Research Paper

# Effect of microRNA-515-5p on Biological Behavior of Colorectal Cancer Cells through Phosphoinositide 3-Kinase/ Protein Kinase B Signaling Pathway

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**This study aims to examine the impact and underlying mechanism of micro ribonucleic acid-515-5p on the biological behavior of colorectal cancer cells. The study conducted an experiment categorizing into control (blank), colorectal cancer, and micro ribonucleic acid-515-5p overexpression groups. Western blot, quantitative polymerase chain reaction techniques, cell counting kit-8, and Transwell methods were used to detect the growth and metastasis ability of the cells, the expression level of the related protein and messenger ribonucleic acid. The micro ribonucleic acid-515-5p messenger ribonucleic acid was found to be significantly reduced in colorectal cancer group cells compared to control group cells. The proliferation activity and migration number of the cells in micro ribonucleic acid-515-5p overexpression group were reduced compared to the colorectal cancer group cells. The first apoptosis signal, B-cell lymphoma 2-associated X protein, and first apoptosis signal ligand in the micro ribonucleic acid-515-5p overexpression group were higher compared to the colorectal cancer group, while the expression level of B-cell lymphoma 2 was lower compared to the colorectal cancer group. Protein kinase B, phosphoinositide 3-kinase, messenger ribonucleic acid and protein in the micro ribonucleic acid-515-5p overexpression group were reduced than the colorectal cancer group. The overexpression of micro ribonucleic acid-515-5p has been observed to exert inhibitory effects on proliferation, migration, and invasion of colorectal cancer cells, while induce apoptosis of colorectal cancer cells by inhibiting phosphoinositide 3-kinase/protein kinase B signaling pathway.**

**Key words: microRNA-515-5p, phosphoinositide 3-kinase/protein kinase B, colorectal cancer, apoptosis**

The incidence of Colorectal Cancer (CRC) on the rise due to shifts in individual's lifestyle choices, dietary patterns, and occupational settings, has become a common malignant tumor of digestive tract in clinic[1]. At present, the clinical treatment of patients with CRC is still lack of radical treatment mainly radiotherapy, chemotherapy and surgical resection[2]. With the progress of targeted therapy, more and more malignant tumors begin to use targeted therapy in clinical treatment[3]. Therefore, in-depth study of the pathogenesis and etiology of CRC, looking for new treatment targets, to improve patient's clinical symptoms, impeding the advancement of cancer, and ameliorating the quality of life of the patient hold significant clinical implications.

Micro Ribonucleic Acid (miRNA) is a kind of highly conserved non-coding small interfering (si) RNA, which has critical role in the regulation of cell biological function. miR-515-5p has been proved to be abnormally expressed in patients with CRC and may participate in the occurrence and development of CRC[4]. However, there is a scarcity of literature documenting the impact of miR-515-5p on the biological behaviors of CRC cells and its potential underlying mechanism. Phosphoinositide 3-Kinase/Protein Kinase B (PI3K/AKT) signal pathway is closely related to tumorigenesis and development, and is an important intracellular

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pathway for promoting proliferation and anti-apoptosis[5]. This study aimed to investigate the impact of miR-515-5p on the CRC cells through PI3K/AKT signal pathway, in order to provide a prospective therapeutic target for CRC.

## MATERIALS AND METHODS

### Reagents:

Fetal Human Cells (FHC) and Human Colorectal Carcinoma (HCT116) cells from American Type Culture Collection (ATCC) were obtained. miR-515-5p, AKT, PI3K, messenger RNA (mRNA) primers and Beta (β)-actin primers (American Sigma company); Annexin V-Fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) apoptosis kit (Shanghai Biyantian Co., Ltd.,); Transwell chamber (American Corning company); artificial reconstructed basement membrane glue (Matrigel) were purchased from American BD company; AKT, PI3K, First Apoptosis Signal (FAS), Bcl-2-Associated X (BAX) protein, FAS Ligand (FasL), B-cell lymphoma 2 (Bcl-2) antibodies (Abcam Biotechnology Co., Ltd., UK) were obtained. The over expressive miR-515-5p gene of *Lentivirus* was required for this study (Shanghai Jikai Gene Co., Ltd.,).

### Methods:

**Cell culture and treatment:** The cells were inoculated into T25 culture bottles with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS), and were subsequently cultured at a temperature of 37° with a 5 % Carbon dioxide ($CO_2$) atmosphere. We set up control group (blank), CRC group and miR-515-5p overexpression group. FHC cells without any treatment were included in the control group, HCT116 cells without any treatment were included in the CRC group, and the cells in the overexpression miR-515-5p group were cultured in the offspring of CRC HCT116 cells infected with miR-515-5p gene overexpression *Lentivirus*. The experiment was repeated for 6 times.

**Western blotting assay:** The cell density was adjusted to $1\times10^6$ cells/well and inoculated them into 6-well plates followed by incubating them at a constant temperature incubator (37°, 5 % $CO_2$). Then the solution was homogenized them by adding 10 % cellular protein lysis buffer at 4°, centrifuged them and the supernatant was collected for examination. Bicinchoninic Acid (BCA) method was used to determine the protein concentration, gel was prepared, electrophoresed for 90 min. Then the gel was cut, membrane was rotated for 90 min, non-fat dry milk was sealed, washed, and then sequentially incubated with primary and secondary antibodies. Finally we developed and analyzed the results using Bio-Rad imaging system.

**Quantitative Polymerase Chain Reaction (qPCR):** The cell density was adjusted to $1\times10^6$ cells/well inoculated in 6-well plates and cultured at a constant temperature incubator (37°, 5 % $CO_2$). HCT116 cells were cultured in an incubator for 48 h, after adding 10 µg/ml caffeic acid p-nitrophenylethyl. Then Total RNA Isolation (TRIzol) reagent was added and RNA was reversely transcribed into complementary Deoxyribonucleic Acid (cDNA). We performed the amplification reactions as per the description provided in the TrasStart® Top Green qPCR SuperMix. After the reaction was completed, a software program was used to calculate relative mRNA expression.

**Cell Counting Kit-8 (CCK-8) assay:** 10 µg/ml caffeic acid p-nitrophenylethyl ester and 10 µl of CCK-8 reagent were added to HCT116 cells and were cultured in an incubator for 4 h. The absorbance at 450 nm was measured by a spectrophotometer. The quantified result was equal to the Optical Density (OD) measure of the experimental group-OD measure of the control group.

**Transwell assay:** 15 µg/ml resveratrol was added to HCT116 cells and were cultured for 48 h. Each group was adjusted to have a cell density of $5\times10_5$ cells/well inoculated in the upper chamber of the Transwell. In the lower chamber, a medium consisting of 20 % FBS was introduced. The control group was given the same amount of DMEM culture medium and the cells were fixed, stained with crystal violet. The resulting purple color stained cells were microscopically counted and the cell migration ability was calculated. For the detection of cell invasion ability, the upper chamber of the Transwell was first covered with Matrigel™ in an ultra-clean bench, and the subsequent steps were followed.

### Statistical analysis:

Analysis and processing of data were carried out using the Statistical Package for the Social

Sciences (SPSS) 22.0 statistical software, and the measurement information was expressed by (x±s), and the comparison was made by t-test, where $p<0.05$ was considered to be statistically significant.

## RESULTS AND DISCUSSION

The CRC group exhibited a significantly lower relative expression level of miR-515-5p mRNA than the control group (Table 1).

The cell proliferative activity and cell migration in CRC group were high than the control group, whereas the miR-515-5p overexpression group demonstrated lower levels of these activities than the CRC group (Table 2).

The CRC group exhibited a higher number of cell invasions than the control group, whereas the miR-515-5p overexpression group demonstrated a lower number of cell invasions than the CRC group. Additionally, the overall apoptosis rate was higher in comparison to the CRC group (Table 3).

FAS, BAX and FasL in miR-515-5p overexpression group was higher than that in CRC group, while the overexpression in Bcl-2 group was lower (Table 4).

The mRNA expression levels of AKT and PI3K were found to be higher in the CRC group than the control group. Conversely, mRNA expression levels in the miR-515-5p overexpression group were observed to be significantly lower compared with the CRC group (Table 5).

The effect of miR-515-5p on protein expression of AKT and PI3K in CRC cells was studied. We observed that the AKT and PI3K protein in CRC group was high than that in control group, and miR-515-5p overexpression group (Table 6).

**TABLE 1: miR-515-5P EXPRESSION IN CRC CELLS**

| Group | n | miR-515-5p mRNA |
|---|---|---|
| Control | 6 | 2.05±0.46 |
| CRC | 6 | 1.12±0.35 |
| t | | 3.941 |
| p | | 0.003 |

**TABLE 2: EFFECT OF miR-515-5P ON PROLIFERATION AND MIGRATION OF CRC CELLS**

| Group | n | Cell proliferation activity | Number of migrated cells |
|---|---|---|---|
| Control | 6 | 0.10±0.01 | 73.25±15.70 |
| CRC | 6 | 0.22±0.04[a] | 142.74±24.34[a] |
| miR-515-5p overexpression | 6 | 0.15±0.03[b] | 106.48±17.44[b] |
| F | | 25.154 | 19.022 |
| p | | 0.000 | 0.000 |

Note: [a]$p<0.05$ and [b]$p<0.05$ relative comparison of CRC to the overexpression of miR-515-5p

**TABLE 3: EFFECT OF miR-515-5P ON INVASION AND APOPTOSIS OF CRC CELLS**

| Group | n | Overall apoptosis rate (%) | Number of cell invasion |
|---|---|---|---|
| Control | 6 | 9.97±1.23 | 51.72±10.31 |
| CRC | 6 | 9.51±1.13 | 121.05±20.26[a] |
| miR-515-5p overexpression | 6 | 31.34±4.25[b] | 93.12±16.35[b] |
| F | | 134.293 | 26.157 |
| p | | 0.000 | 0.000 |

Note: [a]$p<0.05$ and [b]$p<0.05$ relative comparison of CRC to the overexpression of miR-515-5p

**TABLE 4: EFFECT OF miR-515-5P ON THE APOPTOTIC PROTEIN IN CRC CELLS**

| Group | n | FAS | BAX | FasL | Bcl-2 |
|---|---|---|---|---|---|
| Control | 6 | 0.35±0.14 | 0.37±0.09 | 0.41±0.14 | 0.54±0.03 |
| CRC | 6 | 0.33±0.17 | 0.43±0.14 | 0.44±0.14 | 0.49±0.04 |
| miR-515-5p overexpression | 6 | 0.84±0.18[b] | 0.86±0.16[b] | 0.78±0.12[b] | 0.31±0.03[b] |
| F | | 13.446 | 20.294 | 18.641 | 72.177 |
| p | | 0.001 | 0.000 | 0.000 | 0.000 |

Note: [b]p<0.05 denotes the comparison of miR-515-5p overexpression

**TABLE 5: EFFECT OF miR-515-5P ON AKT AND PI3K mRNA IN CRC CELLS**

| Group | n | AKT | PI3K |
|---|---|---|---|
| Control | 6 | 1.24±0.32 | 0.83±0.35 |
| CRC | 6 | 2.47±0.54[a] | 2.59±0.67[a] |
| miR-515-5p overexpression | 6 | 1.65±0.40[b] | 1.76±0.43[b] |
| F | | 12.744 | 18.451 |
| p | | 0.000 | 0.000 |

Note: [a]p<0.05 and [b]p<0.05 relative comparison of CRC to the overexpression of miR-515-5p

**TABLE 6: EFFECT OF miR-515-5P ON PROTEIN EXPRESSION OF AKT AND PI3K IN CRC CELLS**

| Group | n | AMPK | (PGC)-1α |
|---|---|---|---|
| Control | 6 | 0.28±0.04 | 0.30±0.0 |
| CRC | 6 | 0.86±0.28[a] | 0.76±0.113[a] |
| miR-515-5p overexpression | 6 | 0.57±0.08[b] | 0.52±0.09[b] |
| F | | 15.521 | 45.156 |
| p | | 0.000 | 0.000 |

Note: [a]p<0.05 and [b]p<0.05 relative comparison of CRC to the overexpression of miR-515-5p, (AMPK): Adenosine Monophosphate-activated Protein Kinase and (PGC): Peroxisome proliferator-activated receptor-Gamma Coactivator

CRC is a prevalent malignancy affecting the digestive system, specifically the colon or rectum, with high morbidity and mortality[6]. The prevalence of CRC is progressively increasing in our country due to the exacerbation of the aging issue and the alteration of individual's lifestyle patterns. This escalating trend poses a significant threat to the well-being and survival of the population[7]. The pathogenesis and etiology of CRC have not been fully explained, but recent studies have found that age, genetic susceptibility, inflammatory reaction, immune disorder, physical and chemical radiation and other factors are related to the incidence and progression of CRC[8]. Radiotherapy, chemotherapy and surgical resection are mainly used in clinic. Although it can help some patients to cure cancer, but some patients who are diagnosed at later stages, lose the opportunity of surgical resection of tumor and are not sensitive to radiotherapy and chemotherapy, resulting in limited clinical treatment effect[9]. CRC has the characteristics of high incidence, invasiveness, metastatic potential and recurrence rate, so it is of great clinical significance to target cancer cells in patients with CRC[10]. Therefore, in-depth study of the pathogenesis and etiology of CRC, determine the molecular mechanism of growth and metastasis of CRC cells, finding out new therapeutic targets. It has important clinical significance to improve and slow down the pathological progress of CRC and enhance the overall well-being of individuals receiving medical care. The objective of this study is to investigate the impact of miR-515-5p on the biological behavior of CRC cells through PI3K/AKT signal pathway, with the aim of identifying a potential therapeutic target for the clinical management of CRC.

In recent years, miRNA has emerged as a prominent area of research within the field of malignant tumors. As a group of non-coding siRNA, it

participates in regulating various biological processes by combining with target genes[11]. MiR-515-5p is highly conserved in vertebrates and is a kind of miRNA involved in the regulation of cancer-related inflammatory response. The comprehensive elucidation of the precise regulatory mechanisms underlying the initiation and progression of cancer remains incomplete[12]. However, it has been confirmed that miR-515-5p can be used as a tumor suppressor to regulate the level of Thyroid Receptor Interacting Protein 13 (TRIP13), thus regulating the growth and metastasis of prostate cancer cells[13]. In addition, the proliferation and invasion of gastric cancer cells can be impeded *via* targeting of Wingless-related integration site 3 (Wnt) by miR-515-5p[14]. The findings indicated a significant decrease in the relative expression level of miR-515-5p in the CRC group than control group. The cell proliferation and migration in CRC group increased than the control group, but those in miR-515-5p overexpression group were reduced. Similarly the number of cell invasion in the CRC group increased than the control group while the number of cell invasion in the overexpression miR-515-5p group was reduced. The overall apoptosis rate was higher than the CRC group. The findings propose a decrease in the expression of miR-515-5p within CRC cells, implying that a reduction in the vitality of CRC cells, as well as the inhibition of their growth and metastasis. Additional research has demonstrated that the aberrant expression of the FAS pathway, plays a significant role in the inhibition of apoptosis. Fas and FasL are classified as cell surface receptors within the tumor necrosis factor family, and their interaction triggers cell death mediated by the ligand. The reduction in FAS and FasL expression or the attenuation of signal transduction pathways associated with them are closely linked to tumor progression[15,16]. BAX is considered as a highly representative pro-apoptotic molecule, while Bcl-2 is an anti-apoptotic molecule that exhibits a close association with tumor cell apoptosis. The Bcl-2/BAX ratio is frequently employed as a means to assess the extent of tumor cell apoptosis[17]. FAS, BAX and FasL in miR-515-5p overexpression group were higher compared to the CRC group. The present study proposes that the modulation of miR-515-5p expression in CRC cells may potentially enhance the induction of apoptosis in these cells.

PI3K/AKT signal pathway is a widely used signal transduction pathway in human body, which plays a critical role in cell growth, differentiation, metastasis and apoptosis[18]. AKT serves as both direct target gene and downstream effector of PI3K. The phosphorylation of AKT can be employed as an indicator of PI3K activity. Furthermore, AKT phosphorylation governs the activity of downstream proteins, thereby modulating the fundamental cellular functions[19,20]. Research has substantiated that the excessive activation of the PI3K/AKT signaling pathway assumes a significant role in regulating of cellular proliferation, survival and invasiveness of many cancer cells. The results showed that AKT and PI3K, mRNA in CRC group was higher than the control group, while these in miR-515-5p overexpression group was low when compared to the CRC group. The expression level of AKT and PI3K protein in CRC group was higher than that in control group, while in miR-515-5p overexpression group these proteins decreased than that in CRC group. It is suggested that miR-515-5p up-regulating in CRC cells can inhibit PI3K/AKT signal pathway, which provides a theoretical basis for clinical treatment of tumor metastasis and invasion.

To sum up, miR-515-5p in CRC cells decreased significantly, and miR-515-5p overexpression has been observed to decrease the viability of CRC cells, impede their growth and metastasis, as well as induce apoptosis. This effect may be attributed to the inhibition of the PI3K/AKT signaling pathway.

**Conflict of interests:**

The authors declared no conflict of interests.

**Authors' Contribution:**

Wenyuan Zhang and Chongling Zhang Have Contributed Equally To This Study

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