Expression profile and biological function of miR-455-5p in colorectal carcinoma

JINQIU WANG¹, YANG LU², YIYONG ZENG², LEMING ZHANG³, KONGLIANG KE³ and YU GUO¹

¹Department of Breast Surgery, Ningbo First Hospital, Ningbo, Zhejiang 315010; ²Medical School of Ningbo University, Ningbo, Zhejiang 315211; ³Department of Proctology, Ningbo First Hospital, Ningbo, Zhejiang 315010, P.R. China

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Abstract. Underexpression of microRNA-455-5p (miR-455-5p) in medullary thyroid carcinoma, melanoma, gastric cancer and additional cancer types has been reported, which may be associated with carcinoma development. The present study aimed to evaluate the expression profile and biological role of miR-455-5p in colorectal carcinoma. Carcinoma tissues and adjacent tissue specimens from 40 patients with colorectal cancer were randomly collected. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis was conducted to detect the expression levels of miR-455-5p in colorectal carcinoma and adjacent normal tissues. The biological effects of miR-455-5p on selected colorectal cancer cells were assessed using bromodeoxyuridine assays, wound healing migration assays and flow cytometry. Bioinformatics analysis was implemented to predict the potential target genes of miR-455-5p in colorectal cancer. The expression levels of target genes were further validated by RT-qPCR and western blot analysis of the mRNA and protein levels. The results of the experiments demonstrated that miR-455-5p expression was downregulated in colorectal cancer tissues compared with adjacent normal tissues. In colorectal cancer cells (SW-480, HT-29 and HCT-116), miR-455-5p was observed to inhibit cell proliferation and migration while promoting cell apoptosis. Bioinformatics analysis predicted that the oncogene phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1) was one of the top ranked target genes of miR-455-5p in colorectal cancer cells. This association was validated by RT-qPCR and western blotting. In vivo studies revealed that the expression level of miR-455-5p was significantly downregulated in human colorectal cancer. Further in vitro studies suggested that miR-455-5p may prevent the development of colorectal cancer by downregulating the oncogene PIK3R1. It was concluded that miR-455-5p may target and downregulate PIK3R1 in colorectal cancer.

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-associated mortality worldwide (1). In China, CRC ranks fifth among the major causes of mortality (2). In recent years, increasing attention has been paid to the diagnosis, treatment and prognosis of CRC, however, CRC remains a challenge. The 5-year survival rate of patients with CRC remains low. Numerous factors account for the increasing incidence of CRC, including environmental pollution, an unhealthy diet and food safety problems. The development of CRC is a complex procedure involving multiple stages and factors; however, the underlying mechanism is not fully understood (3).

MicroRNAs (miRNAs) are endogenous, small non-coding RNAs, 22-25 nucleotides in length, which are widely present in eukaryotes (4). Previous studies revealed that miRNAs have essential functions in the development of cancer, which supports their roles as potential therapeutic targets and prognostic indicators for cancer (5-7). miRNA (miR)-455-5p, located on chromosome 6, has been reported to have low expression levels in numerous cancer types, including medullary thyroid carcinoma (8), melanoma (9) and gastric cancer (10), compared with normal tissues. However, there is a limited number of studies that have investigated the expression levels of miR-455-5p and its potential role in CRC. Whether miR-455-5p may affect metastasis and its regulatory mechanism in CRC remains unclear.

In the present study, the in vivo expression profile of miR-455-5p was studied in CRC, in order to gain insights into the underlying mechanisms of human CRC, and to assess the biological function of miR-455-5p in CRC. The expression levels of miR-455-5p in colorectal cancer tissues and paracancerous tissues were detected and the differences were evaluated. Using bromodeoxyuridine (BrdU) cell proliferation, apoptosis and migration assays, the biological characteristics of miR-455-5p were determined and its target gene identified.

Key words: microRNA-455-5p, colorectal carcinoma, proliferation, apoptosis, phosphoinositide-3-kinase regulatory subunit 1

Correspondence to: Dr Yu Guo, Department of Breast Surgery, Ningbo First Hospital, 59 Liuting Street, Haishu, Ningbo, Zhejiang 315010, P.R. China
E-mail: guoyu308@163.com
Materials and methods

Patients and samples. Carcinoma tissues and adjacent tissue specimens were randomly collected from 40 patients with CRC (21 cases of rectal cancer, 19 cases of colon cancer), who underwent surgery at the anorectal branch of Ningbo First Hospital (Ningbo, China) between March 2016 and July 2016. CRC specimens and the normal specimens were collected and immediately placed into the RNA preservation solution, and stored at -80°C.

The inclusion criteria of patients included the clinical and pathological diagnosis of colorectal cancer with stable vital signs. Patients with a history of chemotherapy or other malignancies prior to surgery were excluded. Patients whose family had a history of similar neoplastic disease were also excluded. Tumors were classified according to the Tumor Node Metastasis classification staging system (11). All post-operative pathology results were reviewed by two pathologists. Informed written consent (no. S2016-055-10) was provided by all patients and the study was approved by the Ningbo First Hospital Ethics Committee. All the procedures were performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Cell culture. The colorectal cancer cell lines (SW-480, HT-29 and HCT-116) were provided by Shanghai Nuobai Pharmaceutical Co., Ltd. (Shanghai, China). Cells were cultured in DMEM (GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (FBS; BioWest, Nuaillé, France) and 1% penicillin/streptomycin (P/S) solution at 37°C in 5% CO2 and 95% air.

RNA extraction and quantitative analysis of miR-455-5p and phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1) using reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues or transfected cells using TRIzol® reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. Reverse transcription was performed with the following parameters: 25°C for 5 min, followed by 50°C for 60 min and 70°C for 15 min. The expression of miR-455-5p in CRC and adjacent healthy tissue, and PIK3R1 in transfected CRC cell lines, was measured using RT-qPCR using a SYBR-Green PCR system (Tiangen Biotech Co., Ltd., Beijing, China). The thermocycling conditions were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 30 sec and 70°C for 45 sec. All the primer sequences are listed in Table I. Relative quantitative analysis was performed using the 2^ΔΔCt method (12).

Cell transfection of miR-455-5p mimics. The three CRC cell lines (SW480, HT-29 and HCT-116) were cultured in DMEM and transfected with miR-455-5p mimics (miRNA mimics, forward, 5'-UUCUCCGAACGUGACUCCGUUTT-3' and reverse, 5'-ACGUAGACACGUUCCGAGATT-3') or non-specific control (NC) miRNA mimic (forward, 5'-UUCUCCGAACGUGACUCCGUUTT-3' and reverse, 5'-ACGUAGACACGUUCCGAGATT-3') using Hieff Trans™ Liposomal Transfection Reagent (Yeasen Biotechnology Co., Ltd., Shanghai, China), according to the manufacturer's protocol. In brief, cells were grown in 12-well plates until 80-90% confluence was reached. miRNA mimics or NC mimic (20 pmol) were mixed with 2 µl transfection reagent in 50 µl Opti-MEM (Invitrogen; Thermo Fisher Scientific, Inc.). Following incubation at room temperature for 20 min, the mixture was added to the cells. After 6 h, the medium was changed to fresh medium supplemented with 10% FBS and PS.

BrdU cell proliferation assays. Following transfection with miR-455-5p mimics or NC mimic for 48 h, the BrdU assay kit (Boster Biological Technology, Pleasanton, USA) was used to evaluate the proliferative ability of the cells, according to the manufacturer's protocol. Fluorescence microscopy was used to observe the fluorescence ratio (magnification, x10).

Apoptosis assays. A total of 48 h following transfection, cell medium was replaced with DMEM supplemented with 2% FBS. A total of 24 h later, the cells were resuspended in 1X Annexin Binding Buffer (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). A total of 5 µl of 7-Aminoactinomycin D (7-AAD) viability Dye and 1 µl of Annexin conjugated to phycoerythrin (PE) solution (Nanjing KeyGen Biotech Co., Ltd.) were added to 100 µl of cell suspension solution. The tubes were maintained on ice and incubated for 15 min in the dark. Subsequently, 400 µl of ice-cold 1X Binding Buffer was added into the tubes followed by flow cytometry analysis on a BD FACS Calibur Flow Cytometry System using BD FACStation™ 6.1 software (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocols. The cells with Annexin V-PE'/7-AAD- were calculated to represent apoptotic cells.

Wound healing migration assays. Wound healing cell migration assays were carried out as described previously (13). Briefly, 48 h following transfection, HT-29 cells (8x104 cells/well) were seeded into a 24-well plate and grown until ~100% confluent. An uninterrupted scratch was made with a sterile P200 pipette tip across the monolayer. Following washing with PBS, cells were cultured in DMEM without FBS for 0, 6, 12 and 24 h. Alterations in the area of the scratch were observed with an inverted microscope (magnification, x10) at different time points (0, 6, 12 and 24 h).

Bioinformatics analysis. To predict the target genes of miR-455-5p, the following computational programs were used: TargetScan (http://www.targetscan.org); miRanda (http://www.microrna.org); PICTAR (http://www.picтар.mdc-berlin.de); and MICROCOSM Targets (http://www.ebi.ac.uk/enright-srv/microcosm).

Western blotting. Western blotting was performed as previously described (14). At 48 h following transfection, cells were harvested and lysed, and the supernatants were collected by centrifugation at 12,000 x g for 15 min at 4°C. The protein concentration was measured using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. The protein was separated by SDS-PAGE on a 12.5% gel and transferred on to a polyvinylidene difluoride membrane. The membrane was blocked and
subsequently incubated overnight at 4˚C with anti-PIK3R1 (dilution, 1:1,000; cat. no. ab191606; Abcam, Cambridge, UK). A goat anti-rabbit HRP-conjugated antibody (dilution, 1:5,000; cat. no. ab97080, Abcam) was used as the secondary antibody. The membrane was developed using an enhanced chemiluminescence system (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

Protein expression was quantified by Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. SPSS 18.0 (SPSS, Inc., Chicago, IL, USA) statistical software was used for all statistical analyses. The Kolmogorov-Smirnov test was used to test the normality of continuous variables. Data were counted and are presented as the number of cases (n, %). The statistical inferences of intergroup differences were evaluated using the \( \chi^2 \) test. Data conforming to a normal distribution were presented as the mean ± standard error of the mean, and one-way analysis of variance followed by the least significant difference post-hoc test was used for multiple comparisons between groups. Data not conforming to a normal distribution are presented as the median with interquartile range, and non-parametric tests, including Kruskal-Wallis tests with Nemenyi post hoc test, and Mann-Whitney tests, were used for multiple comparisons between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Patient characteristics. A total of 40 patients (21 cases of rectal cancer and 19 cases of colon cancer) were included in the present study. These comprised 24 males and 16 females, aged 43-85 years with an average age of 65±11 years. A total of 28 patients were ≥60 years old and 12 patients were <60 years old. Tumors were classified as stage I (n=6), stage II (n=17), stage III (n=13) or stage IV (n=4). There were 33 cases of moderate differentiation and four cases of poor differentiation. Patients with no previous history of malignancies had not received radiotherapy, chemotherapy or immunotherapy prior to surgery.

Expression of miR-455-5p is downregulated in CRC tissues. The expression levels of miR-455-5p were measured using RT-qPCR in the 40 CRC tissues and adjacent non-tumor tissues. Scatter plot of miR-455-5p expression levels in colorectal carcinoma and para-carcinoma tissues. The expression level of miR-455-5p was significantly higher in para-carcinoma tissues. *P<0.01 vs. para-carcinoma tissues. miR, microRNA.

Association between miR-455-5p expression levels and clinicopathological characteristics. Following the observation that miR-455-5p expression levels were significantly decreased in colorectal tumor tissues, the association between miR-455-5p expression levels and the characteristics of CRC patients was investigated. The expression of miR-455-5p was reported to be negatively associated with the differentiation of colorectal tumors (P<0.05; Table II). According to the results of the present study (Table II), the expression level of miR-455-5p was not associated with the following indicators...
miR-455-5p mimics transfection of CRC cells was successful. RT-qPCR experiments were performed to demonstrate that the transfection with miR-455-5p mimics was successful. As presented in Fig. 2, the relative expression levels of mRNA in the miRNA mimics groups were 54,343-, 43,085- and 18,856-times higher compared with the NC mimics groups in the HCT-116, HT-29 and SW480 cell lines. The mRNA expression level of miR-455-5p was significantly upregulated in CRC cell lines transfected with miR-455-5p mimics (P<0.001).

miR-455-5p suppresses the proliferation of CRC cells. According to results from the present study (Fig. 3), it was demonstrated that the number of viable cells in the miRNA mimics group was significantly reduced (P<0.05) compared with the NC group. In addition, inhibition of endogenous miR-455-5p with miR-455-5p inhibitors increased the cell proliferation by ~20% compared with the control group (HT-29, 40% in miRNA inhibitor vs. 28% in NC inhibitor; HCT-116, 60% in miRNA inhibitor vs. 40% in NC inhibitor; SW480, 53% in miRNA inhibitor vs. 30% in NC inhibitor).

miR-455-5p promotes apoptosis in CRC cells. As displayed in Fig. 4, transient transfection of miR-455-5p mimics in HCT-116 cells markedly promoted apoptosis compared with the NC mimic group (P<0.05; 48 and 39%, respectively). Apoptosis was significantly inhibited in HCT-116 cells transfected with the miRNA inhibitor compared with the NC inhibitor group (P<0.05). In addition, similar patterns of apoptosis in an additional two CRC cell lines (HT-29 and SW480) were observed.

miR-455-5p inhibits the migration of CRC cells. The effect of miR-455-5p on CRC cell migration was detected by wound

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Table II. Correlation between the level of miR-455-5p and clinical pathological characteristics of patients with colorectal cancer.

| Clinical characteristic | No. patients | Expression of miR-455-5p | Z/χ² | P-value |
|-------------------------|--------------|--------------------------|------|--------|
| Sex                     |              |                          |      |        |
| Male                    | 24           | 0.879 (0.401, 1.222)     | -0.939 | 0.392a |
| Female                  | 16           | 0.594 (0.226, 1.016)     |       |        |
| Age, years              |              |                          |      |        |
| ≥60                     | 28           | 0.662 (0.328, 1.102)     | -1.107 | 0.348b |
| <60                     | 12           | 1.002 (0.446, 1.635)     |       |        |
| Location                |              |                          |      |        |
| LSCC                    | 6            | 0.934 (0.492, 1.516)     | 2.875 | 0.238c |
| RSCC                    | 13           | 0.736 (0.441, 1.813)     |       |        |
| Rectum                  | 21           | 0.599 (0.263, 1.015)     |       |        |
| Type                    |              |                          |      |        |
| Protuberant             | 13           | 0.550 (0.314, 0.770)     | 2.100 | 0.350d |
| Ulcerative              | 21           | 0.953 (0.361, 1.548)     |       |        |
| Invasive                | 6            | 0.700 (0.399, 1.792)     |       |        |
| Differentiation         |              |                          |      |        |
| Poorly-differentiated   | 7            | 0.477 (0.290, 1.721)     | 8.837 | 0.012e |
| Moderately-poorly-diff. | 5            | 0.174 (0.171, 0.383)     |       |        |
| Moderately-diff.        | 28           | 0.863 (0.501, 1.222)     |       |        |
| Well-differentiated     | 0            | 0                        |       |        |
| TNM                     |              |                          |      |        |
| I/II                    | 17           | 0.588 (0.324, 1.194)     | -0.328 | 0.743f |
| III/IV                  | 23           | 0.818 (0.390, 1.285)     |       |        |
| Lymph node metastases   |              |                          |      |        |
| No                      | 26           | 0.647 (0.380, 1.203)     | -0.128 | 0.898g |
| Yes                     | 14           | 0.863 (0.297, 1.169)     |       |        |
| No. of metastatic lymph nodes |      |                          |      |        |
| 1-3                     | 9            | 0.908 (0.253, 1.239)     | -0.200 | 0.841h |
| >3                      | 5            | 0.477 (0.307, 2.772)     |       |        |

Expression of miR-455-5p is presented as the median and interquartile range. P<0.05 was considered to indicate a statistically significant difference. aWilcoxon test; bKruskal-Wallis test. TNM, Tumor, Node, Metastasis; miR, microRNA; LSCC, left-sided colorectal cancer; RSCC, right-sided colorectal cancer.
healing cell migration assays using HT-29 cells. As displayed in Fig. 5, the distance of cell migration for the miRNA mimics-treated group was smaller compared with that of the NC mimic group at a variety of different time points (6, 12 and 24 h). Specifically, the relative mobility percentages were as follows: i) 6 h, 4% in miRNA mimics vs. 8% in NC mimics; ii) 12 h, 6% in miRNA mimics vs. 14% in NC mimics; and iii) 24 h, 9% in miRNA mimics vs. 12% in NC mimics. By contrast, depletion of miR-455-5p with an miRNA inhibitor enhanced the cell migratory ability (Fig. 5). The comparisons of relative mobility between the two groups were as follows: i) 6 h, 8% in miRNA inhibitor vs. 3% in NC inhibitor; ii) 12 h, 13% in miRNA inhibitor vs. 9% in NC inhibitor; and iii) 24 h, 15% in miRNA inhibitor vs. 14% in NC inhibitor. These results indicated that miR-455-5p may inhibit the migration of HT-29 cells. miR-455-5p negatively regulates the expression levels of the target gene PIK3R1. The above results prompted an investigation into the molecular mechanism underlying the aforementioned differences in the behavior of CRC cells treated with miR-455-5p. The binding region of miR-455-5p in the target gene PIK3R1 mRNA 3'-untranslated region was predicted using target gene prediction databases (Fig. 6A). To validate these predictions, the effect of miR-455-5p on the expression levels of PIK3R1 was determined using RT-qPCR and western blotting experiments. As displayed in Fig. 6B, the expression levels of mRNA in the miRNA mimics group were 25, 19 and 32% lower in HCT-116, HT-29 and SW480 cell lines, respectively, compared with the NC mimics group. By contrast, the mRNA expression levels in the miRNA inhibitor group were 21, 20 and 21% higher compared with the NC inhibitor group in HCT-116, HT-29 and SW480 cell lines, respectively. The mRNA level of PIK3R1 was significantly reduced by the expression of miR-455-5p in CRC cells (P<0.05). Furthermore, the levels of protein expression of PIK3R1 were also observed to be downregulated by miR-455-5p (Fig. 6C). Western blot quantification reported that the protein expression levels in the mimics group (1.21) were lower compared with the NC mimics group (1.55) in the HT-29 cell lines, while the inhibitor groups exhibited the opposite trend. The results of the western blotting were consistent with the RT-qPCR results (Fig. 6B). Together, these results indicated that miR-455-5p may function as a CRC suppressor by negatively regulating the expression levels of the oncogene PIK3R1.

**Discussion**

The present study demonstrated that miR-455-5p may suppress the proliferation and migration of CRC cells and downregulate PIK3R1 in CRC. *Ex vivo* research revealed that the expression level of miR-455-5p was significantly downregulated in human
miRNAs are widely distributed in viruses, plants and higher mammals (17). Since the discovery of the first miRNA gene (lin-4) in 1993 (4,18), the function of miRNAs has been widely investigated (19-22). Given their essential role, even a small alteration in the expression levels of miRNAs may lead to notable differences in the levels of downstream target mRNAs. As a member of the miR-455 family, miR-455-5p has been reported to have differential expression levels in a variety of carcinomas. For instance, Shoshan et al (9) reported that miR-455-5p was overexpressed in melanoma and downregulated the tumor suppressor gene cytoplasmic polyadenylation element binding protein 1, which consequently promoted the proliferation and metastasis of cancer cells. Similarly, Sand et al (23) demonstrated that miR-455-5p was significantly upregulated in basal cell carcinoma. By contrast, the downregulation of miR-455-5p was also reported in other cancer types, including esophageal, gastric and thymic epithelial cancer (10,24,25). Yang et al (26) reported that mir-455-5p is upregulated in colon cancer and functions as an oncogene, promoting the development of colon cancer. This contradicts the results from the present study, and this may be due to the fact that Yang et al (26) only studied 10 cases of colon cancer tissue, while rectal cancer tissues were not included. Furthermore, only one cell line was studied. The number of tissue specimens and cells was lower compared with the present study, and the results were not representative. There are limited studies on the expression levels of miR-455-5p in human CRC tissues, thus the association remains unclear.

CRC is known to be associated with abnormalities in a large collection of genes, which may be reflected by the identification of numerous molecular markers (27-31). In the present study, it was reported that there was significantly low expression levels of miR-455-5p in CRC, which correlated with tumor differentiation, suggesting that miR-455-5p may have tumor suppressing potential in CRC. However, given the relatively limited tissue samples in the present study, an extensive study with a larger sample size is required in the future.

To investigate the potential role of miR-455-5p in CRC, the effects of up- and downregulation of miR-455-5p on cell proliferation, migration and apoptosis were assessed. The results of the present study reported that the upregulation of miR-455-5p may lead to the inhibition of cell proliferation and migration, in addition to promoting apoptosis in CRC cells. Together, these findings support the tumor suppressor role of miR-455-5p in CRC. Furthermore, expression levels of miR-455-5p in HCT-116 cells regulated apoptosis positively. It was demonstrated that apoptosis was not significantly altered in HT-29 and SW-480 cells treated with the miRNA inhibitor and miRNA mimics, respectively. Further investigation is required in order to address this inconsistency.

To gain insights into the underlying molecular mechanism of CRC, the potential target gene of miR-455-5p was investigated using various online miRNA target gene predictors. As a result, the established oncogene PIK3R1 was identified as the downstream target which serves a key role in activating the PI3K-AKT signaling pathway (32,33). Upon activation, AKT is translocated to the nucleus where it phosphorylates a variety of downstream target proteins, including mechanistic target of rapamycin, X-linked inhibitor of apoptosis protein and...
E3 ubiquitin-protein ligase (34). According to recent studies, the PI3K/AKT signaling pathway has been reported to contribute to the pathogenesis of various cancer types, including breast cancer, ovarian cancer and colon cancer (35). In the present study, it was demonstrated that the expression of PIK3R1 at the mRNA and protein level was downregulated by miR-455-5p, which strongly confirmed the bioinformatics prediction that PIK3R1 may be a functional target for miR-455-5p. Notably, it was reported that miR-455 may positively regulate PIK3R1 gene expression in kidney carcinoma cells, suggesting an association between these two molecules (36). Huang et al (37) reported that miR-4686-5p is downregulated in hepatocellular carcinoma, and suppresses tumor growth by directly targeting PIK3R1. In future studies, the association between miR-455-5p and PIK3R1 will be investigated, along with the intermediate role of the PIK pathway in the tumor suppressive function of miR-455-5p in CRC (38).

In conclusion, it was demonstrated that the expression of miR-455-5p was significantly downregulated in human CRC tissues. In addition, miR-455-5p was reported to serve an inhibitory role in cell proliferation and migration, while promoting cell apoptosis in numerous different CRC cell lines. It was demonstrated that the well-established oncogene PIK3R1 was predicted to serve an essentially intermediate role in the molecular mechanism underlying the anti-cancer role of miR-455-5p. This was validated by assessing the mRNA and

Figure 5. miR-455-5p inhibits HT-29 cell migration. Images of colorectal cancer cell migration in HT-29 cell lines with wound healing cell migration assays (scale=100 µm). Compared with the control groups, the distance of cell migration was reduced with the treatment of miR-455-5p mimics, while the miR-455-5p inhibitor increased the cell migration distance of HT-29 cells. miRNA/miR, microRNA; NC, negative control.
protein expression levels. The results of the present study may facilitate future efforts to elucidate the exact role of miR-455-5p in CRC, and thus hold potential for the treatment of CRC by allowing for the identification of novel gene therapy targets.
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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Author’s contributions

JW and YG contributed to the conception and design of the research. JW performed the histological examination of the colorectal cancer, collected the data, and was a major contributor in writing the manuscript. YL analyzed and interpreted the patient data regarding the colorectal cancer. LZ contributed to the experimental analysis of the data; KK and YZ participated in the statistical processing of data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Informed written consent was provided by all patients and the study was approved by the Ningbo First Hospital Ethics Committee (approval no., S2016-055-10). All the procedures were performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Patient consent for publication

Informed written consent was provided by all patients for the publication of the present study.

Competing interests

The authors declare that they have no competing interests.

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