MiR-133a-3p Functions as a Tumor Suppressor in Colorectal Cancer by Targeting PFKFB3

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Abstract

**Background:** Recent studies reveal that PFKFB3 plays an important role in tumorigenesis and tumor progression. Our study aims to identify a novel microRNA which can suppress the expression of PFKFB3 and to provide a potential target for tumor therapy.

**Methods:** Bioinformatics methods were implemented to explore the expression and clinical significance of PFKFB3 and miR-133a-3p in colorectal cancer (CRC). qRT-PCR was performed to detect PFKFB3, miR-133a-3p, KI67 and MMP9 mRNA expression, while western blot was carried out for the detection of protein expression of PFKFB3, miR-133a-3p, KI67 and MMP9. Bioinformatics analysis was used to predict the binding sites of miR-133a-3p on PFKFB3 3’UTR, while dual-luciferase assay was conducted to validate their binding relationship. CCK-8 assay, KI67 detection, Transwell assay and MMP9 detection were employed to measure CRC cell proliferative and invasive abilities.

**Results:** PFKFB3 is up-regulated in CRC and significantly associated with poor prognosis. Overexpressed PFKFB3 promotes CRC cell proliferation and invasion. miR-133a-3p is down-regulated and has diagnostic value in CRC. Dual-luciferase assay confirmed that there was a binding relationship between miR-133a-3p and PFKFB3. Overexpressed miR-133a-3p remarkably reduced PFKFB3 expression in CRC cells, weakened the promoting effect of PFKFB3 on cell proliferation and invasion.

**Conclusions:** Our study suggests that miR-133a-3p functions as a novel tumor suppressor in colorectal cancer by targeting PFKFB3.

Introduction

Colorectal cancer (CRC) was the most commonly diagnosed gastrointestinal cancer, representing 1.8 million cases and 881,000 deaths globally, and constituting 1 in 10 cancer cases and deaths [1]. Advancements in pathophysiological understanding have increased the array of treatment options for local and advanced disease leading to individual treatment plans. Although these new treatment options have doubled overall survival for advanced disease to 3 years, survival is still best for those with non-metastasised disease. As the disease only becomes symptomatic at an advanced stage, worldwide organised screening programmes are being implemented, which aim to increase early detection and reduce morbidity and mortality from colorectal cancer [2]. Additionally, although all-ages rates of colorectal cancer have largely stabilized or been in decline in high-income countries, incidence rates appear to be rising in adults younger than 50 years. Although genetic, lifestyle, obesity, and environmental factors might have some association, the exact reasons for this increase are not completely understood [3].

6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFK-2/FBPase 3, PFKFB3) is a member of a homodimeric and bifunctional enzyme family (PFKFB1-4) [4], which controls the intracellular steady-state concentration of fructose 2,6-bisphosphate (F2,6P2) [5]. F2,6BP is a potent activator of PFK-1, and PFK-1 is essential for the rate-limiting step of glycolysis [6]. The bifunctional isoenzyme encoded by the pfkfb3...
gene has the highest kinase: phosphatase activity ratio, which in turn sustains high glycolytic rates [7]. PFKFB3 expression could be upregulated in response to mitogenic, inflammatory and hypoxia stimuli and during the DNA synthesis phase of the cell cycle. Considering its significance in cancer metabolism, further explanation of the function of PFKFB3 in diverse cancers is necessary. According to previous studies, the expression of PFKFB3 gene can be regulated by different mechanisms such as signal pathways, oncogenes, noncoding RNAs, inflammatory molecules, and the PFKFB3 protein regulated both at the transcriptional and post-transcriptional levels. Although the glycolytic role of PFKFB3 in cancer progression has been the subject of numerous functional studies, some researchers have also focused on the functions of PFKFB3 beyond glycolysis. PFKFB3 expression may not only be essential for the regulation of glycolysis in the cytoplasm, but also in the control of the cell cycle in the nucleus and maintenance of an anti-apoptotic state. PFKFB3 has been suggested to play a crucial role in many types of tumor cells as well as various cells in the tumor microenvironment.

miRNAs, a class of noncoding RNA (ncRNA) about 22-nucleotide RNAs in size, are highly conserved molecules regulating gene expression post-transcriptionally by binding to the 3′-untranslated region (3′-UTR) of target message RNAs (mRNAs) [8] and participating tumorigenesis, proliferation, invasion and drug resistance in cancer [9].

miR-133a-3p, which belongs to the miR-133 family, was first experimentally characterized in mice [10]. In the human genome, miR-133a-3p is a multicopy gene, with two known copies: miR-133a-1 and miR-133a-2, located on chromosomes 18, 20 respectively. To date, multiple functional roles of miR-133a-3p have been elucidated, such as regulating myoblast proliferation and differentiation [11], inhibiting embryonic cardiomyocyte proliferation [12] and avoiding genetic cardiac hypertrophy [13]. Furthermore, it has been reported that miR-133a-3p is among the most frequently downregulated miRNAs in various types of human malignancies, which suggested that miR-133a-3p may serve a critical part in tumor progression of various malignancies, including non-small cell cancer [14], ovarian cancer [15], colorectal cancer [16], bladder cancer [17], breast cancer [18] and prostate cancer [19].

In this study, we predicted PFKFB3 was a target gene of miR-133a-3p by bioinformatics analysis. qRT-PCR, western blot and luciferase assays were used to verify the relationship between PFKFB3 and miR-133a-3p, and CCK8 assay and transwell assay were applied to elucidate the effect of the miR-129-5p/PFKFB3 axis on the occurrence and development of CRC. Overall, our results demonstrated that miR-133a-3p suppresses the progression of GCs by targeting PFKFB3.

Materials And Methods

Bioinformatic analysis

The online databases Oncomine (https://www.oncomine.org/resource/login.html), TCGA (https://portal.gdc.cancer.gov/) and THPA (https://www.proteinatlas.org/) were applied to explore the expression level and clinical significance of PFKFB3 in CRC. Target miRNA prediction for PFKFB3 was performed by the TargetScan (http://www.targetscan.org/vert 72/) (19) and ONCOMIR
The expression and clinical data of miR-133a-3p were obtained from the TCGA-COAD dataset.

**Cell culture**

Five CRC cell lines (LDL1, LOVO, HT29, SW480, CaCO-2) and a normal human normal colonic epithelial cell line (NCM460) were obtained from the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in DMEM medium (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin, and incubated at 37°C and 5% CO2.

**Cell transfection**

The oligonucleotides of miR-133a-3p mimics, mimics control, miR-133a-3p inhibitor, control inhibitor, PFKFB3 overexpression vector and PFKFB3 siRNA were purchased from HIPPOBIO (China) were transfected into CRC cell line SW480 by Lipo2000 (ThermoFisher, USA) kit in accordance with instructions. After 24h of transfection, transfected cells were used for subsequent experiments.

**Reverse transcription-quantitative polymerase chain reaction**

Total RNA was isolated from cells using TRIzol kit (Life Technologies, USA). The cDNA was synthesized using PrimeScript™ RT Master Mix Kit for qPCR (TaKaRa, Japan) and miRNA First Strand cDNA Synthesis (Sangon Biotech, China) according to relevant protocols. miR-133a-3p, PFKFB3, KI67 and MMP9 mRNA levels were quantified using Power SYBR™ Green PCR Master (Invitrogen, USA) with StepOne Plus Realtime PCR system. U6 and Actin were used as an internal standard control for miRNA and mRNA detection, respectively. Primer sequences used in qRT-PCR were listed in Table 1. The relative expression of miR-133a-3p and PFKFB3 mRNA was presented by $2^{-\Delta\Delta Ct}$ method. The experiment was repeated for three times.

**Table 1** Primer sequences used in qRT-PCR
Gene | Primer sequence
--- | ---
mir-133a-3p | Forward: TTTGGTCCCCTTCAACCAGCTG
| Reverse: GGAACGCTTCACGAATTTG
U6 | Forward: ATTGGAACGATACAGAGAAGATT
| Reverse: GGAACGCTTCACGAATTTG
PFKFB3 | Forward: AAACTGACGCCTGTCGCTTA
| Reverse: CCGGGAGCCTTTCATGTGGTGGT
KI67 | Forward: ATGGAGAGGTGGCCAAGAAC
| Reverse: TGTGTGGTCTGTGTGAGCTG
MMP9 | Forward: GGTGATTGACGACGCCTTTG
| Reverse: GGACCACAACCTCGTCATCGT
Actin | Forward: GGACTTCGAGCAAGAGATGG
| Reverse: AGCACTGTGTTGGCGTACAG

**Western blot analysis**

Total proteins were harvested after cells were lysed by RIPA lysis buffer, and protein concentration was assayed by BCA kit (Beyotime, China). After being denatured at a high temperature, proteins were isolated by sodium dodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes (PVDF; Millipore), which were then blocked with 5% skim milk for 2 h. Then the membranes were incubated with primary antibodies overnight at 4℃. Rabbit anti-PFKFB3 (ET1705-66, 1:1000, HUABIO), rabbit anti-KI67 (ab16667, 1:1000, Abcam), rabbit anti-MMP9 (ab76003, 1:1000, Abcam) and mouse anti-β-Actin (EM21002, 1:5000, HUABIO). Antibodies were added and incubated at 4℃ overnight. Subsequently, the membranes were incubated with secondary antibody goat anti-mouse IgG (1:5000, D110058, BBI) and goat anti-rabbit IgG (1:5000, D110057, BBI). After culture for 2 h at room temperature, all protein bands were visualized using an enhanced chemiluminescence kit (GE Healthcare, USA).

**Cell proliferation assay**

The cell proliferation ability of SW480 cells was measured using the cell counting kit-8 assay (CCK-8) (MCE, USA). Cells were counted and seeded into the 96-well plate with 3,000 cells/well, and then incubated with 5% CO2 at 37 °C for 72 h. The absorbance of cultured CRC cells was measured with the micro-plate reader at 450 nm after CCK-8 solution addition for 2 h.

**Transwell invasion assay**
The 24-well transwell chambers (Corning, NY, USA) with or without the Matrigel (Invitrogen) were used to perform the transwell assays. $1 \times 10^5$ SW480 cells were seeded into the upper chambers in serum-free medium, while the serum-supplied medium was added to the lower chamber in 24-well plates. After being cultured for 48 h at 37 °C, the non-invaded cells were removed, while the invaded cells were stained with 0.1% crystal violet. Numbers of stained cells in the bottom chambers were assessed from five randomly selected fields, and the data were summarized from three individual experiments. The experiment was conducted for three times.

**Luciferase reporter assay**

To determine the binding relationship between miR-133a-3p and PFKFB3 3'-UTR, luciferase vectors pmiRGO (Promega, USA) fused with wild type (WT) SPN 3'-UTR or mutant (MUT) SPN 3'-UTR were established. These constructed vectors were cotransfected with miR-133a-3p mimic or mi-NC into SW480 cells using Lipofectamine 2000. Then, 48 h after transfection, the luciferase activity was detected using a dual-luciferase reporter assay system (Promega, United States).

**Statistical analysis**

All experiments were executed at least three independent times. The statistical data are presented as mean ± standard deviation and analyzed using GraphPad Prism 6.0 (CA, United States). Two-tailed Student’s t-tests were conducted to interpret the differences. P value <0.05 was considered statistically significant.

**Results**

**PFKFB3 is up-regulated in CRC and significantly associated with poor prognosis**

To explore the expression of PFKFB3 mRNA and protein in CRC cells, online databases ONCOMINE, TCGA and THPA were used. We found that PFKFB3 mRNA is up-regulated in 7 types of tumors including colorectal cancer (Fig. 1a). The TCGA-COAD data showed that PFKFB3 mRNA was prominently up-regulated in CRC tissue (Fig. 1b). The THPA data showed the PFKFB3 protein is high expressed in colorectal cancer (Fig. 1c). For further confirmation, we detected the expression of PFKFB3 mRNA and protein in five CRC cell lines (LDL1, LOVO, HT29, SW480, CaCO-2) and a normal human normal colonic epithelial cell line (N460). The results showed that PFKFB3 is up-regulated in all the five CRC cell lines (Fig. 1d.e). In addition, according to the K-M survival curve plotted by THPA online tool, the up-regulated PFKFB3 is significantly associated with poor prognosis (Fig. 1f).

**PFKFB3 affects CRC cell proliferation and invasion**

To evaluate whether PFKFB3 contributes to the process of CRC, we established the PFKFB3 overexpressed and silenced CRC cell lines via SW480. We first confirmed the expression of PFKFB3 by
qRT-PCR and western blot (Fig. 2a-b). CCK8 assay and Ki67 detection suggested that overexpression of PFKFB3 markedly enhanced CRC cell proliferative ability, while silencing of PFKFB3 inhibited the proliferation of CRC cells (Fig, 2d-f). Transwell assay and MMP9 detection demonstrated that overexpression of PFKFB3 promotes the invasion of CRC cells, on the contrary silencing of PFKFB3 suppressed the invasion of CRC cells (Fig. 2g-i). Taken together, these results demonstrated that PFKFB3 enhanced CRC cell proliferation and invasion.

Low expression of miR-133a-3p has diagnostic value in CRC

The online databases Targetscan and ONCOMIR were applied to predict the regulating miRNAs of PFKFB3, we combined the prediction results of two databases. As a result, miR-26-5p and miR-133a-3p were selected as potential regulators of PFKFB3 (Additional file2). To explore the expression of these microRNAs in CRC, a TCGA-COAD dataset was analysed. The results showed that miR-26a-5p and miR-26b-5p is up-regulated in colorectal cancer, while miR-133a-3p is down-regulated in colorectal cancer (Fig. 3a-c). Further more, we validated the expression level of miR-133a-3p in normal colonic epithelial cell(NCM460) and CRC cell line SW480. Additionally a ROC diagnostic curver plotted by TCGA data demonstrated that low expression of miR-133a-3p has diagnostic value in colorectal cancer

miR-133a-3p directly targets PFKFB3

To further explore the regulatory effect of miR-133a-3p on PFKFB3, bioinformatics analysis was used to predict the binding sites of miR-133a-3p on PFKFB3 3’-UTR, indicating that there were binding sites between miR-133a-3p and PFKFB3 (Fig. 4a). Subsequently, dual-luciferase assay validated the targeted binding relationship between miR-133a-3p and PFKFB3, finding that overexpression of miR-133a-3p inhibited the luciferase activity of PFKFB3-WT 3’-UTR, while had no influence on that of PFKFB3-MUT 3’-UTR (Fig. 4b). Taken together, these findings elucidated that miR-133a-3p targeted and down-regulated PFKFB3 in CRC cells.

miR-133a-3p Inhibites the Proliferation and Invasion of CRC cells by targeting PFKFB3

To investigate whether miR-133a-3p regulates CRC cell behaviors by targeting PFKFB3, we established miR-mimics (mimics + control), miR-inhibitor (inhibitor + control) and PFKFB3-OE (miR-mimics + PFKFB3-OE) groups. qRT-PCR and western blot were used to assess PFKFB3 expression in different groups, finding that PFKFB3 was potently down-regulated by miR-133a-3p in miR-mimics and PFKFB3-OE groups (Fig. 5a, b). CCK8 assay and Ki67 detection suggested that the proliferative ability of CRC cells was suppressed by miR-133a-3p in miR-mimics and PFKFB3-OE groups (Fig. 5d-f). Transwell assay and MMP9 detection demonstrated that miR-133a-3p mimics can inhibit the invasion of CRC cells in miR-mimics and PFKFB3-OE groups(Fig. 5g-i). On the contrary, when miR-133a-3p was inhibited, its inhibitory effect...
on cell proliferation and invasion disappeared. Collectively, it could seen that miR-133a-3p supressed cell proliferation and invasion via targeting PFKFB3.

Discussion

In recent years, PFKFB3 has been studied in various cancer cells. Most studies have demonstrated that the overexpression of PFKFB3 promoted cancer cell growth, proliferation, migration and metastasis [20]. In addition, inhibition of PFKFB3 expression by siRNA [21], miRNA [22] and inhibitor [23] decreases the growth, proliferation and migration of these cells. In this study we found that PFKFB3 was significantly up-regulated in CRC by bioinformatics analysisi and cell experiments. Besides, according to our study PFKFB3 could act as a promoter to CRC cell proliferation and invasion. These findings provide a better understood to the regulatory mechanism of PFKFB3 in CRC.

Despite several different kinds of miRNAs, such as miR-206, miR-26b, and miR-330 have been reported to decrease the expression of PFKFB3 [23–24]. We identify a novel miRNA targeting PFKFB3 by combining the bioinformatics prediction results and TCGA expression data. Our research showed miR-133a-3p is significantly down-regulated in CRC and SW480 cell line, and the detection of miR-133a-3p has diagnostic value in CRC. miR-133a-3p has been reported as a supressor of several different oncogenes such as COL1A1, CORO1C and CREB1 [25–27], The regulatory mechanism of the miR-133a-3p/PFKFB3 axis in CRC have not been reported to date. As revealed by cell experiments, we found that PFKFB3 can be supressed by miR-133a-3p mimics. Dual-luciferase assay further validated that there was a targeted binding relationship between miR-133a-3p and PFKFB3. Additionally, rescue experiments indicated that miR-133a-3p suppressed CRC cell proliferation and invasion by targeting PFKFB3.

Conclusion

We identify miR-133a-3p as a novel biomarker in CRC, and also an suppressor of cell proliferation and invasion in CRC, wherein the targeted PFKFB3 plays a critical role. These findings illustrate the regulatory mechanism of miR-133a-3p and PFKFB3 in CRC and provide novel potential therapeutic targets for CRC additional insight into the exploration of new biomarkers and target therapies for ccRCC.

However, the molecular mechanisms of miR-133a-3p function in CRC cell proliferation and invasion are not fully unveiled, suggesting that other targets such as lincRNAs may exit to mediate its function. Further research is needed to better exploit miR-133a-3p as a therapeutic target in CRC treatment.

Abbreviations

CRC: Colorectal cancer

COAD: Colon adenocarcinoma
PFKFB3: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets analyzed for this study were obtained from Oncomine (https://www.oncomine.org/resource/login.html), TCGA (https://portal.gdc.cancer.gov/) and THPA (https://www.proteinatlas.org/) and THPA (https://www.proteinatlas.org/).

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Yanjie Zhou and Lu Jiang designed this study and drafted the manuscript. Yuanjun Cai and Wendong Tang performed the experiments. Ke Wang analyzed the data in this study. All authors reviewed the final version of the manuscript.

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References


Figures
Figure 1

PFNFB3 is up-regulated in CRC and significantly associated with poor prognosis. 

- **a** Summary for PFNFB3 mRNA level in pancancer by ONCOMINE database ($P<0.01$, Fold change $>2$).
- **b** Box plots of PFNFB3 expression in normal and tumor group in TCGA-COAD dataset ($P<0.001$).
- **c** PFNFB3 mRNA expression level in normal and CRC cell lines.
- **d** Summary for PFNFB3 protein level in pancancer by THPA database.
e PFKFB3 protein expression level in normal and CRC cell lines. f Survival analysis of PFKFB3 in clone adenocarcinoma (P<0.01).

Figure 2

PFKFB3 affects CRC cell biological functions. The mRNA expression a and protein expression b of PFKFB3 were detected by qRT-PCR and western blot. The mRNA expression c and protein expression d of Ki67 were detected by qRT-PCR and western blot. The mRNA expression e and protein expression f of MMP9 were detected by qRT-PCR and western blot. The mRNA expression g and protein expression h of Actin were detected by qRT-PCR and western blot. The mRNA expression i and protein expression j of cell number were detected by qRT-PCR and western blot.
MMP9 were detected by qRT-PCR and western blot. g Results of western blot. h Cell proliferation ability detected by CCK8 assay. i,j Cell invasion ability detected by Transwell assay. (* p<0.05; ** p<0.01; *** p<0.001)

**Figure 3**

Low expression of miR-133a-3p has diagnostic value in CRC. Relative expression of a miR-26a-5p, b miR-26b-5p and c miR-133a-3p. d Relative expression of 133a-3p in NCM460 and SW480 (P<0.001). e ROC diagnostic curve of miR-133a-3p in CRC (AUC=0.993)
Figure 4

miR-133a-3p directly targets PFKFB3. a The binding sites of miR-133a-3p on PFKFB3’s 3’UTR were predicted by bioinformatics analysis. b The luciferase activity in different groups was detected by dual-luciferase assay.
miR-133a-3p Inhibites the Proliferation and Migration of CRC cells by targeting PFKFB3. The mRNA expression a and protein expression b of PFKFB3 were detected by qRT-PCR and western blot. The mRNA expression c and protein expression d of KL67 were detected by qRT-PCR and western blot. The mRNA expression e and protein expression f of MMP9 were detected by qRT-PCR and western blot. h Cell
proliferation ability detected by CCK8 assay. g Results of western blot i,j Cell invasion ability detected by Transwell assay. (* p<0.05; ** p<0.01; *** p<0.001)

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- relativeexpressionofPFKFB3.pzfx