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MicroRNA-142-3p is frequently upregulated in colorectal cancer and may be involved in the regulation of cell proliferation

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MicroRNAs are small single-stranded RNA molecules consisting of approximately 22 nucleotides (nt), and have post-transcriptional regulatory functions. By gene chip screening, we previously showed that miR-142-3p was significantly upregulated in colorectal cancer tissue and was associated with clinicopathological features, compared with matched non-tumor tissue. In this study, we confirmed significant upregulation of miR-142-3p in 60 colorectal cancer samples and three colorectal cancer cell lines by quantitative real-time PCR (qRT-PCR). Using software and network resources, we predicted *TCF7* as a target of miR-142-3p, which we confirmed with dual-luciferase assays. By RT-PCR and Western blot analysis, we found miR-142-3p negatively regulates *TCF7* expression post-transcriptionally. CCK8 assays and growth curves indicated that overexpression of miR-142-3p in SW480 colorectal cancer cells potently inhibited cell proliferation *in vitro*. The expression of *TCF7* mRNA and protein was upregulated in both colorectal cancer tissues and colorectal cancer cell lines, closely correlating with its function as an oncogene in promoting tumor cell proliferation and inhibiting apoptosis. With *TCF7* as a direct target, our results suggested that miR-142-3p may be involved in the regulation of cell proliferation in colorectal cancer.

microRNA, colorectal cancer, proliferation, microRNA-142-3p, TCF7

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Cancer is the third leading cause of death worldwide, after cardiovascular and infectious diseases. Colorectal cancer is the third most common cancer diagnosed, and is a cause of mortality in both men and women in the United States [1]. In the past few years, many methods such as colonoscopy, blood tests for tumor markers, ultrasound, magnetic resonance imaging (MRI), computed tomography (CT or CAT) scanning, and positron emission tomography (PET) scanning have been developed to improve the diagnostic rate of colorectal cancer. Improvements in treatment modalities such as surgery, radiation therapy, chemotherapy and targeted therapies may also result in a lower death rate due to colorectal cancer. However, the outcomes of colorectal cancer diagnosed at advanced stages remain poor. The progress made in early diagnostic techniques and screening tests has been inadequate, and the majority of the molecular mechanisms of colorectal cancer are still unknown. Thus, there is an urgent requirement to characterize specific molecular changes that could identify patients with early cancer or precursor lesions.

MicroRNAs (miRNAs) are small single-stranded RNA molecules of approximately 22 nucleotides (nt) long that regulate gene expression. They are naturally abundant and evolutionarily conserved non-coding RNA molecules found in both animals and plants. It is estimated that miRNAs regulate 10%–30% of all protein-coding genes by binding to partially complementary sequences in the 3′-untranslated

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regions (3′UTR) of downstream target mRNAs [2,3]. MiRNAs mainly inhibit protein translation of their target genes and infrequently cause degradation or cleavage of mRNA. The first microRNA was identified in 1993 by Lee et al. [4]. Since then, various cloning and bioinformatics studies have predicted that the human genome may contain up to 1000 miRNAs. Growing evidence indicates that miRNAs are key regulators of a variety of fundamental biological processes such as cell proliferation, development, apoptosis, hematopoiesis, energy metabolism, neural development, stress resistance, cell death and, importantly, tumorigenesis [5−8]. The expression data of miRNAs in various cancers demonstrated that cancer cells have different miRNA profiles compared with normal cells. MiRNAs overexpressed in cancer may function as oncogenes or tumor suppressors when downregulated in cancer. The initial evidence of the involvement of miRNAs in human cancer came from molecular studies in human chronic lymphocytic leukemia, which revealed two miRNAs, miR-16 and miR-15 [9]. More recently, miRNA dysregulation was detected in other cancers [10−14], including gastric [15], prostate [16], lung [17], breast [18], ovarian [19] and hepatocellular [20] cancer. These unique properties of miRNAs make them remarkably useful potential agents for clinical diagnostics as well as in future personalized care for individual patients.

In previous studies, we performed miRNA gene chip screening to detect the miRNA profiles of 25 colorectal cancer samples and 10 normal colorectal mucosa tissue samples. The results showed that in all colorectal cancer tissue compared with matched non-tumor tissue, 35 miRNAs were upregulated and 30 miRNAs were downregulated. We observed that the expression levels of miR-142-3p were significantly increased and were associated with clinicopathological features [21]. Recent studies have reported that miR-142-3p, which is located at chromosome 17q22, is important in the tumorigenesis of human T-cell acute lymphoblastic leukemia (T-ALL) [22], hepatocellular carcinoma [23] and esophageal squamous cell carcinoma [24]. However, the function of miR-142-3p in colorectal cancer is largely unknown.

In this study, we confirmed the miR-142-3p expression profile in human colorectal cancer tissues and several colorectal cancer cell lines by quantitative real-time RT-PCR (qRT-PCR). Subsequent experiments predicted and identified the transcription factor 7 (T-cell specific, HMG box; *TCF7*) as a target of miR-142-3p, which was post-transcriptionally downregulated by miR-142-3p. Further study showed that overexpression of miR-142-3p could inhibit *TCF7* functions in the control of cell proliferation *in vitro*. Therefore, we presumed that miR-142-3p may be involved in the regulation of cell proliferation in colorectal cancer. Our findings may help to enhance our understanding of the mechanisms of miRNAs in the tumorigenesis of colorectal cancer.

1 Materials and methods

1.1 Bacterial strain, plasmids, tissue samples and cell lines

E. coli strain DH5α was preserved by the laboratory at our hospital. Gene expression vector GP/S/EGFP/B/CMV-miR plasmid and psiCHECK-2 dual-luciferase vector were purchased from GenePharma (Shanghai, China). Colorectal cancer tissue and adjacent non-tumor tissue were collected from 60 patients who underwent colorectal cancer radical surgery at the Department of Oncosurgery at the Fourth Affiliated Hospital of Soochow University in 2010. All the patients did not receive preoperative treatment. TNM classification was defined according to the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) (2010). The human colorectal cancer cell lines SW480, DLD-1, HCT116, HCT8 and human embryo colon mucosa cell line CCC-HIE-2 were maintained by the laboratory at our hospital. The cells were routinely cultured in RPMI-1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/mL) at 37°C in a humidified atmosphere containing 5% (v/v) CO_2.

1.2 Bioinformatics analysis

MiR-142-3p sequences were obtained in the microRNA authority database miRBase (http://www.mirbase.org/[25]). The miRNA target genes were predicted by miRanda 3.3a, RNAhybrid 2.1 and TargetSpy softwares, and the network resources of miRBase Targets (http://www.mirbase.org/), TargetScan (http://www.targetscan.org [26]) and PicTar (http://pictar.mdc-berlin.de/[27]). The KEGG pathway database [28] and Expression Analysis Systematic Explorer (EASE) database were used to further analyze the overlap of these results. The 3′UTR of target genes were retrieved by UCSC human Gene Sorter (http://genome.ucsc.edu/cgi-bin/hgNear).

1.3 Quantitative real-time PCR

Total RNA was extracted from colorectal cancer cell lines, normal colorectal mucosal epithelial cell lines, cancer tissue and non-tumorous mucosal tissue using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The expression levels of *miR-142-3p* mRNA were examined using qRT-PCR according to the SYBR Real-Time PCR Kit protocol (GenePharma). Reverse transcription reactions were performed with MMLV Reverse Transcriptase (MBI) using the following reaction conditions: 26°C for 30 min; 42°C for 30 min; and 85°C for 10 min. qRT-PCR was conducted on a real-time cycler (FTC2000) with the following cycling conditions: an initial step of 95°C for 3 min, followed by 40 cycles at 95°C for 30 s, 62°C for 20 s and 72°C for 20 s. The threshold cycle (*C*t) values were ana-

lyzed using the comparative *C*t method. The amount of target was obtained by normalizing to the endogenous reference (U6). Each experiment was repeated three times.

1.4 Construction of miR-142-3p-expression plasmid and miR-142-3p-Silencer plasmid

The DNA sequence of miR-142-3p was amplified from cDNA isolated from SW480 cells and cloned into the *Bbs* I and *Bam*H I sites of the GP/S/EGFP/B/CMV-miR plasmid (GenePharma) with the following sequence: hsa-miRAN-142-S: 5′-TGCTGTGTAGTGTTTCCTACTTTATGGAGTTTTGGCCACTGACTGACTCCATAAAAGGAAACACTACA-3′, hsa-miRAN-142-A: 5′-CCTGTGTAGTGTTTCCTTTTATGGAGTCAGTCAGTGGCCAAAACTCCATAAAGTAGGAAACACTACAC-3′. MiR-142-3p Silencer plasmid was constructed with the following sequences: hsa-miRAN-142-S: 5′-TGCTGTCCATAAAGTAGGAAACACTACAGTTTTGGCCACTGACTGACTCCATAAAAGGAAACACTACA-3′, hsa-miRAN-142-A: 5′-CCTGTCCATAAAGTAGGAAACACTACAGTCAGTCAGTGGCCAAAACTCCATAAAGTAGGAAACACTACAC-3′. Finally, the constructs were verified by sequencing and named pPG/miR/eGFP/Blasticidin-miR-142-3p and pPG/miR/eGFP/Blasticidin-miR-142-3p-Silencer, respectively.

1.5 Construction of dual-luciferase vector comprising the *TCF7* 3′UTR

The 3′UTR segment of *TCF7* was amplified from SW480 cDNA using the following primers: 5′-TTCCTCGAGGCTGCCCCGGGTCCCCA-3′ (forward) and 5′-TGCGCGGCCGCTCAGGCTTTGAAAAACAAACCC-3′ (reverse). The mutant *TCF7* 3′UTR segment was obtained by two rounds of PCR. The first round of PCR was performed using the following combination of primers: 5′-TTCCTCGAGGCTGCCCCGGGTCCCCA-3′ (forward) and 5′-TTGCCTGATAAGGCAGATTATTTCTG-3′ (reverse); and 5′-CAGAAATAATCTGCTTATCAGGCAA-3' (forward) and 5′-TGCGCGGCCGCTCAGGCTTTGAAAAACAAACCC-3′ (reverse). The two mutant PCR products were ligated and a second round of PCR was performed with the following primers: 5′-TTCCTCGAGGCTGCCCCGGGTCCCCA-3′ (forward) and 5′-TGCGCGGCCGCTCAGGCTTTGAAAAACAAACCC-3′ (reverse). Finally, the segments of wild-type and mutant *TCF7* 3′UTRs were cloned upstream of the firefly luciferase gene into the psiCHECK-2 vector (Promega). Both constructs were verified by sequencing and named psiCHECK-2/*TCF7* and psiCHECK™-2/TCF7/mut, respectively.

1.6 Transient transfection of miR-142-3p and miR-142-3p-Silencer plasmid

The miR-142-3p and miR-142-3p-Silencer plasmids were constructed as above. The negative control (NC) RNA duplex with a sequence of S: 5′-TGCTGAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT-3′, A: 5′-CCTGAAATGTACTGCGTGGAGACGTCAGTCAGTGGCCAAAACGTCTCCACGCGCAGTACATTTC-3′ was nonhomologous to any human genome sequences, and was purchased from GenePharma.

SW480 cells were cultured in 24-well plates the day before transfection to ensure 60%–70% cell confluence at transfection. Plasmids were extracted, quantified, and transfected into SW480 cells with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. A final concentration of 500 nmol/L miR-142-3p, miR-142-3p inhibitor or negative control was used for each transfection. Moreover, for each transfection, a blank control without plasmid was prepared. The cells were analyzed 48 h after transfection in proliferation assay and target gene experiments. Each experiment included three parallel groups, and the above experiment was performed in triplicate in each parallel group.

1.7 Dual-luciferase assay

Dual-luciferase assays were performed according to the Dual-Luciferase Reporter Assay System Technical Manual (Promega). SW480 cells were plated at 5×10^4 cells per well in 24-well dishes and co-transfected with Lipofectamine 2000 (Invitrogen) 36 h later according to the manufacturer's recommendations. Each co-transfection reaction contained 500 ng of either psiCHECK-2/*TCF7* or psiCHECK-2/*TCF7*/mut and various concentrations of miR-142-3p plasmid. An additional miRNA with no predicting binding site in the *TCF7* 3′UTR was used as a control. The relative luciferase activity (firefly luminescence/Renilla luminescence) of the transfected cells in each group was determined with the Thermo Scientific Fluoroskan Ascent FL, and used to represent the transcriptional activity of the *TCF7* 3′UTR by miR-142-3p.

1.8 Western blot analysis

Western blotting was performed to detect the relative levels of TCF7 protein after transfection with miR-142-3p-expression plasmid, miR-142-3p-Silencer plasmid or miR-142-3p-NC plasmid, and TCF7 protein in four colorectal cancer cell lines and one normal colorectal mucosal epithelial cell line. Total proteins of all cell lines were extracted and quantified by the Bradford method [16]. Proteins were boiled and loaded onto 10% SDS polyacrylamide gels and electrophoretically transferred onto PVDF membranes. Then, membranes were blocked in 5% non-fat milk in TBS with 0.05% Tween-20 (TBST) at room temperature for 1 h and stained with TCF7 monoclonal antibody (1:200, Epitomics) and GAPDH antibody (Abcam). Protein was visualized using the BM Chemiluminescence Western Blotting

Kit according to the manufacturer's instructions.

1.9 RT-PCR

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to detect the relative transcript levels of *TCF7* mRNA after transfection with miR-142-3p-expression plasmid, miR-142-3p-Silencer plasmid or miR-142-3p-NC plasmid, its mRNA expression levels in several colorectal cancer cell lines and colorectal cancer tissues. The cDNA was generated using the methods mentioned above. *TCF7* and *GADPH* were amplified using the following primers: TCF7: 5′-GCTGGTTCACCCACCCATCCTT-3′ (forward) and 5′-GCCTCTTCTTCTTCCCGTAGTTGTC-3′ (reverse); *GADPH*, 5′-TGAACGGGAAGCTCACTGG-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse), generating PCR product sizes of 413 and 307 bp respectively, with the following PCR conditions: 94°C for 5 min followed by 30 cycles of 94°C for 45 s, 56°C for 45 s and 72°C for 1 min. The expression of GAPDH was analyzed as a control. All primers were purchased from Sangon Biotech Co., Ltd. (Shanghai, China).

1.10 Cell proliferation assays

Cell proliferation was measured with a cholecystokinin-octopeptide (CCK-8) assay. Twenty-four hours after transfection with miR-142-3p, miR-142-3p-inhibitor or NC, cells (approximately 1×10^3) were seeded into 96-well dishes for 12, 24, 36, 48, 60 and 72 h. The cells were then incubated with 10 μL CCK-8 for 1 h at 37°C. The optical density was determined with a spectrophotometer at a wavelength of 450 nm. Growth curves were plotted according to the ODs.

1.11 Immunohistochemistry assays

Immunohistochemical analysis was performed to detect TCF7 protein expression in human colorectal cancer tissues compared with normal colorectal mucosa tissues. Paraffin-embedded and formalin-fixed samples were cut into 4-μm sections, which were then processed for immunohistochemical examination. Histopathological diagnosis for tumor tissues and non-tumor tissues was performed using established criteria. After deparaffinization and rehydration, the sections were boiled in 10 mmol/L of citrate buffer (pH 6.0) for 10 min for antigen retrieval. Then endogenous peroxidase activity was inhibited for 30 min with methanol containing 3% H_2O_2, followed by incubation with normal non-immune goat sera for 30 min. Subsequently, sections were incubated with mouse anti-human TCF7 monoclonal antibody (1:200, Epitomics) overnight at 4°C. The immune complex was visualized with the Dako REAL EnVision Detection System Peroxidase/DAB Rabbit/Mouse (Dako). Negative controls were run by replacing the primary antibody with PBS or normal mouse IgG1. The results of TCF7 protein staining were categorized as either negative or positive based on the presence of clear brown color staining in the cytoplasm and cell membrane. Specimens with more than 10% positive cells were graded as positive.

1.12 Statistical analysis

The data from each group were calculated and presented as mean±SD from at least three separate experiments. Statistical analysis was carried out using SPSS 13.0 computer software, and non-parametric test and two-tailed Student's *t*-test were performed to compare groups. $P<0.05$ indicates a statistically significant difference.

2 Results

2.1 Upregulated miR-142-3p expression in colorectal cancer tissues and colorectal cancer cell lines correlates with the degree of tumor differentiation

MiR-142-3p was detected in all 60 (100%) pairs of colorectal cancer tissues, their matched non-tumor adjacent tissues, their normal colorectal mucosal tissues and three colorectal cancer cell lines using qRT-PCR. Our results confirmed that the average expression level of miR-142-3p was significantly upregulated in tumor tissue compared with matched non-tumor adjacent tissue (3.81±1.62 *vs.* 1.43±0.57; $P<0.05$) (Figure 1(a)). The expression level of miR-142-3p was also significantly upregulated in these colorectal cancer cell lines compared with a normal colorectal mucosal epithelial cell line and normal colorectal mucosal tissue (Figure 1(b)). The results are consistent with our previous miRNA microarray results.

Then, we analyzed the relationship between miR-142-3p and clinicopathological characteristics of colorectal cancer. The Mann-Whitney *U* test revealed that the expression level of miR-142-3p was associated with the degree of tumor differentiation ($P=0.002$) in colorectal cancer. The patients tended to have poorly differentiated cancer tissues with high miR-142-3p expression. However, the miR-142-3p expression level had no correlation with tumor site, gender, age, histological type, deeper local invasion, lymph node metastasis and TNM stage.

2.2 *TCF7* is a target of miR-142-3p

Because miR-142-3p has an overexpression profile in colorectal cancer, we studied how this miRNA exerts its function in colorectal cancer. We predicted its potential target genes using miRanda 3.3a, RNAhybrid 2.1 and TargetSpy and the TargetScan, miRBase, PicTar algorithms. The genes predicted by all the algorithms were chosen as the candidate target genes of miR-142-3p. Among them, *TCF7* was found to have a putative miR-142-3p binding site within its 3′UTR. *TCF7* is a member of the HMG box transcription factor

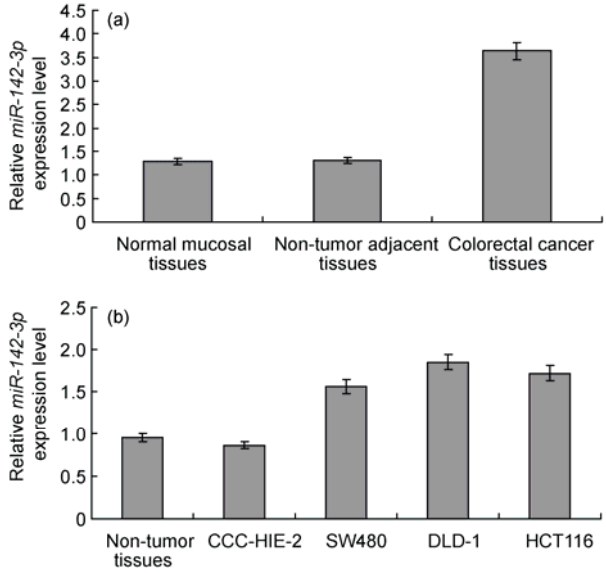

Figure 1 *MiR-142-3p* expression in colorectal cancer tissues and colorectal cell lines. *MiR-142-3p* was detected in 60 colorectal cancer samples, three colorectal cancer cell lines, a human embryo colon mucosa cell line and the corresponding normal colorectal mucosal tissues by qRT-PCR. (a) The relative expression level of *miR-142-3p* in colorectal cancer tissue, their matched non-tumor adjacent tissue and normal colorectal mucosal tissue. (b) The relative expression level of *miR-142-3p* in three colorectal cancer cell lines, a human embryo colon mucosa cell line and normal colorectal mucosal tissue. The mean and standard deviation of *miR-142-3p* expression levels are shown. The data represent triplicate measurements from single RNA samples (P<0.05).

family that plays a key role in the signaling pathways (Figure 2) regulating cell proliferation and adhesion (Table 1). Our subsequent experiments confirmed *TCF7* was an authentic target gene of miR-142-3p in colorectal cancer. In our experiments, the region of the *TCF7* 3′UTR mRNA was cloned downstream of the firefly luciferase gene in the psiCHECK-2 dual-luciferase vector to construct the reporter vector psiCHECK-2-*TCF7*-3′UTR. Then, it was co-transfected into SW480 cells with miR-142- 3p precursor or control precursor. The repression of firefly luciferase activity was observed in SW480 cells co-transfected with psiCHECK-2-*TCF7*-3′UTR and miR-142-3p precursor, but not in SW480 cells co-transfected with psiCHECK-2-*TCF7*-3′UTR and control precursor (Figure 3). To elucidate whether miR-142-3p interacted with a specific target sequence localized in the 3′UTR of *TCF7*, we constructed another reporter vector, psiCHECK-2/*TCF7*/mut, in which the 7-bp "seed" sequence (UGUGAUG) was deleted using PCR. It was co-transfected into SW480 cells with miR-142-3p precursor or control precursor, and the dual-luciferase activity was measured. It was noteworthy that miR-142-3p could no longer decrease the firefly luciferase activity of psiCHECK-2/*TCF7*/mut (P<0.05) (Figure 3(b)). In conclusion, *TCF7* 3′UTR carries direct binding sites for miR-142-3p.

2.3 MiR-142-3p could inhibit *TCF7* expression in a dose-dependent manner

According to the experimental results in the above section, the regulatory effect of different doses of miR-142-3p on *TCF7* 3′UTR was analyzed. We observed that the higher the dose of miR-142-3p, the lower the relative activity of the luciferase. The results demonstrated that the inhibition of *TCF7* by miR-142-3p was dose-dependent (Figure 4).

2.4 MiR-142-3p post-transcriptionally downregulates *TCF7* expression

MiRNAs regulate 10%–30% of all protein-coding genes by binding to partially complementary sequences in the 3′UTR of downstream target mRNAs. miRNAs mainly inhibit protein translation of their target genes and infrequently cause degradation or cleavage of the mRNA. We performed RT-PCR and Western blot assays to verify how miR-142-3p regulates *TCF7*. As shown in Figure 5(a), RT-PCR analysis of *TCF7* showed that miR-142-3p had no effect on *TCF7* mRNA levels. Nevertheless, TCF7 protein levels were decreased in miR-142-3p-transfected SW480 cells but increased in miR-142-3p-Silencer-transfected SW480 cells compared with NC-transfected SW480 cells (Figure 5(b)). These results demonstrate that miR-142-3p negatively regulates *TCF7* expression post-transcriptionally.

2.5 MiR-142-3p inhibits cell proliferation *in vitro*

The significant upregulation of miR-142-3p expression in colorectal cancer samples prompted investigation of the possible biological roles of miR-142-3p in tumorigenesis. First, we detected miR-142-3p expression by qRT-PCR 48 h after transfection of miR-142-3p, miR-142-3p-Silencer, their NC and blank controls. Transfection efficiency was satisfactory. The results of the CCK8 assays and growth curves indicated that proliferation was inhibited at different degrees in the SW480 cells transiently transfected with miR-142-3p (P<0.05), while those cells transfected with miR-142-3p-Silencer had significant growth promotion compared with that of the cells transfected with NC and blank controls (P<0.05). No statistically significant difference was observed between NC and blank controls (P>0.05) (Figure 6).

2.6 *TCF7* mRNA and protein expression was upregulated in four colorectal cancer cell lines

Using RT-PCR, *TCF7* mRNA was detected in four colorectal cancer cell lines and a human embryo colon mucosa cell line. Results showed that the average expression level of *TCF7* mRNA was significantly upregulated in colorectal cancer cells compared with normal colorectal mucosal cells (P<0.05) (Figure 7).

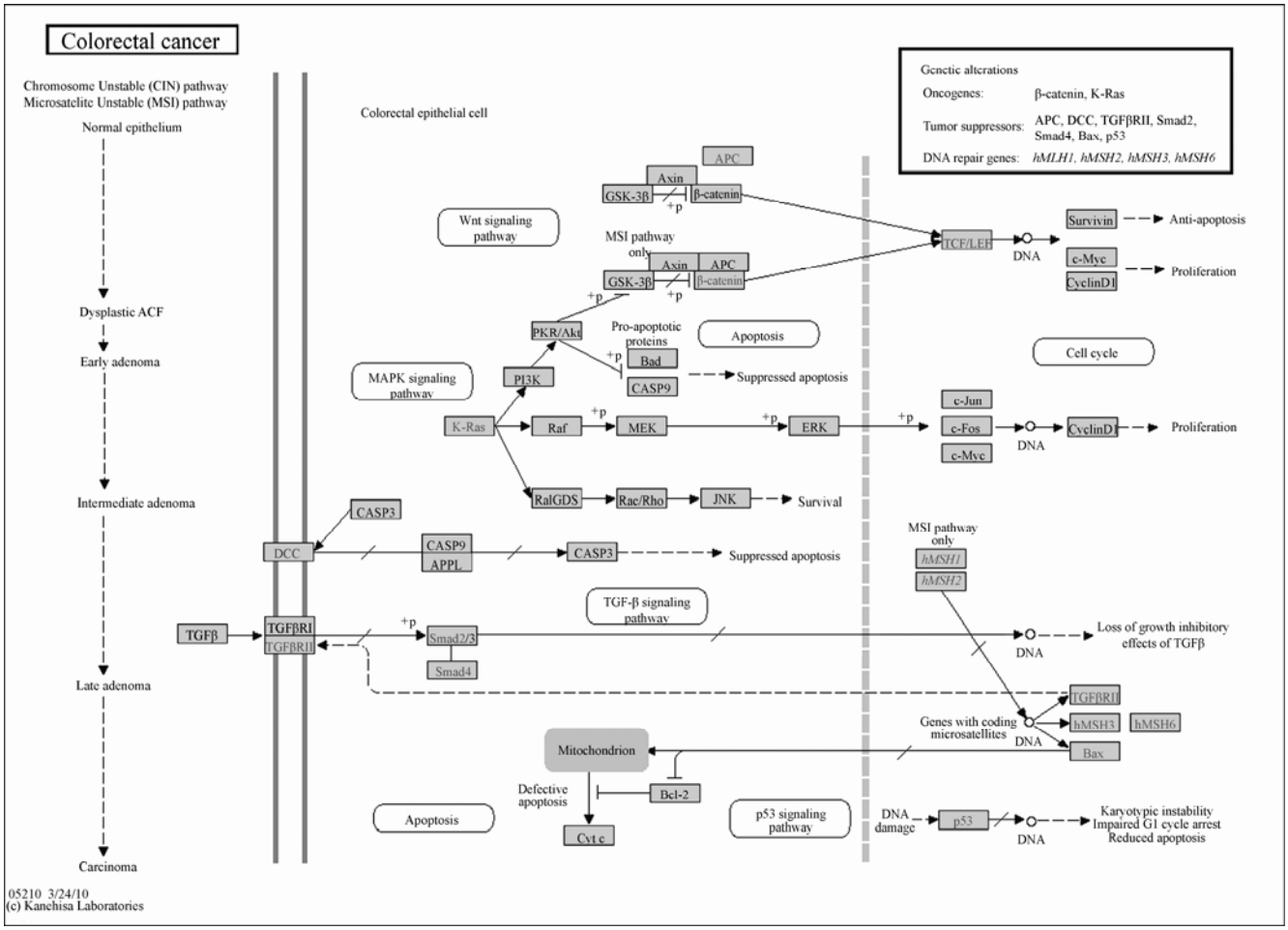

Figure 2 The *TCF7* pathway in colorectal cancer.

Table 1 Target gene prediction for miR-142-3p

Signaling pathway	Gene	Ensembl gene ID
Cell cycle_Initiation of mitosis	*CDC25C*	ENSG00000158402
	MNAT1	ENSG00000020426
Development_WNT signaling pathway. Part 2	*TCF7*	ENSG00000081059
	FZD7	ENSG00000155760
Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling	*TCF7*	ENSG00000081059
	FZD7	ENSG00000155760
Cell adhesion_Role of CDK5 in cell adhesion	*TCF7*	ENSG00000081059
Transport_RAB3 regulation pathway	*DMXL2*	ENSG00000104093

Western blot assays were performed to detect TCF7 protein in four colorectal cancer cell lines and human embryo colon mucosa cell line. As shown in Figure 8, TCF7 protein levels were significantly higher in colorectal cancer cell lines than in human embryo colon mucosa cells (P<0.05).

2.7 *TCF7* mRNA and protein expression in human colorectal cancer tissues

TCF7 mRNA was detected in human colorectal cancer tissues and the corresponding normal colorectal mucosal tissues with RT-PCR. Results showed that the average expression levels of *TCF7* mRNA were significantly upregulated in colorectal cancer tissues compared with the corresponding controls (P<0.05) (Figure 9).

Tissue immunochemistry was carried out with a TCF7 monoclonal antibody to detect TCF7 protein in human colorectal cancer tissues. As shown in Figure 10, *TCF7* expression was +++ in the colorectal cancer tissues, ++ in their matched non-tumor adjacent tissue, while in normal

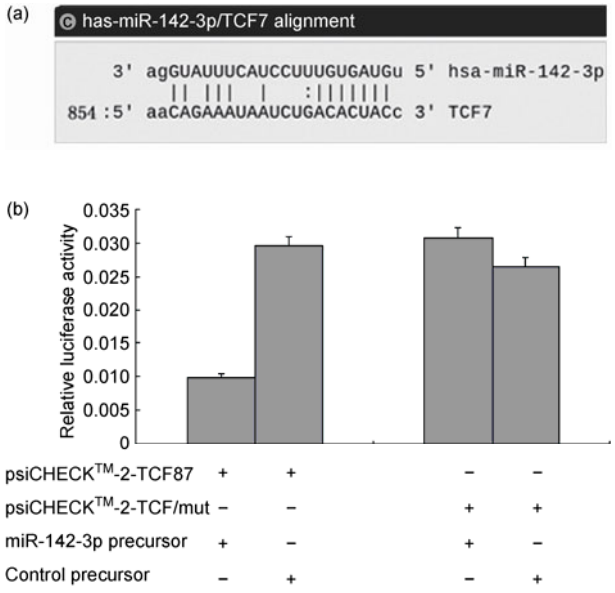

Figure 3 *TCF7* is a validated target of miR-142-3p. (a) Putative binding sites of miR-142-3p in the *TCF7* 3′UTR (ACACUAC). (b) miR-143-3p decreased luciferase activity controlled by wild-type *TCF7* 3′ UTR (P<0.05) but did not affect luciferase activity controlled by mutant *TCF7* 3′UTR (ACACUAC).

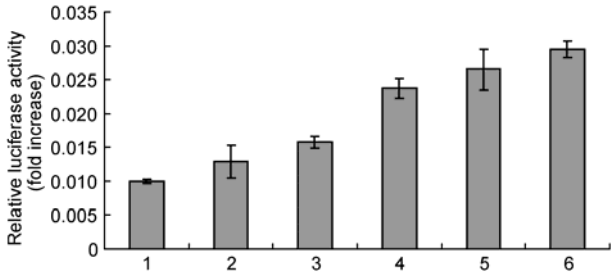

Figure 4 Dose-dependent regulation of *TCF7* 3′UTR by miR-142-3p. The results demonstrated that miR-142-3p could inhibit *TCF7* expression in a dose-dependent manner (the dose of miR-142-3p in each group from 1 to 6 was 500, 400, 300, 200, 100, and 0 ng, respectively).

colorectal tissue it was +.

3 Discussion

In contrast to their tight regulation during development and in normal tissues, miRNAs are abnormally expressed in cancer. MiRNAs that are overexpressed in cancer may function as oncogenes, and may function as tumor suppressors when downregulated in cancer. Thus, they are regarded as important modulators in a variety of fundamental cellular biological processes, and appear to play a pivotal role in human carcinogenesis. For instance, it was reported that high expression levels of miR-21 were significantly correlated with lymph node metastasis, advanced clinical stage, and poor prognosis in patients with breast cancer [29]. MiR-142-3p was recently identified as an oncogenic miRNA in human T-cell acute lymphoblastic leukemia

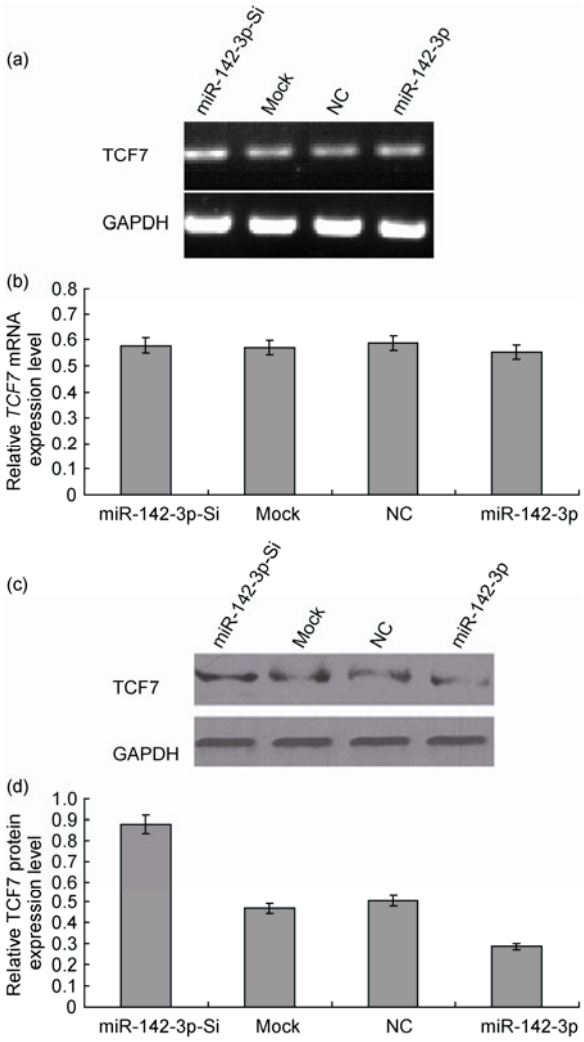

Figure 5 MiR-142-3p post-transcriptionally downregulates *TCF7* expression. (a), (b) *TCF7* mRNA in SW480 cells was detected by RT-PCR at 48 h post-transfection with miR-142-3p, miR-142-3p-Silencer, NC and blank controls (mock). The results are shown as fold changes relative to the control cells. (c), (d) TCF7 protein in SW480 cells was analyzed by western blot at 48 h post-transfection with miR-142-3p, miR-142-3p-Silencer, their respective NCs and blank controls (mock). The expression of GAPDH was analyzed as a control.

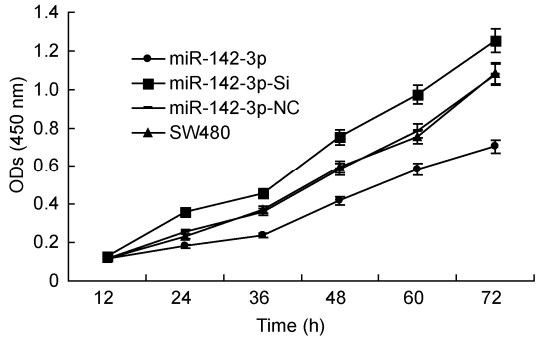

Figure 6 Growth curves of each group as measured by CCK-8 assay. Proliferation was inhibited in SW480 cells transfected with miR-142-3p, and cells transfected with miR-142-3p-Silencer had a significant growth promotion compared with cells transfected with NC and blank controls (mock). All results were reproducible in three independent experiments (P<0.05).

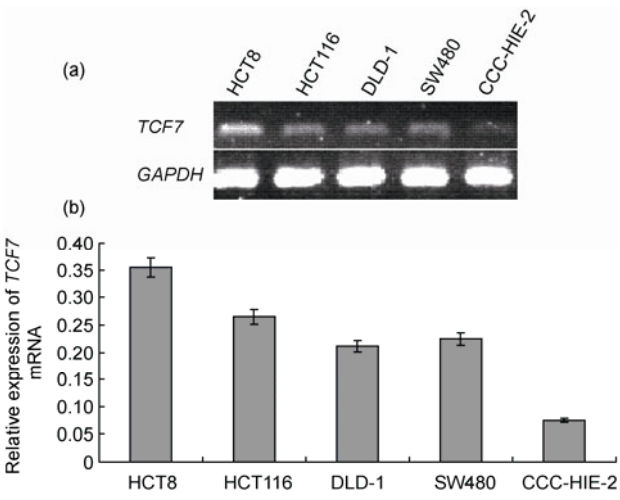

Figure 7 *TCF7* mRNA expression in four colorectal cancer cell lines and human embryo colon mucosa cell lines. (a) *TCF7* mRNA expression in SW480, DLD-1, HCT116, HCT8 human colorectal cell lines and CCC-HIE-2 human embryo colon mucosa cells as demonstrated by RT-PCR. (b) The fold changes in *TCF7* mRNA in these cell lines. The mean and standard deviation of *TCF7* mRNA expression levels are shown.

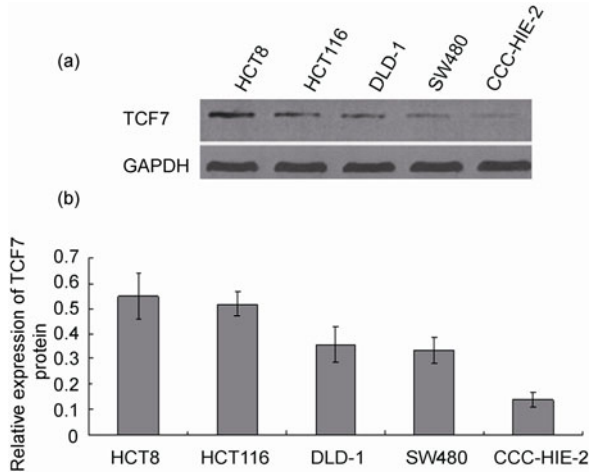

Figure 8 Western blot assays of TCF7 protein in four human colorectal cell lines and human embryo colon mucosa cells. (a) TCF7 protein expression in SW480, DLD-1, HCT116, HCT8 human colorectal cell lines and CCC-HIE-2 human embryo colon mucosa cells. (b) The fold changes of TCF7 protein in these cell lines. The mean and standard deviation of *TCF7* mRNA expression levels are shown.

(T-ALL) by targeting the glucocorticoid receptor-a and the cAMP/PKA pathways [22]. Wu et al. [23] demonstrated that miR-142-3p directly and negatively regulates RAC1 in HCC cells, and suppresses the migration and invasion of hepatocellular carcinoma cells. Another study reported that miR-142-3p was a potential prognostic biomarker for esophageal squamous cell carcinoma [24]. However, the potential function of miR-142-3p in colorectal cancer was not determined. In previous studies, we performed miRNA gene chip screening to detect miRNA profiles in colorectal cancer tissues. We observed that the expression level of miR-142-3p was significantly upregulated and was associated

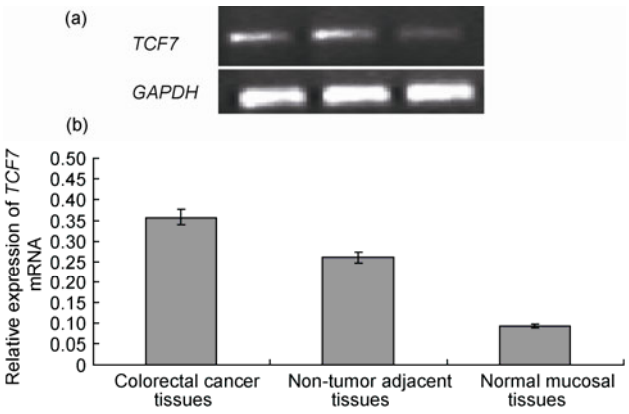

Figure 9 *TCF7* mRNA expression in colorectal cancer tissues. (a) *TCF7* mRNA in colorectal cancer tissues demonstrated by RT-PCR. (b) The fold changes of *TCF7* mRNA in colorectal tissues, non-tumor adjacent tissues and normal mucosal tissues. The mean and standard deviation of *TCF7* mRNA expression levels are shown.

with clinicopathological features; therefore, we focused on miR- 142-3p for further study.

It is clear that the fundamental function of miRNAs is to regulate protein-coding genes. This occurs via two mechanisms: first, miRNAs induce the RNA-mediated interference (RNAi) pathway by binding to protein-coding mRNA sequences [30] that are exactly complementary to the miRNA, leading to cleavage of mRNA. Second, miRNAs bind to imperfect complementary sites within the 3′UTRs of their target protein-coding mRNAs, leading to repression of the expression of these genes at the translational level. In humans, miRNAs mainly inhibit protein translation of their target genes and infrequently cause degradation or cleavage of the mRNA. Nowadays, we use computational algorithms to predict miRNA targets, which are based mainly on base pairing of miRNAs and target gene 3′UTRs [31]. In our study, we predicted the potential target genes of miR-142-3p by using miRanda 3.3a, RNAhybrid 2.1 and TargetSpy and the TargetScan, miRBase, PicTar algorithms. The genes predicted by all the algorithms were chosen as the candidate target genes of miR-142-3p. Among them, *TCF7* was found to have a putative miR-142-3p binding site within its 3′UTR. Further studies provided further evidence to confirm our prediction. First, the repression of firefly luciferase activity was observed in SW480 cells co-transfected with psiCHECK-2-*TCF7*-3′UTR and miR-142-3p precursor, but not in SW480 cells co-transfected with psiCHECK-2-*TCF7*-3′UTR and control precursor. Second, miR-142-3p could not decrease the firefly luciferase activity of psiCHECK-2/*TCF7*/mut. Third, overexpression of miR-142-3p post-transcriptionally downregulates TCF7 protein expression.

TCF7 is a member of the HMG box transcription factor family, which also includes TCF-4, a locus implicated in some colorectal cancers. The *TCF7* gene product is a transcription factor that affects the expression of genes critical to Th1 responses, such as the interleukin (IL)-12R subunit, which is important in cell-mediated immunity. *TCF7*

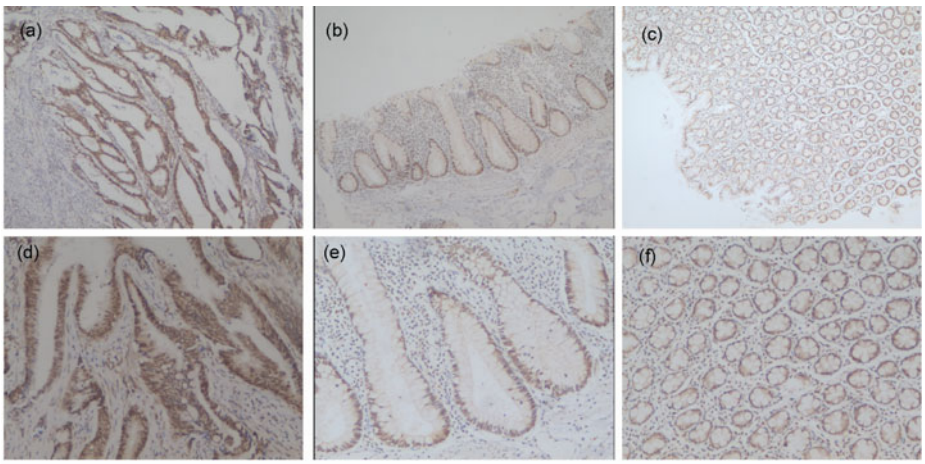

Figure 10 Immunohistochemical analysis of TCF7 protein in human colorectal cancer tissue, adjacent tissue and normal tissue. (a) and (d) human colorectal adenocarcinoma tissue; (b) and (e) adjacent colorectal tissue; (c) and (f) normal human colorectal tissue. Magnifications are ×40 (a), (b), (c), ×200 (d), (e), (f); slice thickness is 4 μm.

expression is limited to T- and NK-cells. Knockout experiments in mice have shown that *TCF7* is necessary for T-cell development in the thymus. The latest findings showed TCF7 protein has crucial functions in signaling pathways such as the Wnt pathway and other pathways involved in regulating cell adhesion, cytoskeleton reorganization, proliferation, apoptosis and migration [32–34]. Accumulating research indicated *TCF7* may function as an oncogene. The expression of *TCF7* is upregulated in many cancers, including colorectal, endometrial, and prostate cancer, and may be correlated with poor prognosis. All this evidence shows that *TCF7* plays a key role in cancer pathogenesis.

In this study, we confirmed significant upregulation of miR-142-3p in colorectal cancer tissues and colorectal cancer cell lines. We predicted and identified *TCF7* as a target of miR-142-3p. Furthermore we found miR-142-3p negatively regulates *TCF7* expression post-transcriptionally, and overexpression of miR-142-3p in SW480 colorectal cancer cell lines potently inhibited cell proliferation *in vitro* by inhibiting the expression level of *TCF7*. *TCF7* mRNA and protein expression is upregulated in colorectal cancer tissues and several colorectal cancer cell lines, and is closely correlated with its function as an oncogene in promoting tumor cell proliferation and inhibiting cell apoptosis, which are known malignant characteristics of tumors. Our results suggested that miR-142-3p may be involved in the regulation of cell proliferation in colorectal cancer, with *TCF7* as a direct target. As each gene is regulated by a variety of regulatory factors, we presumed that there must be other pathways that upregulate the levels of *TCF7* in colorectal cancer. The upregulation of miR-142-3p could not inhibit *TCF7*, highlighting the complicated systems operative in tumorigenesis.

Recent research has enabled the identification of hundreds of human miRNAs, but most of their targets remain unknown and we still know little about their mechanisms of action and the pathways they modulate. In our study, the identification of miR-142-3p and its target gene, *TCF7*, in colorectal cancer may help in understanding the potential molecular mechanisms of colorectal cancer development and may present exciting therapeutic opportunities in the future.

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