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

Yanjie Zhou
The Affiliated Jiangyin Hospital of Southeast University Medical School
Lu Jiang
Wuxi Center for Disease Control and Prevention
Yuanjun Cai
The Affiliated Jiangyin Hospital of Southeast University Medical School
Wendong Tang
The Affiliated Jiangyin Hospital of Southeast University Medical School
Ke Wang (wangk@jyrmyy.com)
The Affiliated Jiangyin Hospital of Southeast University Medical School https://orcid.org/0000-0003-2684-8686

Research Article

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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 an 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 bot 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 expression is up-regulated in colorectal cancer, and is significantly associated with poor prognosis. Silencing PFKFB3 could inhibit the proliferation and invasion of colorectal cancer cells. miR-133a-3p is down regulated in colorectal cancer, which has diagnostic value for colorectal cancer. Dual luciferase assay confirmed that PFKFB3 was the direct acting site of miR-133a-3p. Overexpression of miR-133a-3p could significantly reduce the expression of PFKFB3 and inhibit the effect of PFKFB3 on the proliferation and invasion of colorectal cancer cells.

Conclusions

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

Introduction

Colorectal cancer (CRC) was the most common gastrointestinal cancer, which represented 1.8 million cases and 881,000 deaths globally, accounting for one tenth of cancer cases and deaths [1]. In high income countries, colorectal cancer incidence rate is largely stabilized or been in decreasing, incidence rate seems to be increasing among adults younger than 50 years. This rising trend may be related to genetic, lifestyle, obesity and environmental factors, but the exact reason is not completely clear. [3].Recent studies have provided new treatment options for CRC. Although these new treatments have

doubled the overall survival rate of patients with advanced diseases, the survival rate is still lower than those without metastasis. In order to improve the incidence rate of early colon cancer and reduce its morbidity and mortality, the worldwide screening program is being implemented.[2].

6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFK-2/FBPase 3, PFKFB3) is a member of a bifunctional enzyme family (PFKFB1-4) [4], which contorls the intracellular concentration of fructose 2,6-bisphosphate(F2,6P2) [5]. F2,6BP is a potent actibator 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 helps to maintain a high rate of glycolysis in cells [7, 8]. 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 can be regulated both at the transcriptional and post-transcriptional levels [9]. Although the glycolytic role of PFKFB3 in cancer progression has been the main field of numerous functional studies, some researchers have also focused on the functions of PFKFB3 beyond glycolysis [10, 11]. Recent studies have shown that the expression of PFKFB3 is crucial not only for the regulation of cytoplasmic glycolysis, but also for the control of nuclear cell cycle and anti-apoptosis. [12, 13]. 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 22–24 nucleotide RNAs in size, are involved in the regulation of post transcriptional gene expression in animals and plants by binding to the 3’-untranslated region (3’-UTR) of target mRNAs [14] and participating tumorigenesis, proliferation, invasion and drug resistance in cancer [15].

miR-133a-3p, which belongs to the miR-133 family, was first experimentally characterized in mice [16]. miR-133a-3p is a multicopy gene in the human genome, with two copies: miR-133a-1 and miR-133a-2. To date, multiple functional roles of miR-133a-3p have been elucidated, such as regulating myoblast proliferation and differentiation [17], inhibiting embryonic cardiomyocyte proliferation [18]and avoiding genetic cardiac hypertrophy [19].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 [20], ovarian cancer [21], colorectal cancer [22], bladder cancer [23], breast cancer [24] and prostate cancer [25].

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 (https://www.biosino.org/dbDEMC/index) websites. 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℃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	Foward	TTTGGTCCCCTTCAACCAGCTG
U6	Foward	ATTGGAACGATACAGAGAAGATT
	Reverse	GGAACGCTTCACGAATTTG
PFKFB3	Foward	AAACTGACGCCTGTCGCTTA
	Reverse	CCGGGAGCCTTTCATGTTTTG
KI67	Foward	ATGGAGAGGTGGCCAAGAAC
	Reverse	TGTGTGGTCTGTGTGAGCTG
MMP9	Foward	GGTGATTGACGACGCCTTTG
	Reverse	GGACCACAACTCGTCATCGT
Actin	Foward	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 fuoride 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 ℃ 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. 1×10^5 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 pmirGLO (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 (NCM460). The results showed that PFKFB3 is up-regutated 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).

Silencing of PFKFB3 can inhibit the proliferation and invasion of CRC cells

To evaluate weather PFKFB3 contributes to the progress of CRC, we established the PFKFB3 silenced CRC cell line via SW480. We first detected the expression of PFKFB3, KI67 and MMP9 by RT-PCR(Fig. 2a-c) and western blot(Fig. 2d-g). The results showed that KI67 and MMP9 were downregutated when PFKFB3 was successfully silenced. CCK8 assay suggested that silencing of PFKFB3 inhibited the cell proliferation of SW480 cell line (Fig, 2h). Transwell assay demonstrated silencing of PFKFB3 supressed the invasion of CRC cells (Fig. 2i). Taken together, these results domonstrated that silencing of PFKFB3 can inhibit the proliferation and invasion of CRC cells.

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

The online batabases 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, we predicted the binding site of mir-133a-3p on PFKFB3 3'-UTR by bioinformatics analysis. (Fig. 4a). Subsequently, double luciferase analysis was used to verify the targeted binding relationship between mir-133a-3p and PFKFB3. The results showed that overexpression of mir-133a-3p inhibited the luciferase activity of PFKFB3-WT 3'-UTR, but had no effect on the luciferase activity of PFKFB3-MUT 3'-UTR (Fig. 4b). In conclusion, these results suggest that mir-133a-3p can target PFKFB3 and inhibit its expression in colorectal cancer 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(Fig. 5a-c) and western blot(5d-g) were used to assess PFKFB3, KI67 and MMP9 expression in diferent groups, finding that PFKFB3 was potently down-regulated by miR-133a-3p in different groups. CCK8 assay (fig. h) and KI67 detection suggested that the proliferative ability of CRC cells was supressed by miR-133a-3p in miR-mimics and PFKFB3-OE groups. Transwell assay (fig. i)and MMP9 detection demonstrated that miR-133a-3p mimics can inhibite the invasion ability of CRC cells in miR-mimics and PFKFB3-OE groups. 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.

Disscussion

In recent years, PFKFB3 has been studied in various cancer cells. Most studies have demonstrated that the overexoression of PFKFB3 promoted cancer cell growth, proliferation, migration and metastasis [26]. In addition, inhibition of PFKFB3 expression by siRNA [27], miRNA [28] and inhibitor [29] decreases the growth, proliferation and migration of these cells. In this study we found that PFKFB3 was significantly up-regulated in CRC by bioimformatics 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 [29–31]. 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 [31–33], 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 fndings illustrate the regulatory mechanism of miR-133a-3p and PFKFB3 in CRC and provide novel potential therapeutic targets for CRC.

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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Author details

[1]Department of Clinical Laboratory, The Affiliated Jiangyin Hospital of Southeast University Medical School, Jiangyin, Jiangsu 214400, P.R. China

[2]Laboratory, Wuxi Center for Disease Control and Prevention, No.499 Jincheng Road, Wuxi, Jiangsu 214023, P.R. China

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Figures

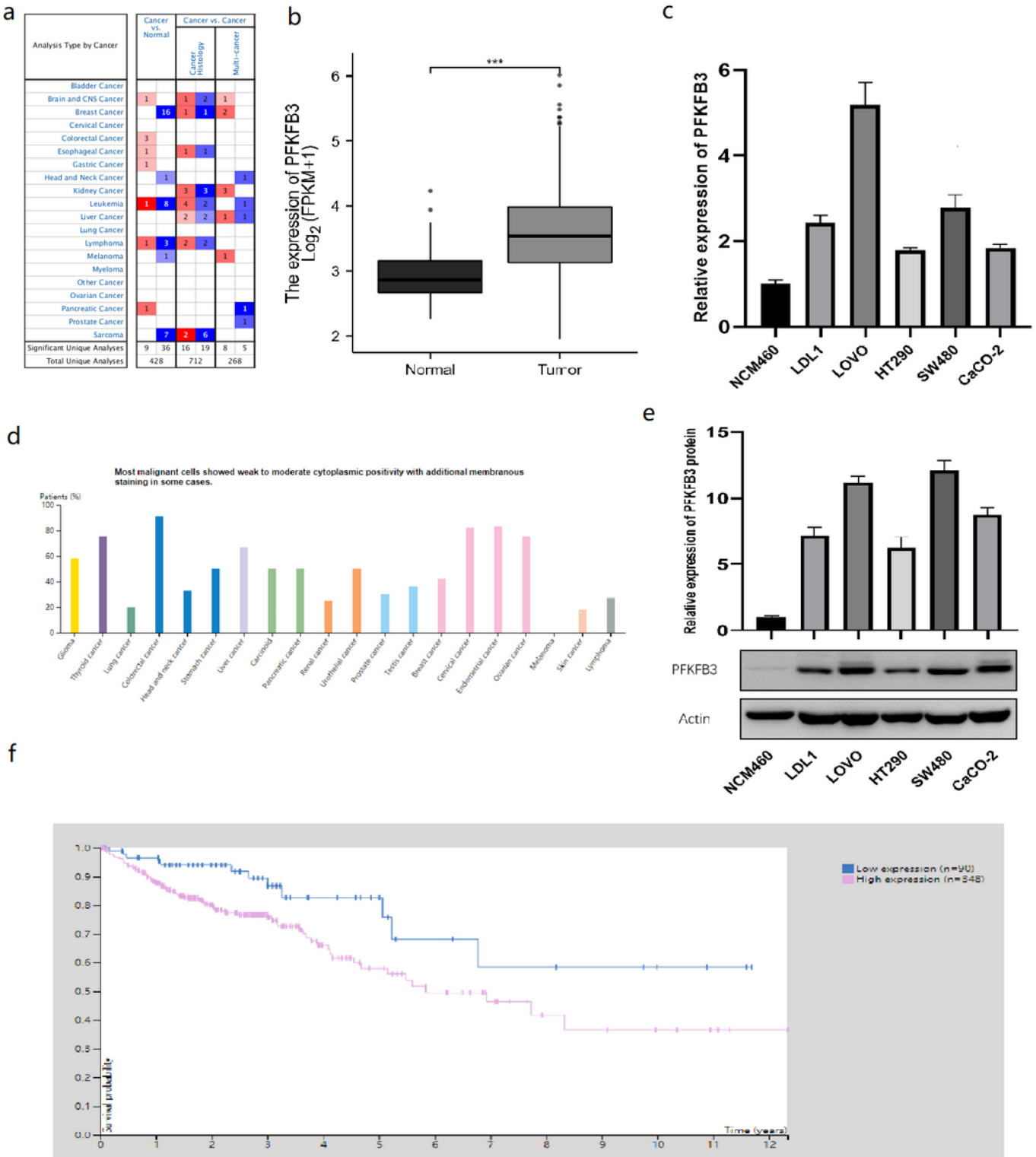

Figure 1

PFKFB3 is up-regulated in CRC and significantly associated with poor prognosis. a Summary for PFKFB3 mRNA level in pancancer by ONCOMINE database(P<0.01, Fold charge>2). b Box plots of PFKFB3 expression in normal and tumor group in TCGA-COAD dataset (P<0.001). c PFKFB3 mRNA expression level in normal and CRC cell lines. d Summary for PFKFBF3 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).

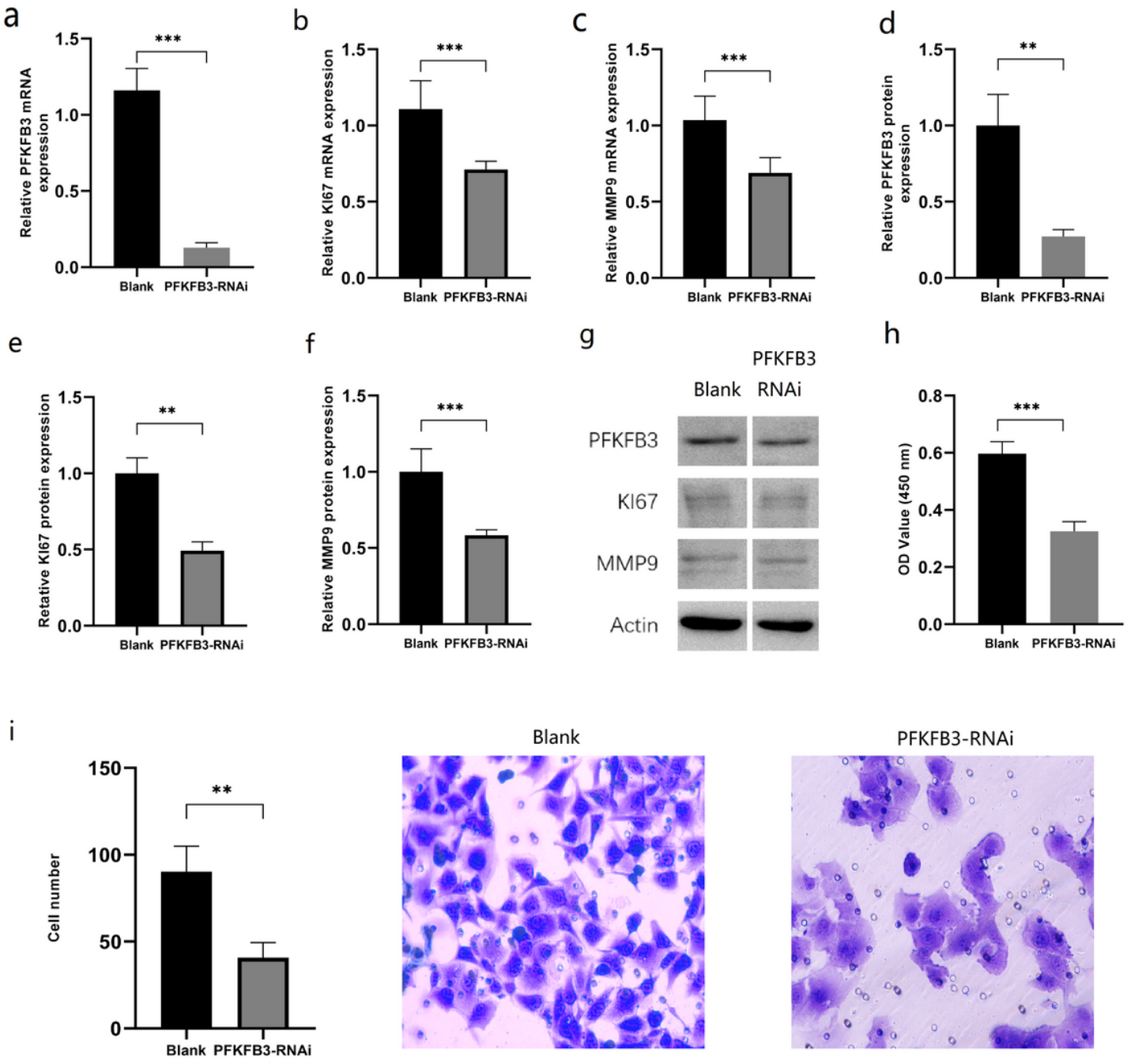

Figure 2

Silencing of PFKFB3 can inhibit the proliferation and invasion of CRC cells. The mRNA a-c and protein expression d-g of PFKFB3, KI67 and MMP9 in CRC cell line SW480 after PFKFB3 was silenced (Western blot results of different groups were from the same membrane). h Cell proliferation ability detected by CCK8 assay. i Cell invasion ability detected by Transwell assay. (* p<0.05; ** p<0.01; *** p<0.001)

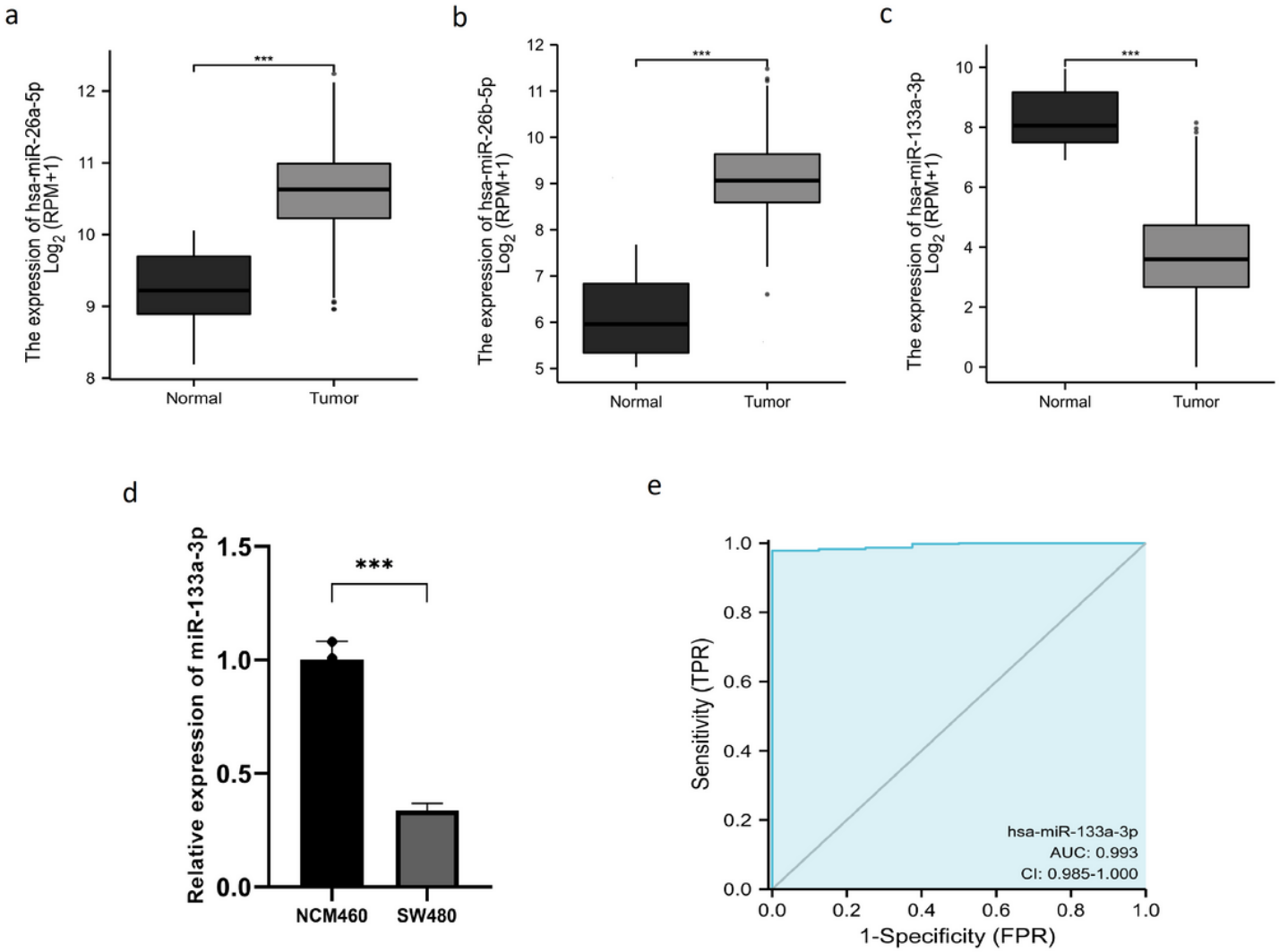

Figure 3

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)

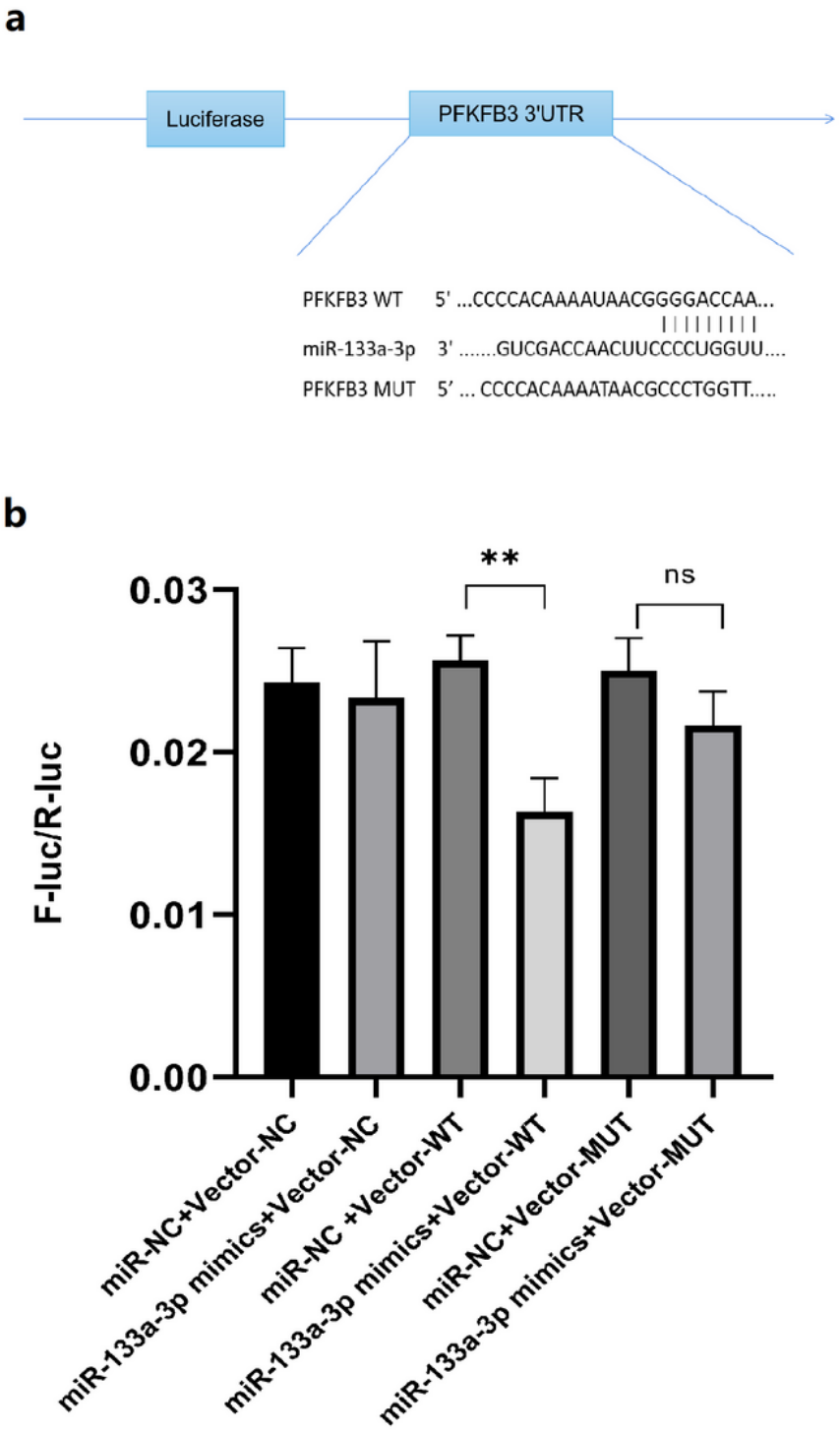

Figure 4

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.

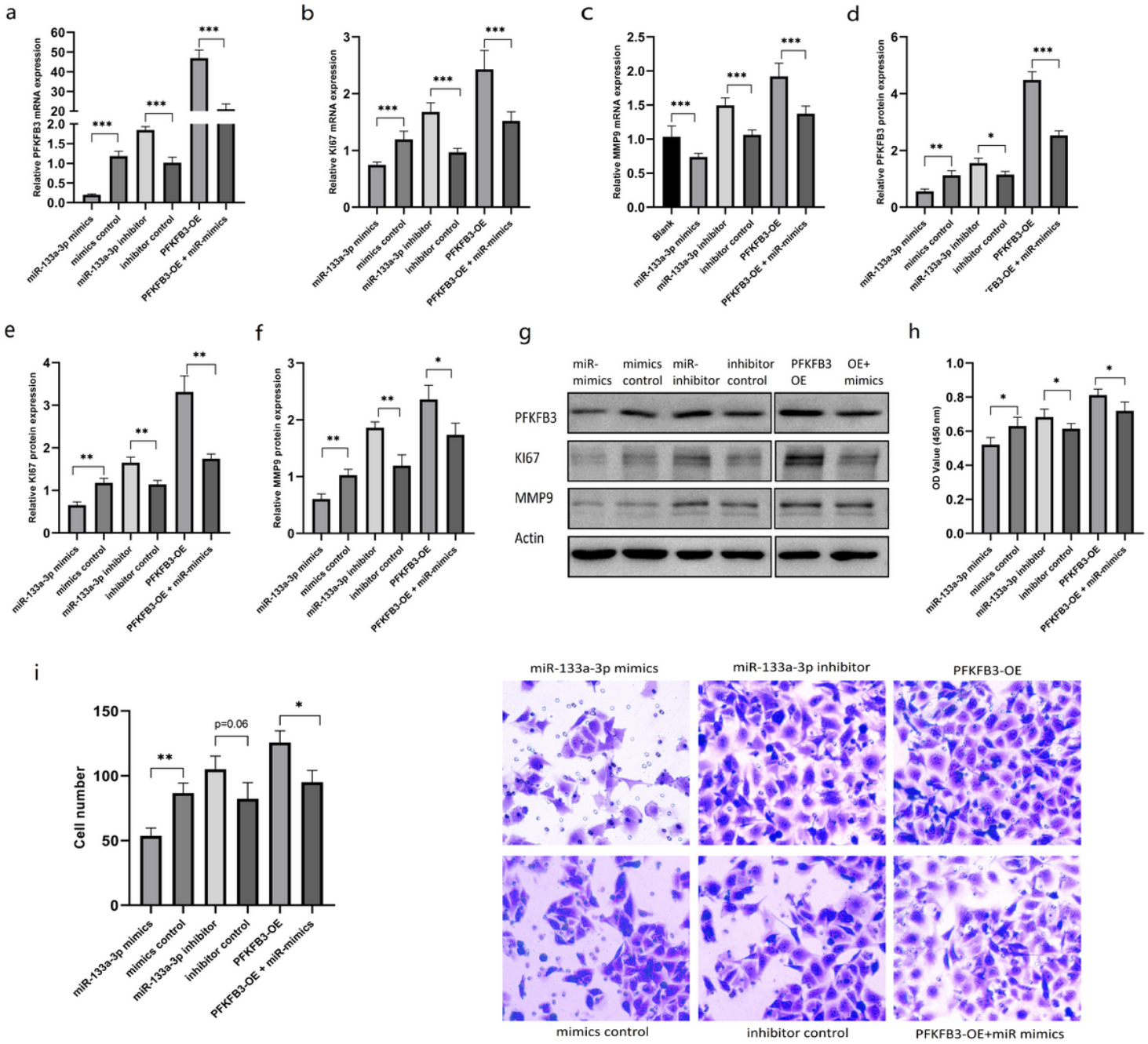

Figure 5

miR-133a-3p inhibites the proliferation and migration of CRC cells by targeting PFKFB3. The mRNA expression a-c and protein expression d-g of PFKFB3, KI67 and MMP9 were detected by qRT-PCR and western blot(Western blot results of different groups were from the same membrane). h Cell proliferation ability detected by CCK8 assay. i Cell invasion ability detected by Transwell assay. (* p<0.05; ** p<0.01; *** p<0.001)