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BASIC RESEARCH

Predicting prognosis of rectal cancer patients with total mesorectal excision using molecular markers

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Abstract

AIM: To explore the prognostic variables in rectal cancer patients undergoing curative total mesorectal excision and the effect of postoperative chemotherapy in advanced rectal cancer.

METHODS: A total of 259 consecutive rectal cancer patients treated with curative total mesorectal excision between 1999 and 2004 were collected. p53, p21, PCNA, and CD44v6 were examined using immunohistochemistry (IHC). The correlation between clinicopathological or molecular variables and clinical outcomes, including local recurrence, metastasis, disease-free survival and overall survival, was analyzed.

RESULTS: The median follow-up was 44 mo. Five-year survival rates and 5-year disease free survival rates were 75.43% and 70.32%, respectively. Multi-analysis revealed TNM staging, preoperative CEA, and CD44v6 level were independent risk factors predicting overall survival or disease free survival. The hazard ratio of peroperative CEA was 2.65 (95% CI 1.4-5) and 3.10 (95% CI 1.37-6.54) for disease free survival and overall survival, respectively. The hazard ratio of CD44v6 was 1.93 (95% CI 1.04-3.61) and 2.21 (95% CI 1.01-4.88) for disease free survival and overall survival, respectively. TNM staging was the only risk factor predicting local recurrence. Postoperative chemotherapy without radiotherapy did not improve patients' outcome.

CONCLUSION: TNM staging, preoperative CEA and CD44v6 were independent prognostic factors for rectal cancer patients with total mesorectal excision. Postoperative chemotherapy may be only used together with radiotherapy for rectal cancer patients.

INTRODUCTION

Colorectal cancer is the third leading cause of cancer death in both males and females. Approximately 35% of colorectal cancers are located in the rectum of patients from Western countries. In China the proportion reached approximately 50%.

New advances such as the standardized surgical technique total mesorectal excision (TME), preoprative or post-oprative radiotherapy and adjuvant chemoradiotherapy have reduced the previously high local recurrence rate and improved overall survival time in rectal cancer patients. Despite these advances, about 40% of patients still die from local or distant recurrence. Hence, new prognostic markers are required to help predict the patients who would benefit from adjuvant treatment.

The knowledge regarding the molecular biology of colorectal cancer has facilitated the study of molecular markers in patients with colorectal cancer. Several tumor associated proteins including p53, p21, p27, cyclin D1, PCNA, CD44, Ki67 may be relevant prognostic markers in rectal cancer. These markers were widely studied in many cancers including colorectal cancer, but the results related to prognosis and implications in colorectal cancer remain controversial, especially in rectal cancer. No single molecular marker has been demonstrated to provide consistent prognostic information yet.

Immunohistochemical (IHC) technique, which is easy, stable with experienced pathologists, and fast with commercially available antibody, is widely used in studies for molecular markers. In this study, the protein expression of p53, p21, PCNA and CD44 was examined with immunohistochemical technique to evaluate their prognostic value in rectal cancer patients undergoing

curative total mesorectal excision (TME).

MATERIALS AND METHODS

Clinicopathological information

A total of 343 rectal cancer patients, who underwent total mesorectal excision in Cancer Hospital of Fudan University from January 1999 to June 2004, were collected retrospectively. The median follow-up time is 44 mo, ranging from 1-90 mo. Twenty-one cases (6.5%) who were lost at the beginning of the surveillance were excluded. Sixty-three patients with simultaneous distant metastases or lesions invading other organs (e.g. bladder, vesicle, prostate, posterior of vagina or urethra) were excluded in this study. All the surgeries were performed by experienced colorectal surgeons. Lateral lymphadenectomy was not performed in our series. A total of 259 patients were available after the screening.

The basic clinicopathological information is presented in Table 1. All cases were histologically confirmed adenocarcinoma and reviewed by two pathologists.

Adjuvant treatment

Adjuvant radiation was not routinely given to stage Ⅱ or stage Ⅲ patients with optimal total mesorectal excision with R0 resection before 2005 in our hospital. Only patients with T4 tumors below peritoneal reflex, which invaded other organs (bladder, prostate, vesicle, vagina, *etc.*) would receive postoperative radiotherapy or chemoradiotherapy. Chemotherapy with 5-Fu based regimenswas given to a part of patients with stage Ⅱ or stage Ⅲ disease and prospective observation was carried out to find out its effect in rectal cancer. None of the patients had received preoperative radiotherapy or chemoradiotherapy.

Immunohistochemistry

Two hundred and fifty-nine formalin fixed paraffin embedded tumor specimens were obtained at the department of pathology in the same hospital. These specimens were cut into 4 μm slides, dewaxed with dimethyl benzene and dehydrated in graded acetone. Tissues previously shown to express the antigen of interest were considered positive controls (i.e. colonic adenocarcinoma for p53, CD44v6, breast carcinoma for p21, normal colon for PCNA), and the primary antibody was replaced by TBS in the negative controls. A minimum of eight sections were examined per case, in which every two slides were used for a single marker.

All the 259 colorectal cancer specimens were collected and specific biological markers were analysed with immunohistochemical procedure, using the enVision two-step visualization technique (DAKO) which was described by Ulrike Kämmerer[1] and Schwandner[2]. The monoclonal antibodies, including anti-p53 (Clone DO-7,code no. M7001, DAKO, dilution, 1/50), anti-p21ras (Clone: NCC-RAS-001,code no. M0637, DAKO, dilution, 1/100), anti-PCNA (Clone PC 10,code no. M 0879,DAKO,dilution 1/300), and anti-CD44 variant 6 (Clone VFF-7,code no. M0130, Antibody Diagnostica,dilution 1/50) were used for immunohistochemical examination.

Table 1 Summary of clinicopathological data (n = 259)

Characteristics	Cases (%)
Gender	
Male	146 (56.4)
Female	113 (43.6)
Age (yr)	
Range	18-80
Median	56
Tumor location	
> 10 cm[1]	62 (23.9)
7-10 cm[1]	115 (44.4)
5-7 cm[1]	82 (31.7)
Mean Max diameter (cm)	4.68
Pathology	
Adenocarcinoma	236 (91.1)
Mucinous aden[3]	18 (6.9)
Signet ring ca[3]	5 (2)
T stage	
T1	18 (7.0)
T2	83 (32.0)
T3	85 (32.8)
T4	73 (28.2)
N stage	
N0	147 (56.8)
N1	62 (23.9)
N2	50 (19.3)
TNM stage (AJCC/UICC)	
Ⅰ	80 (30.9)
Ⅱ	67 (25.9)
Ⅲ	112 (43.2)
Lymphovascular invasion	
Yes	33 (12.7)
No	226 (87.3)
Neural invasion	
Yes	21 (8.1)
No	238 (91.9)
Pre-operative CEA[2]	
Positive	48 (18.5)
Negative	211 (81.5)
Adjuvant therapy	
S	167 (64.5)
S + C	92 (35.5)

[1]Distance of the tumor from anal verge; [2]In our hospital lab, CEA > 10 μg/L is considered positive. S: surgery; C: chemotherapy.

Scoring system and statistics

Immunostained tumor sections were analysed by two experienced pathologists without the knowledge of clinicopathological data. Sections immunostained for p53 and p21 were scored semi-quantitaitvely by scanning the entire section to estimate the percentage of tumor cell nuclear staining, and CD44v6 expression was estimated by the percentage of tumor cell membrane staining. The PCNA staining was expressed as a labeling index (LI) defining the positive nuclei of all the nuclei counted. The median value for the PCNA LI in this tumor series(59.5%,)was used as a cut-off point and tumors were classsified as either less than or greater than the median value.

For statistical analysis, p53 and p21 levels were considered to be positive if over 10% of cancer cells were nuclear immunoreactive; and CD44v6 was defined positive if over 10% of cancer cells were membrane

Table 2 Distribution of stage Ⅱ or Ⅲ patients with or without adjuvant chemotherapy

		Adjuvant chemotherapy (n = 179)				*P*
		No		Yes		
		Cases	%	Cases	%	
N staging	N0	114	68.2	33	35.9	< 0.05
	N1	33	19.8	29	31.5	
	N2	20	12	30	32.4	
T staging	T1-2	90	53.9	11	12	< 0.05
	T3	38	22.7	47	51	
	T4	39	23.4	34	37	
Differentiation	High-Medium	145	86.8	77	83.7	> 0.05
	Low	22	13.2	15	16.3	
Lymphovascular invasion	None	19	11.4	14	15.6	> 0.05
	Yes	148	88.6	78	84.4	
Neural invasion	None	14	8.4	7	7.6	> 0.05
	Yes	154	91.6	85	92.4	

immunoreactive.

Association between these proteins and clinicopathological data, and the univariate analysis between these data and prognosis were both performed by Chi-square test. The overall survival, local recurrence and metastasis rates were calculated using life tables. The multivariate analysis of these proteins and clinicopathological data was made using Cox regression. Significance levels were set at $P < 0.05$.

Follow-up

All patients were followedup every 3 to 6 mo at the Colorectal Cancer Center after surgery by their operative team. Follow-up included a full history and physical examinations including digital rectal examination (DRE) at each session. Chest X-ray, CT or ultrasound of abdomen, and lab tests were performed every 6 mo. And colonoscopy was performed every year for the first three years and then every 2 years. All surviving patients were asked to return to the Colorectal Cancer Center for follow-up for the purpose of this study.

RESULTS

Clinicopathological variables

Forty-eight patients exhibited elevated serum CEA levels. The disease stage, lymphnode metastastic status, lymvascular invasion, neural invasion, histopathology and tumor differentiation were not associated with CEA levels.

Among 179 stage Ⅱ or stage Ⅲ patients including 33 in stage Ⅱ (49.3%) and 59 in stage Ⅲ (52.7%), 92 (51.4%) received 5-Fu based adjuvant chemotherapy,. The detailed clincopathological information for these patientsis presented in Table 2.

Of the 259 rectal carcinomas with anterior resection, 45.6% were p53 postitive (n = 118), 80.7% were p21 positive (n = 209), 49.8% were CD44v6 positive (n = 129), and 61.4% were PCNA positive (n = 159). There was no positive association among these four protein expressions.

The association between these markers and clinicopathological variables were analysed using Chi-square test, including tumor location, histopathological type, TNM staging, invasion depth, lymph node metastasis, neural invasion, lymphovascular invasion and preoperative CEA level. There was no significant difference in the distribution of these proteins and different clinicopathological variables, either (Table 3). But p21 expression was found to have significant association with histopathological type (P = 0.067) and invasion depth (P = 0.052).

Patients' outcome

The median follow-up was 44 mo (range 1-90 mo). Thirty-three patients (12.7%) were dead due to tumor progression. Eleven patients (4.24%) had local recurrence, and 35 patients (13.5%) had distant metastases. The outcome of the patients is shown in Figure 1. Our five-year actual survival rate was 75.43% (Figure 1A), and disease free survival rate was 70.32% (Figure 1B). Overall local recurrence rate was 6.73% (Figure 1C).

In stage Ⅱ and stage Ⅲ locally advanced rectal cancer, our 5-year survival rate was 66.9%, disease free survival rate was 61.1%, and overall local recurrence rate was 8.9%.

Association of clinicopathological variables and immunohistochemistry with recurrence, metastasis and survival

Univariate analysis using Chi-square test as a screening method revealed that possible overall survival related risk factors were histopathological type, TNM staging, invasion depth, lymphnode metastasis, preoperative CEA and CD44v6 levels; possible disease free survival related risk factors were gender, histopathological type, TNM staging, invasion depth, lymph node metastasis and CD44v6 and preoperative CEA levels; possible local recurrence related risk factors were histopathological type, TNM staging, lymphnode metastasis; and possible metastasis related risk factors were TNM staging, invasion depth, lymphnode metastasis and preoperative CEA level (Table 4).

In all the 179 stage Ⅱ or stage Ⅲ patients, adjuvant chemotherapy had negative significant association with overall metastasis and disease free survival. But in stratification for each stage, adjuvant chemotherapy did not have any significance with local recurrence, overall metastasis, disease free survival and overall survival. One reason is that patients with more progressive disease were morelikely to receive adjuvant chemotherapy. For multivariate analysis, these possible risk factors screened in 259 rectal cancer patients by univariate analysis were included in Cox regression model. TNM staging, preoperative CEA, and CD44v6 level were independent risk factors predicting overall survival or disease free survival. The hazard ratio of peroperative CEA was 2.65 (95% CI 1.4-5) and 3.10 (95% CI 1.37-6.54) for disease free survival, and overall survival, respectively. The hazard ratio of CD44v6 was 1.93 (95% CI 1.04-3.61) and 2.21 (95% CI 1.01-4.88). TNM staging was the only risk factor predicting local recurrence.

In 179 stage Ⅱ or stage Ⅲ patients, we added the chemotherapy variable to Cox regression model, the results showed that adjuvant chemotherapy did not improve the overall survival, disease free survival or local recurrence in stage Ⅱ or Ⅲ patients.

Table 3 Association between IHC and clinicopathological data

Characteristics		P53 (*n*)			P21 (*n*)			PCNA (*n*)			CD44 (*n*)		
		+	-	*P*	+	-	*P*	+	-	*P*	+	-	*P*
Tumor location	High	29	33	NS	53	9	NS	36	26	NS	32	30	NS
	Median	53	62		93	22		70	45		63	52	
	Low	36	46		63	19		53	29		34	48	
Pathology	Adeno.	109	127	NS	191	45	< 0.05	144	92	NS	117	119	NS
	Muci	8	10		16	2		13	5		11	7	
	Signet	1	4		2	3		2	3		1	4	
TNM stage	Ⅰ	34	46	NS	59	21	NS	47	33	NS	37	43	NS
	Ⅱ	30	37		58	9		42	25		33	34	
	Ⅲ	54	58		92	20		70	42		59	53	
Invasion depth	T1-2	46	55	NS	74	27	0.052	63	38	NS	46	55	NS
	T3	36	49		72	13		55	30		43	42	
	T4	36	37		63	10		41	32		40	33	
Lymphnode meta.	N0	64	83	NS	117	30	NS	89	58	NS	70	77	NS
	N1-2	54	58		92	20		70	42		59	53	
Lymph-vascular invasion	+	17	16	NS	25	8	NS	21	12	NS	19	14	NS
	-	101	125		184	42		138	88		110	116	
Neural invasion	+	11	10	NS	18	3	NS	15	6	NS	11	10	NS
	-	107	131		191	47		44	94		118	120	
Preoperative CEA	+	18	30	NS	41	7	NS	30	18	NS	109	102	NS
	-	100	111		168	42		129	82		20	28	

Adeno: adenocarcinoma; Muci: mucinous adenocarcinoma; Signet: signet ring adenocarcinoma; Meta: metastasis; NS: not significant.

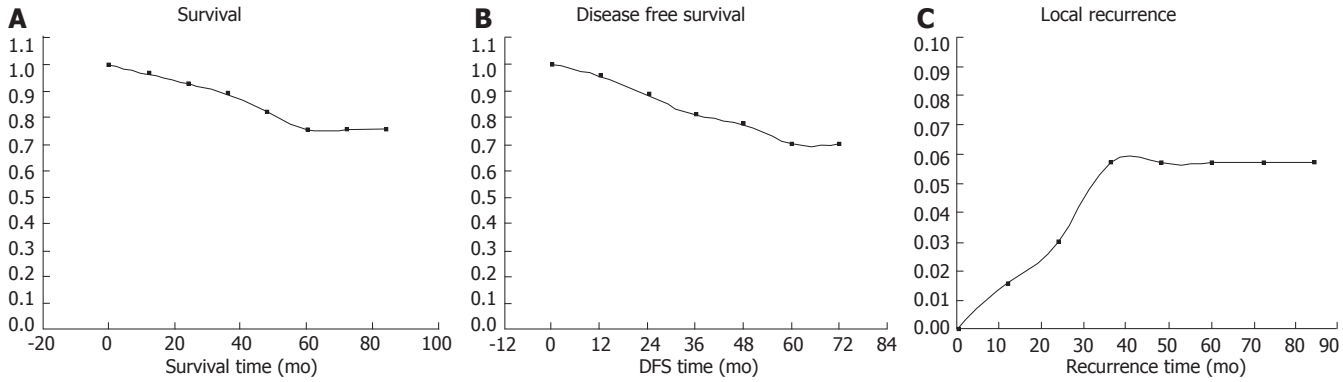

Figure 1 Outcome of 259 rectal cancer patients with TME. **A**: Overall survival curve; **B**: Disease free survival curve; **C**: Local recurrence curve.

DISCUSSION

The surgical management of primary rectal cancer presents unique problems for the surgeon basedlargely on the anatomic constraints of the pelvis. For most of the tumors over 5 cm above the anal verge, anterior resection was increasingly performed in recent years, occupying about 80%-90% of all rectal cancer surgeries in large centers. However, the local and distant recurrence was still challenging. The importance of mesorectum in rectal cancer surgery has been widely recognized. By total mesorectal excision, Heald *et al*[3] and Enker *et al*[4] had reported a lower local recurrence and improved the DFS and overall survival of the patients. In our series, overall local recurrence rate was 6.73%, disease free survival and overall survial were 70.32% and 75.43%, respectively, which was consistent with other studies about TME.

Many factors have been studied in predicting the outcome of the patients with rectal cancer who underwent total mesorectal excision. Whereas the use of clinical and histologic parameters for the determination of prognosis and treatment strategies for patients with rectal cancer is still of great value, they may be distressingly inaccurate in many clinical situations, especially in patients with stage Ⅱ-Ⅲ disease, which may need post- or pre-operative treatment. This may be attributed, at least in part, to differences in the biological behavior of tumors that are determined by altered molecular regulatory mechanisms. Thus, the characterization of molecular changes in colorectal cancer in recent years has been the focus of great interest for both researchers and clinicians, because it may lead to the identification of new prognostic markers more closely resembling the biological nature of the disease. Among the various alterations in gene and protein expression in colorectal cancers, cell-cycle control related genes and proteins (including p53, p21 and PCNA) and cell adhension protein CD44 were widely elucidated in many studies, but the prognostic values were still confusing, and very few studies exclusively focused on rectal cancer patients with anterior resection. In our series, we analyzed the prognostic effect of clinical variables and immnohistochemical markers. TNM staging, preoperative

Table 4 Variables association with overall survival, DFS, recurrence and metastasis

	n	Survival		DFS		Local recurrence		Metastasis	
		%	*P*	%	*P*	%	*P*	%	*P*
Gender									
Male	146	85.6	NS	78.1	< 0.05	5.5	NS	16.4	NS
Female	113	89.4		87.6		2.7		9.7	
Tumor location			NS		NS		NS		
High	62	88.7		85.5		3.2		12.9	
Medium	115	87.8		83.5		6.1		10.4	NS
Low	82	85.4		80.5		2.4		18.3	
Pathology			< 0.05		< 0.05		< 0.05		
Adenocarcinoma	236	87.7		83.9		3.8		13.1	
Mucinous cancer	18	94.4		83.3		5.6		11.1	NS
Signet ring cancer	5	40		40		20		40	
Differentiation									
High-medium	222	87.4	NS	82.9	NS	4.1	NS	13.1	NS
Low	37	86.5		78.4		5.4		16.2	
TNM staging			< 0.05		< 0.05		< 0.05		
I	80	95		92.5		1.3		6.3	
II	67	92.5		89.6		1.5		9.0	< 0.05
III	112	78.6		70.3		8		21.4	
Invasion depth			< 0.05		<0.05		NS		
T1-2	101	95.0		91.1		2		6.9	
T3	85	83.5		78.8		4.7		16.5	< 0.05
T4	73	80.8		75.3		6.8		19.2	
N staging			< 0.05		< 0.05		< 0.05		
N0	147	93.9		91.2		1.4		7.5	< 0.05
N1-2	112	78.6		72.3		8.0		21.4	
Lymphvascular invasion			NS		NS		NS		
+	33	81.8		78.8		6.1		15.2	
-	226	88.1		83.6		4		13.3	NS
Neural invasion					NS		NS		
+	21	85.7	NS	85.7		4.8		14.3	
-	238	87.4		82.8		4.2		13.4	NS
Preoperative CEA			0.06		< 0.05		NS		
+	48	20.8		31.3		6.3		25	
-	211	10.9		14.7		3.8		10.9	< 0.05
P53			NS		NS				
+	118	84.4		80.9		6.4	0.06	13.5	NS
-	141	90.7		85.6		1.7		13.6	
P21			NS		NS		NS		
+	209	87.6		81.8		4.3		13.9	NS
-	50	86		84		4.0		12.0	
PCNA			NS		NS		NS	12.6	
+	159	89.3		84.3		3.1		15.0	NS
-	100	84.0		81.0		6.0			
CD44			< 0.05		< 0.05		NS		
+	129	82.9		76.7		6.2		17.1	0.97
-	130	91.5		87.8		2.3		10.0	

NS: not significant.

CEA and CD44v6 levels were recognized as prognostic factors predicting the disease free survival and overall survival.

Serum CEA level is a common preoperative and follow-up marker in colorectal carcinoma patients. Adenocarcinomas overexpress CEA, which may facilitate metastasis of colorectal carcinoma. Elevated preoperative serum levels are associated with high rates of recurrence and cancer mortality, and it should not be discarded in the current array of prognostic factors. Granell *et al*[5] studied preoperative CEA level and p53 expression in 134 colorectal cancer patients, and found patients with elevated preoperative CEA level were at significant high risk of local recurrence in two years after surgery, whose hazard ratio was 3.26. In our series, preoperative CEA level was an independent prognositic factorin predicting DFS and overall survival, the hazard ratio was 2.65 and 3.10, respectively. Our results suggested that preoperative CEA, like postoperative CEA, may be also a useful prognostic marker for rectal cancer patients.

The expression of specific cell adhesion molecule CD44 splice variants has been shown to be associated with metastasis and poor prognosis in certain human malignancies, such as breast cancer and colorectal cancer, especially the CD44 variant 6 (CD44v6)[6]. In most of these studies, increased levels of CD44 and/or different patterns of splice variants were found in tumors in comparison with their normal counterparts[7,8]. The studies addressing the relationship between CD44 expression at the protein level and clinicopathological variables, such as

tumour grade and stage, have not been uniform. Ishida examined CD44v6 expression in 62 colorectal cancer patients, and the result showed CD44v6 has no correlation with gross type, histology, lymph node involvement, and clinical stage[9]. Bhatavdekar *et al*[10] examined CD44 in 98 Duke's B and C colorectal adenocarcinomas with IHC, and they also found a significantly reduced relapse-free survival in patients with positive CD44. Similary, Yamagnchi *et al*[11] have shown that CD44 is an independent prognostic factor in multivariate analysis. In our study, we did not find any significant association between CD44v6 and clinicopathological parameters either. But in multivariate analysis, we found CD44v6 was the independent biological prognostic marker for disease free survival and overall survival, and the hazard ratio was 1.93 and 2.21, respectively, suggesting CD44v6 is a valuable molecular marker for rectal cancer prognosis.

P53 was studied in colorectal cancer, but the results of IHC p53 rectal tumor status have been inconsistent. Hilska *et al*[12] studied 363 colorectal cancer patients, including 124 with rectal cancers from Duke's stage A to D. The author s used different cut-off values for defining p53 positive, but none of them showed any significance for survival in all colorectal cancer groups. Morgan *et al*[13] studied 171 patients with curative resection of rectal cancer. By immunohistochemical assay for p53 and DCC expression, they found p53 and DCC status of rectal cancers was not associated with other clinical or pathological variables, nor predictive outcomes. The cyclin inhibitors p21 negatively regulates the action of cyclin/CDK complexes, and prevents cell-cycle progression. Lebe *et al* examined IHC p53 p21 and p27 expression in 45 rectal adenocarcinomas, and found p53, p21 and p27 status was not significantly associated with local and distant recurrence. PCNA is an auxiliary factor essential for DNA polymerases activity and exists in a quaternary complex with CDK/cyclin/p21. PCNA is frequently used to measure the proliferative activity of tissues, which was widely studied to evaluate the response of chemotherapy and radiotherapy. PCNA was found associating with improved survival in advanced colorectal cancer by Paradiso *et al*[14]. but several studies discovered no significant association between PCNA expression and prognosis in colorectal cancer[15-17]. In our study, we did not find any association between the three markers and clinicopathological variables. The three markers had no significant prognostic effect for predicting DFS or overall survival, either.

The benefit of adjuvant chemotherapy was of great controversy in rectal cancer patients. The EORTC Radiotherapy Group Trial 22921 found in 253 patients with postoperative chemotherapy, adjuvant chemotherapy was of benefit for local control in T3-4 rectal cancer patients[18]. In that clinical trial, patients were all assigned to receive preoperative radiotherapy or chemoradiotherapy. And the adherance to postoperative chemotherapy was very poor, which made the results accepted. Our patients received adjuvant chemotherapy alone after curative total mesorectal excision. We found in patients with curative excised rectal cancer, postoperative chemotherapy did not improve patients' local control of the tumor or survival. The results suggested that postoperative chemotherapy may only improve the local control by enhancing the effect of radiotherapy. We therefore, do not recommand postoperative chemotherapy for stage Ⅱ or Ⅲ patients without preoperative radiotherapy.

This has been coupled in several series with an improved cancer-specific survival directly attributed to the performance of TME itself[19]. The outcomes are favorable for strictly defined curatively excised rectal cancers with meticulous total mesorectal excision. TNM staging, preoperative CEA, and CD44v6 levels are recognized as independent prognostic facotrs for these patients. And postoperative chemotherapy is not recommanded for curative excised rectal cancer patients without preoperative radiotherapy.

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