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World J Gastroenterol 2007 February 7; 13(5): 699-708
World Journal of Gastroenterology ISSN 1007-9327

Survey of molecular profiling during human colon cancer development and progression by immunohistochemical staining on tissue microarray

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Supported by grant from the National 863 Project about Functional Genomic and Biochip, No. 2002AA2Z2021; and 135 Medical Important Talent Foundation of Jiangsu Province, No. 37RC2002037
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Received: 2006-09-28 Accepted: 2006-12-14

Abstract

AIM: To explore the molecular events taking place during human colon cancer development and progression through high-throughput tissue microarray analysis.

METHODS: We constructed two separate tissue microarrays containing 1.0 mm or 1.5 mm cylindrical samples acquired from 112 formalin-fixed and paraffin-embedded blocks, including carcinomas (n = 85), adenomatous polyps (n = 18), as well as normal para-cancerous colon tissues (n = 9). Immunohistochemical staining was applied to the analysis of the consecutive tissue microarray sections with antibodies for 11 different proteins, including p53, p21, bcl-2, bax, cyclin D1, PTEN, p-Akt1, β-catenin, c-myc, nm23-h1 and Cox-2.

RESULTS: The protein expressions of p53, bcl-2, bax, cyclin D1, β-catenin, c-myc, Cox-2 and nm23-h1 varied significantly among tissues from cancer, adenomatous polyps and normal colon mucosa (P = 0.003, P = 0.001, P = 0.000, P = 0.000, P = 0.034, P = 0.003, P = 0.002, and P = 0.007, respectively). Chi-square analysis showed that the statistically significant variables were p53, p21, bax, β-catenin, c-myc, PTEN, p-Akt1, Cox-2 and nm23-h1 for histological grade (P = 0.005, P = 0.013, P = 0.044, P = 0.000, P = 0.000, P = 0.029, P = 0.000, P = 0.008, and P = 0.000, respectively), β-catenin, c-myc and p-Akt1 for lymph node metastasis (P = 0.011, P = 0.005, and P = 0.032, respectively), β-catenin, c-myc, Cox-2 and nm23-h1 for distance metastasis (P = 0.020, P = 0.000, P = 0.026, and P = 0.008, respectively), and cyclin D1, β-catenin, c-myc, Cox-2 and nm23h1 for clinical stages (P = 0.038, P = 0.008, P = 0.000, P = 0.016, and P = 0.014, respectively).

CONCLUSION: Tissue microarray immunohistochemical staining enables high-throughput analysis of genetic alterations contributing to human colon cancer development and progression. Our results implicate the potential roles of p53, cyclin D1, bcl-2, bax, Cox-2, β-catenin and c-myc in development of human colon cancer and that of bcl-2, nm23-h1, PTEN and p-Akt1 in progression of human colon cancer.

Key words: Colon cancer; Immunohistochemistry; Tissue microarray

Chen WC, Lin MS, Zhang BF, Fang J, Zhou Q, Hu Y, Gao HJ. Survey of molecular profiling during human colon cancer development and progression by immunohistochemical staining on tissue microarray. *World J Gastroenterol* 2007; 13(5): 699-708

http://www.wjgnet.com/1007-9327/13/699.asp

INTRODUCTION

Colorectal cancer (CRC) is one of the most frequent cancers in the Western world. At present time, with the development of living conditions and changes of life behaviors, CRC has become more and more frequent in China. The prognosis in advanced cases is poor, and more than one-third of the patients will die from progressive disease because the overall survival is about 40% (15%-65%) after 5 years[1]. The development and progression of CRC, like others cancers, are results of multiple genetic alterations, so investigation of molecular changes in tumors representing the entire disease spectrum may enhance our understanding of mechanism involved in CRC tumorigenesis. Because CRC is one of the first major epithelial cancers in which molecular alterations were

described to occur in a systematic fashion during disease progression, studies of single molecular markers have not been successful in defining the biology of this disease[2]. This has prompted investigators to explore multiple molecules regulating the tumorigenesis in an effort to identify biologically aggressive tumors and appropriately select patients for adjuvant systemic or targeted therapies. However, with the progression of molecular biology and the development of oncogene research, the cancer-related genes and genetic alterations have been found rapidly. The evaluation of the clinical utility of each of these genes would require multiple consecutive experiments with hundreds of tumors. This would be both time-consuming and labor-intensive.

As a new biological technique which allows rapid visualization of molecular targets in thousands of tissues specimens at a time, either at the DNA, RNA, or protein level, tissue microarray (TMA) can facilitate rapid translation of molecular discoveries to clinical applications[3]. So it brings us a high-throughput and rapid technique which can help us complete the time- and people-consuming work. Here, we constructed two tissue microarrays containing samples from different stages of human colon cancer, adenomatous polyps and corresponding normal para-cancerous colon tissues to survey the gene alterations that may contribute to clinical behaviors of the colon cancer. We decided to investigate the role of protein expressions which had been shown correlated with the development and progression as well as metastases of colon cancer in previous studies. In this study, 11 different proteins (p53, p21, cyclin D1, bcl-2, bax, β-catenin, c-myc, PTEN p-Akt1, Cox-2 and nm23-h1) expressions were assayed by using immunohistochemical (IHC) staining to consecutive formalin-fixed tissue microarray sections. The aim was to obtain a comprehensive survey of the frequency of the target molecular alterations and the relationship between the alterations and the clinicopathological features in human colon cancer.

A number of proteins have been associated with human carcinogenesis and may be relevant to CRC. Among these molecules, we chose 11 cancer-related genes (p53, p21, cyclin D1, bcl-2, bax, β-catenin, c-myc, PTEN p-Akt1, Cox-2 and nm23-h1) which are altered during the development and progression of CRC according to the previous study reports[4-11]. Among these molecules, p53, p21, cyclin D1, bcl-2 and bax play pivotal roles in cell cycle regulation and apoptosis. Akt/protein kinase B (PKB), which is included in phosphatidyl inositol-3-OH kinase (PI3K) signaling, controls many intracellular processes, such as the suppression of apoptosis and the promotion of the cell cycle[12]. PTEN on 10q23.3 encodes a dual-specificity phosphatase that negatively regulates the phosphoinositol-3-kinase/Akt pathway and mediates cell-cycle arrest and apoptosis[13,14]. β-catenin is a member of the cadherin-catenin complex that mediates homotypic cell-cell adhesion[15]. It also plays a role in the *Wnt* signaling pathway through regulating target genes like *c-myc*. Cox-2 was elevated in human colon cancers, and the Cox-2 inhibitor, celecoxib, inhibited intestinal tumor multiplicity in a mouse model and reduced the number of adenomatous polyps in a familial adenomatous polyposis patient[16,17]. The human *nm23* gene, a candidate metastatic suppressor gene, consists of two genes, *nm23-h1* and *nm23-h2*. *Nm23-h1* aberration has been shown to be correlated with the metastatic potential of colorectal cancer in some studies[9,18]. More of these molecules were studied previously by conventional pathological or molecular biological technologies and the numbers of selected target molecules were lesser, but in this study we would assay 11 proteins at a time by IHC staining on TMA.

Many investigators and clinicians consider cancer of the colon and rectum to be two distinct diseases, thus, we chose to evaluate only the patients with colon cancer treated with surgery alone in an effort to optimize the homogeneity of the study population. In addition, all the tumor specimens selected according our data were from sporadic colon cancer patients.

MATERIALS AND METHODS

Materials

Demographic and clinical data were collected retrospectively. None of the patients received radiotherapy or chemotherapy before surgery. Formalin-fixed and paraffin-embedded tumors, adenomatous polyps and para-cancerous tissues specimens were from the archives of the Department of Gastroenterology, the First Affiliated Hospital of Soochow University and National Engineering Center for Biochip at Shanghai. All specimens were viewed by one pathologist (Jing Fang). The specimens that were interpretable for IHC included: (1) Eighty-five cancers including different grades, such as high (n = 11), moderate (n = 50), low differentiated (n = 24); (2) eighteen adenomatous polyps removed at colonoscopy; (3) nine para-cancerous colon tissues resected from colon tissues at least 5 cm apart from the corresponding cancer tissues.

Construction and sectioning of tissue microarray

The colon cancer microarray was constructed as previously described[3]. Briefly, fresh sections were cut from the donor block and stained with hematoxylin-eosin (HE), these slides were used to guide the samplings from morphologically representative regions of the tissues. A tissue array instrument (Beecher Instruments, Silver Spring, MD) was used to create holes in a recipient paraffin block and to acquire tissue cores from the donor block by a thin-walled needle with an inner diameter of 1.0 mm or 1.5 mm, held in an X-Y precision guide. The cylindrical samples were retrieved from the selected regions in the donors and extruded directly into the recipient blocks with defined array coordinates. After the construction of the array block, multiple 4-μm thick sections were cut with a microtom using an adhesive-coated tape sectioning system (Instrumedics, Hackensack, NJ) (Figure 1).

Tissue loss was a significant factor for tissue array-based analysis with previously reported rates of tissue damage ranging from 15% to 33%[19-21]. In our analysis the rates of lost cases attributable to tissue damage were less than 5% for the different markers and damaged tissues

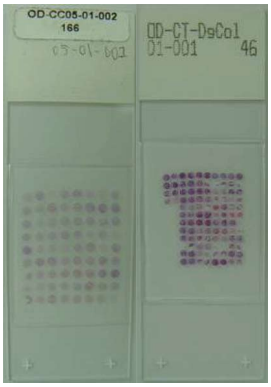

Figure 1 HE staining of 4-μm thick section of the tissue microarray.

were excluded from clinicopathological analyses of the respective markers.

IHC on formalin-fixed tissue microarray sections

IHC staining for the target genes to sections of the formalin-fixed samples on the tissue microarray was carried out by using the Envision ready-to-use methods. Slides were deparaffinized in xylene and rehydrated through graded concentrations of ethanol to distilled water, and endogenous peroxidase activity was blocked by incubation with 30 mL/L H_2O_2 in methanol for 10 min at room temperature. Then sections were submitted to antigen retrieval in a pressure cooker containing 0.01 mmol/L natrium citricium buffer for 10 min. Slides were subsequently incubated in 100 mL/L normal goat serum for 20 min at room temperature. Sections were permeabilized in PBS-Triton and incubated overnight with primary antibody at 4℃. The antibodies were used in PBS-Triton with variable dilution. Mouse anti-human monoclonal antibodies to p53 (clone Do-7; 1:50 dilution; Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd.), p21 (clone DCS-60.2; 1:50 dilution; Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd.), bcl-2 (clone 100/D5; 1:50 dilution; Shanghai Chang-Do Biotechnology Co. Ltd), bax (clone 2D2; 1:50; Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd), β-catenin (clone CAT-5H10; 1:50 dilution; Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd), c-myc (clone 9E11; 1:50 dilution; Shanghai Chang-Do Biotechnology Co. Ltd), Cox-2 (clone COX229; 1:50 dilution; Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd), nm23-h1 (1:50 dilution; Shanghai Chang-Do Biotechnology Co. Ltd) and rabbit anti-human antibody to PTEN (FL-403; 1:100 dilution; Santa Cruz Biotechnology Inc, USA), p-Akt1 (1:50 dilution; Upstate, USA), cyclin D1 (clone SP4; 1:50 dilution; Shanghai Chang-Do Biotechnology Co. Ltd) were used. Each section was then incubated with Envision^{+TM}, peroxidase, mouse or rabbit (GeneTech) for 30 min. Finally, the sections were reacted with 0.02% 3, 3'-diaminoberzidine and 0.005% H_2O_2 in 0.05 mmol/L Tris-Hcl buffer and counterstained by hematoxylin.

The evaluation of the immunohistochemical staining was performed independently by two authors without knowledge of the clinicopathological information. P53, p21 and cyclin D1 immunoreactivities were observed in the nuclei of the cells, while bcl-2, bax, PTEN, p-Akt1, c-myc, Cox-2 and nm23-h1 in the cytoplasm. Only the immunoreactivity in the nucleus or cytoplasm of β-catenin was seemed as positive. The immunoreactive scores besides β-catenin and c-myc were determined by the sum of extension and intensity as reported previously[22] and were modified for some markers according to clinicopathological correlations. The intensity of the staining was scored using the following scale: 0, no staining of the tumor cells; +, mild staining; ++, moderate staining; and +++, marked staining. The area of staining was evaluated and recorded as a percentage: 0, less than 5%; +, 5%-25%; ++, 26%-50%; 3+, 51%-75%; and ++++, more than 75%. The combined score was recorded and graded as follows: -, 0-1; +, 2; ++, 3-5; +++, 6-7. More than 10% of the cancer cells showing elevated β-catenin labeling in the cytoplasm was recorded as positive expression. According to the immunostaining of c-myc in our study, we considered less than 40% cells expressed c-myc as negative.

Statistical analysis

Computerized statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 13.0. Clinical and histopathologic information and the results from the immunohistochemical studies of the tissue microarray were entered into a database. The variances of molecular expressions among different tissues and associations between molecular variables and clinicopathological data were analyzed with χ^2 test, but when the numbers of the cells in crosstables which had expected count less than 5 exceeded 25% or the minimum expected counts were less than 1, the Fisher's exact test was used. The relations among these molecules were analyzed by Spearman's bivariates correlation test. In all statistical analyses, a two-tailed P value $\leqslant$ 0.05 was considered statistically significant.

RESULTS

Clinicopathological data

Complete histological and clinical data of the patients were collected from patients' records. The median age for the study population was 58 years (range, 30-86 years). There were 24 patients with median age less than 58 years. There was a male predominance in the cancer patients (male: female ratio = 48:37). Thirty-two patients had positive lymph node metastasis, whereas 53 had negative. Fourteen patients had distant metastasis, and 71 had no distant metastasis. The clinical stages of these patients were strictly identified as A (n = 15), B (n = 33), C (n = 23) and D (n = 14) according to Dukes stage. After the second diagnostic assessment, there were 24 low, 50 moderately and 11 high differentiated tissues in these cancer tissue blocks according to the histological grades, while 18 tissues were diagnosed as adenomatous polyps and 9 were normal colon mucosa epithelium tissues.

Expression of p53, p21, cyclin D1, bcl-2 and bax

Owing to the short half-life of p53 protein, and its low expression levels in normal cells, wild-type p53 levels cannot be detected by IHC. In cancer cells, most p53 mutations lead to products that accumulate in the nuclei and can easily be detected by IHC. Positive immunostaining most

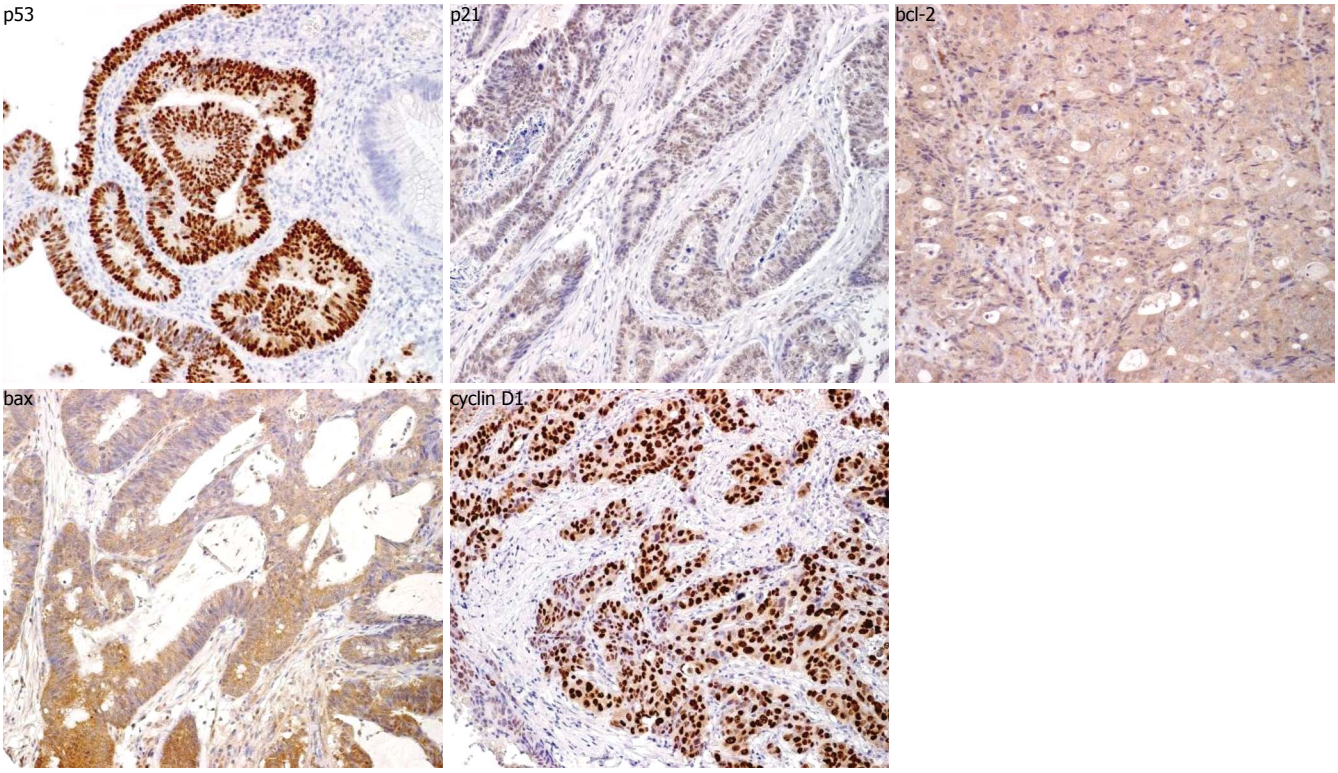

Figure 2 Immunophenotype of the investigated antigens (p53, p21, cyclin D1, bcl-2 and bax) in colon cancer (original magnification x 200). Positive stainings of p53, p21 and cyclin D1 were located in the cell nuclei, while those of bcl-2 and bax were in the cytoplasm.

Table 1 Comparison of IHC results (p53, p21, cyclin D1, bcl-2 and bax) among varying tissues

Groups	*n*	p53				*P*	p21				*P*	Cyclin D1				*P*	bcl-2				*P*	bax				*P*
		-	+	++	+++		-	+	++	+++		-	+	++	+++		-	+	++	+++		-	+	++	+++	
Cancer	85	39	6	19	21		19	9	28	26		17	16	30	22		5	1	46	32		21	9	39	16	
Adenomas	18	15	2	1	0	0.003[2]	8	5	4	1	0.000[1]	3	7	8	0	0.000[1]	1	1	7	8	0.001[2]	0	0	9	9	0.000[1]
Benign tissue	9	9	0	0	0		9	0	0	0		8	1	0	0		2	3	3	0		0	1	1	7	
Total	112	63	8	20	21		36	14	32	27		28	24	38	22		8	5	56	40		21	10	49	32	

[1]Chi-square test; [2]Fisher's exact test.

commonly represents accumulation of the stable protein product of a mutated *p53* gene that has lost its cell cycle-regulatory function. In this study, expression of p53 was identified in 46 of 85 (54%) colon cancer cell nuclei and 3 of 18 (16.66%) adenomas but absent in normal colon mucosa. Positive stainings of p21 and cyclin D1 were also located in the cell nuclei, while bcl-2 and bax were in the cytoplasm (Figure 2). The expression profiles of p21, cyclin D1, bcl-2 and bax are summarized in Table 1, which shows differences among these various tissues (cancer, adenomas, normal mucosa) regarding the IHC results of the cell cycle and apoptosis-associated protein. The relation between the immunohistochemical pattern and clinicopathological features is presented in details in Table 2. We found positive correlation between p53 and bcl-2 (r = 0.245, P = 0.010), while no significant correlation between p53 and bax (r = -0.081, P = 0.395).

Expression of Cox-2 and nm23-h1

Cox-2 and nm23-h1 were all correlated with the colon cancer progression as previously reported[8,9]. The protein expression of Cox-2 was detected in 81 of 85 (95%) colon cancer cytoplasm and 16 of 18 (88.88%) adenomas, and 6 of 9 (66.66%) normal mucosa (Figure 3). Sixty-two colon cancer patients showed a strong positive staining of nm23-h1 (score ++~+++) in the cell cytoplasm. The details of the two genes expression profiles are shown in Table 3 and the relations with clinicopathological parameters are presented in Table 4.

Expression of PTEN, p-Akt1, β-catenin, c-myc

Expressions of PTEN, p-Akt1, β-catenin, c-myc proteins were detected in the cell cytoplasm (Figure 4). Immunohistochemical results of PTEN, p-Akt1No showed no significant difference among the different tissues (data not shown). Significant difference in the expressions of β-catenin and c-myc was found among the benign mucosa, adenomas and malignant tissues (Table 5). β-catenin protein expression had a positive correlation with c-myc expression (r = 0.483, P = 0.000), thereby suggesting their

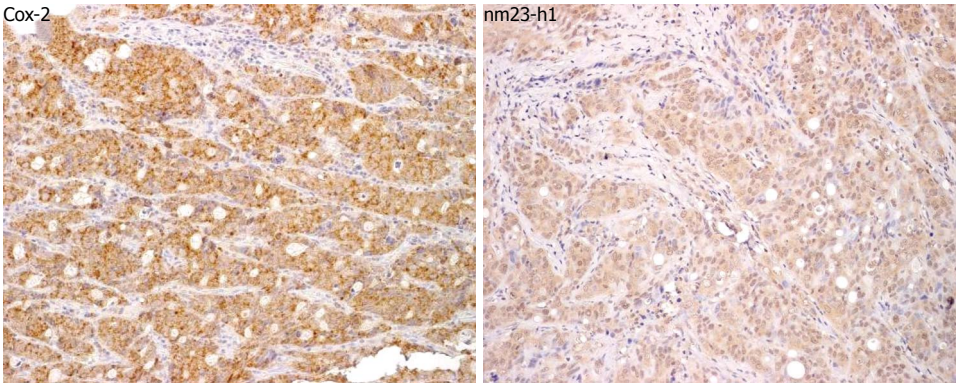

Figure 3 Immunophenotype of the investigated antigens (Cox-2 and nm23-h1) in colon cancer (original magnification x 200). The protein expressions of Cox-2 and nm23-h1 were detected in the cytoplasm.

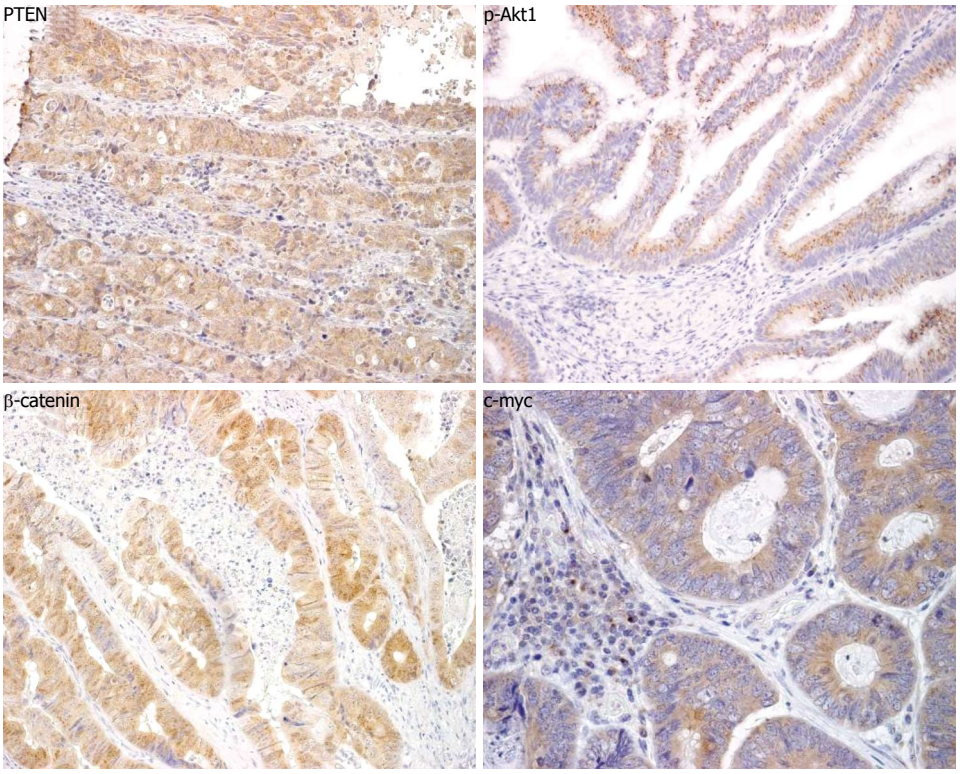

Figure 4 Immunophenotype of the investigated antigens (PTEN, p-Akt1, β-catenin and c-myc) in colon cancer (original magnification x 200). Expressions of PTEN, p-Akt1, β-catenin and c-myc were detected in the cell cytoplasm.

potent roles in *Wnt* pathway during the colon carcinogenesis. Correlations of the protein expression profiles with the clinicopathological features are shown in Tables 6 and 7.

DISCUSSION

Colon carcinogenesis is characterized by distinct morphological, genetic and cellular events. Development and progression of colon cancer to metastasis and lethal state are believed to be driven by multiple genetic alterations, the nature of which has remained poorly understood. Several pathways, such as cell cycle and apoptosis regulation, *Wnt* and PI3K/Akt pathways and so on, have been suggested to be involved in the progression[5,22,23]. However, the specific molecular alterations are largely dependent on the genetic background of the individual tumor. Therefore, investigations of molecular changes in tumors representing the entire disease spectrum may enhance our understanding of mechanisms involved in colon tumorigenesis.

To efficiently investigate the various molecules potentially relevant for colon tumor biology and to determine their potential clinical significance, large-scale analysis of multiple molecules in the same tumor tissues is required. The newly evolved and recently validated tissue microarray technique allows such molecular profiling of cancer specimens by immunohistochemistry[24,25]. In 1998, Kononen *et al*[24] introduced tissue microarrays (TMAs) as a powerful technology to rapidly visualize molecular targets such as genes and gene products in thousands of tissue specimens at a time. Furthermore, the use of TMAs for immunophenotyping of malignant tumors has recently been validated by Hoos *et al*[25] who demonstrated immunohistochemical analysis to characterize the significance of alterations in the p53 pathway and other cell cycle-related molecules in a histopathologically well-characterized cohort of patients with Hurthle cell (HC) neoplasm. In this study, two tumor tissue microarrays were constructed that allowed us to investigate the pattern of protein expressions of multiple genes and the relationships between the gene alterations and biological behaviors of colon cancer. We found that

Table 2 p53, p21, cyclin D1, bcl-2 and bax status in relation to clinicopathological parameters

Clinicopathalogical parameters		Total	p53				P	p21				P	cyclin D1				P	bcl-2				P	bax				P
			-	+	++	+++		-	1+	2+	3+		-	1+	2+	3+		-	+	++	+++		-	+	++	+++	
Age	< 58	39	17	5	8	9	0.301[1]	10	4	13	12	0.966[1]	8	6	13	12	0.752[1]	3	1	21	13	0.654[2]	11	3	17	8	0.772[1]
	⩾ 58	46	22	1	11	12		9	5	15	14		9	10	17	10		2	0	25	19		10	6	22	8	
Gender	Male	48	24	2	10	12	0.608[1]	12	1	17	16	0.040[1]	11	9	16	12	0.891[1]	5	1	26	16	0.139[2]	14	6	20	8	0.579[1]
	Female	37	15	4	9	9		7	8	11	10		6	7	14	10		0	0	20	16		7	3	19	8	
Histological grade	Low	24	18	0	3	3		10	4	3	6		9	4	8	3		3	1	16	4		11	3	9	1	
	Moderate	50	17	3	13	17	0.005[2]	6	3	21	18	0.013[2]	5	9	20	16	0.082[2]	2	0	24	23	0.075[2]	8	6	25	11	0.044[2]
	High	11	4	3	3	1		3	2	4	2		3	3	2	3		0	0	6	5		2	0	5	4	
Lymph node metastasis	Negative	53	19	4	16	14	0.067[1]	9	4	19	19	0.199[1]	11	11	20	11	0.575[1]	2	0	29	21	0.407[2]	10	6	26	11	0.457[1]
	Positive	32	20	2	3	7		10	5	9	7		6	5	10	11		3	1	17	11		11	3	13	5	
Distant metastasis	Negative	71	30	6	18	17	0.292[2]	14	8	23	23	0.644[2]	12	16	24	19	0.100[2]	3	0	39	29	0.052[2]	17	7	32	15	0.694[2]
	Positive	14	9	0	1	4		5	1	5	3		5	0	6	3		2	1	8	3		4	2	7	1	
Dukes stage	A	15	5	2	6	2		3	2	5	5		2	7	4	2		0	0	9	6		2	2	8	3	
	B	33	10	2	9	12	0.061[2]	3	2	13	13	0.286[2]	7	4	15	7	0.038[2]	1	0	18	14	0.487[2]	6	3	16	8	0.716[2]
	C	23	15	2	3	3		8	4	5	5		3	5	5	10		2	0	12	9		9	2	8	4	
	D	14	9	0	1	4		5	1	5	3		5	0	6	3		2	1	8	3		4	2	7	1	

[1] Chi-square test; [2] Fisher's exact test.

Table 3 Comparison of IHC results of Cox-2 and nm23-h1among varying tissues

Histology	n	Cox-2				P	nm23-h1				P
		-	+	++	+++		-	+	++	+++	
Cancer	85	4	4	30	47	0.002	5	7	33	29	0.007
Adenomas	18	2	0	13	3		2	1	13	2	
Benign tissue	9	3	0	1	5		4	1	2	1	
Total	112	9	4	44	55		11	9	48	32	

Fisher's exact test.

Table 4 Cox-2 and nm23-h1 status in relation to clinicopathological data

Groups		n	Cox-2				P	nm23-h1				P
			-	+	++	+++		-	+	++	+++	
Age (yr)	< 58	39	3	1	17	18	0.239	4	3	21	10	0.272
	⩾ 58	46	1	3	13	29		1	4	22	19	
Gender	Male	48	1	2	17	28	0.628	5	5	24	14	0.157
	Female	37	3	2	13	19		0	2	19	15	
Histological grade	Low	24	3	3	12	6		3	6	13	2	
	Moderate	50	1	1	14	33	0.008	2	0	23	24	0.000
	High	11	0	0	4	7		0	1	7	3	
Lymph node metastasis	Negative	53	1	2	17	33	0.223	2	3	29	18	0.434
	Positive	32	3	2	13	14		3	4	14	11	
Distant metastasis	Negative	71	1	3	27	40	0.026	3	3	39	26	0.008
	Positive	14	3	1	3	7		2	4	4	3	
Dukes stage	A	15	0	1	3	11	0.016	1	1	11	2	0.014
	B	33	0	0	12	21		0	1	16	16	
	C	23	1	2	12	8		2	1	12	8	
	D	14	3	1	3	7		2	4	4	3	

Fisher's exact test.

Table 5 Comparison of the IHC results of β-caternin and c-myc among varying tissues

Histological characteristics	Total	β-caternin		P	c-myc		P
		-	+		-	+	
Cancer	85	15	70	0.034	16	68	0.003
Adenomas	18	5	13		4	14	
Benign tissue	9	5	4		6	2	
Total	112	25	87		26	84	

Fisher's exact test.

Table 6 PTEN and p-Akt1 status in relation to clinicopathological data

Clinicopathological parameters		Total	PTEN				P	p-Akt1				P
			-	+	++	+++		-	+	++	+++	
Age	< 58	39	3	2	20	14	0.860[2]	10	0	16	13	0.408[1]
	⩾ 58	46	2	2	22	20		9	3	19	15	
Gender	Male	48	4	4	24	16	0.167[2]	14	0	19	15	0.096[1]
	Female	37	1	0	18	18		5	3	16	13	
Histological grade	Low	24	4	2	14	4		11	3	6	4	
	Moderate	50	1	2	24	23	0.029[2]	8	0	26	16	0.000[2]
	High	11	0	0	4	7		0	0	3	8	
Lymph node metastasis	Negative	53	3	2	22	26	0.132[2]	7	1	26	19	0.032[1]
	Positive	32	2	2	20	8		12	2	9	9	
Distant metastasis	Negative	71	2	3	37	29	0.060[2]	13	3	31	24	0.275[2]
	Positive	14	3	1	5	5		6	0	4	4	
Dukes stage	A	15	0	1	7	7	0.281[2]	1	0	6	8	0.106[2]
	B	33	1	1	15	16		4	1	17	11	
	C	23	1	1	15	6		8	2	8	5	
	D	14	3	1	5	5		6	0	4	4	

[1] Chi-square test; [2] Fisher's exact test.

Table 7 β-catenin and c-myc status in relation to clinicopathological data

Clinicopathological parameters		Total	β-caternin		*P*	c-myc		*P*
			-	+		-	+	
Age	< 58	39	6	33	0.983[1]	9	29	0.117[1]
	⩾ 58	46	7	39		5	41	
Gender	Male	48	8	40	0.689[1]	10	38	0.237[1]
	Female	37	5	32		4	32	
Histological grade	Low	24	10	14		11	13	
	Moderate	50	3	47	0.000[2]	3	46	0.000[2]
	High	11	0	11		0	11	
Lymph node metastasis	Negative	53	4	49	0.011[1]	4	48	0.005[1]
	Positive	32	9	23		10	22	
Distant metastasis	Negative	71	8	63	0.020[1]	6	65	0.000[1]
	Positive	14	5	9		8	5	
Dukes stage	A	15	0	15		0	15	
	B	33	2	31	0.008[2]	2	31	0.000[2]
	C	23	6	17		4	19	
	D	14	5	9		8	5	

[1]Chi-square test; [2]Fisher's exact test.

the 1.0-1.5 mm cores used to create the arrays were easy to work with, included enough tumor tissue that histological relationships were easily evaluated, and focused attention on limited regions of tumor, thus ensuring high reproducibility of scoring. Furthermore, at the same time, we could study the morphous of cells and protein expression parallely and avoid the variance in results in different experiment conditions as seen in the conventional technology.

The cell cycle-regulatory machinery is a complex system of proteins regulating each other's activity and controlling the division of cells[26]. We selected p53, p21, cyclin D1 and bcl-2/bax for analysis as target proteins participating in the regulation of proliferation and apoptosis, which are known to be deranged in cancer cell cycles, and which have been shown to affect survival of colorectal carcinomas. The p53 tumor suppressor plays a pivotal role in cell cycle regulation and apoptosis. Mutations in the *p53* gene are among the most common mutations encountered in human malignancy. Wild-type p53 along with other cellular growth factors activate *p21* gene expression and the corresponding p21 protein triggers cell-cycle arrest in the G_1 phase[27]. In colorectal cancer cells, mutated p21 neither suppressed apoptosis nor affected cell survival[28]. In addition to cell-cycle control, p53 mediates programmed cell death through the bcl-2/bax apoptotic pathway[29]. In this study, we observed that p53 was undetectable in normal colon tissue and over-expressed in only three adenomas and 54% of the adenocarcinomas. When the expression of p53 with clinicopathological parameters was compared, only the significant relation with histological grade could be seen, thereby indicating that p53 may be involved in the late stage of colon carcinogenesis and in the malignant progression of colon cancer. As to the others genes, there were significant differences among the three groups (normal mucosa, adenoma and cancer) regarding the immunohistochemical results (cyclin D1, $P = 0.000$; bcl-2, $P = 0.001$; bax, $P = 0.000$). Comparing these markers with the clinicopathological features, we observed the correlation of histological grades with bax, and Dukes stage with cyclin D1. Thus, we can speculate that cyclin D1, bcl-2 and bax aberrations may involve in the colon cancer development and the decreased expression of bax can accelerate the cancer tissue further differentiate into advanced stage, and malignant colon tissue highly expressed cyclin D1 may acquire the invasive potent. In addition, though the difference of this protein expression between distant metastasis-positive group and -negative group was not significant ($P = 0.052$) in this study, colon cancer with alteration of bcl-2 expression may facilitate to metastasize to distance sites. In our study, p21 over-expression was found in 64 of 82 (78.04%) cancers and was correlated with advanced stage colorectal cancer, which is in agreement with a previous report[30]. Furthermore, IHC revealed that some cancer cells expressed both p21 and p53, suggesting that p21 can also be activated by a p53-independent mechanism. A previous study reported that p53 can inhibit bcl-2 gene expression by transcriptional activation of the pro-apoptotic bax gene. But the relationship between p53 and bax in this cohort tissue was not established. However, our results showed that the expression of p53 had positive correlation with bcl-2. The precise mechanism of regulation of bcl-2/bax pathway through p53 needs further study.

There are two distinct Cox isoenzymes, namely constitutive Cox-1 and inducible Cox-2. It was reported that Cox-2 elevated in human esophagus, skin, and colon cancers, and the Cox-2 inhibitor, celecoxib, inhibited intestinal tumor multiplicity in a mouse model and reduced the number of adenomatous polyps in a familial adenomatous polyposis patient[16,17]. Enhanced Cox-2 expression has been related to tumor differentiation, distant metastasis and Dukes stage[31,32]. In present study, Cox-2 immunoreactivity was increased in the colon carcinoma (91%) and adenomas (88%). However, the expression of Cox-2 was correlated with less advanced grade, fewer distant metastases and lower Dukes stage in these cohort colon cancer tissues. These results indicate that Cox-2 over-expression might be an early event during the tumorigenesis in the colon and its role in progression of colon cancer deserves further investigation. Although a reduced expression of nm23-h1 has been shown to be correlated with a high metastatic potential in some human cancers, such as colorectal cancers, conflicting data have been reported[9,33]. In our study, expression of nm23-h1 was detected in 79 of 85 (92.94%) cancers, 16 of 18 (88.88%) adenomas and 4 of 8 (50%) normal tissues. The decreasing tendency of expression of nm23-h1 was found to be associated with the aggravation of differentiation, distant metastasis in the cohort patients. Therefore, the exact role of nm23-h1 in development and progression of the colon cancer needs further studies.

β-catenin is known to complex with E-cadherin to form intercellular junctions. It also participates in the *Wnt*-signaling pathway, which frequently is disrupted in colorectal carcinomas by adenomatous polyposis

coli (APC) or β-catenin mutations[15]. Translocation of β-catenin to the nucleus with transcription start of cyclin D1 and metalloproteinase 7 could lead to more aggressive colorectal carcinomas[34]. C-myc was also found to be involved in the *Wnt* pathway and seemed as a target gene of β-catenin/TCF[35]. It has been extensively documented that c-myc is over-expressed at RNA and protein levels at both early and late stages of the colorectal tumorigenesis[36]. Previous immunohistochemical studies with β-catenin showed distinct subcellular expression patterns in the colorectal carcinomas, adenomas, and benign epithelium, whereas benign tissue almost universally expressed β-catenin on the plasma membrane only, adenomas and carcinomas expressed β-catenin to varying degrees in the cytoplasm and in the nucleus as well[6,36,37]. This finding is not surprising, given the intracellular pathway described above. However, correlations between alterations of β-catenin expression in colorectal cancer and outcome variables have not been consistent[38-40]. In this study, different expressions of these two molecules were found among the varying types of tissues. β-catenin was expressed in 70 of 85 (82.83%) cancer tissues, 13 of 18 (72.22%) adenomas and only 4 of 9 (44.44%) para-cancerous normal mucosa. Similarly, positive expression of c-myc was detected in 68 of 84 (80.95%) colon cancers, 14 of 18 (77.77%) adenomas as well as 25% benign tissues. In addition to the results which showed positive correlation between the β-catenin and c-myc in our study ($r = 0.483$, $P = 0.000$), we can assume that over-expression of β-catenin in the cytoplasm and c-myc might be early events during the carcinogenesis in human colon *via* involving in the *Wnt* pathway which always distorts during colon tumorigenesis. However, the cancer tissues in the cohort with over-expression of β-catenin or c-myc exhibited less capacity to differentiate into advanced grade and invasive potential, which are contradicted to some previous reports[40,41]. The exact roles of β-catenin and c-myc aberration in progression of colon cancer requires further investigations.

The phosphatidylinositol-3 kinase (PI3K)/Akt is an important survival signal pathway that has been shown to be crucial in the regulation of balance between pro-apoptotic and survival (anti-apoptotic) signal[42]. The phosphorylated Akt level can monitor cell growth and resistance to apoptosis, indicating that activation of Akt plays an important role during the progression of colorectal carcinomas by helping promote cell growth and rescue cells from apoptosis. PTEN is a phosphatase that negatively regulates the phosphoinositol-3-kinase/Akt pathway and mediates cell-cycle arrest and apoptosis[43]. One study reported that PTEN protein expression was abnormal when compared the cancer with benign tissue[44], but same phenomenon was not found among the cohort patients including in this study. However, at the same time, PTEN expression detected by us was found to be related to tumor histological grade and p-Akt1 was related to lymph node metastasis. In this study, the expression profiles demonstrated that though did not involve in the early development of tumorigenesis, these two molecules may conduce to the lately progression of colon cancer which showed lymph node or distant metastasis. Contradiction between the results of p-Akt1 expression in our study and a previous study[11] which reported that Akt over-expression occurred frequently during human colon carcinogenesis might be because the gene background (FAP, HNPCC or sporadic CRC) of the patients studied were not strictly identified and the antibody used which was a polyclonal antibody in our study.

In summary, tissue microarrays (TMAs) combined with immunohistochemical staining for colon cancer, adenomas and normal mucosa showed that TMAs technology with 1.0 to 1.5 mm core tissue adequately represents the immunohistochemical pattern of the colon tissue. It considerably reduces the cost and labor needed to process tissue slides and enrich the technical spectrum of histopathology. The over-expressions of p53, cyclin D1, bcl-2, Cox-2, β-catenin and c-myc and the low or no expression of bax might be involved in the colon tumorigenesis and PTEN, p-Akt1 may contribute to the progression of colon cancer at late stage. No or low expression of nm23-h1 in colon cancer may contribute to the cancer cells acquired the invasion and distant metastasis potential. Further study needs to investigate the precise mechanism of colon cancer development and progression.

COMMENTS

Background

Studies of single molecular marker have not been successful in defining the biology of the colon cancer, so we explored multiple molecules regulating the colon tumorigenesis by using tissue microarray combining with immunohistochemical staining which can help us complete the time- and people-consuming work.

Research frontiers

In this study, the most worthwhile hotspot I think was investigating 11 molecules expressions by using the tissue microarray which allows rapid visualization of molecular targets at a time in protein level.

Innovations and breakthroughs

Firstly I should mention that in this study, we explored 11 molecular expressions at a time during colon carcinogenesis which was seldom found in our country. In addition, the expressions of these molecules were detected by using tissue micoarray which is a new high-throughput biological technique.

Applications

Tissue microarray can facilitate rapid translation of molecular discoveries to clinical applications used, so we can speculate that the tissue microarray technology which is fast, convenient and economic may have potential dominant position in macro-scale detection of tissue specimens in cancer studies.

Terminology

In 1998, Kononen and Kalloiniemi developed tissue microarrays (TMAs) whereby an ordered array of tissue samples are placed on a single slide. Once constructed, the TMA can be probed with a molecular target (DNA, RNA or protein) for analysis by immunohistochemistry, fluorescence *in situ* hybridization (FISH) or other molecular detection methods, enabling high-throughput *in situ* analysis of specific molecular targets in hundreds or even thousands of tissue specimens.

Peer review

In this study, the authors investigated the expressions of 11 cancer related genes involving cell proliferation, cell cycle, apoptosis, as well as signal pathway in human colon cancer, adenoma and para-cancerous mucosa by using tissue microarray, a new biological technique, combining with immunohistochemical staining at a time. The results from this study may manifest the colon cancer situation at some level and have some reference values in our country.

REFERENCES

1 **Pohl C**, Hombach A, Kruis W. Chronic inflammatory bowel disease and cancer. *Hepatogastroenterology* 2000; **47**: 57-70
2 **Fearon ER**, Hamilton SR, Vogelstein B. Clonal analysis of human colorectal tumors. *Science* 1987; **238**: 193-197
3 **Kallioniemi OP**, Wagner U, Kononen J, Sauter G. Tissue microarray technology for high-throughput molecular profiling of cancer. *Hum Mol Genet* 2001; **10**: 657-662
4 **Serrano R**, Gómez M, Farre X, Méndez M, De La Haba J, Morales R, Sanchez L, Barneto I, Aranda E. Tissue microarrays (TMAS) in colorectal cancer: Study of clinical and molecular markers. *ASCO Meeting Abstracts* 2004; **22**: 9665
5 **Chung DC**. The genetic basis of colorectal cancer: insights into critical pathways of tumorigenesis. *Gastroenterology* 2000; **119**: 854-865
6 **Hao X**, Tomlinson I, Ilyas M, Palazzo JP, Talbot IC. Reciprocity between membranous and nuclear expression of beta-catenin in colorectal tumours. *Virchows Arch* 1997; **431**: 167-172
7 **He TC**, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. Identification of c-MYC as a target of the APC pathway. *Science* 1998; **281**: 1509-1512
8 **Kim JY**, Lim SJ, Park K. Cyclooxygenase-2 and c-erbB-2 expression in colorectal carcinoma assessed using tissue microarrays. *Appl Immunohistochem Mol Morphol* 2004; **12**: 67-70
9 **Messinetti S**, Giacomelli L, Fabrizio G, Giarnieri E, Gabatel R, Manno A, Feroci D, Guerriero G, Masci E, Vecchione A. CD44v6 and Nm23-H1 protein expression related to clinicopathological parameters in colorectal cancer. *Ann Ital Chir* 2003; **74**: 45-51
10 **Zhou XP**, Loukola A, Salovaara R, Nystrom-Lahti M, Peltomäki P, de la Chapelle A, Aaltonen LA, Eng C. PTEN mutational spectra, expression levels, and subcellular localization in microsatellite stable and unstable colorectal cancers. *Am J Pathol* 2002; **161**: 439-447
11 **Roy HK**, Olusola BF, Clemens DL, Karolski WJ, Ratashak A, Lynch HT, Smyrk TC. AKT proto-oncogene overexpression is an early event during sporadic colon carcinogenesis. *Carcinogenesis* 2002; **23**: 201-205
12 **Itoh N**, Semba S, Ito M, Takeda H, Kawata S, Yamakawa M. Phosphorylation of Akt/PKB is required for suppression of cancer cell apoptosis and tumor progression in human colorectal carcinoma. *Cancer* 2002; **94**: 3127-3134
13 **Di Cristofano A**, Pandolfi PP. The multiple roles of PTEN in tumor suppression. *Cell* 2000; **100**: 387-390
14 **Weng L**, Brown J, Eng C. PTEN induces apoptosis and cell cycle arrest through phosphoinositol-3-kinase/Akt-dependent and -independent pathways. *Hum Mol Genet* 2001; **10**: 237-242
15 **Wong NA**, Pignatelli M. Beta-catenin-a linchpin in colorectal carcinogenesis? *Am J Pathol* 2002; **160**: 389-401
16 **Soslow RA**, Dannenberg AJ, Rush D, Woerner BM, Khan KN, Masferrer J, Koki AT. COX-2 is expressed in human pulmonary, colonic, and mammary tumors. *Cancer* 2000; **89**: 2637-2645
17 **Masferrer JL**, Leahy KM, Koki AT, Zweifel BS, Settle SL, Woerner BM, Edwards DA, Flickinger AG, Moore RJ, Seibert K. Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. *Cancer Res* 2000; **60**: 1306-1311
18 **Martinez JA,** Prevot S, Nordlinger B, Nguyen TM, Lacarriere Y, Munier A, Lascu I, Vaillant JC, Capeau J, Lacombe ML. Overexpression of nm23-H1 and nm23-H2 genes in colorectal carcinomas and loss of nm23-H1 expression in advanced tumour stages. *Gut* 1995; **37**: 712-720
19 **Mucci NR**, Akdas G, Manely S, Rubin MA. Neuroendocrine expression in metastatic prostate cancer: evaluation of high throughput tissue microarrays to detect heterogeneous protein expression. *Hum Pathol* 2000; **31**: 406-414
20 **Schraml P**, Kononen J, Bubendorf L, Moch H, Bissig H, Nocito A, Mihatsch MJ, Kallioniemi OP, Sauter G. Tissue microarrays for gene amplification surveys in many different tumor types. *Clin Cancer Res* 1999; **5**: 1966-1975
21 **Richter J**, Wagner U, Kononen J, Fijan A, Bruderer J, Schmid U, Ackermann D, Maurer R, Alund G, Knönagel H, Rist M, Wilber K, Anabitarte M, Hering F, Hardmeier T, Schönenberger A, Flury R, Jäger P, Fehr JL, Schraml P, Moch H, Mihatsch MJ, Gasser T, Kallioniemi OP, Sauter G. High-throughput tissue microarray analysis of cyclin E gene amplification and overexpression in urinary bladder cancer. *Am J Pathol* 2000; **157**: 787-794
22 **Lynch HT**, Smyrk TC. Identifying hereditary nonpolyposis colorectal cancer. *N Engl J Med* 1998; **338**: 1537-1538
23 **Bienz M**, Clevers H. Linking colorectal cancer to Wnt signaling. *Cell* 2000; **103**: 311-320
24 **Kononen J**, Bubendorf L, Kallioniemi A, Bärlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 1998; **4**: 844-847
25 **Hoos A**, Urist MJ, Stojadinovic A, Mastorides S, Dudas ME, Leung DH, Kuo D, Brennan MF, Lewis JJ, Cordon-Cardo C. Validation of tissue microarrays for immunohistochemical profiling of cancer specimens using the example of human fibroblastic tumors. *Am J Pathol* 2001; **158**: 1245-1251
26 **Cordon-Cardo C**. Mutations of cell cycle regulators. Biological and clinical implications for human neoplasia. *Am J Pathol* 1995; **147**: 545-560
27 **el-Deiry WS**, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993; **75**: 817-825
28 **Lu Y**, Yamagishi N, Yagi T, Takebe H. Mutated p21(WAF1/CIP1/SDI1) lacking CDK-inhibitory activity fails to prevent apoptosis in human colorectal carcinoma cells. *Oncogene* 1998; **16:** 705-712
29 **Chao DT**, Korsmeyer SJ. BCL-2 family: regulators of cell death. *Annu Rev Immunol* 1998; **16**: 395-419
30 **Viale G**, Pellegrini C, Mazzarol G, Maisonneuve P, Silverman ML, Bosari S. p21WAF1/CIP1 expression in colorectal carcinoma correlates with advanced disease stage and p53 mutations. *J Pathol* 1999; **187**: 302-307
31 **Fujita T**, Matsui M, Takaku K, Uetake H, Ichikawa W, Taketo MM, Sugihara K. Size- and invasion-dependent increase in cyclooxygenase 2 levels in human colorectal carcinomas. *Cancer Res* 1998; **58:** 4823-4826
32 **Zhang H**, Sun XF. Overexpression of cyclooxygenase-2 correlates with advanced stages of colorectal cancer. *Am J Gastroenterol* 2002; **97**: 1037-1041
33 **Zeng ZS**, Hsu S, Zhang ZF, Cohen AM, Enker WE, Turnbull AA, Guillem JG. High level of Nm23-H1 gene expression is associated with local colorectal cancer progression not with metastases. *Br J Cancer* 1994; **70**: 1025-1030
34 **Brabletz T**, Jung A, Dag S, Hlubek F, Kirchner T. beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. *Am J Pathol* 1999; **155**: 1033-1038
35 **Smith DR**, Myint T, Goh HS. Over-expression of the c-myc proto-oncogene in colorectal carcinoma. *Br J Cancer* 1993; **68**: 407-413
36 **Kobayashi M**, Honma T, Matsuda Y, Suzuki Y, Narisawa R, Ajioka Y, Asakura H. Nuclear translocation of beta-catenin in colorectal cancer. *Br J Cancer* 2000; **82**: 1689-1693
37 **Iwamoto M**, Ahnen DJ, Franklin WA, Maltzman TH. Expression of beta-catenin and full-length APC protein in normal and neoplastic colonic tissues. *Carcinogenesis* 2000; **21**: 1935-1940
38 **Günther K**, Brabletz T, Kraus C, Dworak O, Reymond MA, Jung A, Hohenberger W, Köckerling T, Kockerling F, Ballhausen WG. Predictive value of nuclear beta-catenin expression for the occurrence of distant metastases in rectal cancer. *Dis Colon Rectum* 1998; **41**: 1256-1261
39 **Hugh TJ**, Dillon SA, Taylor BA, Pignatelli M, Poston GJ, Kinsella AR. Cadherin-catenin expression in primary colorectal cancer: a survival analysis. *Br J Cancer* 1999; **80**: 1046-1051
40 **Maruyama K**, Ochiai A, Akimoto S, Nakamura S, Baba S, Moriya Y, Hirohashi S. Cytoplasmic beta-catenin accumulation as a predictor of hematogenous metastasis in human

colorectal cancer. *Oncology* 2000; **59**: 302-309

41 **Masramon L**, Arribas R, Tórtola S, Perucho M, Peinado MA. Moderate amplifications of the c-myc gene correlate with molecular and clinicopathological parameters in colorectal cancer. *Br J Cancer* 1998; **77**: 2349-2356

42 **Khor TO**, Gul YA, Ithnin H, Seow HF. Positive correlation between overexpression of phospho-BAD with phosphorylated Akt at serine 473 but not threonine 308 in colorectal carcinoma. *Cancer Lett* 2004; **210**: 139-150

43 **Stambolic V**, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 1998; **95**: 29-39

44 **Taniyama K**, Goodison S, Ito R, Bookstein R, Miyoshi N, Tahara E, Tarin D, Urquidi V. PTEN expression is maintained in sporadic colorectal tumours. *J Pathol* 2001; **194**: 341-348

S- Editor Liu Y **L- Editor** Kumar M **E- Editor** Bai SH