: grade A (claudin 7-high and PNI-), grade B (claudin 7-low and PNI-, claudin 7-high and PNI+), and grade C (claudin 7-low and PNI+). Subgroup analysis showed that the prognosis of patients with different grades differed significantly.

Research conclusions

Our study identifies the prognostic significance of claudin 7 in stage II CRC. Furthermore, we also confirm that the prognostic grading system can better classify patient survival.

Research perspectives

The prognostic grading system can be used as an effective prognostic predictive tool to help clinicians more accurately predict survival.

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