The Expression and Clinical Significance of *Hook1* in Colorectal Cancer

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Abstract: Purpose — Colorectal cancer is one of the most common gastrointestinal malignancies around the world. Early diagnosis of colon cancer is still a difficult problem for clinicians. This study aimed to determine the expression of *Hook1* in colon cancer and evaluate its clinical role. Methods — Real-time Quantitative PCR was performed to characterize the difference of *Hook1* mRNA expression between colon cancer specimens and normal colon specimens. Immunohistochemistry was used to evaluate the *Hook1* expression in colon cancer tissue. Analyses of correlation between *Hook1* level and clinicopathologic features and prognosis were also performed. Results — In total, 70 pair of surgical samples of patients with colorectal cancer were collected. And 70 patients were received postoperative following-up with the median follow-up being 38 months. Enhanced *Hook1* mRNA levels were observed in colorectal cancer tissues (1.49%) compared to normal tissues (0.76%). IHC also revealed significantly higher rates of *Hook1* expression amongst 58 cases in colon cancer tissues versus normal colon. Higher *Hook1* protein levels were associated with better differentiation degree and easier likelihood of lymph node metastasis. Patients with relatively lower *Hook1* level survived extended period without disease progression. And age, distant metastasis, TNM stage and *Hook1* protein expression were marginally identified as poor prognosis indicator. Conclusion — *Hook1* is highly expressed in colorectal cancer tissues, which correlate to well differentiated degree and lymph node metastasis tendency of colorectal cancer and potentially identified as a poor prognosis predictor.

***Keywords*:** Hook1, colorectal cancer, tumor biomarkers, differentiated degree, lymph node metastasis, prognosis

1. Introduction

Colorectal cancer (CRC) is one of the most common gastrointestinal malignancies worldwide, with the relatively high incidence and mortality [1]. In China, CRC ranks the second commonly diagnosed cancer types. Recently, the incidence and mortality of CRC are still on the rise though advances in diagnosis and treatment. Surgical resection is an effective approach for its treatment, enabling the improvement of 5-year OS [2, 3]. However, some patients were diagnosed with metastasis at the first time and lost the chance of radical surgery [4, 5]. Both metastasis and recurrence are reasons for poor prognosis after operation [6-9]. Besides, although advanced treatment methods developed, there are still little improvement in prognosis [10].Thus, early diagnosis of CRC can provide patients with good treatment chances and further improve prognosis.

Pathological biopsy is considered as the gold standard for diagnosing CRC. However, due to complications and patients' discomfort, its popularity is limited as screening method for CRC [11]. Fecal occult blood test was a complication-free screening modality but with either low accuracy [12, 13]. Recently, liquid-biopsy was proposed as favorable approach for the identifying CRC patients, reducing unnecessary invasive procedures [14]. There are numerous studies on biomarkers for the diagnosis and prognosis of CRC, which still need to be validated.

Hook gene was first reported by Mohr [15] nearly a century ago. It has been found that *Hook1* and their homologues Hook2 and Hook3 play an important role in endocytosis transport [16] and correlate to the dynamic equilibrium of microtubule cytoskeleton [17]. Hook family members can regulate tumor progression as cytoskeleton is significant in the biological behavior of tumor cells such as proliferation and migration [18]. Keyuehai's team first reported that *Hook1* is involved in the negative regulation of epithelial-mesenchymal transformation (EMT) in non-small cell lung cancer (NSCLC) [19]. Later studies have reported that *Hook1* present the same effect in liver cancer [20]. However, the role of *Hook1* in CRC was scarcely understood.

Our research group has been dedicated to the research of CRC. In this study, *Hook1* expression in CRC was detected and the clinical effect in CRC patients was evaluated.

* Lei Yue and Yingchao Sun jointly acted as first authors of this work.

2. Materials and methods

2.1 Participants population and tissue samples

Surgical specimens of CRC were obtained during operation of the Department of Gastroenterological Surgery, Sir Run Run Shaw Hospital, Medical College, Zhejiang University, Hangzhou, China, between June 2017 and July 2018. Patients were included accordingly and electronic medical record system was retrospectively consulted for retrieving clinical information. No patients had preoperative operation history and family history of malignancies. Moreover, no other malignancies and major physical diseases were found before surgery. Besides, no preoperative adjuvant therapy was performed.

The study was conducted from April 2019 to May 2021 in compliance with Helsinki Declaration and approved by the Institutional Review Board of Sir Run Run Shaw Hospital (Scientific research 20190409-24). The Institutional Review Board approved the exemption application of informed consent.

2.2 Real-Time Quantitative-PCR (q-PCR)

Real-time quantitative PCR was performed to quantify *Hook1* mRNA levels in specimens. Total RNA was extracted from fresh frozen samples with Trizol reagent (Hangzhou fude biological technology co., ltd.). First step was reverse transcription by using reverse transcription reagent kit (Takara). After that, PCR was performed in total 35 to 40 cycles, containing Initial denaturation at 95°C for 10min followed by denaturation at 95°C for 15s combined with annealing/extension at 65°C for 1 min. The internal control was used by U6 with forward primer (5′-TGCTGCTGAGATTATGCCAGTGGA-3′) and reverse primer (5′-TCAGCCTCTGCTCAGTTTCCAGTT-3′). The relative expression of the target gene was calculated according to the ΔCt value, defined as follows, Ct of the target gene minus Ct of the endogenous control (U6). Finally, the result was calculated as 2 -ΔCt.

2.3 Immunohistochemistry (IHC)

Pathological samples were analyzed by routine immunohistochemistry. The paraffin sections were deparaffinized and then put into another dye vat containing xylene. Rehydration was performed after the deparaffinization. The tissue slides were sequentially placed into ethanol with different concentration gradients followed by immerging the sections into pure water and phosphate buffered saline (PBS; pH 7.4). Sections were incubated in 3% hydrogen peroxide solution followed by PBS washing and antigen retrieval. Then sections were blocked with 10% normal goat serum and then incubated with a rabbit monoclonal antihuman *Hook1* antibody (ab151756, Abcam, USA, 1:150 dilution) at 4℃ overnight. Then, slides were incubated with biotin-conjugated goat anti-rabbit secondary antibody at RT. Finally, the slides were stained with diaminobenzidine and counterstained with hematoxylin followed by dehydration and sealing.

The expression level of protein of *Hook1* was defined according to the following criteria: -, +, 2+ or 3+ were indicative of slides with 0, 1-25%,26-50% or greater than 50% positive cells stained. [21] *Hook1* protein expression was classified as five categories (tumor tissues minus normal tissues): equal, -/+, +, +/++, ++. model 1: *: -/+, +; **: +/++, ++. model 2: $: -/+; $$: +; $$$: +/++; $$$$: ++.

2.4 Statistics

All statistical analyses were performed using the SPSS 23.0 statistical software. Differences between groups were calculated using pairwise Wilcoxon rank sum test. Chi-square test and Kruskal-wallis H test was used to determine correlation of *Hook1* levels with baseline features of patients. Binary logistic regression, ordinal logistic regression and Spearman rank correlation were used simultaneously for correlation analyses. Survival analyses were performed using Kaplan-Meier method and GraphPad Prism 8 was used for the plotting. Cox regression was conducted for evaluating prognosis related factors. All P values are two-sided and $P < 0.05$ was indicative of statistical significance.

3. Results

Collectively, 70 pair of samples of CRC patients were collected. The baseline features of patients are showed in Table 1.

Table 1. The Baseline Features of Patients

Variables		Participants numbers (%)
Gender		
	Male	39 (56%)
	Female	31(44%)
Age (years)		
	Range	40-95
	Means ± SD	65.8 ± 11.8

	< 60	20 (29%)
	≥60	50 (71%)
Tumor location		
	Colon	44 (63%)
	Rectum	26 (37%)
Tumor size		
	< 5 cm	44 (63%)
	≥5 cm	26 (37%)
Differentiated degree		
	Well	7 (10%)
	Moderately	57 (82%)
	Poorly	6 (8%)
Lymph node metastasis		
	Positive	36 (51%)
	Negative	34 (49%)
Distant metastasis		
	Positive	11 (16%)
	Negative	59 (84%)
TNM stage		
	I	9 (13%)
	II	24 (34%)
	III	26 (37%)
	IV	11 (16%)

3.1 *Hook1* Expression distribution between tissues

Hook1 mRNA were over-expressed in CRC specimens compared to adjacent nontumorous specimens (1.49% vs 0.76%) (Figure 1) ($P < 0.001$). The expression quantity of *Hook1* protein was also upregulated in CRC than in nontumorous specimens (Figure 2; $P < 0.001$). Increased staining intensities of IHC were observed in Figure 3.

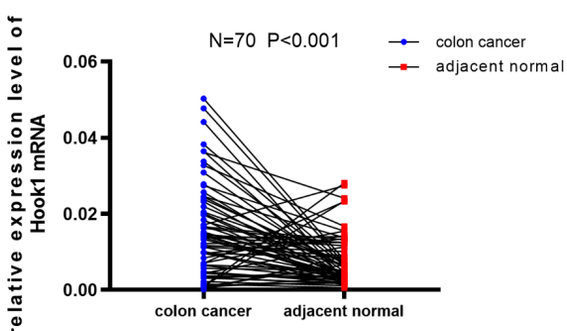

Figure 1. *Hook1* mRNA level in cancer tissues and adjacent nontumorous tissues

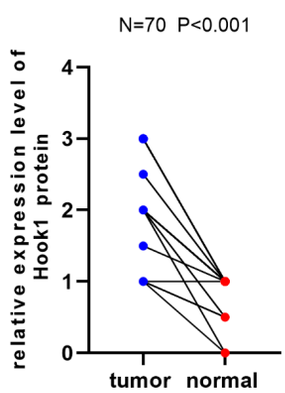

Figure 2. *Hook1* protein level in cancer tissues and adjacent nontumorous tissues

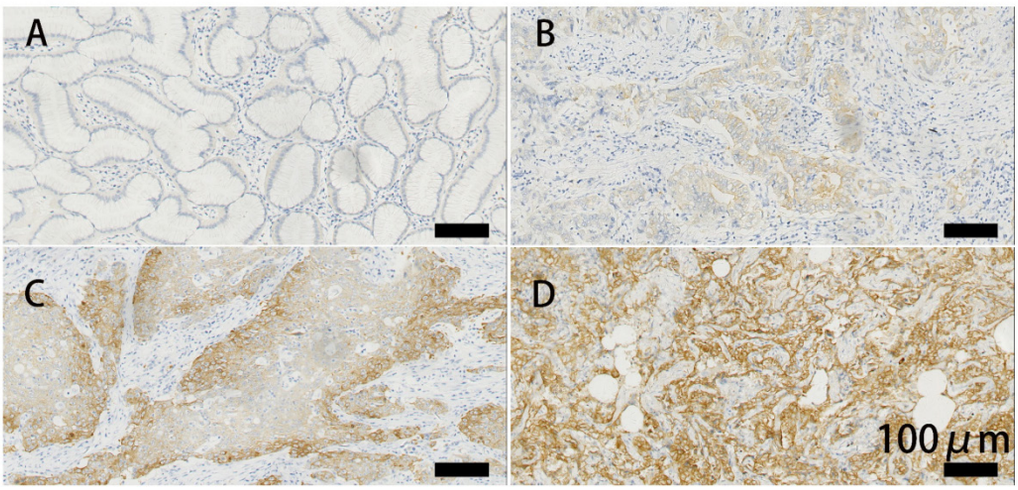

Figure 3. *Hook1* protein expression in tumor and adjacent nontumorous specimens. (Magnification, × 200). A, B, C and D: staining intensity with -, +, 2+ and 3+ respectively

3.2 Correlation between *Hook1* Expression and cohort features

Sequential differentiated degree presented between-group differences (P=0.046) (Table2). Significant difference was observed of *Hook1* protein expression in model 2 in differentiated degree(P=0.039) (Table 3). Logistic regression showed the association of easier likelihood of lymph node metastasis with higher *Hook1* protein level (OR:27.220, 95%CI: 1.110-667.253, P=0.043). Ordinal logistic regression showed that differentiated degree was one of influencing factors for *Hook1* protein both in model 1 (P=0.033) and in model 2 (P= 0.008). Besides, differentiated degree was negatively correlated to *Hook1* protein expression concerning Spearman rank correlation in model 2(P=0.030, γ=-0.295), indicating that as the degree of differentiation deteriorated, the gene expression level decreased. (Table4)

Table 2. Difference of *Hook1* levels in Baseline Features of CRC Patients

			mRNA level			Protein level		
Features		Number of patients	Low	High	P	Equal	High	P
Total	70	18 (26%)	52 (74%)		12 (17%)	58 (83%)		
Gender					0.594			1.000
	Male	39	9 (23%)	30 (77%)		7 (18%)	32 (82%)	
	Female	31	9 (29%)	22 (71%)		5 (16%)	26 (84%)	
Age (years)					0.560			1.000
	<60	20	4 (20%)	16 (80%)		3 (15%)	17 (85%)	
	≥60	50	14 (28%)	36 (72%)		9 (18%)	41 (82%)	
Tumor location					0.259			0.341
	Colon	44	9 (20%)	35 (80%)		6 (14%)	38 (86%)	
	Rectum	26	9 (35%)	17 (65%)		6 (23%)	20 (77%)	
Tumor size					0.406			1.000
	<5 cm	44	13 (30%)	31 (70%)		8 (18%)	36 (82%)	
	≥5 cm	26	5 (19%)	21 (81%)		4 (15%)	22 (85%)	
Differentiated degree					0.637			0.046*
	Well	7	1 (14%)	6 (86%)		2 (29%)	5 (71%)	
	Moderately	57	16 (28%)	41 (72%)		7 (12%)	50 (88%)	
	Poorly	6	1 (17%)	5 (83%)		3 (50%)	3 (50%)	
Lymph node metastasis					0.787			0.345
	Positive	34	8 (24%)	26 (76%)		4 (12%)	30 (88%)	
	Negative	36	10 (28%)	26 (72%)		8 (22%)	28 (78%)	
Distant metastasis					0.135			0.675
	Positive	11	5 (45%)	6 (55%)		1 (9%)	10 (91%)	
	Negative	59	13 (22%)	46 (78%)		11 (19%)	48 (81%)	
TNM stage				0.272			0.608	

	I	9	2 (22%)	7 (78%)		1 (11%)	8 (89%)	
	II	24	7 (29%)	17 (71%)		6 (25%)	18 (75%)	
	III	26	4 (15%)	22 (85%)		4 (15%)	22 (85%)	
	IV	11	5 (45%)	6 (55%)		1 (9%)	10 (91%)	
Living state (outcome 1)					0.692			0.719
	Survival	54	14 (26%)	40 (74%)		10 (19%)	44 (81%)	
	Death	9	3 (33%)	6 (67%)		0 (0%)	9 (100%)	
Living state (outcome 2)					0.332			0.186
	Progression-free survival	51	13 (25%)	38 (75%)		10 (20%)	41 (80%)	
	Metastases/ Relapse/Death	12	4 (33%)	8 (67%)		0 (0%)	12 (100%)	

*, $P < 0.05$.

Table 3. Difference of *Hook1* mRNA and Protein Expression in Clinicopathological Features of CRC Patients

		Protein level (model 1)				Protein level (model 2)						
Features	Number of patients	Equal	High*	High**	Pα	Equal	High$^{\$}$	High$^{\$\$}$	High$^{\$\$\$}$	High$^{\$\$\$\$}$	P^{α}	
Total	70	12	32	26		12	7	25	6	20		
Gender						0.787						1.000
	Male	39	7	18	14		7	2	16	4	10	
	Female	31	5	14	12		5	5	9	2	10	
Age (years)						0.966						0.946
	<60	20	3	10	7		3	3	7	0	7	
	≥60	50	9	22	19		9	4	18	6	13	
Tumor location						0.947						0.757
	Colon	44	6	23	15		6	5	18	5	10	
	Rectum	26	6	9	11		6	2	7	1	10	
Tumor size						0.792						0.527
	<5 cm	44	8	20	16		8	6	14	4	12	
	≥5 cm	26	4	12	10		4	1	11	2	8	
Differentiated degree						0.189						0.039*
	Well	7	2	1	4		2	0	1	0	4	
	Moderately	57	7	29	21		7	5	24	5	16	
	Poorly	6	3	2	1		3	2	0	1	0	
Lymph node metastasis						0.929						0.629
	Positive	34	4	19	11		4	5	14	4	7	
	Negative	36	8	13	15		8	2	11	2	13	
Distant metastasis						0.420						0.540
	Positive	11	1	5	5		1	1	4	2	3	
	Negative	59	11	27	21		11	6	21	4	17	
TNM stage						0.716						0.680
	I	9	1	4	4		1	1	3	0	4	
	II	24	6	8	10		6	1	7	2	8	
	III	26	4	15	7		4	4	11	2	5	
	IV	11	1	5	5		1	1	4	2	3	
						Pβ						Pβ
Living state (outcome 1)						0.370						0.320
	Survival	54	10	25	19		10	6	19	3	16	
	Death	9	0	5	4		0	1	4	2	2	
Living state (outcome 2)						0.211						0.081
	Progression-free survival	51	10	24	17		10	5	19	2	15	
	Metastases/Relapse/Death	12	0	6	6		0	2	4	3	3	

α, Kruskal-wallis H test; β, chi-square test. *, $P < 0.05$.

Table 4. Correlation Between *Hook1* Expression and Features of CRC Patients

	Binary Logistic Regression						Ordinal Logistic Regression				Spearman Rank Correlation			
							model 1		model 2		model 1		model 2	
	mRNA level			protein level										
Features	OR	95%CI	P	OR	95%CI	P	95%CI	P	95%CI	P	γ	P	γ	P
Gender	1.009	0.273-3.721	0.990	2.578	0.511-12.997	0.251	-1.712-0.448	0.251	-1.431-0.612	0.432	0.032	0.790	0.001	1.000
Age	1.010	0.959-1.064	0.709	1.006	0.947-1.069	0.839	-1.188-0.967	0.841	-1.108-0.957	0.886	0.011	0.927	-0.018	0.883
Tumor location	0.455	0.139-1.494	0.194	0.328	0.076-1.426	0.137	-0.961-1.032	0.945	-1.158-0.748	0.673	0.008	0.948	0.037	0.759
Tumor size	1.201	0.759-1.899	0.434	1.237	0.726-2.108	0.435	-1.520-0.573	0.375	-1.635-0.381	0.222	0.074	0.542	0.105	0.388
Differentiated degree	0.668	0.161-2.768	0.578	0.292	0.058-1.471	0.136	0.207-4.920	0.033*	0.818-5.459	0.008*	-0.181	0.134	-0.259	0.030*
Lymph node metastasis	2.291	0.227-23.146	0.483	27.220	1.110-667.253	0.043*	-4.787-0.749	0.153	-4.009-1.193	0.289	-0.011	0.930	-0.058	0.633
Distant metastasis	0.278	0.021-3.765	0.336	33.312	0.318-3491.052	0.140	-2.924-0.390	0.134	-2.573-0.528	0.196	0.097	0.424	0.074	0.544
TNM stage	0.920	0.184-4.607	0.920	0.148	0.013-1.748	0.130	-0.737-5.133	0.142	-1.109-4.423	0.240	-0.007	0.951	-0.045	0.711

*, $P < 0.05$; OR, odds ratio; CI, confidence interval; γ, correlation coefficient.

3.3 Survival analyses and prognosis relevance of *Hook1* in CRC

70 participants were received postoperative follow-up from 1 months to 47 months with median follow-up being 38 months. Patients' survival status was categorized as outcome 1 and outcome 2 (the endpoint event is defined as death in outcome 1 and metastases or relapse or death in outcome 2). In the *Hook1* protein-equal group, the median survival time was 36.5 months with a 4-year OS of 100%. In the *Hook1* protein-high group, the median survival time was 38 months, with a 4-year OS of 83% and the median PFS time was 37 months, with a 4-year PFS of 77%.

Hook1 expression in CRC potentially elucidated poor prognosis (P = 0.081) (Table 3). As depicted in Figure 4A, PFS in patients with higher protein level was lower than that in those with lower protein level (P=0.098). Similarly, patients with lower *Hook1* level survived an extended period without disease progression (Figure 4B, P=0.069). Additionally, univariate analyses indicated that age, distant metastasis and TNM stage were influence factors of poor prognosis in outcome 1. Concerning PFS, age, distant metastasis, TNM stage and especially *Hook1* (model 1) level were marginally identified as poor prognosis indicator (Table 5). Independent prognosis influence factor was distant metastasis both in outcome 1 and in outcome 2 according to multivariate Cox model (Table 6)

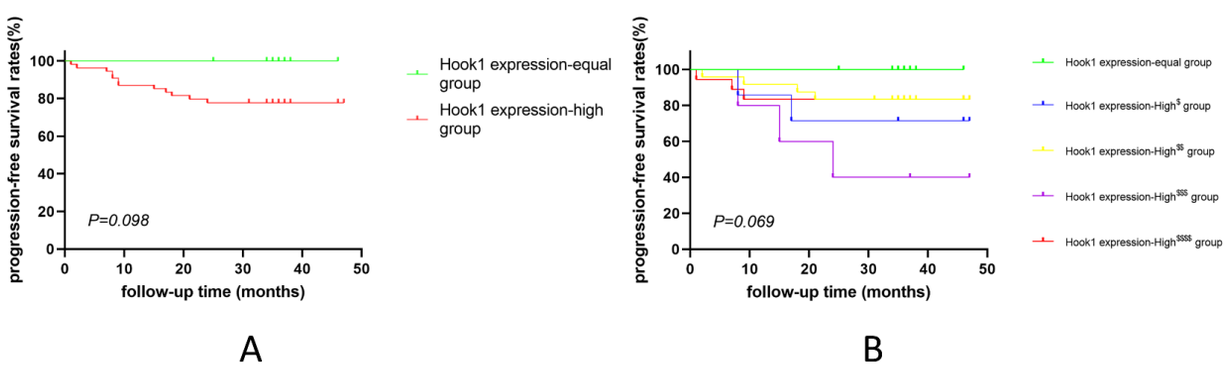

Figure 4. PFS curves of *Hook1* protein level (model 1 (A) and model 2 (B)) in CRC patients.

Table 5. Association of Variables and *Hook1* Expression with Prognosis of CRC Patients by Univariate Cox Regression

Variables	Living state (outcome 1)			Living state (outcome 2)		
	HR	95%CI	P	HR	95%CI	P
Gender	1.653	0.444-6.155	0.454	0.936	0.297-2.948	0.909
Age	1.067	1.014-1.124	0.013*	1.057	1.011-1.105	0.014*
Tumor location	0.802	0.201-3.208	0.755	0.520	0.141-1.922	0.327

Tumor size	0.964	0.632-1.473	0.867	0.963	0.669-1.387	0.84
Differentiated degree	3.270	0.850-12.584	0.085	2.631	0.793-8.730	0.114
Lymph node metastasis	2.124	0.531-8.494	0.287	3.319	0.898-12.268	0.072
Distant metastasis	8.302	2.220-31.043	0.002*	11.380	3.541-36.573	0.001*
TNM stage	2.282	1.002-5.199	0.05	3.192	1.459-6.986	0.004*
mRNA expression	0.696	0.174-2.784	0.609	0.691	0.208-2.296	0.546
protein expression	27.332	0.018-42065.779	0.377	27.601	0.051-14843.978	0.301
protein expression (model 1)	1.869	0.671-5.211	0.232	2.193	0.876-5.491	0.094
protein expression (model 2)	1.243	0.766-2.016	0.378	1.270	0.834-1.933	0.266

HR; hazard ratio; 95% CI; 95% confidence interval. *, $P < 0.05$.

Table 6. Association of Variables and *Hook1* Expression with Prognosis of CRC Patients by multivariate Cox Regression

Variables	Living state (outcome 1)			Living state (outcome 2)		
	HR	95%CI	P	HR	95%CI	P
Age	1.057	0.988-1.130	0.108	1.045	0.987-1.106	0.130
Differentiated degree	2.257	0.521-9.777	0.276	1.504	0.397-5.696	0.548
Lymph node metastasis	5.773	0.356-93.644	0.217	10.507	0.795-138.946	0.074
Distant metastasis	35.089	1.572-783.212	0.025*	41.768	2.938-593.817	0.006*
TNM stage	0.214	0.033-1.385	0.106	0.243	0.044-1.343	0.105
protein expression (model 1)	1.197	0.373-3.847	0.763	1.782	0.612-5.191	0.289

HR; hazard ratio; 95% CI; 95% confidence interval. *, $P < 0.05$.

4. Discussion

Commonly used biomarkers based on liquid-biopsy of CRC include CEA and CA19-9. However, the insufficiency of accuracy limited their popularization [13]. Furthermore, serum CEA levels were recognized only to be related with a high risk of postoperative recurrence [22]. To the best of our knowledge, this is the first estimation of the role *Hook1* play in CRC and its clinical application value. In this study, *Hook1* was identified as a promising biomarker both in the prediction of progression and prognosis assessment of CRC suffers.

Hook1 might play essential roles in pathophysiological processes of various tumors. It is reported that *Hook1* protein expression correlate to clinical stages of NSCLC. However, no significant association was observed between it and patients' prognosis [23]. Cao et al identified a negative association of Hook1with long OS in thyroid carcinoma [21].Thus, *Hook1* might play various roles in diverse carcinoma. We observed that higher *Hook1* expression was related to better degree of differentiation and easier likelihood of lymph node metastasis, showing that *Hook1* might be correlated with the development and progression of CRC.

Our research findings have profound clinical implications. Regarding research outcomes of Yang et al, the positive expression rate of *Hook1* proteins were 57.4% in NSCLC samples [23]. However, the positive rate of *Hook1* comes to 100% in CRC tissues in this study. The tendency to be overexpressed suggest that *Hook1* might play a pivotal role in CRC. Recently, NGS has become an emerging research hotspot, which contribute to guiding target-oriented regimens [24]. Based on our investigation, *Hook1* can be used as a candidate gene for instructing individualized treatment in CRC. Combined with distant metastasis being independently poor prognosis predictor, prognostic prediction value of *Hook1* in CRC lay a solid foundation for its application in clinical practice.

Potential limitations warrant consideration in this study. Firstly, nature of single-center and insufficient sample capacity could not compensate observer variation. Future studies in larger populations may be preferable. In addition, there is no specific analysis of the mechanism of action of *Hook1* and in vivo model assessment. And further researches are necessary for illuminating potential mechanism of action in CRC. Finally, this study only analyzed the histological expression level of *Hook1* lack of body fluids level. However, since *Hook1* has shown to be associated with lymph node metastasis inclination and unfavorable prognosis, *Hook1* is expected to become a novel biomarker for the prediction the occurrence and progression of CRC in the foreseeable future.

5. Conclusions

Hook1 is highly expressed in colorectal cancer tissues, which correlate to well differentiated degree and lymph node metastasis tendency of colorectal cancer and potentially identified as a poor prognosis predictor.

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