Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

MiR-200b-3p is involved in colorectal cancer progression by targeting DDIT4

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Article Info

Article history:

Received: March 13, 2025
Accepted: September 26, 2025
Published: November 30, 2025

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Abstract

This study aimed to explore the functional dynamics between microRNA-200b-3p (miR-200b-3p) and DNA damage-induced transcript 4 (DDIT4) in colorectal cancer (CRC) and their potential as therapeutic targets. Pan-cancer analysis was conducted to evaluate DDIT4 expression across multiple cancer types. Immunohistochemical staining of CRC clinical samples was performed to confirm DDIT4 protein levels. Functional assays, including cell proliferation, migration, and invasion analyses, were used to assess the effects of DDIT4 silencing in CRC cells. Bioinformatics and experimental validation identified microRNAs targeting DDIT4 and their prognostic significance using GEPIA, HPA and ENCORI databases. Pan-cancer analysis showed DDIT4 was highly expressed in CRC compared to other cancers. Immunohistochemistry confirmed moderate to high DDIT4 expression in CRC patient samples. Knockdown of DDIT4 significantly reduced proliferation, migration, and invasion of SW480 CRC cells. miRNA analysis identified miR-200b-3p as a potential regulator of DDIT4. Low expression of miR-200b-3p correlated with poor prognosis in CRC patients. Luciferase reporter assays confirmed direct binding of miR-200b-3p to DDIT4 mRNA. Furthermore, overexpression of DDIT4 was shown to mitigate the tumor-suppressive effects of miR-200b-3p, restoring proliferation, migration, and invasion. DDIT4 promotes CRC progression and is regulated by miR-200b-3p. Targeting the miR-200b-3p/DDIT4 axis may represent a novel therapeutic approach for CRC treatment.

Keywords: Colorectal cancer, miR-200b-3p, DDIT4, Proliferation, Migration, Invasion.

1. Introduction

Colorectal cancer (CRC) is the most common malignant tumor of the digestive system and a leading cause of cancer-related deaths worldwide [1]. It ranks third in incidence and second in mortality among cancers globally, with an increasing trend in younger populations [2, 3]. Projections estimate more than 2.2 million new cases of CRC and 1.1 million deaths worldwide by 2030 [4]. The complex molecular heterogeneity of CRC contributes to the poor therapeutic efficacy of current chemotherapy [5]. Therefore, a deeper understanding of the complex molecular regulatory network of CRC is essential to provide effective therapeutic strategies for clinical treatment.

DNA damage-induced transcript 4 (DDIT4) is a gene that plays a critical role in cellular response to stress, particularly in DNA damage and hypoxia [6]. Under normal conditions, DDIT4 expression is relatively low across various human tissues, including brain, heart, liver, lung, intestine and stomach. However, it is finely tuned to act as a sensor for cellular stress, with its expression dramatically increasing in response to adverse conditions [7]. DDIT4 functions as part of the intricate network that regulates cell survival and apoptosis [6]. By inhibiting the mechanistic target of rapamycin (mTOR) pathway, it mediates the cell's adaptive response to nutrient availability and environmental stresses, balancing the promotion of growth under favorable conditions against the conservation of energy during stress. This inhibition is crucial for the modulation of cellular growth, proliferation, and angiogenesis, as well as for maintaining genomic stability in response to DNA damage [6, 8, 9]. Recent studies have shown that DDIT4 dysregulation is present in various human malignant tumors, including CRC, indicating its role in cancer progression [10-13].

MicroRNAs (miRNAs) are a class of non-coding RNA molecules that play a pivotal role in the regulation of gene expression. These small, approximately 22-nucleotide-long RNA strands exert their influence by binding to the mRNAs of genes, typically leading to the repression of protein synthesis or the degradation of the mRNA itself. As post-transcriptional regulators, miRNAs are instrumental in a wide array of biological processes, including development, cell proliferation, differentiation, and apoptosis [14-16]. miR-200b-3p has garnered significant attention. It is known for its potent ability to suppress epithelial-to-mesenchymal transition

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Doi: http://dx.doi.org/10.14715/cmb/2025.71.11.1

(EMT) by targeting the transcriptional repressors of E-cadherin, ZEB1 and ZEB2, which are key mediators in the transition towards a mesenchymal phenotype [17-20]. miR-200b-3p has been identified as a tumor suppressor due to its role in inhibiting the invasive and migratory capacities of cancer cells. It is involved in the intricate balance of cellular signaling pathways that govern cancer cell phenotype, influencing not only EMT but also angiogenesis, stemness, and chemoresistance [21, 22]. The expression levels of miR-200b-3p have been correlated with patient prognosis, with lower levels often indicating a higher grade of malignancy and a poorer overall outcome [23]. However, the role of miR-200b-3p in CRC is poorly understood.

In this study, we focus on the interplay between miR-200b-3p and DDIT4, suggesting that this interaction has significant implications for CRC development. By investigating the functional outcomes of modulating DDIT4 expression *in vitro* and analyzing its relationship with miR-200b-3p in CRC patient samples, we aim to elucidate the potential of the miR-200b-3p/DDIT4 axis as a target for therapeutic intervention. Our findings offer novel insights into CRC biology and present opportunities for the development of miRNA-based therapeutic strategies.

2. Materials and methods

2.1. Gene expression analysis

To observe the transcriptional expression level of DDIT4 in cancer patients, the Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/detail.php) database was used for pan-cancer expression analysis, focusing on its expression levels in various CRC subtypes. We employed the ANOVA method for statistical analysis, setting the |log2 fold change (FC)| threshold to 1 and the P threshold to 0.01. Gene with higher |log2 FC| value and lower P value than the preset threshold was considered differentially expressed.

2.2. Protein expression analysis

To further investigate the protein expression level of DDIT4 in CRC patients, we used the Human Protein Atlas (HPA, https://www.proteinatlas.org/) database to analyze its expression in 11 clinical tissue samples and quantify its expression intensity. The analyzed patient IDs were 2735, 3062, 1958, 2707, 1454, 1898, 2616, 2960, 3041, 2117 and 2947.

2.3. Gene survival analysis

For the prognosis of 6 candidate miRNAs in CRC patients, we analyzed their overall survival using the Encyclopedia of RNA Interactomes (ENCORI, https://starbase. sysu.edu.cn/) database. Based on the gene expression, patients were divided into low and high number groups in a ratio of 1:1, and P (Log-rank) value less than 0.05 was considered statistically significant for survival analysis.

2.4. Clinical tissue sample collection

We obtained paraffin-embedded specimens of cancer and normal tissues from 5 CRC patients diagnosed and surgically resected (none of whom received chemotherapy or radiotherapy before surgery) at the Anorectal Department of the Sixth Affiliated Hospital of Sun Yat-Sen University. The study was approved by the Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-sen University, with the approval number: F2021051.

2.5. Cell line culture

Human colorectal cancer cell line SW480 was purchased from BNCC (Beijing, China). Cells were maintained in DMEM (Gibco, USA) with 10% FBS (Gibco, USA) and 100 μg/ml penicillin/streptomycin (Gibco, USA) in a humidified atmosphere containing 5% CO2 at 37°C.

2.6. siRNA construction and transfection

DDIT4-specific siRNAs (siDDIT4-1 and siDDIT4-2) and non-targeting control (siNC) were synthesized by GENERAL BIOL (Anhui, China). SW480 cells (1×10^6 cells/ml) were seeded in 6-well culture plate and transiently transfected with 2ml OPTI-MEM medium (Gibco, USA) using the siRNA transfection reagent (Santa Cruz, USA). siRNA sequences were as follow: siDDIT4-1: 5'-CGGAACAGCTGCTCATTGA-3', 5'-GCCTTGTCGACGAGTAACT-3'; siDDIT4-2: 5'-GTGACCCTGAGGATGAAC A-3', 5'-CACTGGGACTC CTACTTGT-3'; siNC, 5'-TTCTCCGAACGTGTCACGT-3', 5'-AAGAGGCTTGCACAGTGCA-3'.

2.7. Plasmid construction and transfection

miR-200b-3p mimics and inhibitors were synthesized by GenePharma (Hangzhou, China) to up-regulate or down-regulate the expression of miR-200b-3p. pIRES2-ZsGreen1-DDIT4 plasmid for overexpressing DDIT4 (oeDDIT4) and an empty plasmid as control (oeNC) were synthesized by HanBio (Shanghai, China). SW480 cells (1×10^6 cells/ml) were seeded in 6-well culture plate with 2ml serum medium. Lipofectamine 2000 (Thermo Fisher, USA) was used for transfection according to the manufacturer's instructions.

2.8. Cell proliferation assay

After transfection for 6h, SW480 cells (1×10^3 cells/well) were seeded into a 96-well culture plate with 100μl serum medium. After incubation for 6, 24, 48, 72 and 96h, cell proliferation was measured by detecting OD values at 450nm with 10μl CCK-8 reagent (Beyotime, China). The cell proliferation curve was generated based on the CCK-8 method.

2.9. Cell migration assay

After transfection for 6h, SW480 cells (5×10^5 cells/well) were seeded into 6-well culture plate with 2ml serum medium. Parallel lines were scratched at the bottom of the culture plate using the pipette tip, and the dislodged cells were removed. The migrating cells at 0 and 48h were imaged and the migration distances were measured using Image J v1.8.0.

2.10. Cell invasion assay

Cell invasion was analyzed using Transwell with 8μm Pore Polyester Membrane Insert. The upper chamber was pre-coated with 80μl matrix solution and incubated at 37°C for 1 hour. After transfection for 6h, SW480 cells (5×10^4 cells/well) were seeded into the upper chamber in 200μl serum-free medium, while the lower chamber was filled with 500μl serum medium. After culturing for 48h, inserts were fixed with 100% methanol and stained with 0.1% crystal violet (Biosharp, China). The invaded cells on the inserts

were imaged and quantified using Image J v1.8.0.

2.11. Luciferase reporter assay

To assess the direct binding effect of miR-200b-5p on DDIT4, we used the TargetScan (http://www.targetscan.org) database to predict the potential miR-200b-5p binding targets. The entire 3'-untranslated region (3'-UTR) of human DDIT4 was PCR-amplified using human genomic DNA as the template. The PCR products were cloned into the psiCHECK2 reporter plasmid (Promega, Madison, WI, USA) and confirmed by sequencing. To confirm binding specificity, the miR-200b-5p binding sites were mutated (from UCAUAAU to GACCGCG), and the mutant DDIT4 3'-UTR was inserted into an equivalent luciferase report. SW480 cells were co-transfected with reporter plasmid (80ng) and miR-200b-5p mimics or NC mimics (50nM) using Lipofectamine 2000. After transfection for 48h, luciferase activity was measured using a dual luciferase reporter assay kit (FulenGen, China) according to the manufacturer's instructions.

2.12. qRT-PCR analysis

Total RNA was extracted from cells and tissues using Trizol reagent (BioTeke, China). After determining the concentration and purity of RNA samples, RNA reverse transcription was performed using steps of reverse transcription kit (Vazyme, China). qRT-PCR was performed using SYBR Green qPCR detection kit (Biosharp, China). The results were quantified using the relative quantitative $2^{-\Delta\Delta CT}$ method. The relative miR-200b-5p expression was normalized to U6 expression, and DDIT4 expression was normalized to β-actin expression. Primer sequences (forward): miR-200b-3p, 5'-TAATACTGCC TGGTAATGATGA-3'; U6, 5'-ATGGACTATCATATGCTTACCGTA-3'; DDIT4, 5'-C TGTTTAGCTCCGCCAACTC-3', 5'-TCAATGAGCAGCTGTTCCGA-3'; β-actin: 5'-AGCGAGCATCCCCCAAAGTT-3', 5'-GGGCACGAAGGCTCATCATT-3'.

2.13. Western blot analysis

Cells were lysed using RIPA lysis buffer (Sigma, USA) supplemented with 0.1% protease inhibitor (Sigma, USA). The isolated proteins (40μg) were separated on 12% sodium dodecyl sulfate (SDS) gels and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with primary antibodies against DDIT4 (1:800, Abcam, USA) and β-actin (1:1000, Proteintech, China) at 4°C overnight, followed by a horseradish peroxidase (HRP)-conjugated secondary antibody (1:3000, BOSTER, China). Protein bands were visualized using a chemiluminescent luminol enhancer solution (YEASEN, China) and analyzed by Image J v1.8.0.

2.14. Statistical analysis

All experiments were performed in triplicate. Statistical analysis was performed by SPSS v18.0. Experimental data were presented as mean ± SD. Student's unpaired t-test or one-way ANOVA (with Tukey's post-hoc test) was employed for statistical comparisons. P-value less than 0.05 was considered statistically significant.

3. Results

3.1. DDIT4 is highly expressed in CRC

A pan-cancer analysis of 33 types of cancer indicated that DDIT4 expression varied considerably. High levels of DDIT4 were observed in seven cancer types, including glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), kidney renal clear cell carcinoma (KIRC), brain lower-grade glioma (LGG), testicular germ cell tumors (TGCT), thymoma (THYM) and CRC. Conversely, low DDIT4 expression was observed in breast invasive carcinoma (BRCA), esophageal carcinoma (ESCA), kidney chromophobe (KICH), acute myeloid leukemia (LAML), ovarian serous cystadenocarcinoma (OV), prostate adenocarcinoma (PRAD) and skin cutaneous melanoma (SKCM) (Figure 1A). Further analysis revealed that DDIT4 was highly expressed in different subtypes of CRC, including microsatellite instability-high (MSI-H), microsatellite instability-low (MSI-L), and microsatellite-stable (MSS) (Figure 1B). Immunohistochemical analysis of clinical samples from 11 CRC patients showed high DDIT4 expression in 54.5% (6/11) of cases and medium expression in 45.5% (5/11), with no cases exhibiting low expression (Figure 1C-D). This suggests that the majority of the CRC samples have medium to high levels of DDIT4 protein, with a significant portion showing high expression, which may have prognostic or diagnostic relevance in CRC.

3.2. Silencing DDIT4 inhibits proliferation, migration and invasion of SW480 cells

To investigate the role of DDIT4 in CRC, we silenced it in SW480 cells by transfecting siRNAs. Western blot analysis showed a significant decrease in DDIT4 protein levels when compared to the siNC, with siDDIT4-1 achieving a notably more substantial reduction than siDDIT4-2 (Figure 2A). Next, we examined the impact of DDIT4 knockout on cell function. CCK-8 assay revealed that the downregulation of DDIT4 led to a significant decrease in cell proliferation over 72 hours (Figure 2B). Cell motility was also assessed through wound healing assays, where the decreased migratory capacity was observed as a reduced closure of the introduced scratch over 48 hours. Cells transfected with siDDIT4 and siDDIT4-2 exhibited significantly less migration compared to the control group (Figure 2C and D). Furthermore, the invasion potential of SW480 cells was

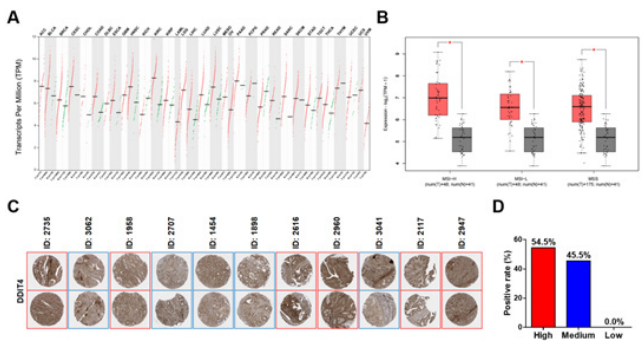

Fig. 1. Elevated DDIT4 expression in CRC and its distribution across cancer types. **A:** The transcript per million (TPM) values across a range of cancer types demonstrated varying levels of DDIT4 expression. **B:** Box plots compare the expression levels of DDIT4 between mutant and wild-type TP53 within three groups: MUT-MSS, MUT-NES, and WT-NES. **C:** Immunohistochemical staining images for DDIT4 in various clinical samples from CRC patients displayed a range of staining intensities. **D:** Quantification analysis showed the percentage of samples with high, medium, and low DDIT4 staining intensities.

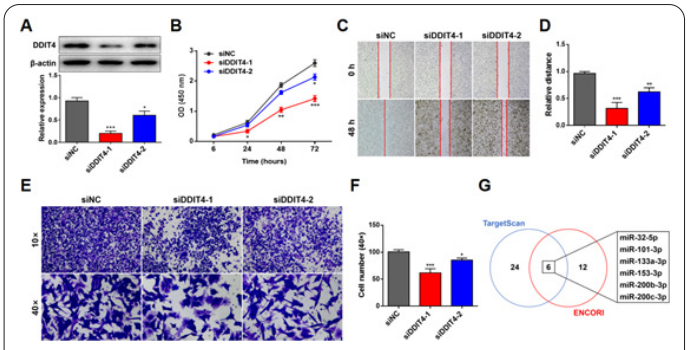

Fig. 2. DDIT4 knockdown suppresses proliferation, migration, and invasion of SW480 cells. **A:** Western blot analysis of DDIT4 expression in SW480 cells post-transfection with non-targeting control siRNA (siNC) and two DDIT4-targeting siRNAs (siDDIT4-1 and siDDIT4-2). Relative expression levels were quantified with β-actin serving as the loading control. **B:** Proliferation of SW480 cells assessed by CCK-8 assay over 72 hours post-transfection with siNC, siDDIT4-1, and siDDIT4-2. **C:** Wound healing assay depicting the migration ability of SW480 cells at 0 and 48 hours post-transfection. **D:** Quantification of the relative migration distances in the wound healing assay. **E:** Representative images of the Transwell invasion assay for SW480 cells post-transfection. **F:** Quantification of invading cells in the Transwell invasion assay. **G:** Venn diagram illustrating the predicted miRNAs targeting DDIT4 based on TargetScan and ENCORI databases. *, P<0.05; **, P<0.01; ***, P<0.001.

evaluated using a Matrigel invasion assay. Both siDDIT4-1 and siDDIT4-2 showed a significant suppressive effect on invasion compared to the control (Figure 2E and F).

To complement the functional studies, we explored the regulatory network surrounding DDIT4, focusing on miRNAs that could potentially target this gene. Utilizing the RNA interaction databases from TargetScan and ENCORI, we identified miRNAs predicted to interact with DDIT4. Cross-analysis of these databases revealed a set of 6 miRNAs that were consistently predicted by both databases as potential regulators of DDIT4 (Figure 2G). These miRNAs, which included miR-32-5p, miR-101-3p, miR-133a-3p, miR-153-3p, miR-200b-3p, and miR-200c-3p, were identified as the candidates for further investigation into the post-transcriptional control of DDIT4 expression in CRC.

3.3. Low expression of miR-200b-3p has poor prognosis for CRC patients

In our comprehensive analysis of the prognostic significance of miRNA expression in CRC patients, we used the ENCORI database's pan-cancer analysis platform. Our investigation centered on six miRNAs as potential prognostic biomarkers. We found that CRC patients with high miR-200b-3p expression (n=224) had better survival compared to those with low expression (n=223). Specifically, the hazard ratio for the high-expression group was 0.66, indicating a 34% lower risk of poor outcomes compared to the low-expression group, suggesting miR-200b-3p's potential role as a protective factor in the CRC. On the other hand, the expression of other miRNAs (miR-32-5p, miR-101-3p, miR-133a-3p, miR-153-3p and miR-200c-3p) did not show a statistically significant association with overall survival in CRC patients (Figure 3A-F). Therefore, the differential impact of miR-200b-3p expression on patient outcomes led us to focus on the interaction between miR-200b-3p and DDIT4 and their mediating role in CRC.

3.4. DDIT4 is the downstream target of miR-200b-3p

The TargetScan database was used to identify potential miR-200b-3p binding sites on the DDIT4 mRNA. We selected the target sequence with the highest score for validation. Additionally, a mutation was introduced into the binding site sequence to create a mutated control, thereby negating any specific miR-200b-3p interaction (Figure 4A). Luciferase reporter assay showed a significant decrease in luciferase activity when miR-200b-3p was co-transfected with a reporter plasmid containing the wild-type DDIT4 3'-UTR sequence. This inhibitory effect was not observed with the mutated sequence, indicating specific binding between miR-200b-3p and the target sequence on DDIT4 mRNA (Figure 4B). To extend these findings to a clinical setting, we analyzed tissue samples from 5 CRC patients. The data revealed that DDIT4 was upregulated in tumor tissues, while miR-200b-3p levels were conversely downregulated compared with normal tissues (Figure 4C-D).

3.5. Overexpression of DDIT4 weakens the suppressive effect of miR-200b-3p on proliferation, migration and invasion of SW480 cells

We further explored the regulatory interplay between

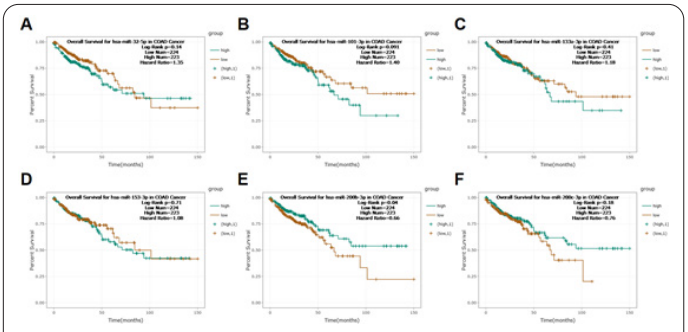

Fig. 3. Prognostic significance of candidate miRNAs in CRC patients. **A:** Overall survival of miR-32-5p (P=0.14). **B:** Overall survival of miR-101-3p (P=0.091). **C:** Overall survival of miR-133a-3p (*P*=0.41). **D:** Overall survival of miR-153-3p (*P*=0.71). **E:** Overall survival of miR-200b-3p (P=0.04). **F:** Overall survival of miR-200c-3p (*P*=0.18).

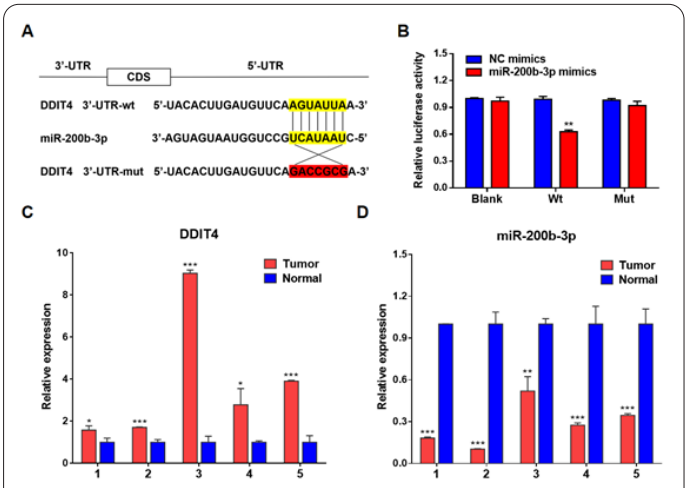

Fig. 4. miR-200b-3p directly binds to DDIT4 and is significantly decreased in CRC. **A:** Schematic diagram of predicted site of miR-200b-3p binding to DDIT4. **B:** Fluorescence expression efficiency of miR-200b-3p binding to target sequence of DDIT4. **C:** Expression of DDIT4 in clinical tissues of CRC patients. **D:** Expression of miR-200b-3p in clinical tissues of CRC patients. *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001.

miR-200b-3p and DDIT4 within SW480 cells using miR-200b-3p mimics and DDIT4 overexpression vectors. The efficiency of miR-200b-3p and DDIT4 overexpression was quantitatively measured through Western blot for DDIT4 and qRT-PCR for miR-200b-3p (Figure 5A-B). miR-200b-3p mimics inhibited cell proliferation, migration, and invasion (Figure 5C-G). Overexpression of DDIT4 promoted these cellular functions and mitigated the suppressive effect of miR-200b-3p on cellular functions (Figure 5C-G). These results suggest the potential of targeting the miR-200b-3p/DDIT4 axis as a multifaceted strategy in the treatment of CRC.

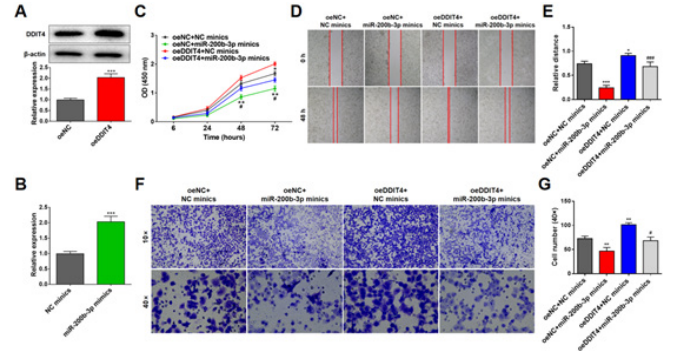

Fig. 5. miR-200b-3p suppresses CRC cell proliferation, migration, and invasion by targeting DDIT4 in SW480 cells. **A:** Western blot analysis showing the expression of DDIT4 following the overexpression of DDIT4. **B:** qRT-PCR showing the expression levels of miR-200b-3p after transfection with miR-200b-3p mimics compared to non-targeting control (NC) mimics. **C:** Cell proliferation results indicating the proliferative capacity of SW480 cells overexpressing DDIT4, miR-200b-3p mimics, or control mimics at 6, 24, 48 and 72 hours post-transfection. **D:** Wound healing assay images captured at 0 and 48 hours post-transfection. **E:** Quantification of the wound healing assay showing the cell migration ability. **F:** Transwell invasion assay displaying the invasive potential of SW480 cells overexpressing DDIT4, miR-200b-3p mimics, or control mimics. **G:** Quantitative analysis of the Transwell invasion assay. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

4. Discussion

CRC persists as a leading cause of mortality, characterized by its high heterogeneity within the digestive system. Despite continuous improvements in detection and treatment, including colonoscopy, colectomy, chemotherapy, and immunotherapy, recurrence remains a significant concern, and the 5-year survival rate for patients is still distressingly low [24, 25]. A major contributor to CRC's clinical challenges is the complexity of TME. TME comprises a diverse range of cell types, including cancerous cells and stromal cells, such as inflammatory cells, smooth muscle cells, adipocytes, fibroblasts, neuroendocrine cells, endothelial cells and microvessels. This environment is also enriched with cytokines, growth factors, and characterized by conditions such as low pH, hypoxia and extracellular substrates [26, 27]. Hypoxia is considered a key regulatory factor and driving force for tumor progression [28]. DDIT4, a gene activated under stress conditions such as hypoxia, dexamethasone, β-mercaptoethanol, carotene, heat stimulation, and osmotic pressure, has emerged as a critical player in the response of cancer cells to these environmental challenges [6, 29]. Our research found that DDIT4 was differentially expressed in various cancers, with patterns of expression that appear to correlate with the degree of heterogeneity in different TMEs. In CRC, we found that DDIT4 was commonly overexpressed, which aligned with findings in GBM, HNSC, KIRC, LGG, TGCT, THYM and CRC, and contrasts with the lower expression in BRCA, ESCA, KICH, LAML, OV, PRAD and SKCM.

The function of DDIT4 has been shown to vary across different cancer types. In breast cancer, DDIT4 regulates miR-495-mediated tumorigenesis and hypoxia tolerance [30], while in prostate cancer, DDIT4 enhances C/EBP-β mediated autophagosome fusion and desensitizes prostate cancer cells to bortezomib [31]. In ovarian cancer, DDIT4 is positively correlated with p-AKT and highly expressed in serous adenocarcinoma and advanced FIGO cancer tissues, suggesting that DDIT4 is a tumor-driving gene [32]. In our in vitro studies utilizing SW480 cells, we observed that silencing DDIT4 significantly inhibited the proliferation, migration and invasion of SW480 cells, suggesting that DDIT4 was actively involved in the progression of CRC and facilitating tumor growth and metastasis. To further understand the regulatory mechanisms upstream of DDIT4, we identified 6 candidate miRNAs (miR-32-5p, miR-101-3p, miR-133a-3p, miR-153-3p, miR-200b-3p and miR-200c-3p) using TargetScan and ENCOR databases. Among them, low expression of miR-200b-3p was associated with a poorer prognosis in CRC patients. Our findings were consistent with an extensive analysis of biological information from 1141 CRC cases, which identified 121 miRNAs associated with disease stage and survival. Of these, 5 miRNAs are associated with advanced disease stage. miR-145-5p and miR-31-5p are positively associated with the progression of tumor stage, while miR-200b-3p, miR-215 and miR-451a demonstrate an inverse relationship [33].

miR-200b-3p is known to target the transcriptional repressors of E-cadherin, such as ZEB1 and ZEB2, and thus acts to maintain the epithelial phenotype of cells. By controlling these pathways, miR-200b-3p has been implicated in inhibiting the EMT, a critical step in tumor cell migration and invasion [19, 33]. A recent study has shown that miR-200b-3p has tumor suppressive effect in various tumors. In gastric cancer, miR-200b-3p restrains cell proliferation, migration and invasion via CXCL12/CXCR7 regulatory axis [34]. In glioma, miR-200b-3p suppresses epithelial-mesenchymal transition (EMT) and inhibits tumor growth by downregulating ERK5 [35]. For CRC, miR-200b-3p negatively regulates MAGP2 to inhibit tumor cell proliferation and metastasis, suggesting that miR-200b-3p/MAGP2 regulatory axis could serve as a potential biomarker and therapeutic target for CRC [36]. These studies indicate that high expression of miR-200b-3p inhibits tumor biological function, especially in terms of growth and metastasis. In CRC, we found that miR-200b-3p expression was positively associated with disease survival, suggesting that higher levels of this miRNA could be protective and potentially beneficial as a therapeutic target. The interplay between miR-200b-3p and DDIT4 was further elucidated through luciferase reporter assays. These assays confirmed the direct binding of miR-200b-3p to the 3'-UTR of DDIT4, negatively regulating gene expression. The clinical significance of this interaction was highlighted by the contrasting expression patterns of miR-200b-3p and

DDIT4 in CRC patient tissues. Additionally, we found that while miR-200b-3p could suppress the malignant properties of SW480 cells, the overexpression of DDIT4 could counteract this effect to some extent.

Our study suggests a complex regulatory network in CRC, where DDIT4 acts as an oncogene whose expression and function can be modulated by miR-200b-3p. The direct interaction between miR-200b-3p and DDIT4 opens up new avenues for targeted therapy, potentially offering a dual approach to suppress DDIT4 expression while enhancing miR-200b-3p activity to combat CRC progression. We also confirm the clinical relevance of monitoring DDIT4 and miR-200b-3p levels in CRC patients. The therapeutic modulation of this regulatory axis holds promise for improving patient outcomes, warranting further investigation into the development of miRNA-based therapies and DDIT4 inhibitors as part of a targeted treatment strategy for CRC.

Conflict of interest
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
No human or animals were used in the present research.

Informed consent
The authors declare that no patients were used in this study.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions
Huang Dandan: conception and design of the study; Liu Zhimin: the database organization;
Li Yiping: statistical analysis; Xie Shangkui: writing first draft of the manuscript; Ren Donglin: writing sections of the manuscript; Liu Zhimin and Li Yiping contributed equally to this work.

Funding
This work was supported by grant from the Natural Science Foundation of Guangdong Province, China (Grant No. 2020A1515010071).

Acknowledgements
This study was supported by the National Key Clinical Discipline.

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