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The correlation between miR -34a-3p, miR -31, PLEK2 and the occurrence, development and prognosis of colorectal cancer

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ABSTRACT

The current study aimed to explore the correlation between Mir-34A-3p, Mir-31, PLEK2 and the occurrence, development and prognosis of colorectal cancer. For this paper, 120 patients with colorectal cancer were selected as the study group, and their adjacent normal tissues were selected as the control group. The quantitative real-time PCR (QRT-PCR) method was used to detect miR-34a-3p and miR-31 in tissues, and the immunohistochemistry EnVision two-step method was used to detect PLEK2 positive expression. The expressions of miR -34a-3p, miR -31, and PLEK2 in colon cancer tissues and normal cancer tissues were compared, and the correlation between miR -34a-3p, miR -31, and PLEK2 and clinic-pathological characteristics of colorectal cancer patients were analyzed. The results showed that expression of miR -34a-3p, miR -31 and positive expression rate of PLEK2 in colorectal cancer tissues were higher than those in normal adjacent tissues ($P<0.05$). The expression of miR -34a-3p was related to tumor size, degree of tissue differentiation, lymph node metastasis and TNM stage ($P < 0.05$). The 3-year survival rate of miR -34a-3p with low expression was lower than miR -34a-3p with high expression, which was a protective factor affecting the poor prognosis of colorectal cancer ($P < 0.05$). The expression of miR -31 was related to tumor size and TNM stage. The 3-year survival rate of the group with high expression of miR -31 was lower than the group with low expression of miR -31, which was a risk factor affecting the poor prognosis of colorectal cancer ($P < 0.05$). PLEK2 positive expression was associated with lymph node metastasis, and the 3-year survival rate of the PLEK2 positive group was lower than the PLEK2 low expression group, which was a risk factor for poor prognosis of colorectal cancer ($P < 0.05$). In general, miR -34a-3p, miR -31, and PLEK2 are closely associated with the occurrence and development of colorectal cancer, and they are all influential factors affecting the prognosis of patients with colorectal cancer, which can provide a basis for the evaluation and treatment of patients, and are worthy of widespread clinical application.

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Introduction

Colorectal cancer (CRC) is a malignant tumor of the digestive system. Its incidence is one of the top three cancers globally, and its fatality rate is the fourth. Due to the change of diet structure and improved living standards, China's morbidity and mortality are increasing (1). Colorectal cancer is caused by dietary structure, living habits, and heredity. There are no specific symptoms in the early stage. Still, with the proliferation and differentiation of cancer cells, patients mainly present with changes in stool traits, abdominal pain accompanied by mass, and systemic involvement of anemia and fatigue in the late stage, which seriously affect life health and quality (2). The clinical treatment effect is not significant, mainly due to the problematic early diagnosis of the disease, uncomplicated metastasis of cancer tissue, high postoperative recurrence rate. The mechanism of occurrence and development mentioned above is still unclear, so it is particularly critical to find biological indicators related to THE development, diagnosis, and metastasis of CRC (3, 4).

It has been found that biomarkers are essential factors in the pathogenesis, development, and prognosis of colorectal cancer. MicroRNAs (miRNAs) are single-stranded RNA regulators in eukaryotic cells and are endogenous non-coding small molecules composed of 18 to 25 bases. It is rich in content and can be degraded or inhibited by the formation of messenger RNA after the tissue interaction with related proteins, participating in the physiological or pathological process of the body (5).

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Relevant studies have shown that miRNA is closely related to the occurrence, proliferation, differentiation, and metastasis of cancer tissues, and it is abnormally expressed in the body (6, 7). Therefore, miRNA can be used as biomarkers for clinical diagnosis, treatment, and prognosis of colorectal cancer. Mir-34a-3p promotes cancer development through different target cell pathways associated with the predicted survival of colorectal cancer patients and can effectively predict cancer proliferation and metastasis. There is an abnormal expression of Mir-31 in colorectal cancer, and the specific correlation remains controversial. Leukocyte C-kinase substrate 2(PLeckstrin-2, PLEK2) is abnormally expressed by participating in abnormal cell apoptosis, but the mechanism is still unclear (8, 9). This study analyzed the differential expressions of Mir-34A-3p, Mir-31, and PLEK2 in colorectal cancer tissues and normal tissues to explore their correlation in cancer occurrence, development, and prognosis.

Materials and methods

General Information

A total of 120 patients with colorectal cancer who underwent surgical resection in our hospital were selected as the research subjects from October 2018 to October 2021. The tumor specimens were taken as the colorectal cancer group, and the normal tissues more than 5cm away from the tumor edge were taken as the control group. All isolated tissue specimens were washed with appropriate normal saline (0.9%) and immediately placed in liquid nitrogen and stored at -80℃. The samples were diagnosed and confirmed by two qualified pathologists. This study was approved by the Medical Ethics Committee of our hospital, and all patients understood the study content and voluntarily signed informed consent.

Inclusion and exclusion criteria

Inclusion criteria were (i): The diagnostic criteria were based on colorectal cancer in Chinese Society of Clinical Oncology (CSCO) Guidelines for the Diagnosis and Treatment of Colorectal cancer 2020 edition (10). (ii): Colorectal cancer was diagnosed by digital rectal examination, colonoscopy and other imaging examinations. (iii): The pathological criteria of TNM staging was based on the NATIONAL Comprehensive Cancer Network guidelines (11). (iv): Preoperative radiotherapy, chemotherapy and immunotherapy were not received. (v): All patients understood the study content and voluntarily signed informed consent.

Exclusion criteria were (i): Patients with serious injury of heart, liver, kidney and other functions. (ii): Differentiation and distant metastasis of other malignant tumors. (iii): Non-primary tumors. (iv): Abdominal cavity and digestive system inflammation. (v): Estimated survival time < 1 month. (vi): Incomplete Clinical data.

Research Methods

Basic information, age, pathological classification, tumor diameter, TNM stage, degree of differentiation, lymph node metastasis and other related data of patients were collected.

Cell culture and transfection

Tissue samples were cultured in a medium containing 10% fetal bovine serum, 1×105 U/L penicillin and 100mg/L streptomycin at 37℃ in 5% CO_2 incubator. For cell transfection, 2.5×10^5 cells were inoculated into 6-well plates. The Mir-34A-3p and Mir-31 overexpressed no-load viruses were infected according to MOI value, and then Lipofectamine TM 3000 was added. After three to four days of infection, the transfection situation was observed. When the transfection rate reached over 90%, cells were collected and expanded for culture.

Total RNA extraction

Trizol reagent (Invitrogen, USA) was used to extract total RNA from colorectal cancer tissues, normal tissues and cultured tissues. The UV spectrophotometer (Qingdao Jingcheng Instrument Co., LTD., Model UV1901) was used to determine the concentration, purity and absorbance of RNA, A260/A280 >1.8.

Detection of Mir-34A-3p, Mir-31 and PLEK2 content

Quantitative real-time polymerase chain reaction (QRT-PCR) was used to detect Mir-34A-3p and Mir-31 in tissues. Total RNA was extracted for reverse transcription and gene amplification by RT-PCR, and the product was analyzed by 1% agarose gel electrophoresis. The primer sequences U6 snRNA (as

an internal reference gene), of Mir-34A-3p, Mir-31, and PDCD4 are listed in Table 1. PCR reaction conditions were 95℃ for 30 s, 60℃ for 30 s, 72℃ for 60 s, a total of 30 cycles. The relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method.

Table1. Primer Sequences of U6 snRNA (as an internal reference gene), Mir-34A-3p, Mir-31, and PDCD4

Primers		Sequences
U6 snRNA	Forward	5'-TCGCTTCGGCAGCACa-3'
	Reverse	5'-AACGCTTCACGAATTTGCG-3'
Mir-34A-3p	Forward	5'- CTCGCCGCGCTCTACCTACCTA- 3'
	Reverse	5'-ATGAGCCATTCGCAGTTTCACTGTA-3'
Mir-31	Forward	5'-ACGCG-GCAAGatGCTGGCA-3'
	Reverse	5'-CAGTGCT-GGGTCCGAGtGA-3'
PDCD4	Forward	5'-CAGTTGGTGGGCCAGTTTATTG-3'
	Reverse	5'-AGAAGCACGGTAGCCTTATCCA-3'

Immunohistochemistry EnVision two-step method was used to detect PLEK2 positive expression: After dewaxing and hydration, the slices were washed with PBS (pH 7.5) three times. The washed slices were placed into high-temperature plastic slices and placed into a boiling buffer solution. After microwaving for 8min, the slices were cooled naturally. After removing PBS, relevant antibodies were dropped and incubated for 2h. The HRP-complex was added and incubated for 20min. Then, DAB solution was dropped and observed in OLYMPUS optical microscope for 5min. The positive expression of PLEK2 was calculated by observing and photographing under an optical microscope.

The transfected cells were inoculated into a 96-well plate with 5×10^3 cells/well, placed in 100μl cell suspension, cultured in a 37 °C 5% CO_2 incubator for 24h, 10μl of the substance was added, and incubated for 12h, then 10μl CCK-8 solution was added to each well. After continued culture for 2 hours, the absorbance at 450mm was measured with a microplate reader. Six multiple wells were set for each group, and the average value was repeated three times. The cell growth curve was plotted with time as the horizontal axis and absorbance at 450mm as the vertical axis.

Western Blot detection of tissue protein expression

0.25% trypsin and 5μl cell protein lysis solution were added to each tissue and the proteins were isolated by electrophoresis. The proteins were then sealed by membrane transfer and skim milk powder. The diluted specific protein primary antibody was added and incubated overnight at 4 °C. The diluted HRP labeled specific protein secondary antibody was added and incubated. The exposure and development of film scanning were adopted. Histoprotein expression was reflected by gray value.

Detection of cell apoptosis and cycle by flow cytometry

The cells were washed with cold PBS 48h after transfection and centrifuged for 10min before the sublayers were taken. Cells were suspended with 200μl Annexin V-FITC binding buffer and Annexin ⅴ-FITC 105 μl were mixed evenly. Then, 5μl propidium iodide staining solution was added and incubated for 15min at room temperature, and a binding buffer was added. Cell apoptosis was detected by FACSCalibur flow cytometry after the mixture was evenly mixed. The cells were collected and washed with cold PBS, and the concentration was adjusted to 1×10^6 /ml. Single cells were centrifuged for 10min to obtain the lower layer. Then, 100μl RNase A (25 mg/ml) and 300μl PI (50mg/ml) were added successively after the ethanol was washed away by PBS. The cell cycle was detected by FACSCalibur flow cytometry after re-suspending at 4 °C for 30min.

Observation Indicators

The levels of Mir-34A-3p, Mir-31 and PLEK2 in colon cancer tissues and normal cancer tissues were statistically analyzed. Kaplan-Meier method was used to analyze the correlation between the expression levels of Mir-34A-3p, Mir-31 and PLEK2 in colorectal cancer tissues and the 3-year survival rate of patients. Multivariate Cox regression was used to analyze the prognostic factors of colorectal cancer: tumor size, degree of tissue differentiation, lymph node metastasis, TNM stage.

Expression criteria of Mir-34A-3p, Mir-31 and PLEK2

The expression standard of each tissue level (12) combined with the ratio of negative and positive cells of cancer cells in the section, the results were divided into no expression (-): all tumor cells were negative without positive signal; Low expression (+): most tumor cells were negative, positive signal < 25%; Medium expression (++): the ratio of negative and positive cells of tumor cells was similar, 25% < positive signal < 50%; High expression (+++): most tumor cells were positive, positive signal > 50%.

Statistical Treatment

SPSS 24.0 statistical software was used. The measurement data conforming to normal distribution were expressed as ± S, and t-test was used for comparison between groups. The statistical data were expressed as the number of cases (n) and percentage (%), and the comparison between groups was performed by $\chi 2$ test, P<0.05 indicated a statistically significant difference. Kaplan-Meier method was used to analyze the correlation between the expression levels of Mir-34A-3p, Mir-31 and PLEK2 in colorectal cancer tissues and the survival rate of patients. Multivariate Cox regression was used to analyze the prognostic factors of colorectal cancer, and P<0.05 indicated a statistically significant difference.

Results and discussion

Levels of Mir-34A-3p, Mir-31 and PLEK2 in different tissues

The results showed that the expression rates of Mir-34A-3p, Mir-31 and PLEK2 in colorectal cancer tissues were higher than those in normal adjacent tissues. The difference was statistically significant (P<0.05), as shown in Table 2.

Table 2. Mir-34A-3p, Mir-31 and PLEK2 levels in different tissues (±s)

Group	Tissue adjacent to carcinoma	Colon cancer tissue	T	P
miR-34a-3p	0.59±2.05	2.38±1.93	0.192	0.004
miR-31	0.69±0.24	1.86±0.72	-0.134	0.036
PLEK2	0.03±0.00	0.07±0.01	0.526	0.017

Note: Compared with the control group before treatment, *#P<0.05.

Relationship between the relative expression levels of Mir-34A-3p, Mir-31 and PLEK2 in colon cancer tissues and pathological factors

The results showed that the expression of Mir-34A-3p had no statistical significance with gender, age and pathological classification (P > 0.05), but had statistical significance with tumor size, degree of tissue differentiation, lymph node metastasis and TNM stage (P < 0.05). There was no significant difference in Mir-31 expression with gender, age, pathological classification, degree of tissue differentiation and lymph node metastasis (P > 0.05). There were significant differences in tumor size and TNM stage (P < 0.05). PLEK2 positive expression had no statistical significance with gender, age, pathological classification, tumor size, TNM stage and degree of tissue differentiation (P > 0.05), but was related to lymph node metastasis (P < 0.05), as shown in Table 3.

Kaplan-Meier method for analyzing the relationship between Mir-34A-3p, Mir-31, PLEK2 and 3-year survival rate

Kaplan-Meier analysis showed that the survival rate of patients with colorectal cancer within three years after surgery was closely related to the expression levels of Mir-34A-3p, Mir-31 and PLEK2 in colorectal cancer. The three-year survival/mortality was used as the evaluation index and the prognostic model was established. The results showed that the three-year survival rate of the low expression group of Mir-34A-3p was lower than that of the high expression group, and the three-year survival rate of the high expression group of Mir-31 and the positive high expression group of PLEK2 was lower than that of the low expression group. The difference was statistically significant (P < 0.05), as shown in Table 4.

Multivariate Cox regression model for analyzing prognostic risk factors of colorectal cancer

The results showed that tumor size, degree of tissue differentiation, lymph node metastasis and TNM stage were the prognostic risk factors of colorectal cancer (P<0.05) by multivariate Cox regression model for statistically significant indexes in general data analysis, as shown in Table 5.

Colorectal cancer is one of the common clinical malignant tumors. Its morbidity and mortality increase year by year, seriously threatening people's lives and health. The disease has no typical symptoms in the early stage, but with the development of the disease, the body's immune system and gastrointestinal function are impaired. The accidental diagnosis has been found in the late stage.

Table 3. Relationship between the relative expression levels of Mir-34A-3p, Mir-31 and PLEK2 and pathological factors (±s)

General information		Number of cases	miR -34a -3p			miR-31			PLEK2		
			Relative expression value	T	P	Relative expression value	T	P	Relative expression value	T	P
Gender	Male	67	2.05±0.43	0.513	0.125	8.32±2.04	0.412	0.063	0.08±0.02	0.116	0.203
	Female	53	0.69±0.24			7.04±1.97			0.07±0.01		
Age (years old)	＜50	34	1.32±0.55	0.162	0.107	7.25±2.13	0.136	0.064	0.05±0.00	0.394	0.134
	≥50	86	1.45±0.60			7.64±2.38			0.06±0.01		
Pathological classification	Colon	69	1.73±0.61	0.305	0.051	7.31±1.56	0.150	0.059	0.07±0.02	1.276	0.062
	Rectum	51	0.97±0.34			7.09±1.24			0.06±0.01		
Tumor size (cm)	＜3.5	58	0.15±0.03	0.337	0.032	7.44±1.55	0.721	0.025	0.05±0.02	0.338	0.097
	≥3.5	62	2.13±0.73			7.87±1.82			0.08±0.02		
TNM staging	Ⅰ + Ⅱ	56	1.07±0.20	0.419	0.013	6.98±1.49	0.139	0.009	0.02±0.00	0.942	0.067
	Ⅲ	34	1.31±0.26			7.33±1.52			0.04±0.01		
	Ⅳ	30	2.28±0.69			8.37±1.96			0.08±0.02		
Degree of differentiation	Low Middle High	36 41 43	0.35±0.15 1.64±0.43 2.43±0.74	1.021	0.005	7.09±1.50 7.57±1.63 7.99±1.85	0.108	0.131	0.02±0.00 0.05±0.01 0.07±0.01	1.076	0.053
Lymph node metastasis	Yes	53	2.13±0.58	0.131	0.027	8.62±2.07	0.437	0.074	0.08±0.01	0.765	0.040
	No	67	0.20±0.04			6.64±1.53			0.04±0.00		

Table note: Compared with the same group, *P<0.05

Table 4. Relationship between different expressions of Mir-34A-3p, Mir-31, PLEK2 and 3-year survival rate

Group	miR -34a -3p					miR-31					PLEK2				
	Low	Middle	High	F	P	Low	Middle	High	F	P	Low	Middle	High	F	P
Number of cases	46	43	31	-	-	52	38	30	-	-	49	45	26	-	-
Survival	25	27	22	-	-	37	25	18	-	-	37	32	16	-	-
Death	21	16	9	-	-	15	13	12	-	-	12	13	10	-	-
Survival rate (%)	54.35	62.79	70.97	0.513	0.008	71.15	65.79	60.00	0.397	0.033	75.51	71.11	61.54	0.507	0.016

Table 5. Cox regression model analysis of prognosis of colorectal cancer

Related factors	*β*	*S.E.*	*Wald* χ^2	*P*	*RR*	*95% CI*
Tumor size	5.230	1.020	0.617	0.046	0.872	0.549~9.642
Degree of tissue differentiation	2.064	0.032	0.284	0.007	0.615	0.375~1.137
Lymph node metastasis	8.261	106.324	5.263	0.015	349.235	0.156~1.520
TNM staging	-6.347	98.641	3.105	0.034	0.067	0.364~9.523

In addition, the condition is prone to distant metastasis, missing the best opportunity for surgical treatment. Therefore, it is necessary to discover and clarify the markers and mechanisms of cancer cell occurrence and development (13). Mir-34a-3p, Mir-31 and PLEK2 are key factors in the development of cancer cells. Among them, the expression of Mir-34a-3p is lost and decreased in pancreatic cancer, breast cancer and other diseases, and there are few studies on colorectal cancer. Mir-31 is generally considered to be abnormally expressed in colorectal cancer and is correlated with the expression level of tumor tissues. Clinical studies of PLEK2 are limited, and its mechanism of action in colorectal cancer is still unclear (14-16). In this study, the levels of Mir-34A-3p, Mir-31 and PLEK2 in colorectal cancer tissues and normal cancer tissues were statistically analyzed to explore their correlation in the occurrence, development and prognosis of colorectal cancer.

MiRNA is a non-coding molecule in eukaryotes. When combined with corresponding target genes, miRNA interferes with gene degradation and translation, thus affecting the growth, development and prognosis of tumor cells. Wang *et al*. (17) observed the expression of Mir-34A in the proliferation, apoptosis and cycle of human colon cancer cells, and the expression of Mir-34A in colorectal cancer tissues was significantly higher than that in the control group. Yang *et al*. (18) detected the expression of Mir-31 in colorectal cancer and normal para-cancer tissues by fluorescence reduction hybridization and RT-PCR, and data showed that the expression level of Mir-31 in colorectal cancer was significantly higher than that in normal para-cancer tissues. The results of this study showed that the expression rates of Mir-34A-3p, Mir-31 and PLEK2 in colorectal cancer tissues were higher than those in normal adjacent tissues. This is consistent with the results of Wang *et al*. and Yang *et al*., indicating that

the expression of Mir-34A-3p, Mir-31 and PLEK2 were significantly increased in the process of cancer occurrence and development. Mir-34a-3p, Mir-31 and PLEK2 are tumor regulatory factors, which may regulate KLF4, E2F2, MMP-9 and other channels to participate in tumor growth, apoptosis and cycle, and may be factors influencing the poor prognosis of colorectal cancer.

As tumor-related factors, miRNA may play an important role in inhibiting tumor genes and their genesis, development and metastasis. Jun *et al*. (19) explored the horizontal expression of Mir-34a methylation in colorectal cancer, and the results showed that mir-34a promoter methylation was correlated with tumor tissue occurrence and pathogenesis. Siemens *et al*. (20) explored the correlation between methylation in the mir-34a promoter region and pathological features in colorectal cancer, and the results were closely related to lymph node metastasis and the TNM stage. The results of this study showed that mir-34A-3p expression was related to tumor size, degree of tissue differentiation, lymph node metastasis and TNM stage. The 3-year survival rate of mir-34A-3p with low expression was lower than that of Mir-34A-3p with high expression, which was a protective factor affecting the poor prognosis of colorectal cancer. Similar to the results of Jun *et al*. and Siemens *et al*., mir-34A-3p expression is related to the occurrence, development and prognosis of colorectal cancer. Mir-34a exists on chromosome 1Q36.22 and is involved in the genesis, proliferation, migration and metastasis of colorectal cancer cells. Mir-34a-3p is widely expressed outside lung tissues with tissue specificity, mainly by regulating p53 and HT-29. It may indirectly affect tumor cell growth, metastasis, proliferation, and prognosis and survival rate by

inhibiting the expression of YAP1 signal in colon cancer, which can be used as a predictor of poor prognosis of colorectal cancer.

Eneh *et al*. (21) explored the role of Mir-31-5p in the proliferation, cycle, migration and invasion of colorectal cancer cells by targeting NUMB, which can be used as an influencing factor to regulate the occurrence, development and prognosis of colorectal cancer cells. Tian *et al*. (22) studied that mir-31 levels in inflammation and colorectal cancer tissues were significantly higher than those in normal tissues. In addition, Mir-31 can promote the regeneration of colon epithelial tissue and inhibit the occurrence and development of tumor tissue by regulating the expression of inflammatory receptors and proteins. The results of this study showed that mir-31 expression was related to tumor size and TNM stage, and the 3-year survival rate of the group with high Mir-31 expression was lower than that of the group with low Mir-31 expression, which was a risk factor affecting the poor prognosis of colorectal cancer. Consistent with the results of Eneh *et al*. and Tian *et al*., mir-31 is correlated with the occurrence, development, prognosis and survival rate of colorectal cancer, and is an independent predictor of cancer. The abnormal expression of Mir-31 in colorectal cancer tissues varies with tumor tissue size and stage, indicating that Mir-31 is related to the growth and proliferation of colorectal cancer. Its mechanism of action may be through the RAS signaling pathway to inhibit tumor gene translation, leading to the growth of colorectal cancer cells, etc., in addition, it can enhance the activity of tissue cells, promote their growth and cycle, etc. It may promote tissue development and prognosis of colorectal cancer by down-regulating RhoBTB1 protein expression, suggesting that Mir-31 can be used as a biomarker for the diagnosis of colorectal cancer.

Biological factors can regulate the stage, differentiation and metastasis of malignant tumor cells through different pathways, and thus are related to tumor prognosis and survival rate. Wang *et al*. (23) controlled different doses of atherosclerotic mice and found that the loss of PLEK2 would reduce the activity of protein kinase, participate in the division and apoptosis of tissue cell cycle, and indirectly affect the occurrence and development of tumor cells. Shen *et al*. (24) data showed that PLEK2 positive expression was significantly higher in gallbladder cancer tumors than in adjacent tissues, which was correlated with tumor lymph node metastasis and was an independent risk factor for survival of patients. The results of this study showed that positive PLEK2 expression was associated with lymph node metastasis, and the 3-year survival rate of the PLEK2 positive group was lower than that of the low PLEK2 expression group, which was a risk factor for poor prognosis of colorectal cancer. It is consistent with the study of Wang *et al*. and Shen *et al*., indicating that PLEK2 positive expression level can evaluate tumor development, prognosis and survival rate, and can be used as an independent risk factor for colorectal cancer. The positive expression of PLEK2 in colorectal cancer was significantly higher than that in adjacent tissues, and in patients with lymphatic metastasis was significantly higher than that in patients without metastasis, suggesting that PLEK2 is involved in the development and metastasis of colorectal cancer. Its promoting effect on tumor metastasis may be achieved through the Wnt pathway in colorectal epithelial tissue, and its level of expression is correlated with the 3-year survival rate, which can be used as a tumor marker or prognostic factor for colorectal cancer.

In conclusion, the positive expressions of Mir-34A-3p, Mir-31 and PLEK2 are correlated with the occurrence, development, staging, metastasis and prognosis of colorectal cancer, and can be used as biological targets for tumor diagnosis and treatment. However, the sample size of this study is limited, and the specific mechanism of action has not been analyzed, so it is necessary to further expand the sample size and explore its development process.

Interest conflict

The authors declare no conflicts of interest.

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