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RESEARCH

Expression of p53, Ki-67 and c-Myc Proteins is Predictive of the Surgical Molecular Margin in Colorectal Carcinoma

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Abstract Surgical resection is the mainstay of treatment for colorectal carcinoma, however, the overall survival is modest due to frequent local recurrence from residual cancer cells after "curative" resection. Therefore, the status of surgical margin (tumor free or positive) has a significant influence on patient's survival. The difference in molecular profile between mucosa neighboring tumor lesions and remote area (surgical margin) may aid in evaluating resection status. 44 colorectal tumor tissues with corresponding adjacent non-neoplastic mucosa (within 3 cm from tumor tissues), and 110 tumor tissues with corresponding surgical margin mucosa (5 cm from tumor tissues) were randomly collected, fixed in 10% formalin and followed by embedding in paraffin. And the expression of p53, Ki-67 and c-Myc were investigated by tissue microarray (TMA) and immunohistochmistry. The expression of p53, Ki-67 and c-Myc were decreased in both adjacent non-neoplastic mucosa and mucosa of surgical margin, comparing to their expression in corresponding cancer cells. Furthermore, the expression of these proteins in mucosa of remote area (surgical margin) was significantly lower than those adjacent to tumor lesions. The expression of p53, Ki-67 and c-Myc in mucosa can be used as molecular marker for assessing surgical margin status in colorectal carcinoma.

Keywords Colorectal carcinoma · Surgical molecular margin · p53 · Ki-67 · c-Myc

Introduction

Colorectal carcinoma is the third most common cause of cancer-related mortality in the world, and the most frequent malignancy of the gastrointestinal tract. Approximately 150,000 new cases are diagnosed annually [1]. So far, surgical resection remains the mainstay of treatment for colorectal carcinoma, however, the overall survival is rather modest after so-called "curative" resection. This might be due to a fact that many of these "curative" resections actually leave behind residual cancer cells or preneoplastic cells either as primary micro-invaded cancer cells or hidden in a dormant state as remote micro-metastasis. The presence of tumor cells in the surgical margin is a reliable marker for residual primary cancer cells which constitutes an important source for local recurrence of disease later on. However, it is quite frequent that patients still develop local recurrence, even though the initial conventional histological examination of surgical margin report as "tumor-free". Recent evidence suggests that there are two different mechanisms potentially responsible for this: 1) the small clusters of residual tumor cells that are undetectable on routine histopathological examination form the new detectable tumor mass; 2) additional genetic mutations in remaining preneoplastic cells lead to de novo transformation [2].

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Therefore, a thorough molecular characterization of "normal" tissue directly next to tumor tissues as well as those in remote site (surgical margin) may help pathologist to better evaluate resection status.

Recent genome-wide genetic characterization efforts have revealed that nearly all colorectal cancer cells carry genetic alterations in cell growth and apoptosis pathways [3]. Specifically, recent studies revealed that Ki-67 (a surrogate marker of proliferation) expression is gradually increasing from initial hyperplasia, to different degrees of hysplasia of adenoma, and to final adenocarcinoma. In contrast, the apoptosis rate showed an opposite tendency: it is high in the hyperplastic polyps and adenomas lesions but rather low in adenocarcinomas. Interestingly, p53 (central coordinator of cell proliferation and apoptosis) expression was found in 77% of adenocarcinomas but in only 3% of adenomas. Additionally, c-Myc-another regulator of cell proliferation and apoptosis-was also increased in adenomas as well as in adenocarcinoma [4]. All these data suggest that proliferation and apoptosis related genes (*e.g.* Ki-67, p53 and c-Myc) may step-wisely participate into the carcinogenesis process of colorectal cancer. Therefore, we hypothesize that these genes might also be altered in those above mentioned "invisible tumor clusters" and preneoplastic cells. In the current study, the expression of ki67, p53 and c-Myc were characterized in colorectal tissues with corresponding adjacent mucosa as well as surgical margin mucosa by using tissue microarray (TMA).

Materials and Methods

Tissue Specimen

Between September 2007 and January 2008, 44 colorectal tumor tissue specimens with corresponding adjacent non-neoplastic mucosa (1, 2 and 3 cm from tumor tissue) were randomly collected at the pathological department of Drum Tower Hospital. 110 preserved colorectal tumor tissue specimens with corresponding surgical margin mucosa (5 cm from tumor tissue) from 2002 to 2004 were also collected. All specimens were fixed in 10% formalin and embedded in paraffin, and corresponding hematoxylin-eosin (HE) stained sections were made conventionally.

Tissue Microarray

All HE stained sections were observed by optical microscope for rechecking their pathological diagnosis, and typical regions were marked and followed TMAs manually. Firstly, a block was made by beeswax as the recipient block (Volume: 35 mm×27 mm×6 mm); Secondly, donor paraffin blocks were arranged and compared with corresponding HE stained sections. Then typical regions were marked on the donor paraffin blocks, and the cores of typical regions with 1 mm in diameter were dislodged. Subsequently, the recipient block was perforated with 1 mm in diameter, and the

Table 1 Expression of p53, Ki-67 and c-Myc proteins in 44 colorectal tumor tissues and corresponding adjacent non-neoplastic mucosa

Molecular marker	location	(-)	(+)	(++)	(+++)
p53	tumor tissue	2	24	14	4
	1 cm from tumor tissue	26	18	0	0
	2 cm from tumor tissue	35	9	0	0
	3 cm from tumor tissue	39	5	0	0
Ki-67	tumor tissue	0	3	24	17
	1 cm from tumor tissue	1	19	22	2
	2 cm from tumor tissue	3	34	7	0
	3 cm from tumor tissue	9	33	2	0
c-Myc	tumor tissue	0	11	31	2
	1 cm from tumor tissue	1	29	14	0
	2 cm from tumor tissue	5	30	9	0
	3 cm from tumor tissue	10	26	8	0

$P<0.001$ $P<0.05$ $P<0.001$ $P<0.001$ $P<0.05$

cores were implanted. Finally, two different TMAs were completed: one contained 44 colorectal tumor tissue specimens and corresponding adjacent non-neoplastic mucosa (1, 2 and 3 cm from tumor tissue); the other contained 110 colorectal tumor tissue specimens and corresponding surgical margin mucosa (5 cm from tumor tissue). Two TMAs were respectively duplicated for repeating the experiment [5–7].

Immunohistochemistry

Four μm thick TMA sections were stained using EnVision System [8, 9]. The primary antibodies used were monoclonal rabbit antihuman p53 antibody (SP5, Prediluted, Zhongshan Scientific, China), monoclonal rabbit antihuman Ki-67 antibody (SP6, Prediluted, Zhongshan Scientific, China), and monoclonal mouse antihuman c-Myc antibody (9E10.3, 1:300, Thermo Fisher Scientific, USA). Preparations in which the primary antibody was omitted were used as negative controls, and established positive stained sections of breast carcinoma were considered as positive controls.

p53, Ki-67 and c-Myc positivity was determined by the presence of brown reaction product within the nuclei of epithelial cells. An immunohistochemical assessment was made according to the semiquantitative method by optical microscope [10]. In summary, a score of 0–4 was assigned according to the percentage of positively stained cells: 0, ≤5% stained; 1, 6–25% stained; 2, 26–50% stained; 3, 51–75% stained; 4, >75% stained. A further score was assigned according to the intensity of staining: 0, colorless; 1, weak; 2, moderate; 3, intense. The percentage and intensity scores were subsequently multiplied to produce a weighted score for each specimen: 0, negative (-); 1–4, weak positive (+); 5–8, positive (++); 9–12, intense positive (+++). Scoring of two same TMAs was independently performed by two observers, and the final score of each specimen was determined by the mean value of two same ones. Single expression of p53, Ki-67 and c-Myc proteins was statistically analyzed.

Then, the assessment listed above was reclassified. Weak positive, positive and intense positive were all classified as positive (+). Coexpression of p53, Ki-67 and c-Myc proteins was statistically analyzed. When the expression of p53, Ki-67 and c-Myc proteins was respectively positive, coexpression was positive (+/+, +/+/+). Other coexpression was negative (-).

Statistical Analysis

The $\chi 2$ test and Fisher's exact test were used for statistical analysis.

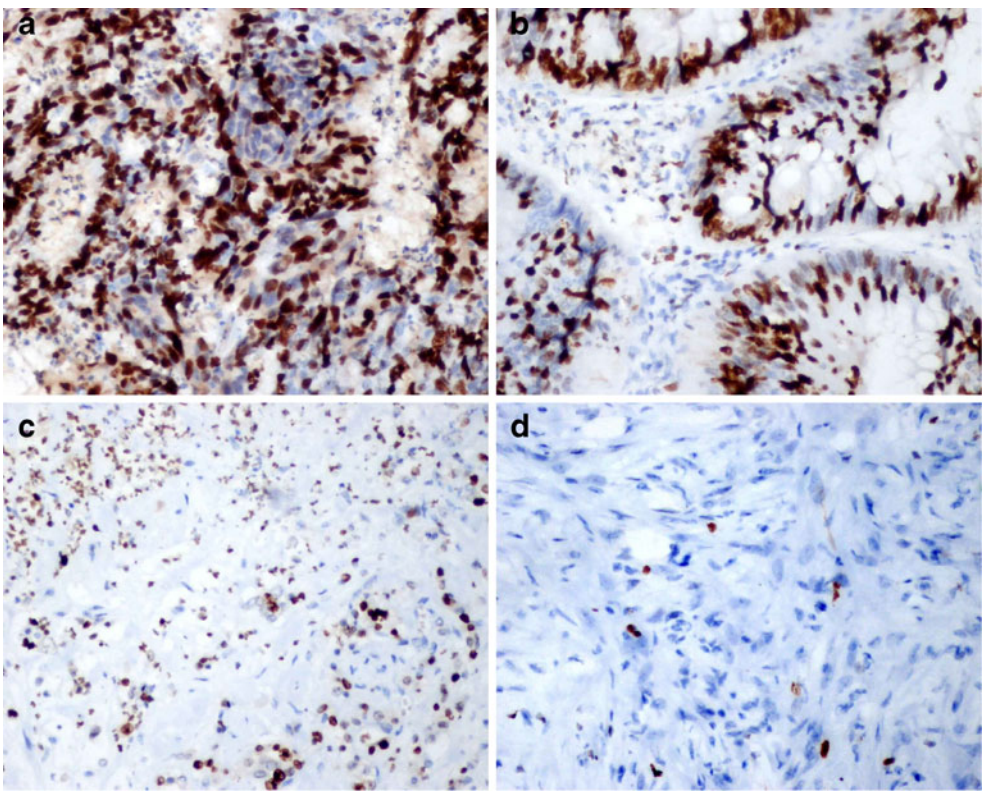

Fig. 1 Expression of p53 protein in colorectal tumor tissues (immunohistochemistry, ×200). **a** intense positive (+++). **b** positive (++). **c** weak positive (+). **d** negative (-)

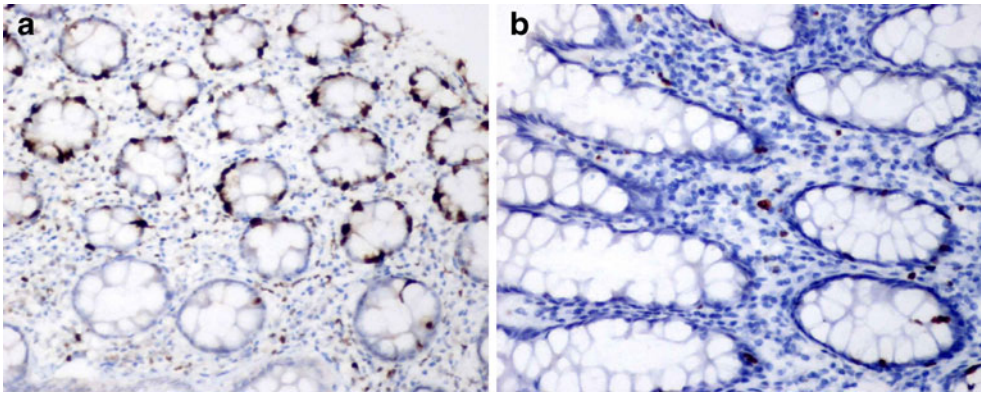

Fig. 2 Expression of p53 protein in adjacent non-neoplastic mucosa and surgical margin mucosa (immunohistochemistry, ×200). **a** weak positive (+), adjacent mucosa 1 cm. **b**) negative (-), adjacent mucosa 5 cm

Results

The Differential Expression of p53, C-Myc and Ki-67 in Cancer Cells, Corresponding Nearby non-neoplastic Mucosa and Mucosa at Surgical Margin

The expression of p53 and c-Myc proteins in 44 colorectal tumor tissues and corresponding adjacent non-neoplastic mucosa (1, 2 and 3 cm from tumor tissue) was quite heterogenous. Though p53 and c-Myc expression level in non-neoplastic mucosa at 1 cm is higher than that at 3 cm from tumor tissue ($P<0.05$; Table 1), there are no difference in staining intensity in other intergroup comparison (1 cm *vs* 2 cm and 2 cm *vs* 3 cm, $P>0.05$; Table 1, Figs. 1–2, Figs. 3–4). Accordingly, the expression of Ki-67 protein in cancer cells of 44 colorectal tissues was significantly increased compared to that in corresponding adjacent non-neoplastic mucosa. And Ki-67 staining shows a similar pattern as p53 and c-Myc in intergroup comparison (Table 1, Figs. 5–6). Additionally, the expression of p53, Ki-67 and c-Myc in cancer cells of 110 colorectal cancer tissues was collectively increased comparing to mucosa in corresponding surgical margin (5 cm from tumor tissue) ($P<0.001$; Table 2).

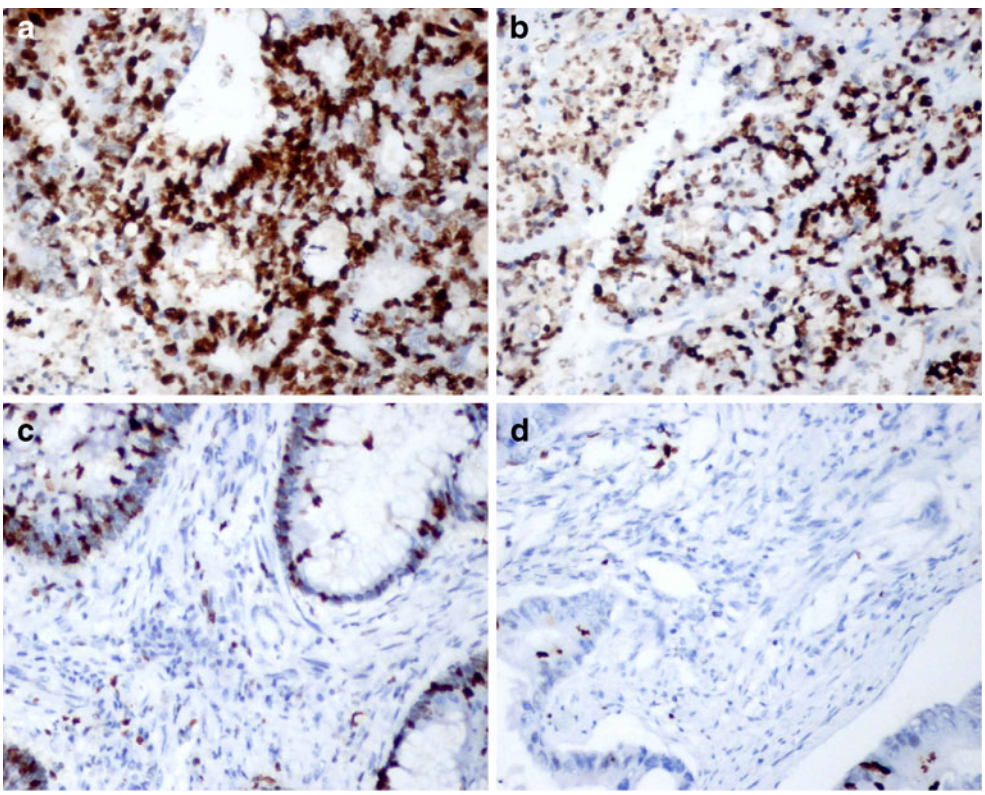

Fig. 3 Expression of c-Myc protein in colorectal tumor tissues (immunohistochemistry, ×200). **a** intense positive (+++). **b** positive (++). **c** weak positive (+). **d** negative (-)

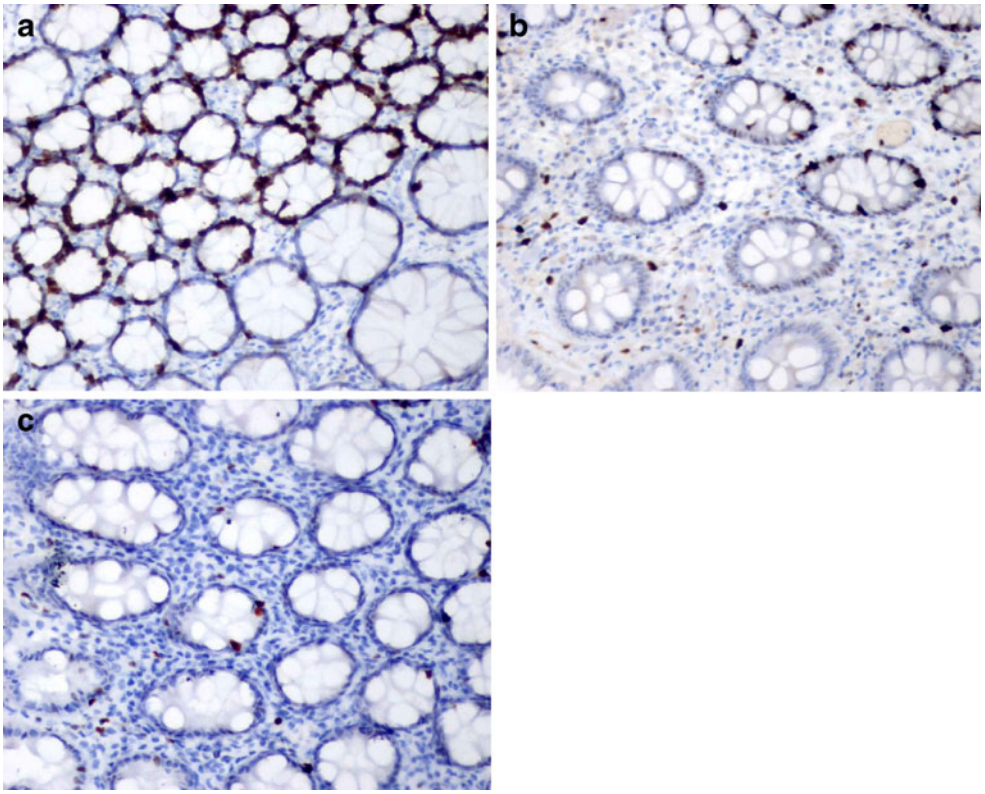

Fig. 4 Expression of c-Myc protein in adjacent non-neoplastic mucosa and surgical margin mucosa (immunohistochemistry, ×200). **a** positive (++), adjacent mucosa 1 cm. **b** weak positive (+), adjacent mucosa 2 cm. **c** negative (-), adjacent mucosa 5 cm

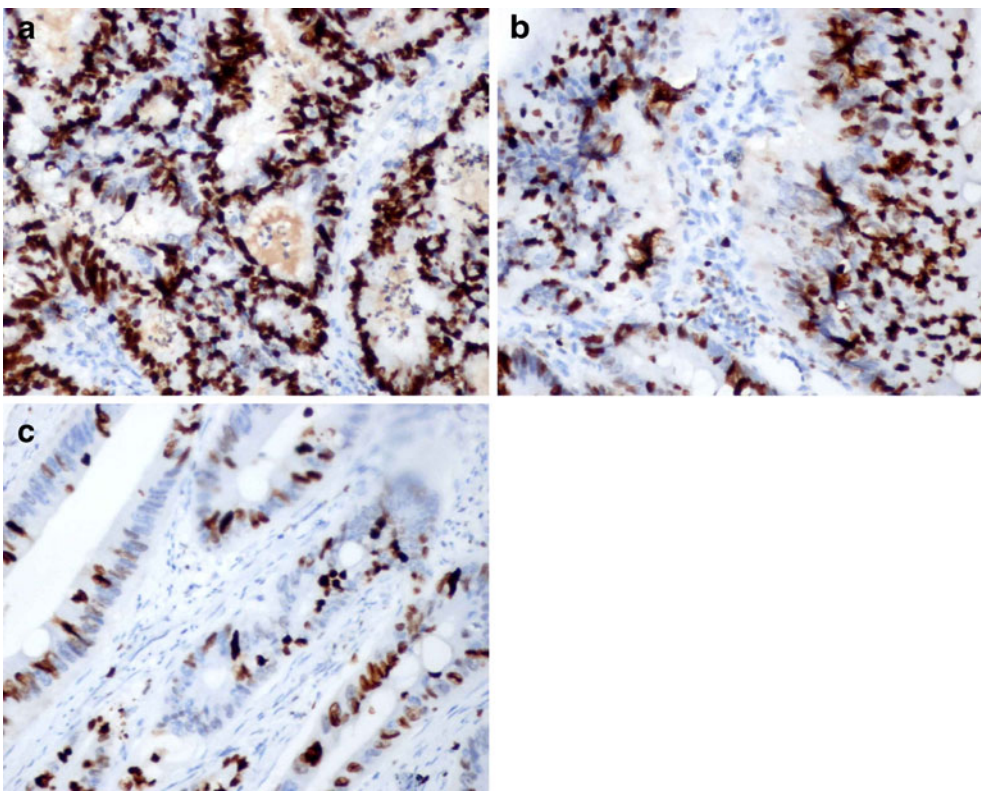

Fig. 5 Expression of Ki-67 protein in colorectal tumor tissues (immunohistochemistry, ×200). **a** intense positive (+++). **b** positive (++). **c** weak positive (+)

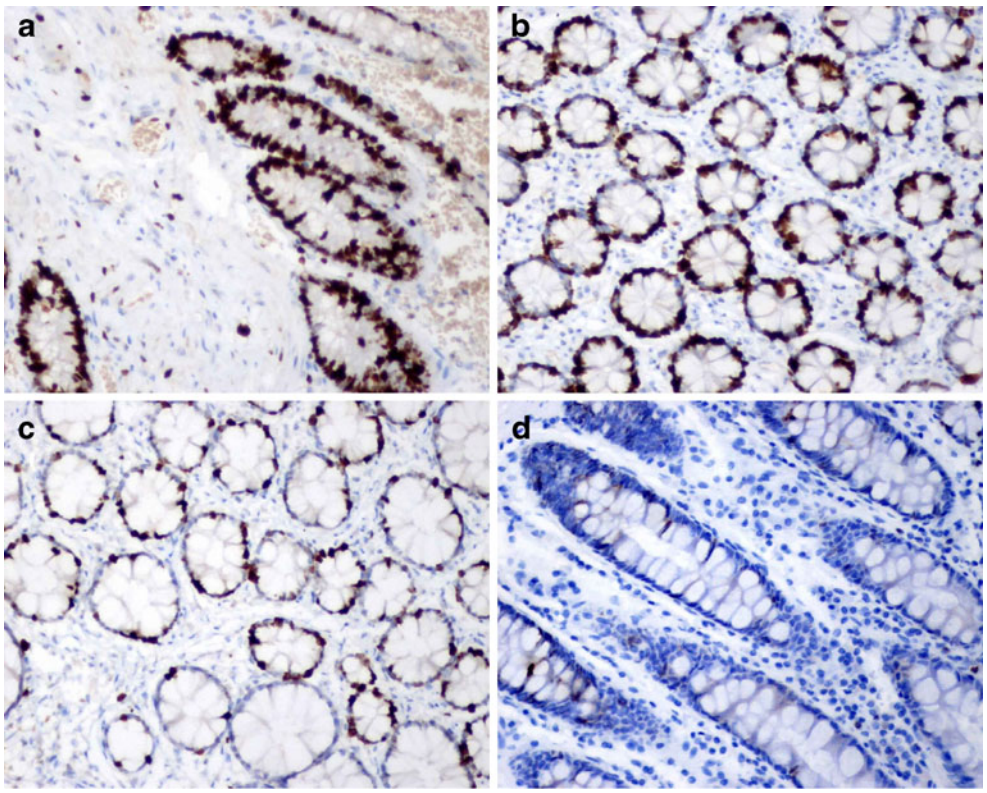

Fig. 6 Expression of Ki-67 protein in adjacent non-neoplastic mucosa and surgical margin mucosa (immunohistochemistry, ×200). **a** intense positive (+++), adjacent mucosa 1 cm. **b** positive (++), adjacent mucosa 2 cm. **c** weak positive (+), adjacent mucosa 3 cm. **d** negative (-), adjacent mucosa 5 cm

Correlation Analysis of p53, Ki-67 and c-Myc Staining Intensities in Different Colorectal Tissues

Compared with corresponding adjacent non-neoplastic mucosa (1, 2 and 3 cm from tumor tissue), coexpression of p53 and Ki-67 proteins (p53/Ki-67), p53 and c-Myc proteins (p53/c-Myc), and p53, Ki-67 and c-Myc proteins (p53/Ki-67/c-Myc) in 44 colorectal tumor tissues was evidently intensified. Moreover, that in adjacent non-neoplastic mucosa at 1 cm and 3 cm from tumor tissue was also obviously different ($P<0.05$; Table 3). But the difference was not observed in adjacent non-neoplastic mucosa between 1 cm and 2 cm from tumor tissue, and between 2 cm and 3 cm, separatively ($P>0.05$; Table 3).

Coexpression of Ki-67 and c-Myc proteins (Ki-67/c-Myc) in 44 colorectal tumor tissues and corresponding adjacent non-neoplastic mucosa (2 cm and 3 cm from

Table 2 Expression of p53, Ki-67 and c-Myc proteins in 110 colorectal tumor tissues and corresponding surgical margin mucosa

Molecular marker	location	(-)	(+)	(++)	(+++)	
p53	tumor tissue	6	61	37	6	$P<0.001$
	5 cm from tumor tissue	77	33	0	0	
Ki-67	tumor tissue	0	28	52	30	$P<0.001$
	5 cm from tumor tissue	21	79	10	0	
c-Myc	tumor tissue	7	75	27	1	$P<0.001$
	5 cm from tumor tissue	58	50	2	0	

Table 3 Coexpression of p53, Ki-67 and c-Myc proteins in 44 colorectal tumor tissues and corresponding adjacent non-neoplastic mucosa

Molecular marker	location	(-)	(+/+)		
p53/Ki-67	tumor tissue	2	42		
	1 cm from tumor tissue	27	17		
	2 cm from tumor tissue	35	9	$P<0.001$	$P<0.05$
	3 cm from tumor tissue	39	5		
p53/c-Myc	tumor tissue	2	42		
	1 cm from tumor tissue	27	17	$P<0.001$	
	2 cm from tumor tissue	36	8		$P<0.05$
	3 cm from tumor tissue	39	5		
Ki-67/c-Myc	tumor tissue	0	44		
	1 cm from tumor tissue	2	42	$P<0.05$	
	2 cm from tumor tissue	7	37		$P<0.001$
	3 cm from tumor tissue	16	28		
		(-)	(+/+/+)		
p53/Ki-67/c-Myc	tumor tissue	2	42		
	1 cm from tumor tissue	28	16	$P<0.001$	
	2 cm from tumor tissue	36	8		$P<0.05$
	3 cm from tumor tissue	39	5		

tumor tissue), and that in adjacent non-neoplastic mucosa at 1 cm and 3 cm from tumor tissue was respectively different ($P<0.05$; Table 3), but the coexpression exhibited similar pattern in 44 colorectal tumor tissues and corresponding adjacent non-neoplastic mucosa at 1 cm from tumor tissue, in adjacent non-neoplastic mucosa at 1 cm and 2 cm, and at 2 cm and 3 cm from tumor tissue, respectively ($P>0.05$; Table 3).

Coexpression of p53/Ki-67, p53/c-Myc, Ki-67/c-Myc, and p53/Ki-67/c-Myc in 110 colorectal tumor tissues was all strongly increased compared to that in corresponding surgical margin mucosa (5 cm from tumor tissue) ($P<0.001$; Table 4).

Discussion

Colorectal carcinoma is one of the most common cancerous diseases with worldwide distribution, and its prevalence has gradually increased. Up to now, surgical resection constitutes the only curative treatment for colorectal carcinoma. Despite significant advances in surgical techniques over the years, the 5-year survival rate of colorectal cancer patients is still low due to a high local recurrence rate. The positive surgical margin is considered to be one significant source of recurrence. However, even in those patients with histologically negative margins, a high recurrence rate is also observed [11]. To improve the detection rate of cancerous or preneoplastic cells (hiding) in histologically negative margins, molecular margin analysis was proposed and has been used to predict local recurrence of various carcinomas [12–15]. Furthermore, the molecular margin assay is considered to be more sensitive than conventional histological margin examination in detecting preneoplastic lesions, giving well-established stepwise genetic/epigenetic molecular events during the transformation of colorectal cancer [16].

In this preliminary work, we have described a simple, rapid and high-throughput technique to analyze the expression of p53, Ki-67 and c-Myc proteins for investigating molecular margin of colorectal cancer. TMAs consisting of tumor tissues with corresponding adjacent non-neoplastic mucosa as well as surgical margin mucosa were used in our studies. Our studies revealed that expression of these

Table 4 Coexpression of p53, Ki-67 and c-Myc proteins in 110 colorectal tumor tissues and corresponding surgical margin mucosa

Molecular marker	location	(-)	(+/+)	
p53/Ki-67	tumor tissue	6	104	$P<0.001$
	5 cm from tumor tissue	83	27	
p53/c-Myc	tumor tissue	13	97	$P<0.001$
	5 cm from tumor tissue	87	23	
Ki-67/c-Myc	tumor tissue	7	103	$P<0.001$
	5 cm from tumor tissue	66	44	
		(-)	(+/+/+)	
p53/Ki-67/c-Myc	tumor tissue	13	97	$P<0.001$
	5 cm from tumor tissue	91	19	

proteins in tumor tissues were universally higher than that in adjacent non-neoplastic and surgical margin mucosa ($P<0.05$; Figs. 7–8). Moreover, the expression level of these proteins in adjacent non-neoplastic mucosa was gradually decreased along the increasing distance from tumor tissue ($P<0.05$; Fig. 7). It demonstrates the feasibility to use proliferation and apoptosis related molecules as molecular marker to define molecular margin of colorectal cancer. Combing with conventional histological examination, it may enhance the diagnostic specificity of the positive surgical margin.

The precise diagnosis resection status of colorectal cancer would have direct clinical relevance because patients with positive margins are at high risk for recurrence of disease. Hence, additional therapeutical modalities are required in these patients. However, this diagnosis is frequently complicated by the presence of epithelial dysplasia lesions within resection margin, especially when the carcinoma is in close vicinity of surgical margins. Here, basing on the causes of local relapse, we have employed a gradient change in expression level of p53 and Ki67 level to define molecular margin of colorectal cancer. This is supported by previous studies demonstrating that the expression of p53 in tumor tissues was associated with the reduced disease-free interval whereas Ki-67 expression had no such effect [17]. Though it remains unclear whether this gradient change would also influence the prognosis of patient, it provides additional information in stratifying patients into different risks group of local recurrence. However, further studies with a large sample size are required to validate the clinical relevance of this technique.

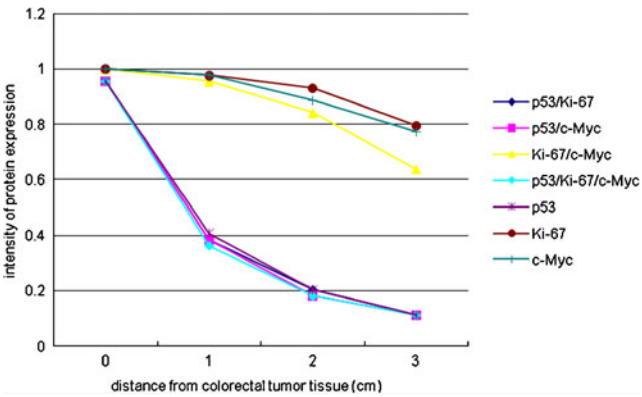

Fig. 7 Expression of p53, Ki-67 and c-Myc proteins in 44 colorectal tumor tissues and corresponding adjacent non-neoplastic mucosa

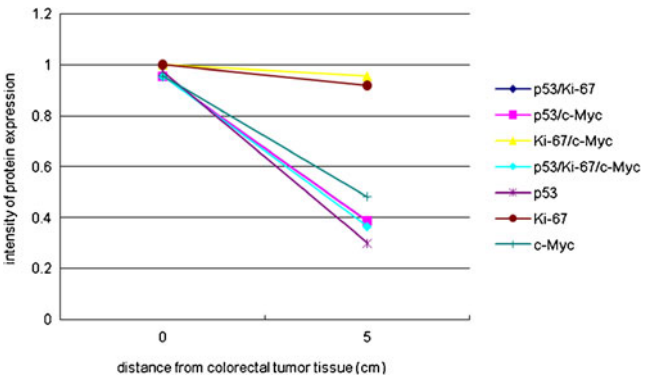

Fig. 8 Expression of p53, Ki-67 and c-Myc proteins in 110 colorectal tumor tissues and corresponding surgical margin mucosa

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