The role of miR-4469 as a tumor suppressor regulating inflammatory cell infiltration in colorectal cancer

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1. Introduction

MicroRNAs (miRNAs) are endogenous single-stranded 22 nt RNAs that can target mRNAs to regulate cleavage or translational repression [1]. miRNAs are first transcribed as long primary transcripts, followed by a series of processing steps to produce the single-stranded mature miRNAs [2]. In the tumor, many miRNAs function by negatively regulating target genes [3,4]. Therefore, miRNAs may inhibit the occurrence and development of tumors by targeting oncogenes in many malignancies [5].

Colorectal cancer (CRC) is a common malignancy of the gastrointestinal tract. According to the Global Cancer Statistics 2020, CRC ranks third in incidence, accounting for 10.0% of all new cancers, and second in mortality, accounting for 9.4% of all causes of cancer death [6]. The reasons for the incidence of CRC are not well understood, it may be associated with obesity, high-fat diet, genetics, lack of exercise, and smoking [7]. Currently, the treatments for CRC mainly include surgery, radiotherapy, chemotherapy, immunotherapy, and Chinese medicine. The identification of new suppressors of CRC is important for the development of new treatment methods.

Several studies have reported that miRNAs are correlated with CRC development. For example, miR-532-3p could suppress growth, metastasis, and epithelial-mesenchymal transition (EMT) in CRC cells [8]. miR-511-5p could inhibit growth, invasion and promote cell apoptosis in CRC cells [9]. Therefore, miRNA can play...
a certain effect in inhibiting the growth of CRC cells and may be used in the development of drugs.

In tumor tissues, the immune microenvironment constituted by immune cells plays an important role in the development of tumors. In this case, neutrophils play an important role [10]. Neutrophils are able to form Neutrophil Extracellular Traps (NETs), which promote proliferation and stimulate tumor metastasis in CRC [11,12]. Moreover, the production of NETs is significantly increased in CRC patients and is significantly associated with poor patient prognosis [13]. Therefore, targeting NETs may be one of the strategies to treat CRC.

In this study, we identified miR-4469 as a tumor suppressor in CRC by constructing a miRNA-target gene negative regulatory network, and further clarified the role of miR-4469 in CRC via bioinformatics analysis and experimental validation.

2. Materials and methods

2.1. Screening differentially expressed miRNAs related to colorectal cancer progression

The miRNA expression profile in CRC was obtained from the Gene Expression Omnibus (GEO) database in the National Center for Biotechnology Information (NCBI) [14], the dataset ID is GSE115513. The GSE115513 dataset includes 649 normal colorectal cases and 731 CRC cases. According to the American Joint Committee on Cancer (AJCC) staging categories, the 731 CRC cases comprised 231 cases in stage I, 181 cases in stage II, 219 cases in stage III, and 100 cases in stage IV. We performed differential expressed miRNAs screening in the following order: stage I vs normal; stage II vs stage I; stage III vs stage II; stage IV vs stage III. The up-regulated miRNAs and down-regulated miRNAs from each group were intersected separately, and miRNAs with continuously increasing expression or continuously decreasing expression were obtained according to the order of development of CRC (normal → stage I → stage II → stage III → stage IV). The software used for differentially expressed miRNA screening was Genespring, and the relevant parameters were chosen: One Way ANOVA for the statistical methods section, asymptotic for the calculated p-value, Bonferroni FWER [15] for the multiple testing correction, the p-value was set to <0.05 and folder change was set to greater than 1.

2.2. Construction of miRNA-target gene negative regulatory network

In a previous study [16], we analyzed the CRC expression profile dataset GSE41258 from the GEO database and obtained 295 genes with continuously increasing expression and 290 genes with continuously decreasing expression according to the order of development of CRC (normal → stage I → stage II → stage III → stage IV). While in this study, we obtained miRNAs with continuously increasing expression or continuously decreasing expression at those same CRC stages. Due to the negative regulatory relationship of miRNA and target genes, we constructed a negative regulatory network of miRNA-target genes related to CRC progression. We obtained the target gene of miRNA using two experimentally supported miRNA-target gene databases: Diana TarBase v8, a reference database devoted to indexing experimentally supported miRNA-gene interactions that correspond to 670,000 unique miRNA-target gene pairs with interactions supported by more than 33 experimental methods [17,18], and miTarBase, another database devoted to indexing experimentally supported miRNA-target gene interactions, including 422,517 miRNA-target gene pairs involving 4076 miRNAs [19,20]. By intersecting the target genes of continuously increasing miRNAs with continuously decreasing genes and intersecting the target genes of continuously decreasing miRNAs with continuously increasing genes, we obtained the negative regulatory relationship between miRNAs and target genes associated with CRC progression (Fig. 1A). Cytoscape was used to construct the miRNA-target gene negative regulatory network [21,22].

2.3. Expression and survival analysis of miR-4469 in pan-cancer

To verify that miR-4469 has suppressor properties in CRC, we analyzed the expression level and relationship with patient survival time of miR-4469 in pan-cancer. We first analyzed the expression levels of miR-4469 in pan-cancer from the TCGA database by the EVAtlas tool [23], and then visually compared the expression differences of miR-4469 in cancerous and normal tissues. Then we analyzed the relationship between miR-4469 expression levels and the overall survival time of patients in pan-cancer by the Kaplan-Meier Plotter tool [24,25]. Kaplan-Meier Plotter data were derived from GEO, EGA, and TCGA, integrating gene expression, relapse-free and overall survival information, thus by analysis we obtained the cancer types associated with miR-4469.

2.4. Experimental validation of miR-4469 to inhibit colorectal cancer cells

2.4.1. Cell lines and cell culture

The human CRC cell line including NCM460, HCT116, RKO, HT29, SW480, SW620, and LoVo was from the pathology laboratory at Southern Medical University and cultured with the different media as followed: RPMI-1640 medium, DMEM-H, McCoy’s 5A Medium, Leibovitz’s L-15 Medium, Ham’s F-12 K (Gibco, USA). All of these culture mediums were supplemented with 10% fetal calf serum (Gibco, USA).

2.4.2. RNA extraction and quantitative polymerase chain reaction

Total RNA in cells was extracted using Trizol (TaKaRa, Japan). Mir-X™miRNA First Strand Synthesis Kit (TaKaRa, Japan) was used to reverse transcribe miRNA into cDNA. A quantitative polymerase chain reaction (qPCR) was conducted using the 7500 Real-Time PCR Systems (Applied Biosystems, USA) with TB Green® Premix Ex Taq™ II (TaKaRa, Japan) as the reaction reagent. The upstream miR-4469 primer sequence was 5’-CTCTAGGGTGCCTGCGAAA-3’. The upstream and downstream primers of U6 and the downstream primer of miR-4469 were derived from the Mir-X™miRNA First Strand Synthesis Kit (TaKaRa, Japan).

2.4.3. Transient transfection

Lipofectamine 3000 (Invitrogen, USA), miR-4469 mimics, and miR-4469 inhibitor were purchased from Guangzhou Ribo Biotechnology Co., Ltd. (Guangzhou, China). Lipofectamine and transfected RNA fragments were mixed and incubated for 10 min then added to cells and cultured for 24 h in a cell incubator until further use. The relevant sequences are as follows.

mimic sequence: 5’-GCUCUCUCUCUGGUCUCGAGGA-3’ (positive-sense strand), 5’-UCCGAGCCCAAGGAGAAGG-3’ (antisense strand).

inhibitor sequence: 5’-UCCGAGCCCAAGGAGAACG-3’.

2.4.4. Cell proliferation identified by Cell Counting Kit 8

After 24 h of transfection, the cells were counted after digestion by trypsin and seeded in a 96-well plate at a density of 1500 cells/well. The cells were measured for 5 d with five replicates each. To each well was added 10 μL Cell Counting Kit 8 (CCK8) reagent (Dojindo, Japan) and each was incubated for 2 h. The optical density (OD) at 450 nm in each well was measured using a microplate reader, and a growth curve was drawn.
2.4.5. Cell migration identified using Transwell
To each chamber (Corning Transwell, USA; 8-um-pore membrane) was added 100,000–200,000 transfected cells (200 μL cell suspension with serum-free medium) and placed in a 24-well plate. The lower chamber containing 600 μL medium with 20% serum was incubated for 48 h, after which the medium was removed. The cells at the upper surface of membranes were removed using a cotton swab, fixed with methanol, stained with 0.1% crystal violet, washed with phosphate-buffered saline, and photographed under a microscope.

2.4.6. Statistical analyses
SPSS 19.0 was used to analyze the data. Data between the two groups were analyzed by Student’s t-test for independent samples. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001, respectively, were considered statistically significant.

2.5. Enrichment analysis of miR-4469 expression-related genes
To further clarify the potential regulatory mechanism of miR-4469 in CRC, we analyzed the CRC dataset GSE104836 from the GEO database [14]. The dataset is RNA-seq data obtained by Illumina HiSeq 3000, including 10 cases of CRC samples. According to miR-4469 expression, we divided those 10 cases into the high miR-4469 expression group and the low miR-4469 expression group by median value, with 5 samples in each group. Differentially expressed genes were screened by Genespring using unpaired Student’s t-test parameters. Gene ontology enrichment analysis of differentially expressed genes was performed using the ToppGene Suite tool [26] to clarify the biological functions involved in miR-4469 expression-related genes (Fig. 4A).

2.6. Analysis of the regulatory mechanism for miR-4469
To screen for target genes that play a core role in the regulatory mechanism of miR-4469, we selected genes that are down-regulated in the miR-4469 high expression group from section 3.5 and took the intersection with miR-4469 target genes to obtain target genes with opposite expression to miR-4469. Here the target genes of miR-4469 were derived from miRTarBase [19,20] and the miRDB database [27,28]. We performed biological process enrichment analysis of the screened target genes by ToppGene Suite tool [26], and for five of the key genes that play an enrichment function, we performed immune cell infiltration analysis by TIMER tool [29,30] to clarify the correlation between the expression of these five genes and inflammatory cell infiltration in CRC tissues. Finally, we used miR-4469 mimics to overexpress miR-4469 in three CRC cell lines HCT116, SW620, and LoVo, and verified the expression alteration of five target genes by qPCR.

3. Results
3.1. Differentially expressed miRNA
For screening the differentially expressed miRNAs in CRC progression, we need to analyze miRNA expression changes in normal colorectum as well as in CRC tissues at each stage. We analyzed differentially expressed miRNAs at each stage of CRC and screened for continuously increasing miRNAs and continuously decreasing miRNAs according to the order of CRC progression. Among them, eight miRNAs (hsa-miR-135b-5p, hsa-miR-183-5p, hsa-miR-31-5p, hsa-miR-3147, hsa-miR-3648, hsa-miR-4417, hsa-miR-4482-3p, and hsa-miR-663a) with continuously increasing expression and six miRNAs (hsa-miR-194-3p, hsa-miR-1973, hsa-miR-4469, hsa-miR-451a, hsa-miR-4728-5p, and hsa-miR-5696) with...
continuously decreasing expression according to the order of CRC progression (normal → stage I → stage II → stage III → stage IV).

3.2. miRNA-target gene negative regulatory network

We retrieved the target genes of miRNAs via two experimentally validated databases, and since we obtained 290 decreasing and 295 increasing genes associated with CRC progression in a previous study, we intersected the target genes of 8 increasing miRNAs with 290 decreasing genes, and the target genes of 6 decreasing miRNAs with 295 increasing genes. Based on this negative regulatory relationship, we constructed a negative regulatory network of miRNAs and target genes, and since both are expressed in increasing or decreasing order, the miRNAs and target genes in the network are all associated with the progression of CRC (Fig. 1A). From the network, we found that the number of target genes of hsa-miR-135b-5p, hsa-miR-183-5p, and hsa-miR-31-5p was higher among the increasing miRNAs and more reports about these miRNAs, while the number of target genes of hsa-miR-4728-5p, hsa-miR-4469, and hsa-miR-194-3p was higher among the decreasing miRNAs (Fig. 1B). Among these miRNAs, miR-4469 is relatively new and less reported in the literature, more importantly, we found that the target gene types regulated by miR-4469 are consistent with the previous study [16]. Therefore, we selected miR-4469 for further study. More details can be found in result section 3.5.

3.3. Results of miR-4469 expression and survival analysis

From the above analysis, we knew that miR-4469 expression decreased continuously with the progression of CRC. In order to verify the potential inhibitory property of miR-4469 in CRC, we analyzed the expression difference of miR-4469 in cancerous and normal tissues in pan-cancer by TCGA database. We found that only in colon cancer (COAD) and rectal cancer (READ), the expression level of miR-4469 was significantly lower than that of the corresponding normal tissues, and the difference was not significant in other types of cancers (Fig. 2A), which was consistent with our expectation. By analyzing the overall survival of miR-4469 in pan-cancer we found that miR-4469 in Bladder carcinoma, Kidney renal clear cell carcinoma, Liver hepatocellular carcinoma, Lung squamous cell carcinoma, Thyroid carcinoma, and Rectum adenocarcinoma showed a positive correlation between the expression of miR-4469 and the survival time of patients (Fig. 2B), that is,

Fig. 2. Expression and survival analysis of miR-4469 in pan-cancer. A. Expression levels of miR-4469 in cancerous and normal tissues in pan-cancer; Red represents cancerous tissues, dark blue represents normal tissues, and yellow arrows show miR-4469 expression levels in colon cancer and rectal cancer. B. Survival curves of miR-4469 expression levels and survival times of multiple cancer patients; Cancer types include Bladder carcinoma, Kidney renal clear cell carcinoma, Liver hepatocellular carcinoma, Lung squamous cell carcinoma, Thyroid carcinoma, and Rectum adenocarcinoma. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
the overall survival rate of cancer patients was better when miR-4469 was highly expressed. Among them, the log-rank P-value of the survival curves of the top 5 cancer types was <0.05, while Rectum adenocarcinoma, despite the log-rank P-value >0.05, due to the fact that the number of Rectum adenocarcinoma samples was the smallest among the above cancer types and it was also positively correlated with patient survival time, which indicated that miR-4469 in Rectum adenocarcinoma has suppressor properties.

3.4. Effect of miR-4469 on the proliferation and migration in colorectal cancer cells

To verify the inhibitory effect of miR-4469 on CRC cells, we first detected the endogenous expression level of miR-4469 in CRC cells and normal colon cells by qPCR. After we detected miR-4469 expression in NCM460, HCT116, RKO, HT29, SW480, SW620, and LoVo cells, we found that miR-4469 expression was higher in HCT116 and RKO cells, while lower in SW620 and LoVo cells (Fig. 3A). In addition, the expression of miR-4469 was found to be much higher in normal colon cell NCM460 than in CRC cell HCT116, demonstrating the expression of miR-4469 is relatively low in CRC cells (Fig. 3A). SW620 and LoVo are human CRC cell lines with high invasion activity. The expression of miR-4469 was lower in the LoVo cell line (cell derived from distant organ metastasis [31]) and the SW620 cell line (cell derived from lymph node metastasis [32]), indicating a possible association of miR-4469 with CRC invasiveness.

Transient transfection was used to alter the expression of miR-4469 in CRC cells. We selected two cell lines with high miR-4469 expression, HCT116, and RKO, for miR-4469 silencing, and two cell

Fig. 3. The expression of miR-4469 in colorectal cancer cells and normal colon cell, and the effect on cell proliferation and migration ability. A. Upper: Expression levels of miR-4469 in six CRC cell lines, including HCT116, RKO, HT29, SW480, SW620, LoVo, and one normal colon cell line NCM460; Lower: Validation of miR-4469 interference and overexpression efficiency in CRC cells. B. Proliferation curves showing alterations in the proliferative ability of CRC cells after miR-4469 interference or overexpression. In which the proliferation curve after miR-4469 alteration is red and unaltered is blue. C. The number of cells penetrating the well showed alterations in the migration ability of CRC cells after miR-4469 interference or overexpression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
lines with low miR-4469 expression, SW620, and LoVo, for miR-4469 overexpression. A qPCR was used to verify the silenced and overexpression efficiencies (Fig. 3A). CCK8 and Transwell were used to detect CRC cells proliferation and migration. After overexpressing miR-4469, the proliferation of SW620 and LoVo decreased compared with that in the control group (Fig. 3B). Similarly, after miR-4469 was over-expressed, the amount of SW620 and LoVo passing through Transwell chambers decreased obviously (Fig. 3C). The above results indicated that overexpression of miR-4469 could inhibit proliferation and migration of CRC cells. In contrast, after silenced miR-4469, the proliferation of HCT116 and RKO increased compared with that in the control group (Fig. 3B). Similarly, after miR-4469 was silenced, the amount of HCT116 and RKO passing through Transwell chambers increased obviously (Fig. 3C). The above results confirmed that miR-4469 could inhibit CRC cell growth and migration, which were consistent with the bioinformatics analysis.

3.5. Results of enrichment analysis for miR-4469 expression-related genes

To clarify the biological functions involved in miR-4469 expression-related genes. We divided the 10 CRC cases from GSE104836 into the high and low miR-4469 expression groups by median value. After screening the differentially expressed genes, we obtained 861 upregulated genes and 282 downregulated genes in the high miR-4469 expression group (Fig. 4A). Enrichment analysis of biological processes showed that the upregulated genes were involved mainly in metabolic processes, such as fatty acid metabolism, organic acid metabolism, and nucleotide metabolism. The enrichment of the cell components showed that the upregulated genes were localized mainly in the mitochondria (Fig. 4B). Downregulated genes were involved mainly in biological processes related to inflammatory cell activation, such as the inflammatory response, the immune system process, leukocyte activation, and cytokine response. Enrichment of cell components showed that downregulated genes were located mainly in secretory vesicles, cell surfaces, and the extracellular matrix (Fig. 4B). These results indicate that high expression of miR-4469 could inhibit the activation of inflammatory cells, and the affected genes were mainly located in the extracellular matrix, on the other hand, high expression of miR-4469 could promote metabolic processes and the affected genes were mainly located in the mitochondria. This characteristic is consistent with our previous study [16].

In the previous study, we analyzed the continuous increasing and decreasing genes associated with CRC progression. We identified the key molecular events at each stage, among which, the key molecular events of increasing genes were mainly inflammatory cell chemotaxis, which was mainly located in the extracellular matrix. The key molecular events of the decreasing genes were metabolic processes, which were mainly localized in mitochondria [16]. The results of the enrichment analysis for miR-4469 expression-related genes in this study were coincident with molecular events related to CRC progression and were negatively correlated. This reflects that the high expression of miR-4469 is indeed negatively correlated with CRC progression. This is the main reason for our selection of miR-4469 from the negative regulatory network for further study.

The retrieval using the University of California Santa Cruz (UCSC) Genome Browser [33–35] revealed that miR-4469 was located in the intron of the RNF170 gene. Therefore, the expression of miR-4469 might be related to the RNF170 gene. While the 861 upregulated genes in the high miR-4469 expression group indeed contain RNF170, this further demonstrates the reliability of screening for miR-4469 expression-related genes.

3.6. Molecular mechanism of miR-4469 regulation of inflammatory cell infiltration

From the above analysis, we found that genes expressed opposite to the miR-4469 high expression group are mainly involved in biological processes such as inflammatory response, and leukocyte activation. Since miR-4469 is negatively regulated with the target genes, in order to further clarify the key target genes for miR-4469 functioning, we obtained 900 target genes of miR-4469 via two databases, and took the intersection of these genes with 282 genes whose expression is opposite to miR-4469 and we obtained 10 target genes (Fig. 5A). We performed enrichment analysis of these target genes and found that these genes are mainly involved
in biological processes such as neutrophil activation, among which there are five target genes mainly involved in enrichment, which are CD300A, SLC2A3, FGR, PLEKHO2, and MYO1F (Fig. 5B). Since the enrichment results correlated with neutrophil activation, we further analyzed the correlation between the expression of these five target genes and the level of neutrophil infiltration in CRC tissues, we found that these five target genes were indeed highly correlated with neutrophil infiltration in CRC tissues (Fig. 5C). In addition, on the basis of the negative regulatory relationship between miRNA and target genes, we overexpressed miR-4469 in three CRC cell lines and observed the mRNA expression alterations of these five target genes. We found that, except for CD300A, overexpression of miR-4469 downregulated the expression of the target genes SLC2A3, FGR, PLEKHO2, and MYO1F in three cells, demonstrating that SLC2A3, FGR, PLEKHO2, and MYO1F were indeed the target genes of miR-4469. Therefore miR-4469 might be targeting these four key target genes to inhibit the progression of CRC.

4. Discussion

MiRNA for tumor treatment has made progress in recent years. For the ability to target mRNA sequences of specific oncogenes in cells, some tumor-suppressor miRNAs have the potential for anti-tumor progression. The main method in tumor treatment is to recover the expression of those tumor-suppressor miRNAs resulting from their low expression. Therefore, synthesizing mimics of
these miRNAs and wrapping them in lipid carriers may be directly applicable in the treatment of tumors. Some reports indicated that MRX34, a mimic of miR-34a wrapping in liposomal, can suppress a solid tumor [36]. Another study has reported that miR-16 mimic MesomiR-1 and might be useful in the treatment of thoracic cancer [37]. Therefore, our study focused on the miRNA that can inhibit CRC progression and aimed to provide a theory for CRC treatment.

In this study, we analyzed the miRNA expression profile of CRC and screened several miRNAs related to CRC progression. We constructed a negative regulatory network of miRNA-target genes and screened a key tumor suppressor miRNA: miR-4469. There are not many studies on miR-4469. One study identified miR-4469 as a possible significant miRNA in the network by constructing a miRNA network in high-grade serous ovarian cancer [38]. Another study showed that the long non-coding RNAs HCG11 can inhibit laryngeal carcinoma progression by sponging miR-4469 followed by upregulating the expression of the suppressor gene APOM [39]. Also, circRNA_141539 was found to promote proliferation and invasion of esophageal squamous cell carcinoma cells by sponging miR-4469 followed by activation of CDK3 [40]. Some studies showed that CDK3 is a target of miR-4469, and miR-4469 is able to directly target the 3’ UTR region of CDK3, which in turn negatively regulates CDK3 [41], moreover, CDK3 could promote CRC metastasis by promoting EMT [42], which indirectly confirms that miR-4469 could suppress CRC progression by targeting CDK3. In addition, in the negative regulatory network, many target genes of miR-4469 were closely related to CRC development. For example, BACH1 could promote CRC progression via the BACH1/CXR4 pathway [43], and THBS2 was related to a poor prognosis of CRC [44,45].

In this study, we identified miR-4469 as a suppressor of CRC. Therefore, miR-4469 expression deficiency may be a risk factor for CRC. In tumors, the expression deficiency of miRNA is related to many factors. A study has shown that many miRNAs are located at fragile sites in the genome. The deletion of these sites may cause the down-regulation of miRNA [46]. Also, methylation at the miRNA gene locus can inhibit the expression of miRNA [47]. Another study has suggested that regulating the expression levels of transcription factors of miRNA can also affect the expression of miRNA. For example, miR-34a is a tumor-suppressive miRNA, which can be regulated by antioncogene P53, whereas the down-regulation of P53 in tumor tissues can inhibit the expression of miR-34a [48]. Thus, the regulation of miR-4469 expression in CRC may be affected by one or more factors.

In addition to miR-4469, several miRNAs with continuously increasing or decreasing expression during colorectal cancer progression were screened in this study, and many of these miRNAs are closely associated with cancer development. For example, among the eight continuously increased or decreased miRNAs screened in this study, it was demonstrated that miR-135b-5p was able to promote the proliferation, migration, and invasive ability of gastric cancer cells [49]. Similarly, miR-183-5p can promote the proliferation and migration of breast cancer cells [50], and miR-183-5p in exosomes can promote angiogenesis in colorectal cancer by regulating FOXO1 [51]. In addition, it has been demonstrated that miR-31-5p is able to promote proliferation, migration, and invasion of colorectal cancer cells by targeting NUMB [52]. Moreover, overexpression of miR-3147 was able to promote proliferation, migration, invasion, and G1/S transition of vulvar cancer cells [53]. Some studies have reported that miR-3648 can promote the proliferation of prostate cancer cells [54], and miR-663a was found to be significantly upregulated in the serum expression of bladder cancer patients and correlated with poor prognosis of patients [55]. Among the six continuously decreasing expressed miRNAs screened in this study, it was demonstrated that miR-194-3p was able to inhibit the proliferation, migration, and invasion of colorectal cancer cells by suppressing KLK10 [56]. In addition, miR-451a was able to inhibit colorectal cancer cell proliferation and promote apoptosis by inducing endoplasmic reticulum stress response [57]. Moreover, the expression of miR-4728-5p was down-regulated in colorectal cancer tissues [58]. These studies further confirm the close relationship between the miRNAs screened in our study and tumors.

In this study, we identified miR-4469 as an inhibitory factor associated with CRC progression and clarified the inhibitory effect of miR-4469 on CRC by bioinformatics analysis and experimental validation. In addition, we found that the molecular events involved in miR-4469 expression-related genes were consistent with the molecular events related to CRC progression in our previous study. In particular, down-regulated genes in the high miR-4469 expression group were mainly involved in inflammatory cell activation, indicating that there are some key targets in miR-4469 target genes that regulate inflammatory cells. Finally, we combined miR-4469 target genes and identified five key target genes CD300A, SLC2A3, FGR, PLEKH2, and MYO1F that were highly associated with neutrophil infiltration in CRC tissues, and demonstrated the negative regulation relationship of miR-4469 with target genes SLC2A3, FGR, PLEKH2 and MYO1F by experiments, which further clarified the molecular mechanism of miR-4469 in CRC. Therefore, we expect that this study can play a positive effect on the clinical application research of miRNA in the future.

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CRediT authorship contribution statement
Lu Qi: Conceptualization, Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. Lu Wang: Investigation. Fuyao Song: Data curation. Zhenhua Ding: Supervision. Ying Zhang: Validation, Supervision, Funding acquisition.

Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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