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Expression and significance of miR-34 with PI3K, AKT and mTOR proteins in colorectal adenocarcinoma tissues

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ABSTRACT

This research was carried out to investigate the expression of miR-34a, miR-34b and p-PI3K, p-AKT, and mTOR proteins in colorectal adenocarcinoma and corresponding distal cutaneous normal mucosal tissues and their relationship with the clinicopathological parameters of colorectal adenocarcinoma as well as the correlation between miR-34a, miR-34b and PI3K/AKT/mTOR signaling pathway. The expression of p-PI3K, p-AKT, and mTOR proteins in 67 colorectal adenocarcinomas and the corresponding distal cut-off normal mucosa were assayed by immunohistochemistry. Their relationship with clinicopathological parameters and the correlation of the three proteins were evaluated. The expression of miR-34a and miR-34b in colorectal adenocarcinoma and the corresponding distal cutaneous normal mucosa was detected by applying real-time quantitative PCR. The correlation between colorectal adenocarcinoma tissue miR-34a, miR-34b and p-PI3K, p-AKT, and mTOR proteins, respectively, was analyzed. Results showed that the expression of p-PI3K, p-AKT and mTOR proteins in colorectal adenocarcinoma tissues was higher than that in the corresponding distal cutaneous normal mucosa (P=0.000), and there was a positive correlation between the expression of the three proteins in colorectal adenocarcinoma tissues. The expression of p-PI3K and p-AKT protein in colorectal adenocarcinoma tissues were correlated with tumor size, differentiation degree, infiltration degree, lymph node metastasis and TNM stage (P<0.05). The expression of mTOR protein was related to tumor size and differentiation degree (P<0.05). The relative expression of miR-34a and miR-34b in colorectal adenocarcinoma tissues was less than that in the corresponding distal cutaneous normal mucosa (P<0.05), and the expression of miR-34a and miR-34b was positively correlated. The expression of miR-34a and miR-34b in colorectal adenocarcinoma tissues was negatively correlated with the expression of p-PI3K, p-AKT and mTOR proteins. In conclusion, the PI3K/AKT/mTOR signaling pathway may promote colorectal adenocarcinoma and differentially participate in differentiation, infiltration and lymph node metastasis. Also, miR-34a and miR-34b may inhibit colorectal adenocarcinoma. Importantly, miR-34a and miR-34b may affect the development and progression of colorectal adenocarcinoma by regulating PI3K/AKT/mTOR signaling pathway.

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Introduction

Rectal cancer ranks fourth and third in the world in terms of incidence and mortality, with an increasing trend year by year (1). Colorectal carcinogenesis is the result of a multifactorial and multi-step process involving multiple signaling pathways. PI3K/AKT/mTOR signaling pathway plays a central role in cell growth, survival, apoptosis and migration, as well as being closely associated with the development of many malignancies (2). PI3K/AKT/mTOR is activated by upstream signaling molecules and is involved in the regulation of metabolism, protein synthesis, apoptosis, cell cycle and transcription factors, which are key factors in determining and maintaining the oncogenic phenotype (3). Currently, it has been identified as a critical target for a variety of tumor-targeted therapies (4).

MicroRNAs (miRNAs) are small, non-protein-coding RNA molecules that are key regulators of gene expression. Studies have shown that miRNAs play a key role in a variety of cancers. Their deletion, amplification or aberrant expression may affect a range of developmental processes by post-transcriptionally regulating the expression of their downstream target mRNAs (5). miR-34 is a type of miRNA, and its family consists of miR-34a, miR-34b and miR-34c. In the human genome, miR-34a is located on chromosome 1p36.22 and is encoded by a separate transcript. While miR-34b and miR-34c are located together on chromosome 11q23.1 and in the same exon where they are transcribed by the same multiple cis-trans (6). The dysregulation of miR-34 is related to a variety of tumors and diseases, including lung adenocarcinoma, prostate cancer, pancreatic cancer, and osteosarcoma (7,8). However, miR-34 expression in colorectal cancer has opposite results in different studies. miR-34b and miR-34c were reported to be highly expressed by Hiyoshi Y et al (9), while some other studies (10-12) showed low miR-34 expression and

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suppressed cancer progression. Moreover, miR-34a has been reported to regulate the progression of malignant tumors such as gastric cancer and bladder cancer through PI3K/AKT signaling pathway (13,14). However, how miR-34 is expressed in colorectal cancer and whether it can regulate the progression of colorectal cancer through the PI3K/AKT signaling pathway has not been investigated in the literature. Therefore, this study examined the expression of miR-34a, miR-34b and PI3K/AKT/mTOR signaling pathway-related proteins p-PI3K, p-AKT, mTOR in colorectal adenocarcinoma and corresponding distal mucosal tissues to investigate the relationship among miR-34a, miR-34b and PI3K/AKT/mTOR signaling pathway-related proteins with colorectal adenocarcinoma development. Meanwhile, the correlation between miR-34a, miR-34b and PI3K/AKT/mTOR signaling pathway was analyzed to provide experimental data and a theoretical basis for the mechanism of colorectal carcinogenesis and targeted therapy.

Materials and Methods

Sample selection

A total of 67 colorectal adenocarcinoma tissue specimens, 40 males and 27 females, surgically resected from January 2016 to December 2018 at the Clinical Pathology Diagnostic Center of Qiqihar Medical College were selected. The age of onset was >60 years old in 33 cases and ≤60 years old in 34 cases. The diameter of cancer was >6 cm in 21 cases and ≤6 cm in 46 cases. There were 4 cases with high differentiation, 51 cases with middle differentiation and 12 cases with low differentiation. The depth of infiltration was <mylohyoid layer in 16 cases, mylohyoid-plasma layer in 38 cases, and extra-plasma layer in 13 cases. There were 28 cases with lymph node metastasis and 39 cases without lymph node metastasis. According to the WHO TNM staging criteria, there were 38 cases with TNM stage I+II and 29 cases with TNM stage III+IV, and none of them received other treatments such as radiotherapy or chemotherapy before surgery. The control group was normal mucosal tissue corresponding to the distal section end (>5 cm from the cancer margin and no cancer tissue infiltration confirmed by hematoxylin-eosin (HE) staining).

Experimental methods

Immunohistochemical staining

The EGVisionTM Super kit and DAB kit for immunohistochemical staining were purchased from Fuzhou Meixin Biotechnology Co. The specific operation steps were performed according to the instructions. Sections were routinely dewaxed to water and citrate (PH 6.0 ± 0.1) buffered for high-pressure repair. Then, endogenous peroxidase was blocked by dropwise addition of 3% H2O2, rabbit anti-human p-PI3K, p-AKT (both working concentration 1:200) and mTOR antibody (working concentration 1:50)) working solution was added dropwise overnight at 4°C, reaction amplifiers were incubated for 10 min at room temperature, highly sensitive enzyme-labeled mouse/rabbit IgG polymer was added dropwise for 10 min at room temperature, and PBS was rinsed. DAB quilt was for color development for 3-5 min at room temperature, hematoxylin quilt was used for re-staining for 2-3 min, gradient alcohol was employed for dehydration, xylene was used for transparency, and neutral gum was applied to seal the slices for microscopic observation. PBS buffer was used instead of the primary antibody as a negative control and positive slices of gastric cancer were treated as a positive control. The results were determined by double-blind reading by two experienced pathologists. p-PI3K, p-AKT and mTOR positive expressions were located in the cytoplasm with brownish-yellow granules. The percentage of positive cells and the intensity of staining were scored and judged comprehensively under the microscope, respectively. The scoring criteria for the percentage of positive cells were as follows: 5 high magnification fields were observed in each section, and the percentage of positive cells <5% was scored as 0, 5%-25% as 1, 26%-50% as 2, 51%-75% as 3, and 76%-100% as 4. The intensity of cell staining was scored as 0 for colorless, 1 for yellowish, 2 for brownish, and 3 for tan. The composite score was multiplied by the two scores for positive grade: 0-1 was negative (-), 2-4 was weakly positive (+), 5-7 was positive (++), and 8 and above was strongly positive (++++).

Real-time quantitative PCR

The experimental materials were soaked in 1% DEPC water for more than 12 hours and prepared for use after 30 minutes of high pressure. Total RNA was extracted and assayed as follows: Total RNA was extracted according to RNAeasy™ Plus tissue RNA extraction kit (Biyuntian Biotechnology Co., Ltd.). The total RNA concentration and purity were detected by ultra-micro spectrophotometer. The samples met 1.7 <OD260/OD280 <2.0 and OD260/OD230>2. Reverse transcription cDNA acquisition was performed on a PCR instrument. The reverse transcription system was 100μl and the reaction conditions were 37°C for 1 hour and 85°C for 5 minutes. The Real-time PCR reaction system was 25μl. The reaction conditions were 95°C for 10 sec, 95°C for 5 sec, 60°C for 20 sec, with 40 cycles. The primer sequences were Hsa-miR-34a-5p TGGCAGTGTAGCTGGTTGT and Hsa-miR-34b-5p TAGGCAGTGTCACTAGCTGATTG. Three replicate wells were set up each time, and the experiment was repeated three times with U6 as the reference gene. The 2-ΔΔCT method was used to analyze the relative fold changes and calculate the results.

Statistical analysis

The obtained data were analyzed using SPSS 21.0 software and the plots were drawn by GraphPad Prism 8.0. The measurement data that conformed to a normal distribution with uniform variance were expressed as mean ± standard deviation, and one-way ANOVA was used for comparison between multiple groups. The non-parametric test was used for non-normally distributed or count data. The calibration level was α=0.05.

Results

Expression of p-PI3K, p-AKT and mTOR proteins in colorectal adenocarcinoma and normal mucosal tissue at the distal cut end

The expression of p-PI3K, p-AKT and mTOR proteins in colorectal adenocarcinoma and normal mucosal tissues at the distal cut-off were located in the cytoplasm and intercellular stroma, while the nucleus was not colored (Fi-

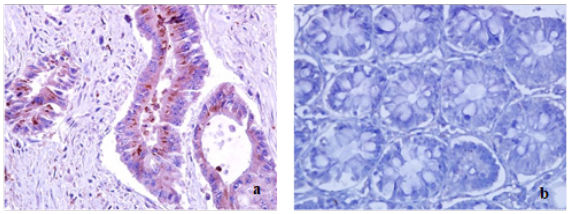

Figure 1. Colorectal adenocarcinoma and distal normal mucosal tissue p-PI3K protein expression (immunohistochemical staining 200x). a Colorectal adenocarcinoma and b Distal normal mucosa.

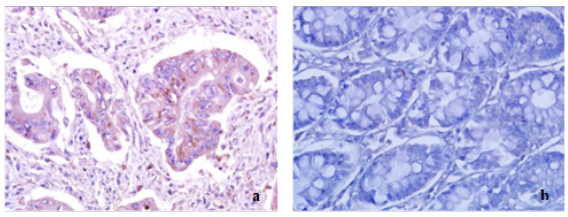

Figure 2. Colorectal adenocarcinoma and distal normal mucosal tissue p-AKT protein expression (immunohistochemical staining 200x). a Colorectal adenocarcinoma and b Distal normal mucosa.

gure 1-3). Colorectal adenocarcinoma tissues had higher p-PI3K, p-AKT and mTOR protein expression than distally cut normal mucosal tissues, and the differences were statistically significant (P < 0.001) (Table 1).

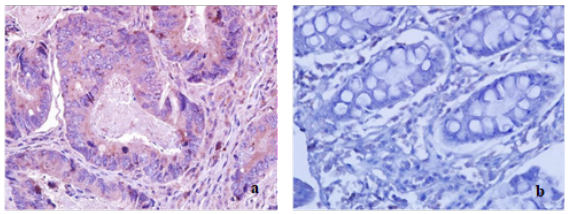

Figure 3. Colorectal adenocarcinoma and distal normal mucosal tissue mTOR protein expression (immunohistochemical staining 200x). a Colorectal adenocarcinoma and b Distal normal mucosa.

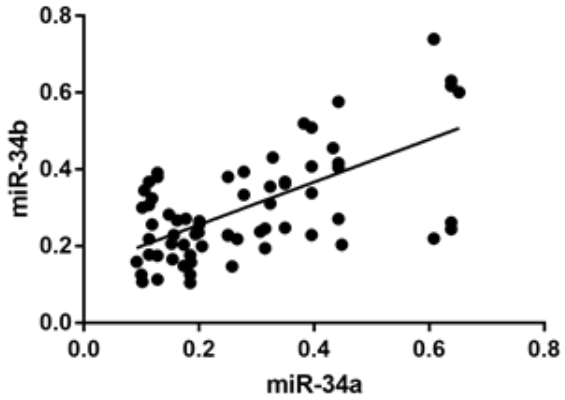

Figure 4. Correlation of miR-34a and miR-34b expression.

Relationship between p-PI3K, p-AKT and mTOR proteins and clinicopathological parameters in colorectal adenocarcinoma tissues

The expression of p-PI3K, p-AKT and mTOR proteins in colorectal adenocarcinoma tissues was independent of patient age and gender. The p-PI3K and p-AKT protein expression showed significant differences in tumor size, degree of differentiation, degree of infiltration, lymph node metastasis, and TNM stage (P<0.05). The expression of mTOR protein was significantly correlated with the degree of differentiation, degree of infiltration, lymph node metastasis, and TNM stage (P<0.05) (Table 2-5).

Expression and correlation of miR-34a and miR-34b in colorectal adenocarcinoma and distantly cut normal mucosa

Colorectal adenocarcinoma tissue miR-34a and miR-34b expression were significantly lower than that of normal mucosa at the distal end, and the differences were statistically significant (miR-34a: Z=-9.989, P<0.0001; miR-34b: Z=-9.984, P<0.0001). Spearman correlation analysis showed that miR-34a was positively correlated with miR-34b (r=0.510, P<0.01).

Correlation of colorectal adenocarcinoma miR-34a and miR-34b with p-PI3K, p-AKT and mTOR proteins

Based on the above experimental results of colorectal adenocarcinoma, the data were analyzed using SPSS21.0 statistical software. miR-34a, miR-34b and the three protein correlations were analyzed by two-by-two rank correlation. The results showed that miR-34a and miR-34b were negatively correlated with the three proteins (P<0.05).

Discussion

Current studies have shown that the PI3K/AKT/mTOR signaling pathway is involved in the regulation of multiple biological functions and is also critical for tumorigenesis

Table 1. Expression of p-PI3K, p-AKT and mTOR proteins in colorectal adenocarcinoma and normal mucosal tissues of distal cut-off.

Variables	Grade	Colorectal adenocarcinoma (%)	Normal mucosa at distal cut-off (%)	*Z*	*P*
p-PI3K	-	3 (4.5)	59 (88.1)	-9.987	0.000
	+	7 (10.4)	8 (11.9)		
	++	24 (35.8)	0 (0.0)		
	+++	33 (49.3)	0 (0.0)		
p-AKT	-	3 (4.5)	57 (85.1)	-9.664	0.000
	+	10 (14.9)	8 (11.9)		
	++	22 (32.8)	2 (3.0)		
	+++	32 (47.8)	0 (0.0)		
mTOR	-	2 (3.0)	54 (80.6)	-9.966	0.000
	+	6 (9.0)	13 (19.4)		
	++	24 (35.8)	0 (3.0)		
	+++	35 (52.2)	0 (0.0)		

Table 2. Relationship between p-PI3K protein expression and clinicopathological parameters in colorectal adenocarcinoma tissues.

Clinicopathological parameters		N	-	+	++	+++	X^2	*P value*
Age	> 60	33	3	3	14	13	2.683	0.108
	≤60	34	0	4	10	20		
Sex	Male	40	2	4	15	19	0.093	0.761
	Female	27	1	3	9	14		
Tumor diameter	> 6cm	21	0	0	6	15	7.582	0.006
	≤6cm	46	3	7	18	18		
Degree of differentiation	High	4	2	1	1	0	10.443	0.005
	Medium	51	1	6	19	25		
	Low	12	0	0	4	8		
Depth of infiltration	<mylohyoid layer	16	2	3	7	4	11.441	0.003
	myxomatous-plasma layer	38	1	4	15	18		
	extra-plasma membrane	13	0	0	2	11		
Lymph node metastasis	Yes	28	1	2	6	19	5.472	0.019
	No	39	2	5	16	14		
TNM stage	I+II	38	2	6	19	11	12.739	0.000
	III+IV	29	1	1	5	22		

Table 3. Relationship between p-AKT protein expression and clinicopathological parameters in colorectal adenocarcinoma tissues.

Clinicopathological parameters		N	-	+	++	+++	X^2	*P value*
Age	> 60	33	2	6	12	13	1.977	0.160
	≤60	34	1	4	10	19		
Sex	Male	40	3	8	12	17	2.602	0.107
	Female	27	0	2	10	15		
Tumor diameter	>6cm	21	0	1	5	15	7.696	0.006
	≤6cm	46	3	9	17	17		
Degree of differentiation	High	4	1	3	0	0	10.091	0.006
	Medium	51	0	7	20	24		
	Low	12	2	0	2	8		
Depth of infiltration	<mylohyoid layer	16	3	4	7	2	14.550	0.001
	myxomatous-plasma layer	38	0	6	11	21		
	extra-plasma membrane	13	0	0	4	9		
Lymph node metastasis	Yes	28	0	3	4	21	12.135	0.000
	No	35	3	7	18	11		
TNM stage	I+II	38	3	8	18	9	18.463	0.000
	III+IV	29	0	2	4	23		

Table 4. Relationship between mTOR protein expression and clinicopathological parameters in colorectal adenocarcinoma tissues.

Clinicopathological parameters		N	-	+	++	+++	X^2	*P value*
Age	> 60	33	1	5	11	16	0.884	0.347
	≤60	34	1	1	13	19		
Sex	Male	40	2	1	17	20	0.002	0.996
	Female	27	0	5	7	15		
Tumor diameter	> 6cm	21	0	1	8	12	0.694	0.405
	≤6cm	46	2	5	16	23		
Degree of differentiation	High	4	1	2	1	0	10.150	0.006
	Medium	51	1	4	19	27		
	Low	12	0	0	4	8		
Depth of infiltration	<mylohyoid layer	16	2	4	8	2	17.348	0.000
	myxomatous-plasma layer	38	0	2	11	25		
	extra-plasma membrane	13	0	0	5	8		
Lymph node metastasis	Yes	28	0	0	10	18	4.665	0.031
	No	39	2	6	14	17		
TNM stage	I+II	38	2	5	15	16	6.714	0.010
	III+IV	29	0	1	9	19		

Table 5. Correlation of miR-34a, miR-34b with p-PI3K/p-AKT/mTOR.

	p-PI3K	p-AKT	mTOR
miR-34a	- 0.492**	- 0.638**	- 0.469**
miR-34b	- 0.498**	- 0.562**	- 0.418**

** P<0.05

and development (2). PI3K is divided into three classes based on its different structures and lipid substrates. Class I PI3K is a heterodimer of 110 kDa catalytic (p110) and regulatory subunits (p85), including four p110 isoforms p110α, p110β, p110γ, and p110δ and seven regulatory subunits p85α, p85β, p55α, p55γ, p50α, p101, and p87 (15). Activation of PI3K leads to the production of a second messenger PIP in the cell membrane. PIP3 binds to AKT, a signaling protein with a PH structural domain, and to phosphatidylinositol-dependent kinase 1 (PDK1), thus promoting PDK1 phosphorylation of the thr308 site of AKT proteins, or it can be fully phosphorylated by PDK2 (e.g. integral protein) on Ser473 to activation (16). Activated AKT phosphorylates mTORC1 on Ser2,448 to activate its kinase activity, activating multiple anabolic biosynthetic pathways that control cell proliferation (17). Increased PI3K/AKT has been shown to play a crucial role in the development and progression of hepatocellular carcinoma (18), renal tumors (19), nasopharyngeal carcinoma (20), glioblastoma (21), breast cancer (22), osteosarcoma (23), breast cancer, and colorectal cancer (24), and is closely associated with tumor size, degree of differentiation, depth of infiltration, and lymph node metastasis. The results of this experiment showed that the expression of p-PI3K, p-AKT and mTOR proteins in colorectal adenocarcinoma tissues was higher than that in the distal cut-off normal mucosa, which is consistent with previous studies (24), suggesting that the PI3K/AKT/mTOR signaling pathway is involved in colorectal adenocarcinoma development. The results of this experiment also showed that the three protein expressions did not differ significantly with patient age and gender, and showed significant differences in the degree of tumor differentiation. The p-PI3K protein expression was correlated with tumor TNM stage and lymph node metastasis. p-AKT protein expression was correlated with tumor TNM stage, lymph node metastasis and tumor diameter size. mTOR protein expression was associated with diameter tumor size. It is suggested that p-PI3K, p-AKT and mTOR proteins are involved in the differentiation, infiltration and metastasis of colorectal adenocarcinoma to different degrees.

miRNAs are a class of highly conserved small single-stranded non-coding RNAs that bind to the 3'-untranslated region (UTR) of messenger RNAs to inhibit mRNA translation or induce mRNA degradation, thereby silencing gene expression at the post-transcriptional level and control the expression of more than 30% of human structural genes (25). In addition, miRNA regulates gene expression by translationally repressing or destabilizing mRNA. In rare cases, it also causes translational activation. miRNAs can regulate the expression of target genes and thus regulate many important biological processes, such as cell proliferation, metastasis, apoptosis, senescence, differentiation, autophagy and immune response (26,27). The three members of the miR-34 family are encoded by two different transcription units, and a comparison of the sequences of these three members revealed that miR-34a is highly homologous to miR-34b and miR-34c (6). Most current studies have shown that miR-34 is significantly down-regulated in tissues and cells of cervical, breast, pancreatic, thyroid, nasopharyngeal, gastric, ovarian, and bladder cancers and is very closely related to tumor size, degree of differentiation, invasion, and migration. In addition, there is also some literature suggesting that miR-34 not only also influence patient resistance to chemotherapeutic agents and prognosis in the clinical setting, but also may serve as a clinical diagnostic indicator for some cancers (28,29). However, miR-34 expression in colorectal cancer studies had opposite results (9-12). The results of this study showed that miR-34a and miR-34b expression was significantly down-regulated in colorectal cancer tissues, which is consistent with the majority of studies, suggesting that miR-34 may inhibit the development of colorectal adenocarcinoma. The correlation of miR-34a and miR-34b expression in colorectal adenocarcinoma tissues were analyzed, and the results showed a positive correlation, suggesting that miR-34a and miR-34b expression in colorectal adenocarcinoma tissues are homogeneous and jointly involved in the development of colorectal adenocarcinoma.

Current studies have confirmed that miR-34 intervenes in tumorigenesis, invasion and metastasis through the regulation of multiple signaling pathways. These include signaling pathways such as wnt (30), EMT (31), TGF-β (32) and PI3K/AKT (3). Downregulation of miR-34a can lead to loss of inhibition of the PI3K/AKT/mTOR signaling pathway, which can cause tumorigenesis and affect tumor proliferation and invasion (10). In this study, we analyzed the correlation between miR-34a, miR-34b and three proteins. The results showed that miR-34a and miR-34b were significantly and negatively correlated with the three proteins. It is suggested that miR-34a and miR-34b may be involved in the development and progression of colorectal adenocarcinoma through the regulation of the PI3K /AKT/mTOR signaling pathway. miR-34 regulates multiple signaling pathways and is also regulated by upstream genes, such as P53, lncRNA, etc. Subsequent experiments can look for these genes, signaling pathways and direct targets of miR-34 in the database to further determine the regulatory mechanism of miR-34 in colorectal cancer.

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