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Original Article

MiR-27a-3p exacerbates cell migration and invasion in right-sided/left-sided colorectal cancer by targeting TGFBR2/TCF7L2

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Abstract

Left-sided colorectal cancer (LSCC) and right-sided colorectal cancer (RSCC) belong to colorectal cancer happening at different positions, which exhibit different pathogenesis. MicroRNA (miRNA)s are widely known regulators in diverse carcinomas. This research aims to identify a differentially expressed miRNA that simultaneously regulates genes associated with LSCC and RSCC and reveal their regulatory relation in cell migration and invasion. Bioinformatics analyses were conducted to uncover the dysregulated functional genes in LSCC/RSCC and obtain their common targeted miRNAs. The expression pattern of miR-27a-3p, TCF7L2, and TGFBR2 in cancerous and adjacent tissues from LSCC/RSCC patients was assessed through qRT-PCR, followed by Pearson's correlation coefficients analysis. The interaction of miR-27a-3p with TCF7L2 or TGFBR2 was thereafter confirmed through luciferase reporter assay. TCF7L2 and TGFBR2 protein levels were assessed by western blotting after overexpressing level of miR-27a-3p. Cell migration and invasion were routinely examined by wound healing and transwell experiments, respectively. TCF7L2 and TGFBR2 were respectively identified and verified to be lowly expressed in LSCC and RSCC, both of them were predicted and confirmed as targets of miR-27a-3p. MiR-27a-3p elevation exacerbated migration and invasion of both LSCC and RSCC cells. The impacts of miR-27a-3p on migration and invasion could be blocked by overexpressing TCF7L2 in LSCC cells and also reversed by up-regulating TGFBR2 in RSCC cells. In general, miR-27a-3p accelerated the migration and invasion capabilities of LSCC and RSCC cells through negatively regulating TCF7L2 and TGFBR2, respectively, which might be an effective molecular target for the treatment of LSCC/RSCC.

Keywords: Left-sided colorectal cancer, Right-sided colorectal cancer, miR-27a-3p, TCF7L2, TGFBR2

1. Introduction

Colorectal cancer (CRC) is the third most commonly identified carcinoma with the incidence rate rising with age [1, 2], which is also a principal cause of cancer death around the globe [3, 4]. CRC pathogenesis is different due to its anatomical location on the left side and right side of the colon [5, 6]. Left-sided colorectal cancer (LSCC), as well as right-sided colorectal cancer (RSCC), are regarded as two disease entities for their distinct clinical and biological features [7]. Thus, searching for molecules targeting functional proteins in both LSCC and RSCC is meaningful for the treatment of CRC.

Past research has illustrated that the chromosomal instability (CIN) pathway and microsatellite instability (MSI) pathway are principal carcinogenic pathways determining the molecular profile of CRC [8, 9]. The CIN pathway is featured by loss of heterozygosity and imbalances in chromosome number and it correlates with poor survival and metastasis in cancer progression [10]. The CIN pathway usually begins with APC mutation and is typified by TP53 and KRAS mutations [11]. PIK3CA mutation may serve as a biomarker for anti-EGFR therapy in CRC [12]. FBXW7, TCF7L2, and SMAD4 are frequently mutated genes in CIN-high tumors [13]. Owing to DNA polymerase slippage, MSI pathway is featured by length polymorphisms of microsatellite sequences, frequently linked to germline mutations in mismatch repair genes, such as MLH1, MSH2, MSH3, and MSH6 [14]. $BRAF^{V600E}$ mutation has been found as a poor prognostic factor for metastatic CRC patients [15]. The inactivation of TGFBR2 frameshift mutation is common in >90% of CRC cases with MSI [16]. As reported by previous research, CIN and MSI pathways are crucial pathways in LSCC and RSCC [5]. Hence, the genes involved in the CIN and MSI pathways may be the therapeutic targets for LSCC and RSCC, respectively.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that post-transcriptionally mediate genes by targeting message RNAs (mRNAs) on their 3' untranslated region (3'UTR) [17-19]. Mounting reports have demonstrated the function of miRNAs in diverse malignancies, CRC contained. For instance, miR-31 decreases radio-resistance of CRC cells via inhibiting STK40 [20]. MiR-144 also resists proliferation and migration in CRC through decreasing GSPT1 levels [21]. MiR-4319 plays a suppressive role in CRC through mediating ABTB1 [22].

Given that the pathogenesis of LSCC and RSCC was different, the present study was conducted with an attempt to identify a miRNA that has been implicated in CRC, which can target genes that respectively regulate LSCC and RSCC to act as the common therapeutic target for pa-

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tients with LSCC or RSCC.

2. Materials and methods

2.1. Sample collection and cell isolation

Twenty-five LSCC, 25 RSCC, and 25 colonic polyps tissues collected from Zibo Central Hospital were stored in liquid nitrogen at −80°C prior to experiments. All cancer patients were diagnosed by histopathology without treatment of radiotherapy or chemotherapy and signed informed consent prior to surgery. This work has been approved by the Ethics Committee of Zibo Central Hospital. LSCC/RSCC cells were separately harvested from LSCC/RSCC tissues with Tumor Cell Isolation Kit (Miltenyi) for subsequent experiments. Cells were grown in an incubator with 5% CO_2 at 37°C with the addition of serum-free medium.

2.2. Cell transfection

For cell transfection, sequences of TCF7L2 or TGFBR2 were routinely amplified by qRT-PCR, which were subsequently cloned into the pcDNA3.1 vector (Thermo Fisher, Waltham, MA, USA) to establish the TCF7L2 or TGFBR2 overexpression vector (TCF7L2 OE or TGFBR2 OE) in LSCC or RSCC cells. miR-27a-3p mimic with its negative control (miR-NC) was synthesized via RiboBio (Guangzhou, China). All plasmids were thereafter transfected into cells utilizing 10-μL Lipofectamine™ 2000 (Thermo Fisher, Waltham, MA, USA).

2.3. qRT-PCR

To test RNA expression, TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) for harvesting total RNA was applied in carcinoma tissues and cells. Following reverse transcription of RNA into cDNA via a PrimeScript RT reagent kit (TaKaRa, Dalian, China), this experiment was conducted utilizing SYBR Green Kit (TaKaRa, Dalian China) in fluorescent qPCR 7500 system (Applied Biosystems; Foster City, CA, USA). Additionally, a miRcute miRNA qPCR kit (Tiangen Biotech Co., Ltd., Shanghai, China) was prepared in advance for miRNA detection. Primer sequences were listed in Table 1, with U6 and GAPDH serving as internal references. The $2^{-\Delta\Delta Cq}$ method was applied to calculate RNA expression.

2.4. Bioinformatics tools

The GSE14333 dataset [23] was exploited to discover differentially expressed genes (DEGs) between LSCC and RSCC with a threshold of $P < 0.05$ and $\log2|FC|>0.263$, the results of which were visualized as volcano plots. CIN-related genes, MSI-related genes, and DEGs were intersected to obtain genes that might function in LSCC and RSCC. Subsequently, the GSE25609 dataset [24] was also downloaded to find out dysregulated miRNAs ($P < 0.05$, $\log2|FC|>0.263$) in CRC. The specific binding sequences of miR-27a-3p and TCF7L2 or TGFBR2 were separately predicted by Targetscan (https://www.targetscan.org/vert_80/) [25] or the starBase (https://starbase.sysu.edu.cn/) [26] website. The expression profile of miR-27a-3p between CRC and normal subjects was also explored through starBase website.

2.5. Luciferase reporter assay

First, wild-type (WT) binding sequences of TCF7L2 or TGFBR2 by miR-27a-3p were exhibited and corresponding mutant-type (MUT) sequences were designed, which were named as TCF7L2 WT, TCF7L2 MUT, TGFBR2 WT, and TGFBR2 MUT, followed by subclone into luciferase reporter vector pmirGLO (Promega, Madison, WI, USA). Transfection of plasmids with miR-27a-3p mimic or NC mimic was performed and then a Dual Luciferase Assay Kit (Promega, Madison, WI, USA) assessed luciferase activities.

2.6. Wound healing assay for cell migration

After isolation, 2×10^5 cells were inoculated into each well of a 6-pore plate. A sterilized 200-μL pipette tip was prepared and utilized to generate scratch wounds, and floating cells were subsequently scrapped off by washing with PBS. Scratches were photographed to capture images at 0 and 24 h, respectively, using an inverted microscope (Olympus, Tokyo, Japan).

2.7. Transwell assay for cell invasion

Matrigel (5 mg/mL) was plated in the upper chambers of 8-μm pore inserts which were thereafter placed into 24-well plates. The upper chambers were added with 1×10^5 cells and the lower chambers were placed with RPMI-1640 medium including 20% FBS. Post incubation for 1 d at 37°C with 5% CO_2, a large number of cells invaded the lower chambers. A cotton swab was applied to remove cells left on the upper chambers. Migratory cells were immobilized utilizing methanol and dyed using crystal violet at 37°C for just 1 h. Eventually, an inverted microscope was supported to quantify the number of migratory cells.

2.8. Western blot

Total proteins were isolated from cells that were lysed by RIPA buffer (Solarbio, Beijing, China). After that, sonication and centrifugation were implemented, followed by separation by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as well as transferring onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Subsequently, 5% skimmed milk was applied for blocking at room temperature for just 1 h. Primary antibodies were added at 4°C overnight, followed by cultivation with secondary antibodies Finally, the membrane was probed with HRP-labeled Goat (1:1000) secondary antibodies for another 2 h at room temperature.

Table 1. Primer sequence (5'-3').

Gene	Forward Primer	Reverse Primer
miR-27a-3p	GCGGAACTTAGCCACTGTGA	TGAGGAGCAGGGCTTAGCTG
TCF7L2	AGAAACGAATCAAAACAGCTCCT	CGGGATTTGTCTCGGAAACTT
TGFBR2	GTAGCTCTGATGAGTGCAATGAC	CAGATATGGCAACTCCCAGTG
U6	CTCGCTTCGGCAGCACATATACT	ACGCTTCAGAATTTGCGTGTC
GAPDH	GTGGCTGGCTCAGAAAAAGG	GGGGAGATTCAGTGTGGTGG

Table 2. The information of antibodies used in Western blot.

Antibody	Manufacturers	Cat.no
TCF7L2	Beyotime	AF2089
TGFBR2	Thermo Scientific	PA5-36115
GAPDH	Beyotime	AF1186
HRP-labeled Goat Anti-Human IgG (H+L)	Beyotime	A0201

All antibodies contained anti-TCF7L2 (1:500), anti-TGFBR2 (1:500), and anti-GAPDH (1:1000), information of which was listed in Table 2. Protein bands were visualized via enhanced chemiluminescence (ECL) reagent from BeyoECL Moon, Shanghai, China as per the instructions.

2.9. Statistical analysis

Data obtained from triplicated assays were dissected through Statistic Package for Social Science (SPSS) 19.0 (IBM, SPSS, Chicago, IL, USA). The correlations were explored utilizing Pearson's correlation coefficients. Meanwhile, student's t-test or one-way ANOVA was utilized to analyze differences between two groups or among groups. Data with $P < 0.05$ were regarded as statistically significant and represented as the mean ± standard deviation (SD).

3. Results

3.1. miR-27a-3p regulates TCF7L2 and TGFBR2 in LSCC and RSCC, respectively

GSE14333 dataset was used to analyze DEGs in LSCC and RSCC, which discovered 1216 upregulated genes and 1018 downregulated genes (Figure 1A). Since the CIN pathway is related to LSCC development and the MSI pathway is related to RSCC progression, the intersection of MSI-associated genes, CIN-associated genes, and the above DEGs was conducted by drawing a Venn diagram to find out potentially functional genes in LSCC and RSCC (Figure 1B). To explore dysregulated miRNAs in CRC, we analyzed the GSE25609 dataset by differential expression analysis (Figure 1C), and found 48 overexpressed miRNAs and 44 silenced miRNAs. Among the upregulated miRNAs, miR-27a-3p was predicted to bind with TCF7L2 and TGFBR2. Detailed binding sequences of miR-27a-3p with TCF7L2 by starBase or TGFBR2 by Targetscan were exhibited in Figure 1D-E. Based on the public data analyzed by starBase, miR-27a-3p expression was obviously high in CRC (Figure 1F). All data uncovered that miR-27a-3p might be involved in LSCC and RSCC upon simultaneous regulation of TCF7L2 and TGFBR2.

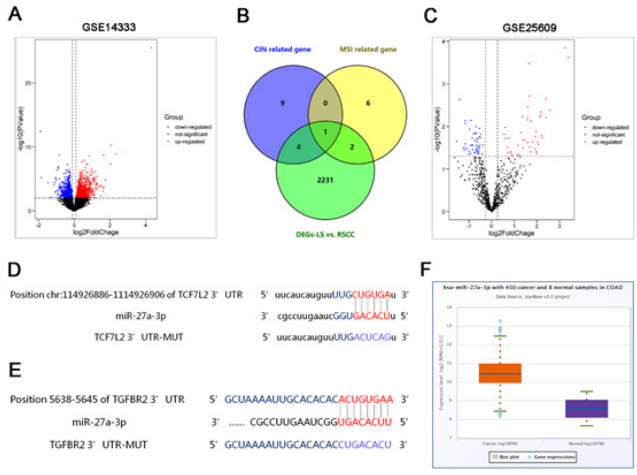

Fig. 1. Identification of miR-27a-3p as a regulator of TCF7L2 and TGFBR2 in LSCC and RSCC, separately. (A) Volcano plots of DEGs in LSCC and RSCC on the basis of the GSE14333 dataset. (B) Venn diagram of CIN-related genes, MSI-related genes, and DEGs in LSCC/RSCC. (C) Identification of differentially expressed miRNAs in CRC and normal tissues based on the GSE25609 dataset. (D) Wild and mutant-type binding sites between miR-27a-3p and TCF7L2 were exhibited based on the starBase database. (E) Wild and mutant-type binding sites between miR-27a-3p and TGFBR2 were presented based on the Targetscan database. (F) The expression of miR-27a-3p between CRC and normal tissues was downloaded from the starBase database.

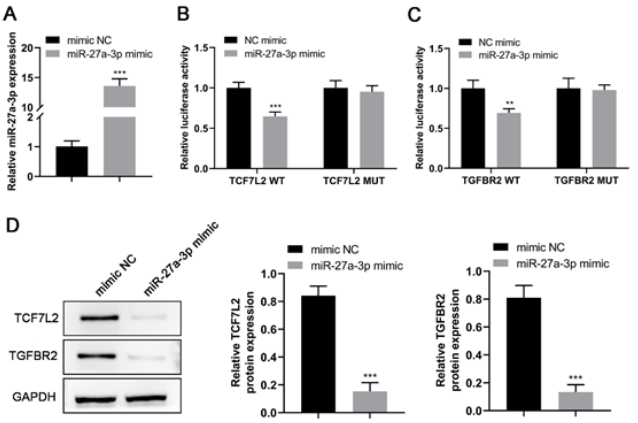

Fig. 2. MiR-27a-3p directly regulates the expression of TCF7L2 and TGFBR2. (A) qRT-PCR experiment explored miR-27a-3p expression under transfection of miR-27a-3p mimic. (B) Luciferase reporter assay assessed the binding between miR-27a-3p and TCF7L2. (C) The binding ability between miR-27a-3p and TGFBR2 was explored via luciferase reporter assay. (D) Western blot was performed to test TCF7L2 and TGFBR2 protein expression under miR-27a-3p mimic transfection. $^{**}P<0.01$ and $^{***}P<0.001$.

3.2. MiR-27a-3p negatively modulates TCF7L2 and TGFBR2

Subsequently, the effects of miR-27a-3p on TCF7L2 and TGFBR2 were investigated. MiR-27a-3p mimic was transfected to promote miR-27a-3p expression, as exhibited via qRT-PCR (Figure 2A). In the luciferase reporter experiment, it was observed that luciferase activity of WT TCF7L2 was distinctly weakened after the miR-27a-3p expression was elevated (Figure 2B). Also, luciferase activity of WT TGFBR2 was dramatically restrained after miR-27a-3p upregulation (Figure 2C). Further, the western blot assay affirmed that TCF7L2 and TGFBR2 protein levels were overtly decreased after the addition of miR-27a-3p (Figure 2D). Taken together, TCF7L2 and TGFBR2 were in negative modulation of miR-27a-3p.

3.3. MiR-27a-3p exacerbates LSCC migration and invasion by targeting TCF7L2

Twenty-five colonic polyps and 25 LSCC tissues were gathered for analyzing the levels of miR-27a-3p and TCF7L2. Results of qRT-PCR illustrated that miR-27a-3p was distinctly heightened and TCF7L2 was obviously decreased in LSCC tissues, compared with the normal group (Figure 3A-B). TCF7L2 expression was in negative relation with miR-27a-3p expression in LSCC tissues (r = -0.6925, P = 0.001) (Figure 3C). In LSCC cells, TCF7L2 expression was overexpressed by transfection of the TCF7L2 overexpression vector (Figure 3D). In wound healing experiment, miR-27a-3p overexpression accelerated cell migration but TCF7L2 upregulation refrained cell migration, either of which was abrogated under co-treatment of miR-27a-3p mimic+TCF7L2 OE (Figure 3E). In the transwell experiment, cell invasion was evidently promoted by miR-27a-3p addition but visibly restricted by TCF7L2 increase, which was neutralized under co-treatment (Figure 3F). All findings revealed that miR-27a-3p inhibited TCF7L2 to exacerbate LSCC migration and invasion.

3.4. MiR-27a-3p facilitates RSCC migration and invasion by restraining TGFBR2

The function of miR-27a-3p/TGFBR2 axis in migration and invasion of RSCC cells was explored. qRT-PCR uncovered high miR-27a-3p expression but low TGFBR2 expression in RSCC tissues in comparison with normal tissues (Figure 4A-B). Moreover, TGFBR2 expression was in negative relation with miR-27a-3p expression in RSCC tissues (r = -0.7887, P = 0.001) (Figure 4C). After that, TGFBR2 expression was elevated by transfecting the TGFBR2 overexpression vector in RSCC cells (Figure 4D). It was viewed that miR-27a-3p elevation accelerated

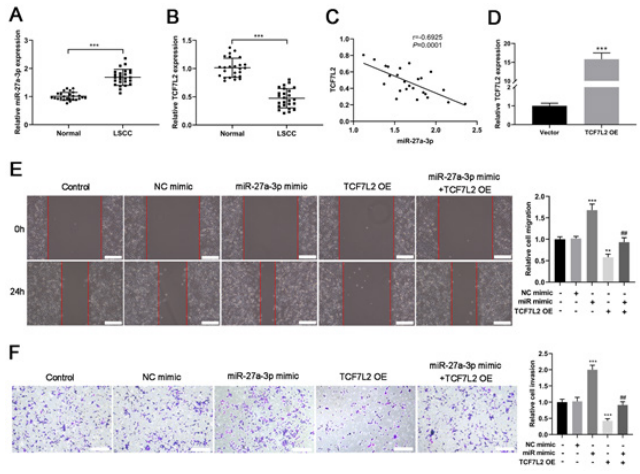

Fig. 3. MiR-27a-3p exacerbates migration and invasion of LSCC cells via TCF7L2. (A-B) Colonic polyps and LSCC tissues were collected and then miR-27a-3p and TCF7L2 levels were separately examined. (C) The association between miR-27a-3p and TCF7L2 in LSCC was analyzed by Pearson's correlation analysis. (D) TCF7L2 overexpression plasmids were transfected into LSCC cells to increase TCF7L2 expression and the results were affirmed through qRT-PCR. (E) LSCC cells were divided into five groups: control, NC mimic, miR-27a-3p mimic, TCF7L2 OE, and miR-27a-3p mimic+TCF7L2 OE. The migration capability of five-group cells was measured via wound healing assay. (F) The invasion ability of five-group cells was evaluated via transwell assay. (Scale bar = 100μm) $^{**}P<0.01$ and $^{***}P<0.001$.

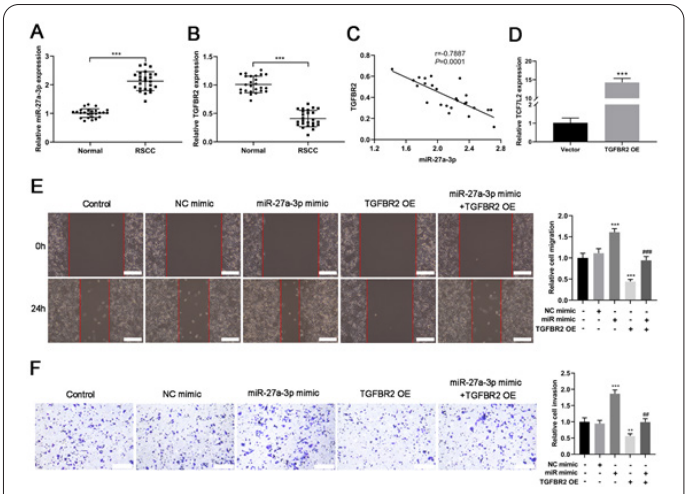

Fig. 4. MiR-27a-3p facilitates migration and invasion of RSCC cells via TGFBR2. (A-B) miR-27a-3p and TGFBR2 levels were separately measured in normal and RSCC tissues. (C) The correlation of miR-27a-3p with TGFBR2 in RSCC tissues was analyzed by Pearson's correlation analysis. (D) TGFBR2 overexpression plasmids were transfected into RSCC cells, which was affirmed through qRT-PCR. (E) RSCC cells were also divided into five groups: control, NC mimic, miR-27a-3p mimic, TGFBR2 OE, and miR-27a-3p mimic+TGFBR2 OE. Cell migration was assessed via wound healing assay. (F) Cell invasion of five groups was evaluated via a transwell experiment. (Scale bar = 100 μm) $^{**}P<0.01$ and $^{***}P<0.001$.

but TGFBR2 increase inhibited cell migration in wound healing assay, which was abolished under miR-27a-3p overexpression and TGFBR2 promotion (Figure 4E). In the transwell experiment, cell invasion was clearly accelerated by miR-27a-3p addition but significantly suppressed by TGFBR2 augment, which was reversed under co-treatment of miR-27a-3p overexpression and TGFBR2 upregulation (Figure 4F). These experiments disclosed that miR-27a-3p restrained TGFBR2 to facilitate RSCC migration and invasion.

4. Discussion

As known to all, miRNAs can modulate multiple cellular activities of carcinomas, such as proliferation, apoptosis, migration as well as invasion, in a promotive or suppressive way [27, 28]. Emerging evidence has disclosed that miRNAs have the capability to mediate gene expression post-transcriptionally via target on their 3'UTRs [29]. Hence, we aimed to find functional miRNAs simultaneously modulating DEGs in LSCC and RSCC.

Bioinformatics tools are often applied to search for potentially functional genes in tumors, thus obtaining potent therapeutic targets for patients with different cancers. In this research, the GSE14333 dataset was applied to identify DEGs between LSCC and RSCC, which contained 1216 upregulated genes and 1018 downregulated genes. Considering that CIN is related to LSCC and MSI pathway is related to RSCC, we intersected CIN-related genes, MSI-related genes, and the above DEGs in order to gain the most possible genes functioning in LSCC and RSCC. Subsequently, the GSE25609 dataset was analyzed to identify dysregulated miRNAs in CRC tissues compared with normal tissues, which revealed 48 upregulated miRNAs and 44 downregulated miRNAs. Among these miRNAs, miR-27a-3p was an overtly upregulated miRNA in CRC, and the bioinformatic tool predicted that it might si-

multaneously target the genes related to LSCC and RSCC, respectively. Consistently, public data has indicated that miR-27a-3p is evidently augmented in CRC compared to the normal. Based on these findings of bioinformatics analysis, we reasonably supposed that miR-27a-3p might exert its function in LSCC and RSCC through TCF7L2 and TGFBR2.

Previous papers unveiled the oncomiR role of miR-27a-3p in several carcinomas. For example, miR-27a-3p accelerates non-small cell lung cancer upon it regulation of SLC7A11-modulated ferroptosis [30]. Also, miR-27a-3p can regulate cervical cancer through the reduction of FBXW7 protein [31]. It is striking to note that miR-27a-3p is oncogenic in CRC. For example, miR-27a-3p decreases BTG1 to regulate proliferation and apoptosis in colon cancer [32]. Also, miR-27a-3p modulates cellular activities through refraining RXRα/Wnt/β-catenin pathway in CRC [33]. Moreover, its predicted targets, TCF7L2 and TGFBR2, are also reported to be tumor repressor genes in CRC. TCF7L2 depletion can facilitate migration and invasion activities in CRC [34]. In colon adenoma cells, TCF7L2 acts as a suppressor through affecting proliferation and tumorigenesis [35]. In CRC, TGFBR2 is negatively mediated via miR-135b, which represses carcinoma progress [36]. MiR-301a targets and restrains TGFBR2 to inhibit migration and invasion in CRC [37-40]. Thus, we further believed that in CRC, miR-27a-3p might promote tumor progression via targeted inhibition of TCF7L2 and TGFBR2.

Luciferase reporter experiment confirmed the interaction between miR-27a-3p and TCF7L2 or TGFBR2. The impacts of miR-27a-3p on TCF7L2 and TGFBR2 expression were confirmed by western blotting. The results validated that miR-27a-3p overexpression dramatically refrained TCF7L2 and TGFBR2 expression. Subsequently, expression of miR-27a-3p and TCF7L2/TGFBR2 as well as their correlation in LSCC/RSCC tissues were measured. Function assays for migration and invasion were performed to interrogate the role of miR-27a-3p/TCF7L2 axis in LSCC cells and also that of miR-27a-3p/TGFBR2 axis in RSCC cells, respectively. In LSCC/RSCC tissues, high miR-27a-3p level, low TCF7L2/TGFBR2 expression as well as the negative association between miR-27a-3p and TCF7L2/TGFBR2 were observed. Function experiments further discovered that miR-27a-3p facilitated migration and invasion via restraining TCF7L2/TGFBR2 in LSCC/RSCC cells.

In conclusion, our research indicated miR-27a-3p as a potent therapeutic target in CRC for its upregulated expression and simultaneous inhibition of TCF7L2 in LSCC or TGFBR2 in RSCC to induce cell migration and invasion. The current paper is of great significance since it unveiled that miR-27a-3p can simultaneously modulate TCF7L2, a CIN pathway downstream gene, in LSCC, and TGFBR2, an MSI pathway downstream gene, in RSCC. This provides a novel and effective molecular target for the treatment of CRC patients. However, we must realize the limitations of our study. No animal experiments were performed and an in-depth investigation on the miR-27a-3p-TCF7L2/TGFBR2 axis in CRC is needed.

5. Conclusion

With the assistance of bioinformatics tools, we proposed that highly expressed miR-27a-3p might function in CRC as it was predicted to target TCF7L2 and TGFBR2, two CRC repressors that might be responsible for LSCC and RSCC, respectively. MiR-27a-3p was proved to bind with and negatively regulate TCF7L2 and TGFBR2. Function experiments affirmed that miR-27a-3p exacerbated both migration and invasion in LSCC via TCF7L2 and it also facilitated these activities upon TGFBR2 in RSCC.

Conflict of Interests

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

This work has been approved by the Ethics Committee of Zibo Central Hospital.

Informed Consent

All cancer patients were diagnosed by histopathology without treatment of radiotherapy or chemotherapy and signed informed consent prior to surgery.

Availability of data and material

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

Authors' contributions

Lei Bi and Xin Zhang designed the study and performed the experiments, Yuntao Zhou collected the data, Yong Zhang analyzed the data, Lei Bi and Xin Zhang prepared the manuscript. All authors read and approved the final manuscript.

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Non.

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