

## RESEARCH ARTICLE

# Expression of Ang-2/Tie-2 and PI3K/AKT in Colorectal Cancer

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### Abstract

**Purpose:** To study the expression of angiogenin-2 (Ang-2) and its receptor Tie-2 in colorectal cancer and discuss the possible mechanisms behind this process. **Materials and Methods:** Using the streptavidin-peroxidase (SP) immunohistochemical method, paraffin sections from 100 colorectal cancer samples and 10 samples from tumor-adjacent normal tissue (> 2 cm from the edge of the gross tumor) were tested for protein expression of Ang-2, Tie-2, PI3K, and AKT. Reverse transcription-polymerase chain reaction and Western blots were further used to measure expression of the 4 genes and proteins in 20 freshly-resected colorectal cancer samples and tumor-adjacent normal tissues. **Results:** In colorectal cancer tissues, the expression of the Ang-2, Tie-2, PI3K, and AKT genes and their proteins was significantly higher than in tumor-adjacent normal tissues. Protein expression in poorly-differentiated adenocarcinoma was higher than that in well and moderately differentiated adenocarcinoma. According to Duke's classification, the protein expression in Stages C and D was significantly higher than that in Stages A and B. In the group with lymphatic metastasis, the protein expression was higher than that without lymphatic metastasis. **Conclusions:** In colorectal cancer, the expression of the Ang-2, Tie-2, PI3K, and AKT genes and their proteins is markedly higher than those in tumor-adjacent normal tissues. No correlation was observed between protein expression and gender, location, or histologic type. Correlations did exist between protein expression and differentiation level, stage of Duke's classification, and lymphatic metastasis; in colorectal cancer tissues with lower differentiation levels, higher stages of Duke's classification, and lymphatic metastasis, the expression of all 4 proteins was higher. The study of their expression patterns and relationships with aggression and metastasis will provide a valuable experimental foundation for assessing prognosis and targeted therapy of colorectal cancer.

**Keywords:** Angiogenin-2 - Tie-2 - colorectal cancer - PI3K - AKT

*Asian Pac J Cancer Prev*, 15 (20), 8651-8656

### Introduction

Colorectal cancer is one of the most common malignant tumors threatening human health. Its pathogenesis and progression are closely related to angiogenesis. Researchers in China and other countries have confirmed that Ang-2 and its receptor Tie-2 are highly expressed in Lung cancer, ovarian cancer, cervical cancer and colorectal cancer (Goede et al., 2010; De Palma et al., 2011; Mazziari et al., 2011; Sun et al., 2011). However, little data on the relationship between Ang-2/Tie-2 and colorectal cancer exists. The phosphatidylinositol 3-kinase/v-akt murine thymoma viral oncogene homolog (PI3K/AKT) signal transduction pathway is an important intracellular signal transduction pathway which plays a vital role in the process of cellular apoptosis, survival, and proliferation (Coelho et al., 2009; Huang et al., 2011). AKT is a serine/threonine kinase (a major downstream effector molecule of PI3K), and through direct phosphorylation of transcription factors

such as NF- $\kappa$ B and the mammalian target of rapamycin (mTOR), it participates in multiple in vitro bioactivities (Sheng et al., 2009). Recent studies have demonstrated that many human tumors, for example ovarian cancer, pancreatic cancer, breast cancer, and non-small cell lung cancer, overexpressed PI3K/AKT (Kang et al., 2010; Cheng et al., 2011). Unfortunately, few reports concerning the relationship between PI3K/AKT and colorectal cancer have been published. In this study, 100 paraffin sections and 20 freshly-resected samples of colorectal cancer were subjected to immunohistochemistry, reverse transcriptase-polymerase chain reaction (RT-PCR), and Western blot. Taking tumor-adjacent tissue as the control, the gene levels of Ang-2, Tie-2, PI3K, and AKT and their protein expressions in colorectal cancer tissue were measured in order to reveal their correlation with clinical pathological characteristics. This study intended to elucidate the possible mechanism behind the occurrence of colorectal cancer.

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## Materials and Methods

### Materials

Ang-2 and PI3K polyclonal antibodies were purchased from Wuhan Boster Bioengineering; and the Tie-2 and AKT1/2 polyclonal antibodies were purchased from Santa Cruz. The protein and DNA markers were purchased from Solarbio, and the reverse transcriptase SuperScript II and Trizol were purchased from Invitrogen. TaqDNA polymerase was purchased from Beijing Tianwei Biotechnic Company. The DAB color developing kit and ready-to-use SP immunohistochemistry kit were purchased from Beijing Zhongshan Goldenbridge Biotechnology Company. All other agents were analytical grade (imported or domestic).

From July 2010 to July 2012, paraffin sections of resected colorectal cancer were collected from 100 patients in the Affiliated Hospital of Beihua University. Patients ranged in age from 28 to 85 years, with an average age of 63.36 years. Sixty patients were male and 40 were female. All cases were confirmed by a pathologist as colorectal cancer and were classified according to WHO Colorectal Cancer Classification in Digestive System criteria (2000): 10 cases of papillary adenocarcinoma, 73 cases of tubular adenocarcinoma, 17 cases of other adenocarcinoma (12 cases of myxoadenocarcinoma, 5 cases of signet ring carcinoma); 65 cases of highly and moderately differentiated tumor, 35 cases of poorly differentiated tumor; 40 cases with lymphatic metastasis, 60 cases without lymphatic metastasis. According to Dukes classification: 40 cases belonged to Stage A, 40 to Stage B, 11 to Stage C, and 9 to stage D. Among the colorectal cancer samples, 57 were located in the left side of the abdominal cavity (sigmoid colon and descending colon), and 43 in the right side (cecum and ascending colon). During the sampling, 10 samples of tumor-adjacent tissue (> 2 cm from the edge of gross tumor) were collected to be used as controls. All samples were fixed with 10% neutral buffered formalin.

From January 2011 to June 2011, 20 freshly-resected samples were collected from 20 patients in the Affiliated Hospital of Beihua University. Tumor tissue and tumor-adjacent tissue were resected, and placed in liquid nitrogen at -180°C within 30 min of excision; then, they were stored at -80°C in a low temperature refrigerator. This set of patients ranged in age from 45 to 67 years, with an average age of 60.24 years. Eleven of these patients were male and 9 were female. Ten highly and moderately differentiated adenocarcinomas and 10 poorly differentiated adenocarcinomas were identified. No patient received chemotherapy, radiotherapy, or other treatment targeting colorectal cancer before the surgeries.

### Methods

**Immunohistochemical Staining (SP method):** After routine paraffin embedment, dehydration, and antigen retrieval, samples were blocked by normal goat serum and then the primary antibody (1:100) was added. The primary antibody-positive colorectal cancer sections were used as positive controls. PBS solution was used in place of the primary antibody in the negative control samples.

All samples were incubated at 4°C overnight. After the biotin-labeled secondary antibody (1:100) was added, samples were incubated with horseradish peroxidase (HRP)-labeled streptavidin. After freshly prepared 3,3'-Diaminobenzidine (DAB) solution was added, the color was observed under a microscope. Samples were processed with hematoxylin re-staining, dehydration, and vitrification, and then sealed in the slide with neutral balsam.

**Criteria for grading immunohistochemistry staining:** Ang-2, Tie-2, PI3K, and AKT were all positively expressed in the cytoplasm, and tumor cells containing yellow or dark brown granules were counted as positive cells. Representative regions were chosen with low magnification, and then high magnification (400×) was used to select the 5 fields with concentrated positive expression. Semi-quantitative evaluation was performed on the positive cells as follows: 1. According to the degree of staining, scoring was 0 score for no color, 1 for yellow, 2 for brown-yellow, 3 for dark brown. 2) According to the percentage of area of positive cells in the field, scoring was 0 for positive cells percentage ≤ 5%, 1 for 6% - 25%, 2 for 26% - 50%, and 3 for > 51%. The scores from the above scoring systems were multiplied, and a score of 0-1 was considered negative, 2 as mild positive, 3-4 as positive, and 5-6 as strongly positive; > 2 was regarded as positive expression (Hu et al., 2009).

**RT-PCR:** According to the RNA sequences from the literature and Genbank, the primers used in RT-PCR are shown in Table 1.

The primers were dissolved in 5 mmol/L Tris-HCl (pH 7.6) after synthesis, and the final concentration was 10 pmol/μl. 5 μl of PCR product was used for electrophoresis. Images were taken under ultraviolet (UV) lamp, and a gel imaging system was used to perform gray-scale scanning on the specific amplified PCR product fragments from each sample. GAPDH density was used as the reference of quantitative standard; the expression amount of amplified product was expressed as the optical density ratio with GAPDH.

**Western blot:** 0.01 g of tissue was homogenized, electrophoresed, and then transferred to a PVDF membrane. The PVDF membrane was blocked, incubated with the primary antibody (1:500), washed with shaking, incubated with the secondary antibody (1:2000), and washed again with shaking. After exposure, gel imaging and analytic systems were used to analyze the absorbance value of each strip (A).

Statistical analysis: SPSS v12.0 was used for statistical analysis, and enumeration data were assessed with Fisher's exact test, Pearson's chi-square test, and Spearman rank correlation; measurement data were analyzed with Student's t-test.  $p < 0.05$  was considered statistically significant.

## Results

### Immunohistochemical staining

Expression of Ang-2, Tie-2, PI3K, and AKT in colorectal cancer and tumor-adjacent tissues: Ang-2 manifested as mildly positive in 2 of 10 tumor-adjacent

tissue samples, and it was expressed in the cytoplasm of glandular epithelial cells; of 100 samples of colorectal cancer, 78 demonstrated positive Ang-2 expression and they had yellow, brown-yellow, or dark brown cytoplasm staining. As shown in Table 2, the positive expression rate of Ang-2 in colorectal cancer samples was significantly higher than that in tumor-adjacent tissues ( $p<0.05$ ). Tie-2 was expressed in the cytoplasm of glandular epithelial cells, and 2 of 10 samples of tumor-adjacent tissues showed mildly positive expression; 70 out of 100 colorectal cancer samples demonstrated positive Tie-2 expression. The positive expression rate of Tie-2 in colorectal cancer tissue was significantly higher than that in tumor-adjacent tissue ( $p<0.05$ ).

**Table 1. Primers Used in RT-PCR**

Name	Sequence	Amplification length (bp)	Annealing temperature (°C)
GAPDH	sense: 5'-ACCACAGTCCATGCCATCAC-3' antisense: 5'-TCCACCACCCTGTTGCTGTA-3'	450	55
Ang-2	sense: 5'-GAGATCAAGGCTACAGTGA-3' antisense: 5'-AAGTTGGAAGGACCACATGC-3'	263	55
Tie-2	sense: 5'-TGTTCTGTGCCACGGCTG-3' antisense: 5'-CACTGTCCCATCCGGCTTCA-3'	721	55
PI3K	sense: 5'-TGGTCTTTCGGAAGTGAGATAG-3' antisense: 5'-AAGTTGGAAGGACCACATGC-3'	117	55
AKT	sense: 5'-TAGGCATCCCTTCTTACAGC-3' antisense: 5'-CACTGTCCCATCCGGCTTCA-3'	114	55

PI3K and AKT were not expressed in tumor-adjacent tissues, but in 100 samples of colorectal cancer, 88 demonstrated positive expression of PI3K and 85 showed positive expression of AKT. Both proteins were mainly expressed in the cytoplasm of tumor cells, as evidenced by a yellow, brown-yellow, or dark brown stained cytoplasm. The positive expression rate of PI3K and AKT in colorectal cancer tissues was higher than that in tumor-adjacent tissues ( $p<0.01$ ).

The relationship between the expression of Ang-2, Tie-2, PI3K, and AKT and clinical pathological changes: the positive expression rate of Ang-2 in papillary adenocarcinoma, tubular adenocarcinoma and other adenocarcinoma was 80%, 71%, and 76.5%, respectively, Tie-2 was 70%, 68.5%, and 70.6%, respectively, PI3K was 80%, 80.8%, and 82.4%, respectively, and AKT was 80%, 79.5%, and 76.5%, respectively. The expression of the 4 proteins in neoplasms in 3 different histological types showed no significant difference ( $p>0.05$ ). The positive expression rate of the 4 proteins was higher in poorly differentiated tumors than in well and moderately differentiated tumors, and there was significant difference between them respectively (both  $p<0.01$ ). Based on Duke's classification, the positive expression rate in Stages C and D was significantly higher than that in Stages A and B ( $p<0.05$ ). The positive expression rate in colorectal

**Table 2. Relationship between Ang-2 and Tie-2 Expression and Clinical Pathological Processes**

Clinical Pathology	Cases	Ang-2 expression				Tie-2 expression				
		(+)	(-)	$\chi^2$	P Value	(+)	(-)	$\chi^2$	P Value	
Gender	Male	60	49	11	2.641	0.104	42	18	0.611	0.435
	Female	40	27	13			25	15		
Location	Left	57	40	17	0.044	0.834	40	17	0.002	0.965
	Right	43	31	12			30	13		
Histological type	Papillary adenocarcinoma	10	8	2	0.337	0.561*	7	3	0.009	0.923*
	Tubular adenocarcinoma	73	52	21	0.045	0.831*	50	23	0.001	0.974*
	Other adenocarcinoma	17	13	4	0.189	0.664#	12	5	0.028	0.867#
Level of differentiation	High and moderate	65	46	19	9.89	0.002	44	21	9.085	0.003
	Poor	35	34	1			33	2		
Dukes classification	Stage A and B	80	55	25	9.967	0.003	54	26	6.139	0.013
	Stage C and D	20	20	0			19	1		
Lymphatic metastasis	Without metastasis	60	42	18	9.375	0.002	40	20	9.044	0.003
	With metastasis	40	38	2			37	3		

Note: \*\* indicates comparison between papillary adenocarcinoma and tubular adenocarcinoma; \* comparison between papillary adenocarcinoma and other adenocarcinoma; #comparison between tubular adenocarcinoma and other adenocarcinoma

**Table 3. Relationship between PI3K and AKT Expression and Clinical Pathological Processes**

Clinical Pathology	Cases	PI3K expression				AKT expression				
		(+)	(-)	$\chi^2$	P Value	(+)	(-)	$\chi^2$	P Value	
Gender	Male	60	50	10	0.531	0.466	49	11	0.643	0.423
	Female	40	31	9			30	10		
Location	Left	57	46	11	0.008	0.93	44	13	0.261	0.609
	Right	43	35	8			35	8		
Histological type	Papillary adenocarcinoma	10	8	2	0.004	0.951*	8	2	0.002	0.968*
	Tubular adenocarcinoma	73	59	14	0.021	0.885*	58	15	0.074	0.786*
	Other adenocarcinoma	17	14	3	0.023	0.879#	13	4	0.045	0.831#
Level of differentiation	High and moderate	65	46	19	12.631	0	44	21	14.314	0
	Poor	35	35	0			35	0		
Dukes classification	Stage A and B	80	61	19	5.864	0.015	59	21	6.646	0.01
	Stage C and D	20	20	0			20	0		
Lymphatic metastasis	Without metastasis	60	41	19	15.638	0	39	21	17.722	0
	With metastasis	40	40	0			40	0		

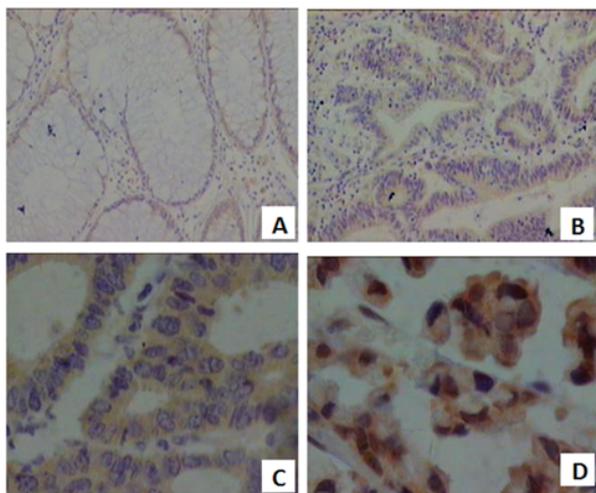
\*comparison between papillary adenocarcinoma and other adenocarcinoma; #comparison between tubular adenocarcinoma and other adenocarcinoma; \*\* indicates comparison between papillary adenocarcinoma and tubular adenocarcinoma

cancer with lymphatic metastasis was significantly higher than that without lymphatic metastasis ( $p < 0.01$ ). See Tables 2-3.

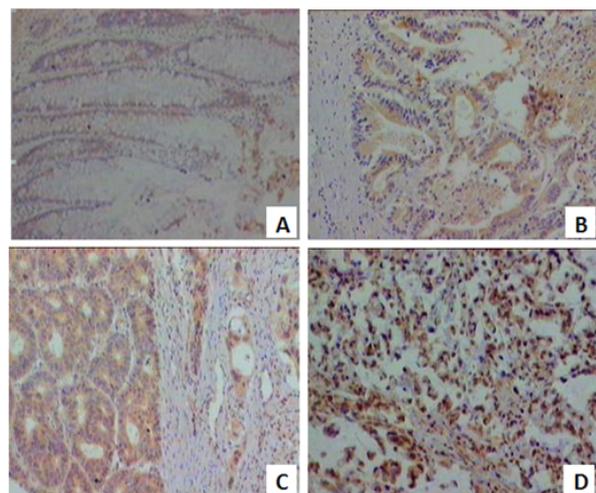
Results of RT-PCR: As can be seen in Figure 1, the mRNA expression of Ang-2, Tie-2, PI3K, and AKT was significantly higher in tumor tissue than in the tumor-adjacent tissue (both  $p < 0.05$ ).

Results of the Western blot: As can be seen in Figure 2, the protein expression of Ang-2, Tie-2, PI3K, and AKT was significantly higher in the tumor tissue than in the tumor-adjacent tissue (both  $p < 0.05$ ).

Correlation analysis of the expression of Ang-2, Tie-



**Figure 1. The mRNA Expression of Ang-2, Tie-2, PI3K and AKT in Colorectal Cancer and Normal Tumor-Adjacent Tissue.** A. RT-PCR showed that the mRNA Expression of Ang-2, Tie-2, PI3K and AKT in colorectal cancer and normal tumor-adjacent tissue. B. The statistical graph on the mRNA Expression of Ang-2, Tie-2, PI3K and AKT in colorectal cancer and normal tumor-adjacent tissue. C: Colorectal cancer, N: Normal tumor-adjacent tissue. \* $p < 0.05$ , \*\* $p < 0.01$  vs N group



**Figure 2. Protein Expression of Ang-2, Tie-2, PI3K, and AKT in Colorectal Cancer and Normal Tumor-Adjacent Tissue.** A. Western-blotting showed that the protein expression of Ang-2, Tie-2, PI3K, and AKT in colorectal cancer and normal tumor-adjacent tissue. B. The statistical graph on the protein expression of Ang-2, Tie-2, PI3K, and AKT in colorectal cancer and normal tumor-adjacent tissue. C: Colorectal Cancer, N: Normal tumor-adjacent tissue. \* $p < 0.05$ , \*\* $p < 0.01$  vs N group

2, PI3K, and AKT in colorectal cancer: In the colorectal cancer tissue, positive expression of Ang-2 and Tie-2, Ang-2 and PI3K, Ang-2 and AKT, Tie-2 and PI3K, Tie-2 and AKT, and PI3K and AKT underwent Spearman correlation analysis. The results suggested positive correlations between the expressed proteins.

## Discussion

In the world, colorectal cancer is one of the most common malignant tumors of the digestive system. Research has suggested that when the tumor volume reaches 2 - 3mm<sup>3</sup>, continued growth depends on angiogenesis inside the tumor. These newly-formed blood vessels not only provide nutrients to the tumor cells, but also serve as pathway for metastasis (Lin et al., 2008). Therefore, the formation of new blood vessels is an important step in the proliferation, infiltration, and metastasis of tumor cells, and it is regulated by multiple factors, including Ang family proteins. Ang family proteins are recently discovered angiogenic factors, a group of proteins closely connected with the formation of blood vessels. Proteins in this family include Ang-1, Ang-2, Ang-3, and Ang-4; however, Ang-2 is the most important member produced by vascular endothelial cells and its receptor is Tie-2.

As a reinforcing factor for tumor angiogenesis, Ang-2 is closely connected with the number and density of blood vessels, and the size, infiltration, and metastatic ability of tumor (Zhou et al., 2008; Pohl et al., 2008; Sun et al., 2011). It was previously confirmed that the expression level of the Ang-2 protein was correlated with the infiltration depth of colorectal cancer in intestinal wall, as well as its metastasis by blood vessels and clinical staging (Wang et al., 2007). In the present study, an immunohistochemical method using paraffin sections was applied, and RT-PCR and Western blotting were performed on freshly-resected colorectal cancer tissue to determine the gene and protein expression levels of Ang-2 and Tie-2. The results were then compared with those from normal tumor-adjacent tissues. We found that the mRNA and protein levels of both Ang-2, Tie-2 were overexpressed in colorectal cancer tissue, and there were significant differences compared with normal tumor-adjacent tissues. These results were consistent with the existing literature, and suggested that the Ang-2/Tie-2 signal transduction pathway plays an important role in the pathogenesis and progression of colorectal cancer (Wang et al., 2007).

When the correlation between the protein expression of Ang-2 and Tie-2 and their clinical pathology in colorectal cancer was examined, the expression of Ang-2 and Tie-2 was associated with the differentiation level of colorectal cancer, stage of Duke's classification, and the presence of lymphatic metastasis. The expression levels of Ang-2 and Tie-2 in poorly differentiated tumors were significantly higher than those in well and moderately differentiated tumors. The expression levels in Stages C and D were significantly higher than those in Stages A and B, and the expression levels in those with lymphatic metastasis were significantly higher than those without lymphatic metastasis. All of the above results were consistent with

existing literature (Wang et al., 2007; Cao et al., 2007; Sarraf-Yazdi et al., 2008). The results suggested that overexpression of Ang-2 could facilitate the metastasis of colorectal cancer and influence its development and prognosis. Therefore, the expression levels of Ang-2 and Tie-2 might be indicators of the malignancy of colorectal cancer and its overall prognosis.

It has been previously reported that Ang-2 and Tie-2 were overexpressed in lung cancer tissues. It is very possible that, as the receptor for Ang-2, Tie-2 (and Ang-2) might participate in the initiation and progression of tumor angiogenesis. Both Ang-2 and Tie-2 can regulate tumor angiogenesis. In this study, the expression distribution of Tie-2 was consistent with Ang-2, and its expression was quite remarkable in the tumor-adjacent tissues, as well as in smooth muscle. It is very likely that they play important roles in the molding of blood vessels. These results were consistent with the results of a previous study (Seval et al., 2008). Most studies on this topic have demonstrated that Ang-2 and Tie-2 had the ability to promote the growth of tumors. In this study both Tie-2 and Ang-2 were more highly expressed in colorectal cancer tissues compared with tumor-adjacent tissues, with a positive correlation between Ang-2 and Tie-2. This suggested that Ang-2 and Tie-2 might play important roles in the progression of colorectal cancer. Therefore, antagonizing Ang-2 and Tie-2 could be a viable treatment option for colorectal cancer.

PI3K is a conservative lipid kinase in signal transduction pathways. When tumor cells are stimulated by growth factors, receptor tyrosine kinases (RTKs) are activated to alter the protein conformation of PI3K, and further alter the protein structure of AKT. AKT translocates to the cellular membrane and is phosphorylated by PDK-1 and PDK-2 on the membrane. Then, a cascade reaction of AKT and its downstream signal is triggered to participate in cellular growth, development, differentiation, and survival. AKT is an important kinase located downstream of PI3K, and both are critical for promoting cellular survival, maintaining normal cellular function, and constituting the signal transduction chain which promotes cellular growth, suppresses cellular apoptosis, and maintains the key functions of cells during the stress reaction to external stimuli.

The PI3K/AKT signal transduction pathway is essential to cellular proliferation and apoptosis. In liver cancer, the phosphorylation of AKT is remarkably intensified; therefore, AKT has become an important target of anti-cancer research (Yap et al., 2008). Studies on breast cancer and prostate cancer suggested that PI3K/AKT could increase the expression of S-phase kinase-associated protein 2 (Skp2) at different levels, transcription, translation, and post-translation, to promote the occurrence of tumors (Gao et al., 2009; Lin et al., 2009; Li et al., 2013). In this study, the expression of PI3K and AKT in colorectal cancer was significantly higher than those in tumor-adjacent tissues, and was closely correlated with the differentiation level of colorectal cancer (Li et al., 2013), Stage of Duke's classification, and presence of metastasis. These results were consistent with the research results of Abubaker (Abubaker et al., 2008) and Johnson (Johnson et al., 2010). It has been suggested that

PI3K/AKT could promote the development of colorectal cancer. By means of genetic intervention to knock out PI3K/AKT-related genes or by means of small molecule drugs to suppress PI3K/AKT-related genes, blocking the activation of downstream multiple anti-apoptosis effector molecules could promote cellular apoptosis to effectively suppress the growth of tumors; it also could increase the sensitivity of tumor cells to radiotherapy and chemotherapy, and improve their effects. This study suggests that the pathogenesis of colorectal cancer was closely related to the hyperactivity of the PI3K/AKT signal transduction pathway. Hence, PI3K/AKT is a promising target for anti-cancer treatment.

Research on Ang-2/Tie-2/PI3K/AKT in the pathogenesis of tumor is critical for further understanding the mechanism of tumor pathogenesis and provides an effective treatment strategy for cancer. Since the gene and protein expression of Ang-2, Tie-2, PI3K, and AKT might play important roles in the progression and metastasis of colorectal cancer, study of their expression patterns and relationships with aggression and metastasis will provide a valuable experimental foundation for the prognosis and targeted therapy of colorectal cancer.

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