miR-675 promotes colorectal cancer cell growth dependent on tumor suppressor DMTF1

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Abstract. Colorectal cancer (CRC) has become a worldwide health concern, particularly in developing countries. Therefore, the present study focuses on the investigation of oncogenic microRNA (miR)-675-3p, and its role in colorectal carcinogenesis. miR-675-3p expression was either overexpressed or inhibited in SW480 CRC cells in order to demonstrate its positive effect on the cell proliferation, as determined by MTS and flow cytometry. Then the present study utilized a luciferase assay to demonstrate that cyclin D binding myb like transcription factor 1 (DMTF1) was modulated by miR-675-3p directly at its 3'untranslated region. Overexpression or inhibition of miR-675-3p affected the expression of DMTF1, as determined by reverse transcription-quantitative polymerase chain reaction and western blotting. In addition, the overexpression of miR-675-3p promoted cell proliferation, whereas the additional introduction of DMTF1 rescued the overgrowth of the SW480 cells. These results were also confirmed in HT29 CRC cells. In summary, the results of the study demonstrated that miR-675-3p directly regulated the expression of DMTF1, which contributed to the further regulation of CRC cell proliferation.

Introduction

Colorectal cancer (CRC) is now commonly diagnosed worldwide, particularly in patients who are in favor of a 'Western diet'. Statistically, >1,400,000 new cases of CRC were diagnosed and a cancer-associated mortality rate of 5% among patients with CRC was reported in 2012. It is estimated that there are likely to be >2,200,000 new cases diagnosed and 1,100,000 cases of cancer-associated mortality by the year 2030 (1). CRC normally begins with noncancerous adenomas and gradually develops into CEC over a period of 10-20 years (2). The presence of inherited genetic abnormalities, (i.e. family history) significantly increases the risk of developing CRC compared with the absence of such an abnormality (3). Studies have identified a substantial number of genes linked to hereditary CRC syndromes (4). In addition, epigenetic aberrations have been found to be implicated in the initiation and development of a variety of tumors, including CRC (5,6). Given the fact that epigenetic modifications are reversible, a number of studies have focused on the potential applications of epigenetic aberration as a therapeutic target.

MicroRNAs (miRNAs), one of the epigenetic regulatory mechanisms, are important in the downregulation of gene expression by complementarily binding to the target mRNA (7,8). miRNAs are small fragments of non-coding RNA with a size of 20-24 nucleotides and are generated through multiple enzymatic excisions of miRNA precursor pri-miRNA. The RNA-induced silencing complex, which is composed of mature miRNAs including dicer and other associated factors, is formed and leads to enzymatic cleavage of the target mRNA, resulting in substantially decreased levels of protein translation (7,9). miR-675 is derived from exon 1 of long noncoding RNA H19 (10). Mice with deficiency of the H19 transcript grow normally. Hao *et al* (11), showed that H19 exhibits tumor suppression activity, and its associated miR-675 has been shown to be oncogenic in gastric (12), liver (13) and lung cancer (14). Therefore, the dysregulation of miR-675 may be used as a potential biomarker for detecting carcinogenesis in multiple types of cancer.

Cyclin D binding myb like transcription factor 1 (DMTF1) is induced by oncogenic Ras-Raf signaling and functions as a tumor suppressor (15). DMTF1-heterozygous and -null mice exhibit accelerated formation of spontaneously-developed or oncogene-induced tumors (16). Of all types of human non-small lung cancer, ~40% have been found to have DMTF1 gene deletion (15). In addition, the expression level of DMTF1 is higher in the colon relative to that in the lung, according to the proteome database (17); this indicates its potential role in CRC. In the present study, it was demonstrated that miR-675-3p directly suppressed DMTF1, which further contributed to the proliferation of CRC cells.

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Materials and methods

Human patients and CRC tissues. CRC tissues and adjacent non-carcinogenic tissues were collected from patients who underwent surgery between 2012 and 2017 at the Affiliated Hospital of Beihua University (Jilin City, China). All patients with CRC were diagnosed by colonoscopy pathology. The total number of patients was 60 with age ranging between 45 and 81 years. The gender ratio was 1.4:1.0 (male:female). All procedures were conducted under the approval of the Ethics Committee of the Affiliated Hospital of Beihua University. The tissues were collected with patients' informed consent. The collected tissues were immediately stored at -80°C for future use.

Cell culture and transfection. The SW480 and HT29 CRC cell lines (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Sigma; Merck KGaA, Darmstadt, Germany). The cell cultures were maintained at 37°C under a humidified atmosphere containing 5% CO_2. Transfections were conducted with either Lipofectamine 3000 (Thermo Fisher Scientific, Inc.) for the overexpression (plasmid) or RNAiMax for the miRNA mimics and inhibitors.

pCDNA3 was used as a vector to construct the full-length DMTF1 overexpression plasmid. An empty pCDNA3 vector was used as the negative control. The miR-675-3p mimics, inhibitors and the corresponding controls were purchased from Sigma; Merck KGaA for transfection. The cells were seeded in antibiotic-free medium prior to transfection to increase the transfection efficiency. The growth medium was replaced 12 h following transfection.

RNA extraction, cDNA synthesis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The cultured cells were washed with cold PBS and then treated with TRIzol (Thermo Fisher Scientific, Inc.). Total RNA was isolated from the TRIzol-lysed cells following the manufacturer's protocol. Following isolation, 500 ng of total RNA was used with 10 μl cDNA synthesis system, including 2 μl of 10X RT buffer, 0.8 μl of 100 mM NTP mix, 2 μl of 10X RT Random Primers, 1 μl of reverse transcriptase and 3.2 μl of nuclease-free water (High-Capacity cDNA Reverse Transcript kit; Thermo Fisher Scientific, Inc.). The temperature protocol for RT-PCR was as follows: 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. The qPCR system included: 5 μl of SYBR master mix (Thermo Fisher Scientific, Inc.), 0.5 μl of synthesized cDNA, 2 μl of primer mix (5 μM each) and 2.5 μl of water. Specific primers were used for SYBR green RT-qPCR analysis, as previously described (18): DMTF1, forward 5'-TGTAGCTGATCCATCCGTTGT-3' and reverse 5'-GGG GTTGCTCCTATTTCTTTG-3'; GAPDH, forward 5'-GCG AGATCGCACTCATCATCT-3' and reverse 5'-TCAGTGGTG GACCTGACC-3'.

The PCR conditions were as follows: 95°C for 3 min, 40 cycles at 95°C for 10 sec and then at 60°C for 30 sec, followed by 72°C for 3 min. The total RNA was isolated with TRIzol, and the qScript microRNA cDNA Synthesis kit (Quantabio) was used for miRNA according to the manufacturer's protocol. RT-qPCR analysis was performed using the miScript SYBR Green PCR kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. The miScript Primer Assay (Qiagen, Inc.) was used for quantification of miR-675-3p and the U6 small RNA Assay (Qiagen, Inc.) was used for the housekeeping control (primer sequences are commercially unavailable). The RT-qPCR was performed as follows: 95°C for 2 min, 40 cycles at 95°C for 5 sec and then at 60°C for 30 sec. Data were quantified using the $2^{-\Delta\Delta Cq}$ method, as previously described (19).

MTS assay. The cells were seeded in 96-well plates (10^5 cells/well) and were maintained in the growth medium (100 μl) at 37°C prior to the addition of sterile MTS dye (0.33 mg/ml, 20 μl). The absorbance was obtained using an enzyme immunoassay analyzer (BioTek Instruments, Inc., Winooski, VT, USA) at a wavelength of 490 nm upon a further incubation period of 2 h.

Crystal violet staining. The cells were washed with cold PBS and fixed with ice-cold methanol for 10 min, followed by staining with 0.5% crystal violet solution for 10 min. The cells were washed with deionized water prior to being examined using the Olympus CX21 light microscope (Olympus Corporation, Tokyo, Japan).

Flow cytometry. The cells were washed with cold PBS and fixed in cold 75% ethanol at 4°C for 30 min. The fixed cells were washed three times and treated with ribonuclease. The resulting cells were stained with propidium iodide (50 μg/ml) at 4°C overnight (Abcam, Cambridge, UK) prior to flow cytometric analysis with excitation at 488 nm (BD Biosciences, Franklin Lakes, NJ, USA).

Vector construction and luciferase assay. The TargetScan database (targetscan.org) was used to predict potential targets of miR-675-3p (20). The 3'untranslated region (UTR) of the DMTF1 sequence was amplified and cloned into a pGL4.10-report vector (Promega Corporation, Madison, WI, USA). Equal quantities of the pGL4.10-3UTR-DMTF1 and *Renilla* expression vector pRL-TK (Promega Corporation) were co-transfected into the SW480 cells. The luciferase activity was measured 48 h post-transfection using the Dual-Glo Luciferase Assay system (Promega Corporation).

Western blot analysis. The cells were lysed with proteinase inhibitors-containing RIPA buffer to extract total protein. The protein concentration was measured using the Bradford assay (Sigma; Merck KGaA). Subsequently, 20 μg of total protein was added to each well of 10% SDS-PAGE. Then proteins were transferred to PVDF membranes and incubated in transferring buffer with 5% non-fat milk at room temperature for 90 min. Membranes were incubated with a 1:2,000 dilution of the primary antibody against human DMTF1 (cat. no. PA5-29466; Thermo Fisher Scientific, Inc.) or Tubulin (cat. no. T9026; Sigma-Aldrich; Merck KGaA) at 4°C overnight and then with secondary antibody (goat anti-rabbit; cat. no. 31460; Thermo Fisher Scientific, Inc.) at room temperature for 60 min.

Statistical analysis. Data were analyzed using unpaired t-tests or two-way analysis of variance with Tukey's post hoc test for multiple comparisons. Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-675-3p promotes proliferation in CRC cells. To demonstrate the effect of miR-675-3p on CRC cell growth, overexpression of miR-675-3p was induced by transfecting miR-675-3p mimics into the human SW480 CRC cells, whereas the inhibition assay was performed by transfecting miR-675-3p inhibitors. The expression level of miR-675-3p was found to be significantly increased or halved upon transfection with the miR-675-3p mimic or miR-675-3p inhibitor, respectively, compared with the corresponding controls (Fig. 1A). Proliferation of the aforementioned cells was evaluated using an MTS assay. Cells transfected with the miR-675-3p mimics showed higher values of OD_{490}, indicating the presence of more viable cells compared with the miR-NC control (Fig. 1B), whereas the inhibition of miR-675-3p notably reduced the number of viable cells compared with its control. To confirm the changes observed were caused by miR-675-3p, crystal violet staining was used to evaluate the formation of cell colonies. Consistent with the results of the MTS assay, a significant increase in the number of cell colonies was observed for the miR-675-3p-overexpressed cells and a decrease was observed for the miR-675-3p-inhibited cells (Fig. 1C). The increase in the number of cell colonies induced by miR-675-3p was considered to be due to enhanced proliferation. Flow cytometry was employed to analyze the cell cycle: For the miR-675-3p-overexpressed cells, the number of cells in the S phase increased but those in the G_0/G_1 phases decreased, whereas the reverse was observed for the miR-675-3p-inhibited cells (Fig. 1D).

To confirm the concept that miR-675-3p promotes cell proliferation, the expression of miR675-3p was manipulated in the HT29 CRC cells. The HT29 cells overexpressing miR675-3p and HT29 cells with suppressed expression of miR675-3p were generated (Fig. 2A). In these two cell lines, consistent data were obtained that the overexpression of miR675-3p significantly increased cell proliferation whereas the inhibition of miR675-3p lowered proliferation of the HT29 cells, determined by the OD_{490} value for viable cells and MTS assay for cell colony (Fig. 2B and C).

miR-675-3p directly modulates the expression of DMTF1. The TargetScan database was used to predict potential targets of miR-675-3p (20). The binding site at the 3'UTR region of DMTF1, a known tumor suppressor gene (15,21,22), was found to be a potential target of miR-675-3p (Fig. 3A). A luciferase assay was conducted to examine the regulation of DMTF1 by miR-675-3p. The overexpression of miR-675-3p in the SW480 cells significantly reduced the relative luciferase activity compared with that in the cells with the mutated DMTF1-3'UTR region, indicating a direct negative regulation of the expression of DMTF1 by miR-675-3p through the DMTF1 3'UTR region (Fig. 3B). Consistently, a decrease in the expression of DMTF1 was found in the SW480 cells with overexpressed miR-675-3p at the mRNA level (Fig. 3C). Such a decrease in the expression of DMTF1 was also confirmed by inhibiting the expression of miR-675-3p in the SW480 cells (Fig. 3D). In addition to the changes of DMTF1 observed at the mRNA level, western blotting was performed to examine the protein level changes of DMTF1 in the miR-675-3p-overexpressed and -inhibited SW480 cells. The results showed that the overexpression of miR-675-3p suppressed the protein level of DMTF1, whereas the inhibition of miR-675-3p increased the level of DMTF1 (Fig. 3E).

Significant reduction in the expression of DMTF1 in human CRC tumors. As miR-675-3p promoted CRC cell proliferation and regulated the downstream expression of DMTF1, it was hypothesized that DMTF1 was also involved in the development of CRC. The expression of DMTF1 was examined and was found to be markedly decreased in the human CRC tumors using immunohistochemistry (Fig. 4A) and western blot analysis (Fig. 4B) compared with that in its adjacent non-carcinogenic tissue. As miR-675-3p was effective in regulating DMTF1 mRNA, the relative mRNA level of DMTF1 in tumors was assessed and was also found to be reduced, consistent with the protein pattern (Fig. 4C).

Overexpression of DMTF1 suppresses cell proliferation in CRC cells. As DMTF1 was downregulated in CRC tumors, the SW480 cells were transfected to overexpress DMTF1 to illustrate its function in carcinogenesis. As shown in Fig. 4D, there was an increase of DMTF1 in the transfected SW480 cells compared with that in the control group. Cell proliferation (Fig. 4E), cell colony formation (Fig. 4F and G) and cell cycle (Fig. 4H) were also examined using this cell line. The results demonstrated significant decreases in all three aforementioned parameters in the DMTF1-overexpressed SW480 cells, indicating the negatively regulatory role of DMTF1 in cell proliferation.

miR675-3p promotes CRC proliferation in a DMTF1-dependent manner. It has been shown that miR675-3p and DMTF1 regulated CRC cell proliferation and that miR675-3p modulated the level of DMTF1 through binding to its 3'UTR region. A rescue assay was performed to further demonstrate the correlation between miR-675-3p and DMTF1 on CRC growth. The SW480 cells were used to overexpress miR-NC, miR-675-3p and miR675-3p + DMTF1, respectively. Consistent with the previous results, the mRNA level of DMTF1 reduced when miR-675-3p was overexpressed (Fig. 5A) and co-transfection of the plasmid expressing DMTF1 normalized its expression (Fig. 5A). In addition, the protein level of DMTF1 was examined by western blotting (Fig. 5B); the expression level of DMTF1 decreased in the presence of exogenous miR-675-3p but normalized with additional expressed DMTF1. Subsequently, crystal violet staining was conducted to evaluate the single cell colony formation. For the miR-675-3p-overexpressed SW480 cells, co-transfection of DMTF1 reduced the number of single colonies (Fig. 5C). It was found that the increased cell proliferation induced by miR675-3p was rescued by the expression of DMTF1, based on the MTS results (Fig. 5D). miR-675-3p significantly amplified the proportion of cells
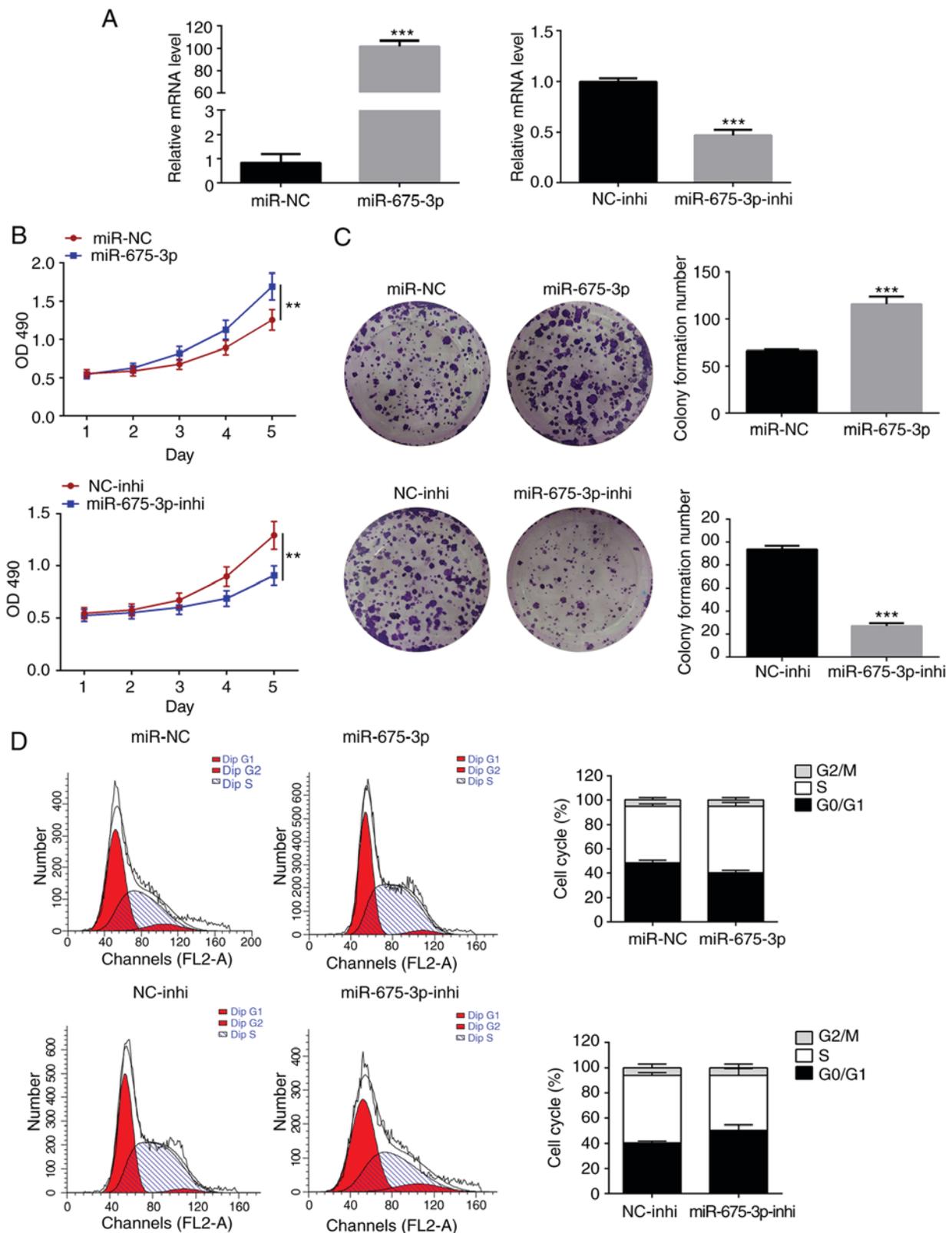
Figure 1. miR-675-3p promotes cell growth. (A) SW480 CRC cells were transfected to overexpress of miR-675-3p or have reduced expression of miR675-3p, confirmed by reverse transcription-quantitative polymerase chain reaction analysis. SW480 cells with either overexpressed and reduced miR-675-3p exhibited (B) increased and decreased cell proliferation, (C) single colony formation and (D) cell cycle alterations, respectively. **P<0.01 and ***P<0.001 vs. NC. miR, microRNA; NC, negative control; inhi, inhibitor.

in the S phase in the cell cycle assay. Upon co-expression of DMTF1, the S phase population decreased back to a level comparable to that of the control group (Fig. 5E).

In addition, the DMTF1 rescue experiment was performed in another CRC cell line (HT29). Consistent with the SW480 cells, the overexpression of miR675-3p inhibited the expression
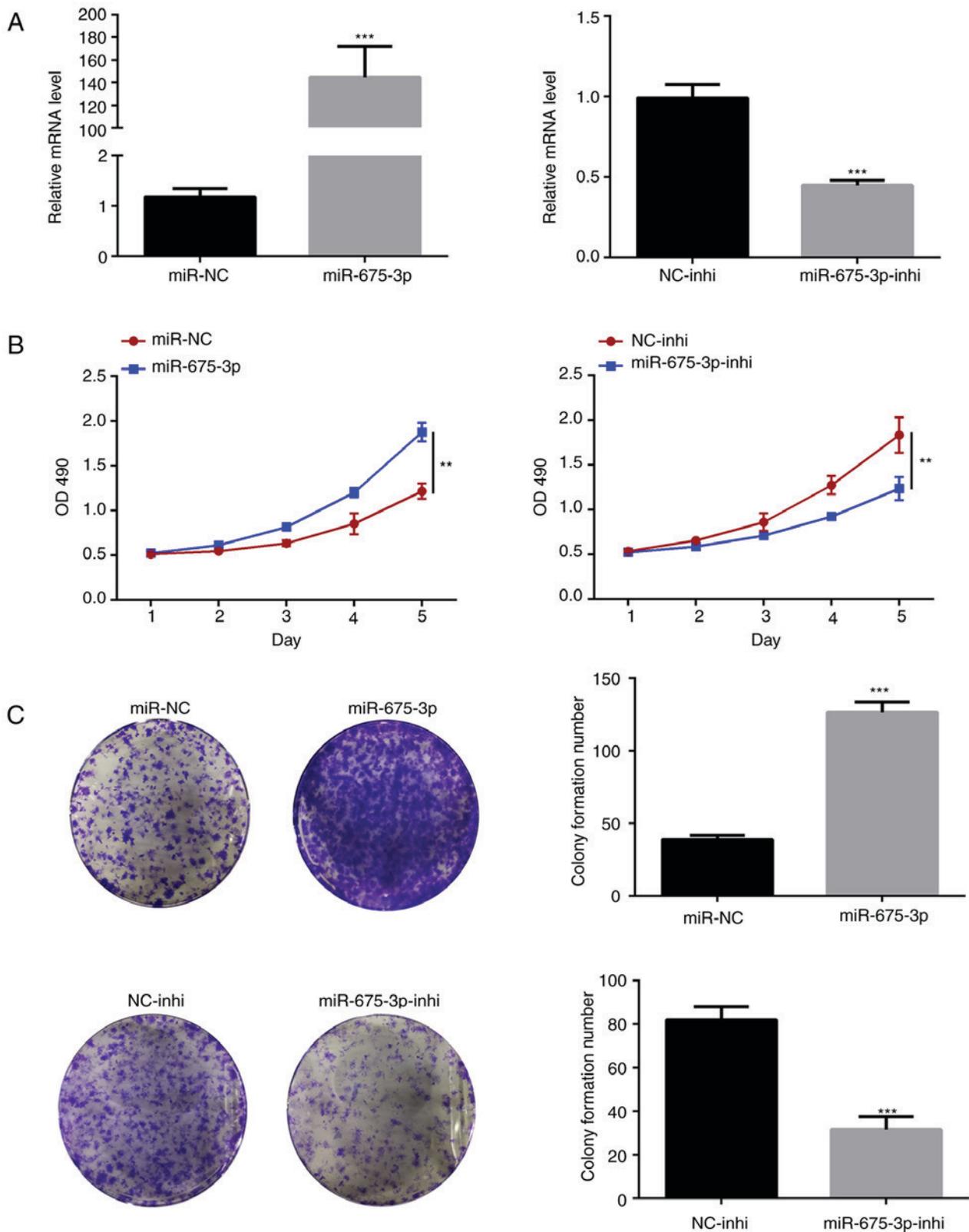
Figure 2. miR-675-3p promotes cell growth in HT29 cells. (A) Confirmation of overexpressed or reduced expression of miR-675-3p in the transfected HT29 cells. (B) HT29 cells with either overexpression or reduced miR-675-3p exhibited increased or decreased cell proliferation. (C) Increased and decreased single colony formation were observed in the HT29 cells with overexpressed miR-675-3p and inhibited miR-675-3p, respectively. **P<0.01 and ***P<0.001 vs. NC. miR, microRNA; NC, negative control; inhi, inhibitor.

of DMTF1 (Fig. 6A). To elucidate the effect of DMTF1, DMTF1 was overexpressed in HT29 cells overexpressing miR675-3p (Fig. 6A). It was found that DMTF1 negatively

regulated the miR675-3p-induced overgrowth of colonic cells, as determined using the MTS assay (Fig. 6B) and via OD_{490} measurement (Fig. 6C).
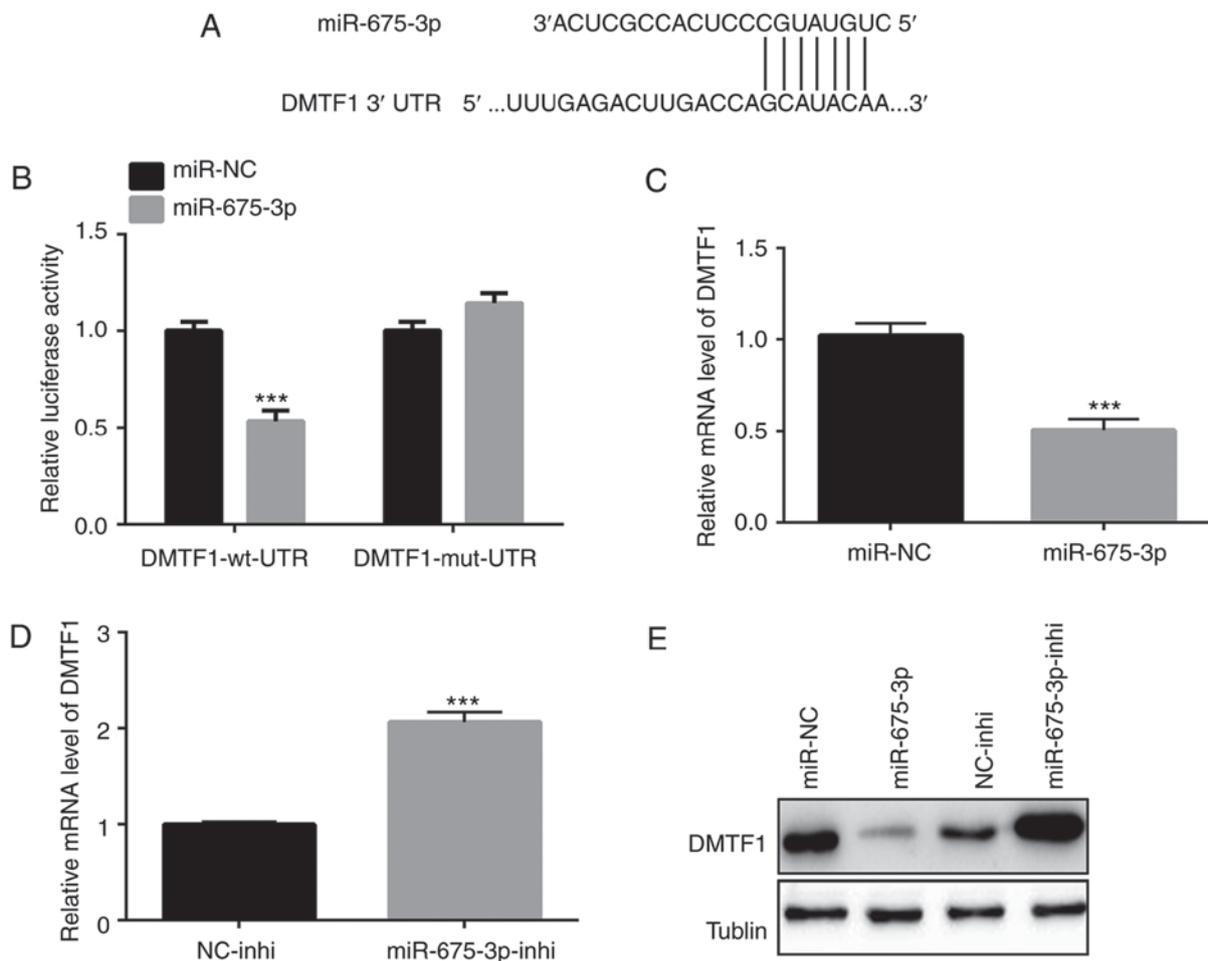
Figure 3. miR675-3p directly regulates the expression of DMTF1 through its 3'UTR. (A) Predicted results from the online resource TargetScan indicated a potential binding site of miR675-3p at the 3'UTR of DMTF1. (B) Luciferase activity assay showed a negative regulatory effect of miR675-3p on DMTF1 through the 3'UTR region. (C) Overexpression of miR675-3p in the SW480 cells led to a reduction in the mRNA level of DMTF1. (D) Inhibition of miR675-3p led to a significant increase in the mRNA level of DMTF1. (E) Western blotting showed consistent protein patterns in the miR-675-3p-overexpressed and miR-675-3p-inhibited cells. ***$P<0.001$ vs. NC. miR, microRNA; NC, negative control; inhi, inhibitor; DMTF1, cyclin D binding myb like transcription factor 1; UTR, untranslated region.

Discussion

The initiation and progression of CRC are driven by multiple factors, including distinct genetic and epigenetic alterations (6). The therapeutic manipulation of aberrations in the epigenome, which include DNA methylation and histone acetylation, has been implicated in the control of cancer progression. In current clinical settings, two classes of epigenetic drugs are mainly used: DNA methyltransferase inhibitors (DNMTis) and histone deacetylase inhibitors (HDACis). These are designed to restore the tumor suppressor gene expression by downregulating promoter methylation and upregulating histone acetylation of the target gene, respectively (23). These two classes of drugs have been approved and used in the treatment of lymphoma and myelodysplastic syndrome (24). However, HDACi failed to generate promising results, based on the clinical phase study evaluation (25-27). Although the combination of DNMTi and HDACi treatment in metastatic CRC in phase II was found to reverse hypermethylation and improve progression-free survival rate (28), the side-effects of DNMTi and HDACi were of concern, due to their relatively high *in vivo* non-specificity. Alternatively,

the modulation of miRNA in cancer provides a novel therapeutic method by introducing specific miRNA mimics to restore tumor-suppressor miRNA or inhibitors to suppress onco-miRNA expression (29).

miR-675-3p has been found to be upregulated in several types of cancer, however, its role in CRC remains to be fully elucidated. The present study focused on miR-675-3p and its downstream factor DMTF1 in their ability to induce CRC cell proliferation. Firstly, the effect of miR675-3p on CRC cell proliferation was examined by either overexpressing or inhibiting its expression in the SW480 cell line. It was found miR-675-3p was able to promote cell growth (Fig. 1); this was consistent with previous studies (12-14). An online resource was then used to predict a potential target of miR-675-3p, DMTF1. It was shown that miR-675-3p regulated the expression of DMTF1 through its 3'UTR binding site (Fig. 2). DMTF1 is induced by activation of the Ras-Raf pathway and functions as a tumor suppressor in a negative feedback-loop (30). The significant reduction of DMTF1 observed in the human CRC tissue, compared with its adjacent normal tissue (Fig. 3C), indicated that the dysregulation of DMTF1 may be responsible for the development of CRC.
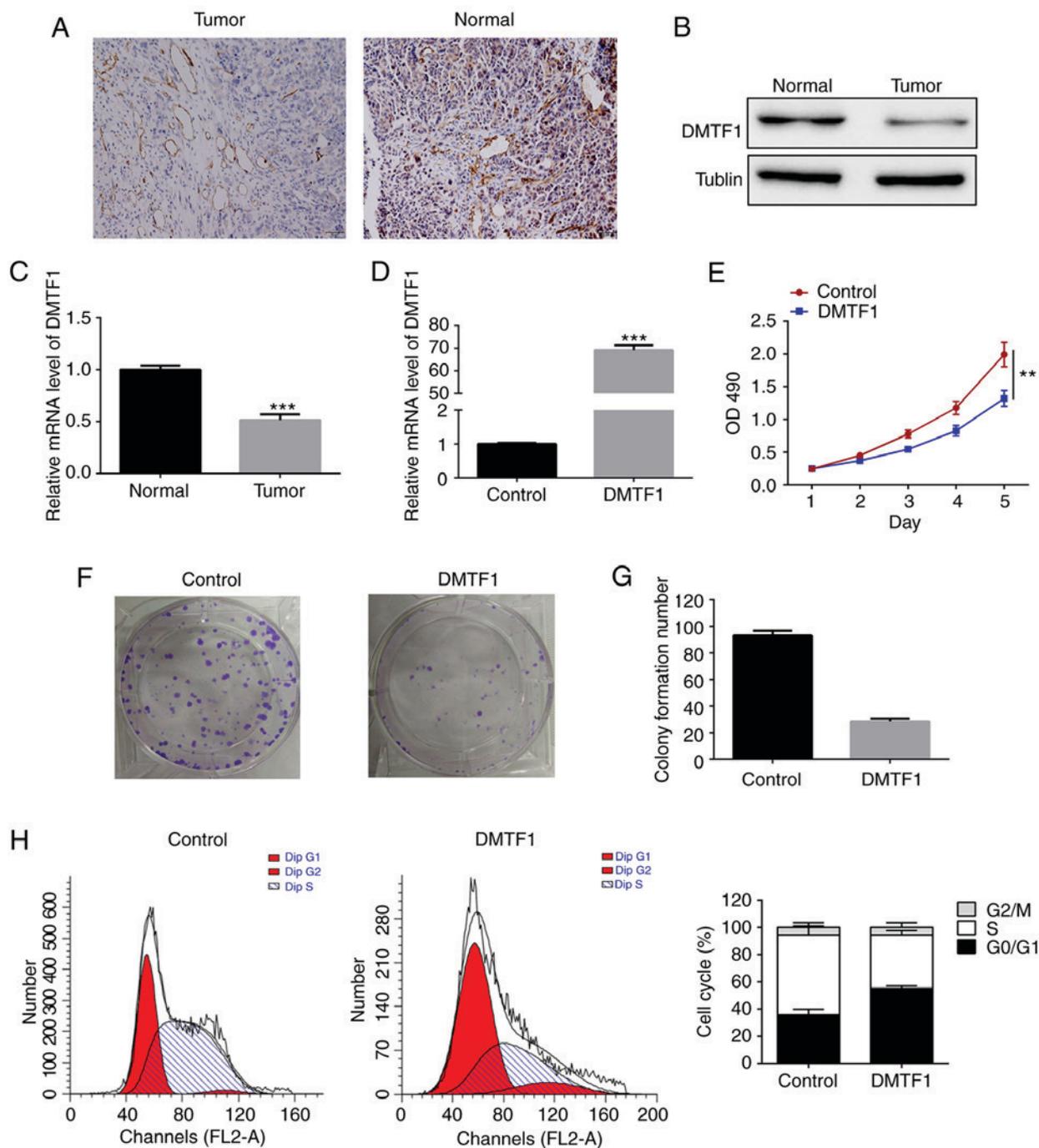
Figure 4. DMTF1 negatively contributes to cell growth. (A) Immunohistochemistry (magnification, x200) and (B) western blot analysis of DMTF1 protein and (C) mRNA levels were examined in human CRC tissue and adjacent normal tissue. (D) Lipofectamine-mediated overexpression of DMTF1 in SW480 was evaluated by reverse transcription-quantitative polymerase chain reaction analysis. In the DMTF1-overexpressing SW480 cells, decreased (E) cell proliferation, (F) single colony formation, (G) colony numbers, and the (H) S phase proportion of the cell cycle were observed. ***P<0.001 vs. control. DMTF1, cyclin D binding myb like transcription factor 1.

By overexpressing DMTF1 in the SW480 cells, the suppressive effect on CRC cell growth confirmed. As DMTF1 was able to induce cell cycle arrest at entry to the S phase (31), a cell cycle assay was performed in the DMTF1-overexpressed SW480 cells. An increased cell population in the G_0/G_1 phase at the expense of a reduced population in the S phase was observed (Fig. 3G). Finally, miR-675-3p and DMTF1 were co-expressed in the SW480 cells to examine their synergistic effect on cell proliferation. The results showed that increased

expression of DMTF1 in the miR-675-3p-overexpressed SW480 cells normalized cell growth (Fig. 4C-E). In summary, it was demonstrated that the oncogenic property of miR-675-3p was dependent on its negative regulation of tumor suppressor DMTF1. miR-675-3p together with DMTF1 were important in CRC proliferation.

miRNA-based biomarkers have been implicated in the prognosis and diagnosis of tumors (32). In particular, miR-675 was one of the miRNAs differentially expressed in benign
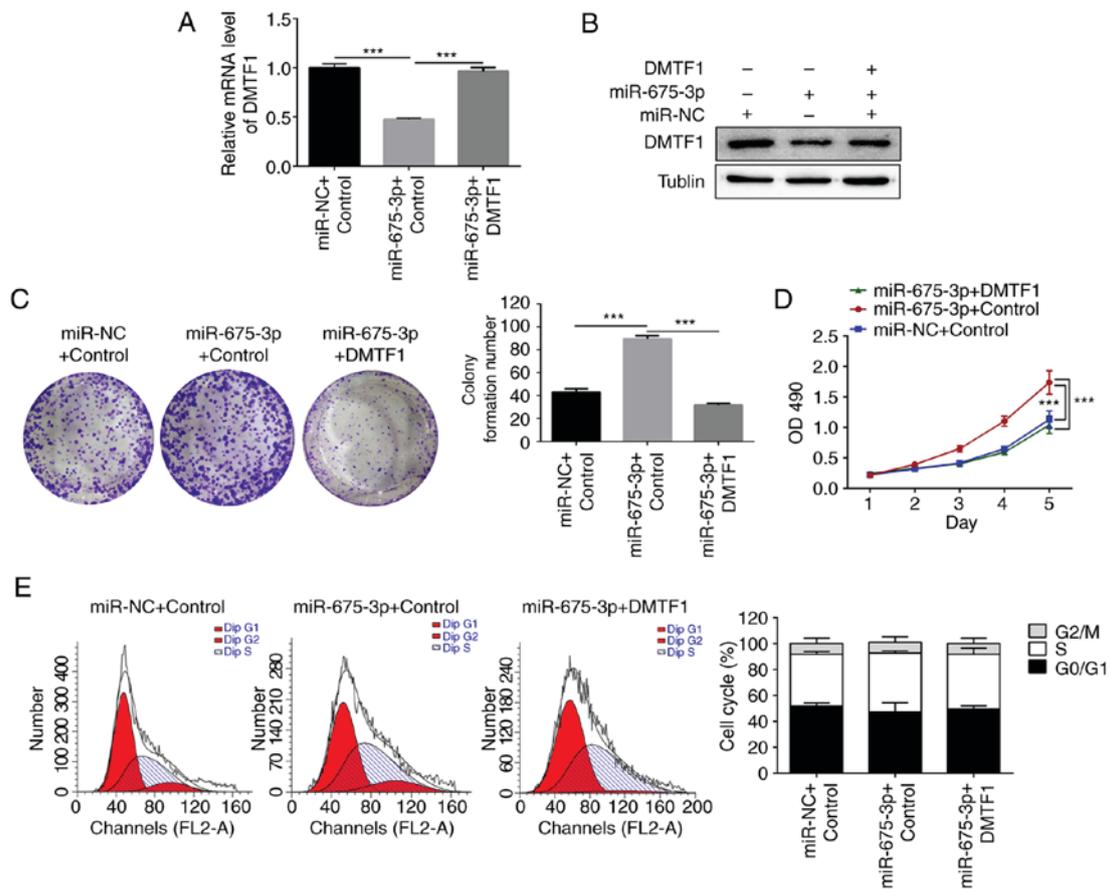
Figure 5. DMTF1 restores normal cell growth in miR-675-3p-overexpressing SW480 cells. (A) mRNA levels of DMTF1 were significantly reduced upon introduction of miR-675-3p, but restored by transfection of the DMTF1-expressing plasmid. (B) Protein levels were measured by western blotting in the SW480 cells transfected with miR-NC, miR-675-3p and miR-675-3p + DMTF1, respectively. (C) DMTF1 lowered the miR675-3p-induced elevation in SW480 single colony formation, (D) viable cell proliferation, (E) and the S phase proportion of the cell cycle. ***$P<0.001$, as indicated. DMTF1, cyclin D binding myb like transcription factor 1; miR, microRNA; NC, negative control.
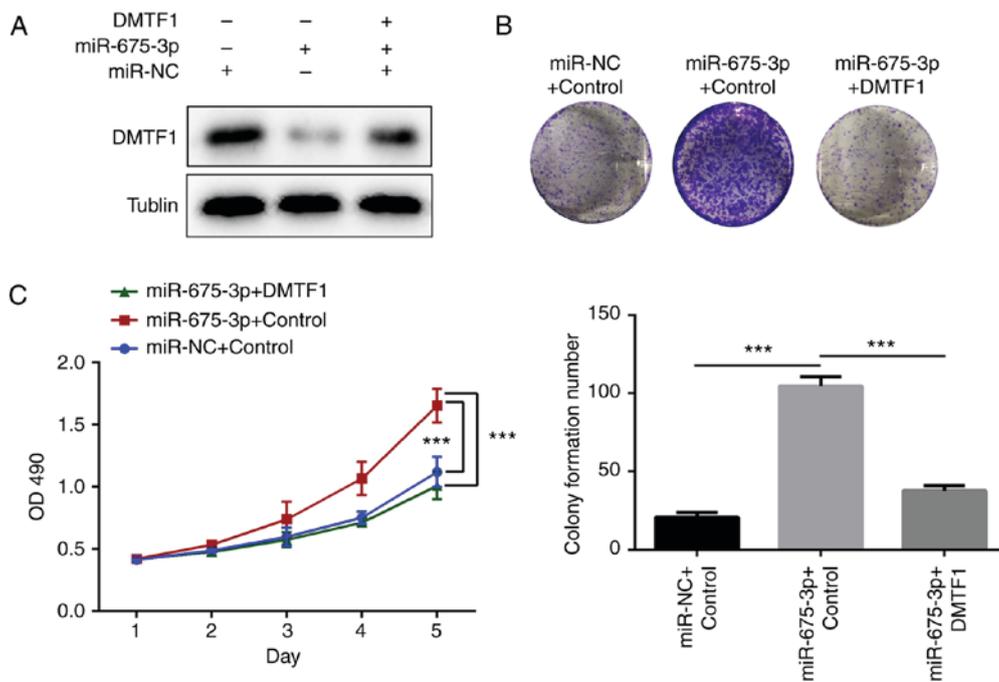
Figure 6. DMTF1 restores normal cell growth in miR-675-3p-overexpressed HT29 cells. (A) Protein levels were measured by western blotting in HT29 cells transfected with miR-NC, miR-675-3p and miR-675-3p + DMTF1, respectively. (B) DMTF1 lowered the miR675-3p-induced increase in single colony formation of HT29 cells. (C) Cell proliferation was induced by the overexpression of miR-675-3p and restored when DMTF-1 was introduced. ***$P<0.001$, as indicated. DMTF1, cyclin D binding myb like transcription factor 1; miR, microRNA; NC, negative control.

and malignant adrenocortical tumors (33). miR-675 was also reported to be dysregulated in lung cancer (14) and significantly correlated with the overall decreased survival rate of patients with pancreatic cancer (34). The significant increase in expression of miR-675-3p and subsequent decrease in DMTF1 in CRC cells suggests that the comprehensive comparison of expression levels of miR-675-3p together with DMTF1 can be useful for developing a biomarker for CRC diagnosis. Accordingly, the introduction of miR-675-3p inhibitor may reduce cell growth rate in CRC, minimizing the side-effects compared with those of other non-specific epigenetic drugs.

Acknowledgements

Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

WH and XY designed all of the experiments and revised the manuscript. YLo and MW performed the experiments, and CL and YLi analyzed and interpreted the data.

Ethics approval and consent to participate

All procedures were conducted under the approval of the Ethics Committee of the Affiliated Hospital of Beihua University (Jilin, China). The study conforms with The Code of Ethics of the World Medical Association (Declaration of Helsinki), printed in the British Medical Journal (18 July 1964). Informed consent was obtained from all individual participants included in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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