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ORIGINAL ARTICLE

Basic Study

Reduced microRNA 375 in colorectal cancer upregulates metadherin-mediated signaling

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Abstract

BACKGROUND

The human microRNA 375 (*MIR375*) is significantly downregulated in human colorectal cancer (CRC) and we have previously shown that *MIR375* is a CRC-associated miRNA. The metadherin (*MTDH*) is a candidate target gene of *MIR375*.

AIM

To investigate the interaction and function between *MIR375* and *MTDH* in human CRC.

METHODS

A luciferase reporter system was used to confirm the effect of *MIR375* on *MTDH* expression. The expression levels of *MIR375* and the target genes were evaluated by quantitative RT-PCR (qRT-PCR), western blotting, or immunohistochemistry.

RESULTS

MTDH expression was found to be upregulated in human CRC tissues compared to that in healthy controls. We show that *MIR375* regulates the expression of many genes involved in the MTDH-mediated signal transduction pathways [BRAF-MAPK and phosphatidylinositol-4,5-biphosphate-3-kinase catalytic subunit alpha (PIK3CA)-AKT] in CRC cells. Upregulated MTDH expression levels were found to inhibit NF-κB inhibitor alpha, which further upregulated NFKB1 and RELA expression in CRC cells.

CONCLUSION

Our findings suggest that suppressing *MIR375* expression in CRC regulates cell proliferation and angiogenesis by increasing MTDH expression. Thus, *MIR375* may be of therapeutic value in treating human CRC.

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Core tip: The microRNA 375 (*MIR375*) is significantly downregulated in human colorectal cancer (CRC) tissues. In this study, we investigated that metadherin (*MTDH*) is a direct target gene of *MIR375* and that MTDH expression levels were upregulated in CRC tissues. Upregulated MTDH expression levels were found to inhibit NF-κB inhibitor alpha expression, which further upregulated NFKB1 and RELA expression in CRC cells. *MIR375* also regulate MTDH-mediated BRAF-MAPK and PIK3CA-AKT signal pathways in CRC cells. Consequently, *MIR375* regulates cell proliferation, cell migration, and angiogenesis by suppressing MTDH expression in CRC progression.

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INTRODUCTION

Colorectal cancer (CRC) is a common malignant tumor and is the third leading cause of cancer-related mortality worldwide[1,2]. The cause of CRC is multifactorial, which includes genetic variation as well as epigenetic factors[3]. Overall survival of patients with CRC has not much improved relative to significant advances in the management of CRC[4]. Thus, it is most importance to understand the molecular mechanisms underlying CRC tumorigenesis and recognize the fundamental genes responsible for such fatal cancer.

MicroRNAs (miRNAs) are endogenously expressed, small noncoding RNAs that bind at the 3′ untranslated region (3′-UTR) of their target mRNAs and promote mRNA degradation or inhibit translation[5]. miRNAs act as tumor suppressors or oncogenes by targeting the genes involved in cell proliferation, cell survival, apoptosis, and metastasis[6-8].

In humans, microRNA 375 (*MIR375*) is located on chromosomal band 2q35. *MIR375* has been shown to have dual functions: As a tumor suppressor[9,10] and as an oncogene[11,12]. The dual characteristic of *MIR375* depends on the target mRNA. In our previous study, we detected *MIR375* in CRC[13] and dextran sulphate sodium (DSS)-induced mice colitis[14] via miRNA expression profiling of CRC tissues versus healthy colorectal tissues and DSS-induced colitis versus healthy colons, respectively. We found that *MIR375* was significantly downregulated in both CRC and DSS-induced colitis tissue samples[13,14]. Additionally, we have shown that downregulation of *MIR375* modulates epidermal growth factor receptor (EGFR) signaling pathways in human CRC cells and tissues by upregulating connective tissue growth factor (CTGF) expression[15].

Metadherin (*MTDH*, also known as *AEG1*, *LYRIC*, or *LYRIC/3D3*) is located on chromosome 8q22.1 and encodes for a 64 kDa protein. It was first detected as an upregulated transcript in primary human fetal astrocytes infected with human immunodeficiency virus 1 (HIV-1)[16]. Brown and Ruoslahti have shown that metadherin mediates tumor cell localization at the metastatic sites[17]. Several studies have shown the role of *MTDH* as an oncogene in different types of human malignant tumors[18] and revealed various functions such as increased tumor growth, invasion and metastasis, angiogenesis, and chemoresistance[19]. Furthermore, our previous research has shown that *MTDH* is one of the putative target genes of *MIR375*[15].

In this study, we show that *MTDH* is a target gene of *MIR375* in CRC and analyze its functions in CRC tissues and cell lines. Additionally, we reveal that *MIR375* regulates cell proliferation and migration in CRC progression by suppressing MTDH-mediated signaling pathways.

MATERIALS AND METHODS

Patients and tissue samples

The tissue samples used in this study were provided by Biobank of Wonkwang University Hospital, a member of National Biobank of Korea. On approval from the institutional review board and obtaining informed consent (WKIRB-201710-BR-012) from the patients, we collected 19 CRC tissue samples from 16 patients with colon cancer (10 males and 6 females) and 3 patients with rectal cancer (2 males and 1 female). Mean age of the patients with colon cancer and rectal cancer was 68.4 years and 67.0 years, respectively. Ten colon cancer tissue samples and matching healthy colon tissue samples (7 males and 3 females) were investigated to confirm the endogenous expression of *MIR375*. Additionally, 12 colon cancer tissue samples with matching healthy colon tissue samples and 3 rectal cancer tissue samples with matching healthy rectal tissue samples were assessed for MTDH expression levels. Four colon cancer tissue samples and matching healthy colon tissue samples (3 males and 1 female) were examined for immunohistochemistry analysis.

Cells culture and reagents

Human CRC cell lines; Caco2, HT29, LoVo, HCT116, and SW48 were obtained from Korea Cell Line Bank (KCLB, Seoul, South Korea) or American Type Culture Collection (ATCC, Manassas, VA, United States). SW48, HT29, Lovo, and HCT116 cells were cultured in RPMI 1640 (HyClone, Logan, UT, United States) supplemented with 10% FBS while Caco2 cells were cultured in α-MEM (HyClone, Logan, UT, United States) supplemented with 20% FBS in a humidified atmosphere containing 5% CO_2 at 37 °C.

MTDH antibody and all secondary antibodies were purchased from Thermo Fisher Scientific (Waltham, MA, United States). NF-κB inhibitor alpha (NFKBIA/IκBα), nuclear factor κ B subunit 1 (NFκB1/p50), RELA (NFkB3/p65), protein kinase B (AKT), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, Texas, United States). RAS, BRAF, p44/42 mitogen-activated protein kinase (MAPK) (Erk1/2), phospho-MAPK, phosphatidylinositol-4,5-biphosphate-3-kinase catalytic subunit alpha (PIK3CA), phospho-AKT, and GAPDH antibodies were purchased from Cell Signaling Technology (Danvers, MA, United States). β catenin (CTNNB1) antibody was purchased from Abcam (Cambridge, United Kingdom). Vascular endothelial growth factor A (VEGFA) antibody was purchased from Novus Biologicals (Centennial, CO, United States). Ez-cytox was obtained from DoGenBio (Seoul, South Korea) and dual luciferase reporter assay system was obtained from Promega (Madison, WI, United States). TRIzol and siPORT NeoFx transfection reagents were purchased from Ambion, Inc. (Waltham, MA, United States). Lipofectamine 2000 reagent was purchased from Invitrogen (Waltham, MA, United States) while Viromer blue transfection agent was purchased from Lipocalyx (Weinbergweg, Halle, Germany). RIPA buffer was obtained from Elpis biotech (Daejeon, South Korea) and DAB substrate kit was purchased from Pierce Biotechnology (Waltham, MA, United States).

RNA extraction and quantitative real-time polymerase chain reaction

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) were performed according to our previously established protocol[13-15]. Total RNA was isolated using TRIzol reagent. After digesting with DNase and performing a sample clean-up, RNA samples were quantified, aliquoted, and stored at -80 °C. qRT-PCR was performed on total RNA samples that were isolated from tissue samples or cultured cells to synthesize cDNA using StepOne Real-time PCR system (Applied Biosystems, Foster City, CA, United States).

Differential miRNA expression patterns were validated by TaqMan qRT-PCR assay (Applied Biosystems, Foster City, CA, United States). qRT-PCR was performed using SYBR Green dye (ELPIS Biotech, Daejeon, Korea) to assess mRNA expression. *RNU48* (for TaqMan qRT-PCR) or 5.8S (for SYBR qRT-PCR), and GAPDH served as endogenous controls for qRT-PCR of miRNA and mRNA, respectively. Each sample was analyzed in triplicates by qRT-PCR. Primers for qRT-PCR and TaqMan analysis are listed in Supplementary Table 1.

Transfection of oligonucleotides

Endogenous *MIR375* mimic [hsa-miR-375, Pre-miR™ miRNA precursor (AM17100)], *MTDH* small interfering RNA (siRNA), and each of the negative controls were synthesized commercially (Ambion, Austin, TX, United States) and transfected at 50 nM. Transfection was performed according to our previously published protocols[13-15].

Luciferase reporter assay

Wild-type (WT) or mutant type (MT) fragments of the 3'-UTR of *MTDH* containing the predicted binding site for *MIR375* were amplified using PCR. The primer set used for the experiment is shown in Supplementary Table 1. Plasmid constructions and analysis of the luciferase assay were executed following our previously published protocols[13-15].

Protein extraction and Western blot analysis

Protein extraction and western blot analysis were performed according to our earlier established methods[13-15]. Briefly, membranes were incubated overnight at 4 °C with primary antibodies to MTDH (1:250), NFKBIA (1:100), NFKB1 (1:50), RELA (1:100), KRAS (1:1000), BRAF (1:500), MAPK (1:1000), p-MAPK (1:500), CTNNB1 (1:2500), PIK3CA (1:1000), AKT (1:100), p-AKT (1:500), and VEGFA (1:500). Subsequently, the membranes were incubated with secondary antibodies (1:1000).

Cell proliferation assay

For cell proliferation assay, cells (2×10^4 cells/well) were transfected with *MIR375* mimic, negative control siRNA, or *MTDH* siRNA (*siMTDH*) in 96-well plates. Cell growth was measured at 72 h after transfection using Ez-Cytox cell viability assay kit following manufacturer's instructions. After incubating for 2 h, absorbance values were measured at 450 nm using SpectraMax (Molecular Devices, CA, United States). Percentage of viable cells was calculated by comparing to the number of viable cells in the untreated controls. Experiments were performed in triplicates. Cell proliferation assay was performed following our previously published protocols[15,20].

Immunohistochemistry

Immunohistochemistry assay was performed according to our previously established protocols[14,15]. The tissue slides were blocked with 3% BSA for 2 h at room temperature followed by overnight incubation at 4 °C with primary antibodies against MTDH (1:50) and RELA (1:50). The following day, the slides were incubated with SignalStain® Boost IHC detection reagent (Cell Signaling Technology; Danvers, MA, United States) for 2 h at room temperature. After washing, chromogenic substrate (Thermo Fisher Scientific; Waltham, MA, United States) was applied to visualize the staining of the target proteins. Following counterstaining with hematoxylin, the sections were dehydrated and mounted using a coverslip.

Statistical analysis

Sample size was estimated using the G*power software (Version 3.1., Heinrich Heine University, Duesseldorf, Germany). Each experiment was repeated at least three times and consistent results were obtained. Data are expressed as mean ± standard deviation (SD). The differences between the groups were evaluated using GraphPad Prism 5.0 statistical software (GraphPad Software Inc., San Diego, CA, United States) or Student's *t*-test. Differences with *P* value less than 0.05 were considered as statistically significant.

RESULTS

Validation of MIR375 expression level in CRC tissues

Previously, we have shown *MIR375* as a colon cancer-associated miRNA using miRNA microarray analysis of colon tumor tissues and matched healthy colon tissues[13]. Additionally, we have shown that *MIR375* expression is downregulated in human CRC tissues. To confirm the result, we compared *MIR375* expression in 10 human CRC tissues and matched healthy colon tissues by qRT-PCR. We found that *MIR375* expression levels were significantly reduced in CRC tissues ($P < 0.01$; Supplementary Figure 1A).

Endogenous expression levels of MIR375 in CRC cell lines

To determine the endogenous expression levels of *MIR375* in different cell lines, we performed qRT-PCR on the total RNA isolated from various cell lines including Caco2, SW480, HT29, HCT116, LoVo, and SW48 cells. As shown in Figure S1B, *MIR375* expression level was highest in HT29 cells while it was lower in HCT116 and Caco2 cells (Supplementary Figure 1B).

MTDH is a direct target of MIR375

To determine the direct interaction between *MTDH* 3'-UTR and *MIR375*, we cloned the WT *MTDH* 3'-UTR region, the putative target sequence of *MIR375*, in a luciferase reporter vector (Figure 1A). We observed that luciferase activity was reduced by approximately 24% when cells were co-transfected with *pre-MIR375* ($P < 0.01$, Figure

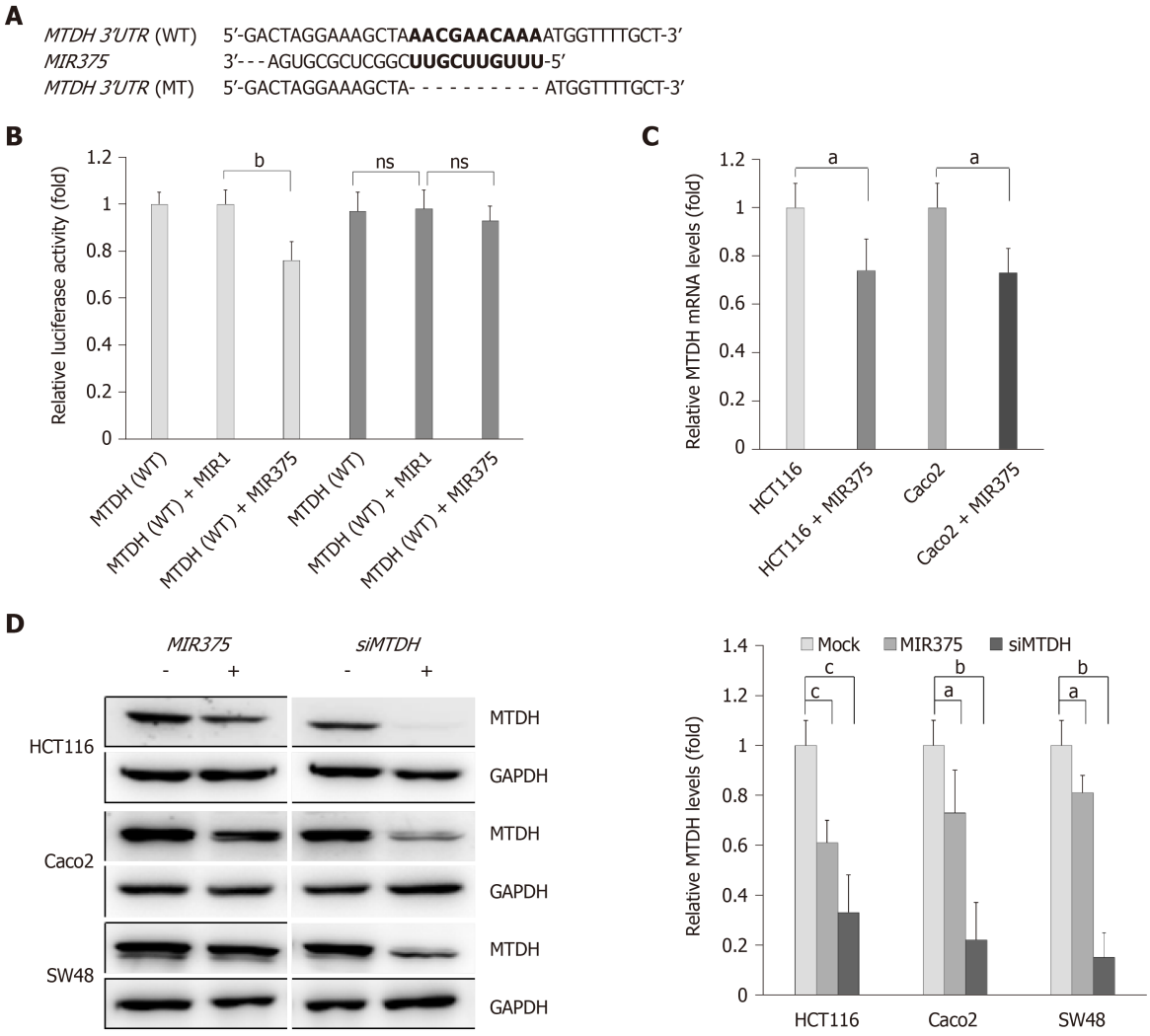

Figure 1 Metadherin is a direct target of *microRNA 375*. A: Sequence alignment of the wild type (WT) and mutated type (MT) microRNA 375 (*MIR375)* target site in the 3'-UTR of metadherin (*MTDH)*; B: A luciferase reporter plasmid containing WT or MT *MTDH* 3'-UTR was co-transfected in HCT116 and Caco2 cells with *pre-MIR1* as a negative control or *pre-MIR375*. Results are shown as relative firefly luciferase activity which is standardized to *Renilla* luciferase activity. Three independent experiments were conducted with duplicates; C: qRT-PCR analysis of *MTDH* mRNA expression in HCT116 and Caco2 cells. The data are presented as the fold change in *MIR375* mimic transfected cells relative to non-transfected cells. Experiment was performed in duplicate and repeated 5 times; D: Cellular MTDH levels in *MIR375* mimic-transfected and *siMTDH*-transfected HCT116, Caco2, and SW48 cells. Three independent experiments were conducted with duplicates. *P* values were calculated using Student's *t*-test ($^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$). *MTDH:* Metadherin; *MIR375:* MicroRNA 375; ns: Not significant.

1B). As a control experiment, we cloned mutated *MTDH* 3'-UTR sequence which lacked ten of the total complementary bases (Figure 1A). As expected, repression of the luciferase activity was revoked when the interaction between *MIR375* and its target 3'-UTR was disrupted (Figure 1B). Additionally, another control experiment was performed where *pre-MIR1* (instead of *pre-MIR375*) was co-transfected with WT and mutated *MTDH* 3'-UTR constructs. We found that transfection with *pre-MIR1* did not affect the luciferase activity of either of the constructs (Figure 1B).

MIR375 regulate MTDH expression in CRC cells

To validate the obtained data, we investigated whether *MIR375* regulates *MTDH* mRNA levels in HCT116 and Caco2 cells. We found that *MTDH* mRNA expression levels were lower in HCT116 as well as Caco2 cells on transfection with *MIR375* mimic compared with that in non-transfected control cells ($P < 0.05$; Figure 1C). Additionally, we investigated MTDH expression levels in *MIR375* mimic or *siMTDH*-transfected HCT116, Caco2, and SW48 cells and found that cellular MTDH expression was significantly reduced in *MIR375*-overexpressing HCT116, Caco2, and SW48 cells. Furthermore, MTDH was significantly downregulated by *siMTDH* transfection (Figure 1D).

MIR375 regulates MTDH-mediated BRAF-MAPK signaling pathways

To determine the functional interaction between *MIR375* and its target gene *MTDH*,

we analyzed the expression levels of KRAS, BRAF, MAPK, pMAPK, and CTNNB1 in HCT116 and Caco2 cells on *MIR375* mimic transfection. Earlier study has shown that HCT116 cells express WT *BRAF* and mutated *KRAS* while Caco2 cells express only WT *KRAS* and WT *BRAF*[21]. Although KRAS expression level was unaltered on *MIR375* transfection, BRAF, MAPK, pMAPK, and CTNNB1 expression levels were significantly downregulated in HCT116 (Figure 2A) and Caco2 (Figure 2B) cells. We observed a similar expression trend in CRC cells on silencing *MTDH* with *siMTDH* (Figure 2A and B). These results suggested that *MIR375* regulates the MTDH-mediated BRAF-MAPK signal pathway in CRC cells.

MIR375 inhibits CRC cells viability by inhibiting MTDH expression

We investigated the biological functions of *MIR375* in CRC cells. MTT assay showed that cell viability was steadily reduced on *MIR375* transfection in the CRC cell lines HCT116 ($P < 0.01$), Caco2 ($P < 0.05$), and SW48 ($P < 0.05$; Figure 2C) cells. Further, we found a similar trend on *siMTDH* transfection in HCT116 ($P < 0.01$), Caco2 ($P < 0.001$), and SW48 cells ($P < 0.05$; Figure 2C). These results suggested that *MIR375* downregulates CRC cell proliferation by inhibiting MTDH expression. The rate of inhibition was lower in SW48 cells compared with that in HCT116 and Caco2 cells. This might be due to relatively high endogenous expression of *MIR375* in SW48 cells than that observed in HCT116 and Caco2 cells (Supplementary Figure 1B).

MIR375 regulates MTDH-mediated PIK3CA-AKT signaling pathways

We investigated the functional correlation between *MIR375* and MTDH expression in HCT116 and Caco2 cells. HCT116 cells are mutated for PIK3CA whereas Caco2 cells express wild type PIK3CA. We found that PIK3CA, AKT, pAKT, and VEGFA expression levels were downregulated on *MIR375* mimic transfection in HCT116 as well as Caco2 cells (Figure 3A and 3B). Similar results were obtained on silencing *MTDH* in HCT116 and Caco2 cells (Figure 3C and 3D). Thus, the results suggested that *MIR375* regulates MTDH-mediated PIK3CA-AKT signaling pathway by inhibiting MTDH expression levels in CRC cells.

MIR375 regulates MTDH-mediated NFKB1 signaling pathways

Furthermore, we investigated another MTDH-mediated signaling pathway in CRC cells. To determine the role of *MIR375* or MTDH-mediated pathways in NFKB1 signaling, we quantified the expression of relevant proteins in HCT116 and Caco2 cells on *MIR375* mimic or *siMTDH* transfection. NFKB1 and RELA expression levels were found to be significantly downregulated in both the cell lines (HCT116 and Caco2 cells) on *MIR375* transfection while NFKBIA expression levels were found to be upregulated (Figure 4A and B). We observed similar results on silencing *MTDH* using *siMTDH* in CRC cells (Figure 4C and D). Overall, the results evidently suggested that NFKBIA expression was upregulated on inhibiting MTDH in *MIR375*-overexpressing CRC cells which further leads to downregulation of NFKB1 and RELA expression in CRC cells. These results showed that *MIR375* regulates MTDH-mediated NFKB1 signaling pathway.

MTDH expression levels in human CRC tissues

Based on the findings of this study, we evaluated MTDH expression in 15 human CRC tissues and matching healthy colon tissues. Western blot analysis showed that MTDH expression levels were upregulated (12 out of 15 samples) in CRC tissue samples compared with that in healthy colon tissues ($P < 0.05$, Figure 5A). Further, we investigated NFKB1 and RELA expression levels in five CRC tissues and four CRC tissue samples, respectively. NFKB1 expression level was significantly upregulated in all CRC tissues while RELA expression level was predominantly upregulated in three (75%) CRC tissues (Figure 5B).

Consistent with the results obtained, we investigated MTDH and RELA expression in four human CRC tissues and matching healthy colon tissues using immunohistochemical analysis. MTDH and RELA expression levels were significantly upregulated in CRC tissues (Figure 5C).

DISCUSSION

Many miRNAs have been detected as associated biomarkers and therapeutic targets in CRC. Several studies have shown that targeting specific miRNAs effectively inhibits cell proliferation and angiogenesis in CRC[22-24]. Thus, a better identification of CRC-associated miRNAs may contribute to the development of efficient miRNA-based therapy for CRC. In our previous study, we used miRNA expression profiling and showed *MIR375* to be associated with human CRC tissue[13] as well as DSS-

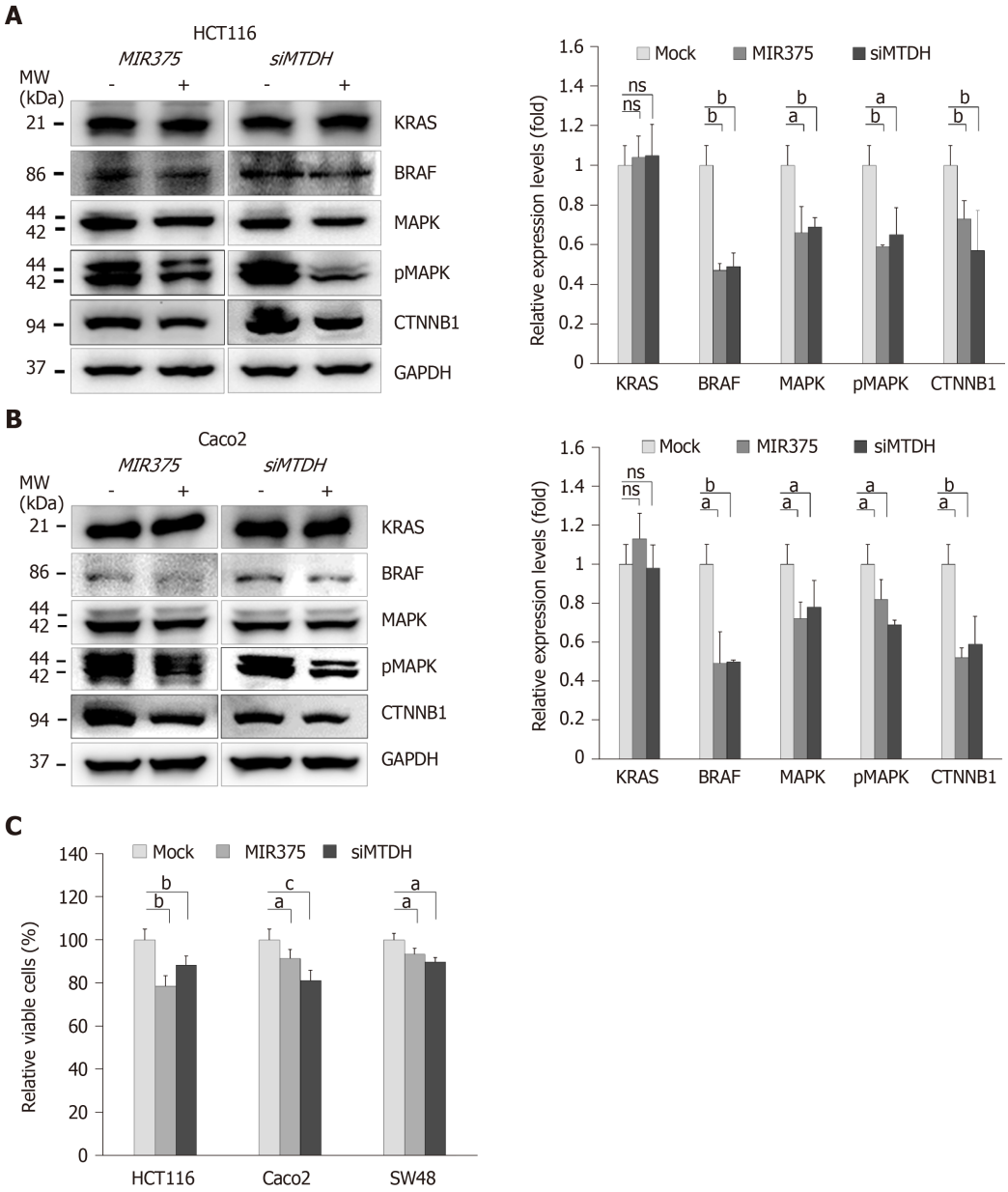

Figure 2 ***MicroRNA 375*** **regulates metadherin-mediated BRAF-MAPK signaling in colorectal cancer cell lines.** A: Western blot analysis of KRAS, BRAF, mitogen-activated protein kinase 3/1 (MAPK3/1), pMAPK3/1 and β catenin (CTNNB1) expression levels in colorectal cancer (CRC) cells. Except for KRAS; BRAF, MAPK3/1, pMAPK3/1 and CTNNB1 expression levels were downregulated on microRNA 375 (*MIR375)* mimic and *siMTDH* transfection in HCT116 cells; B: Western blot analysis of KRAS, BRAF, MAPK3/1, pMAPK3/1 and CTNNB1 expression levels in CRC cells. Except for KRAS; BRAF, MAPK3/1, pMAPK3/1 and CTNNB1 expression levels were downregulated on *MIR375* mimic and *siMTDH* transfection in Caco2 cells. Five independent experiments were performed with duplicates; C: Effects of *MIR375* and *siMTDH* on cell viability in HCT116, Caco2, and SW480 cells. Cell viability was determined by MTT assay. Three independent experiments were performed with duplicates and *P* values were calculated using Student's *t*-test ($^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$). ns: Not significant; *MTDH:* Metadherin; CTNNB1: β catenin; MAPK3/1: Mitogen-activated protein kinase 3/1; *MIR375:* microRNA 375; CRC: Colorectal cancer.

induced mice colitis[14] by comparing the expression pattern in CRC tissues versus matching healthy colorectal tissues and DSS-induced mice colitis versus healthy mice colons, respectively. Hyper-methylation of MIR375 has been demonstrated in CRC cell lines including HCT116 and SW480. Down regulation of MIR375 in HCT116 and SW480 cells compere to HT29 cells is due to hyper-methylation of MIR375 in HCT116 and SW480 cells[25]. In the present study, we confirmed the findings in a larger sample size and showed that *MIR375* expression was downregulated in human CRC tissues compared with that in matching healthy colon tissues (Supplementary Figure 1A). The results of the current study are consistent with the earlier research work by Dai *et al*[26]. In addition to that in our study, *MIR375* downregulation has been observed in several other types of cancer such as hepatocellular carcinoma[27], gastric cancer[28], and glioma[29]. However, some reports have revealed that *MIR375* is upregulated in tumors of prostate cancer[12] and breast cancer[30]. Primarily, miRNA expression levels were believed to be cell type-specific in many cancer tissues[31].

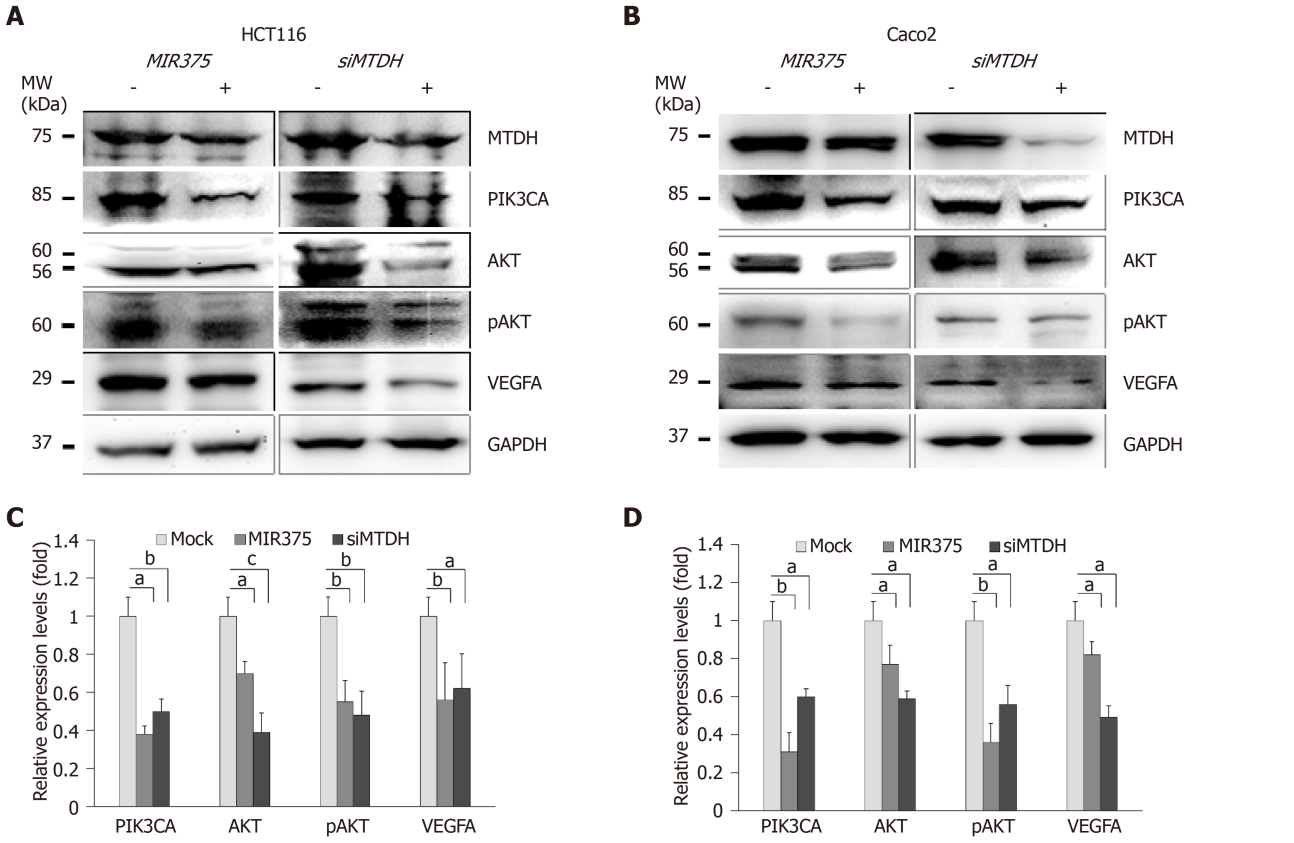

Figure 3 ***MicroRNA 375*** **regulates metadherin-mediated phosphatidylinositol-4,5-biphosphate-3-kinase catalytic subunit alpha-protein kinase B signaling in colorectal cancer cell lines.** A: Western blot analysis of phosphatidylinositol-4,5-biphosphate-3-kinase catalytic subunit alpha (PIK3CA), protein kinase B (AKT), pAKT, and vascular endothelial growth factor A (VEGFA) expression levels in colorectal cancer (CRC) cells; B: Western blot analysis of PIK3CA, AKT, pAKT, and VEGFA expression levels in CRC cells; C: PIK3CA, AKT, pAKT, and VEGFA expression levels were downregulated on microRNA 375 (*MIR375)* mimic and *siMTDH* transfection in HCT116 cells; D: PIK3CA, AKT, pAKT, and VEGFA expression levels were downregulated on *MIR375* mimic and *siMTDH* transfection in Caco2 cells. Five independent experiments were performed with duplicates and *P* values were calculated using Student's *t*-test ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$). *MTDH:* Metadherin; PIK3CA: Phosphatidylinositol-4,5-biphosphate-3-kinase catalytic subunit alpha; AKT: Protein kinase B; VEGFA: Vascular endothelial growth factor A; *MIR375:* microRNA 375; CRC: Colorectal cancer.

In our previous study, we found that *MIR375* regulates the CTGF-mediated EGFR-PIK3CA-AKT signaling pathway by directly downregulating CTGF expression in CRC cells. However, CTGF is not involved in EGFR-KRAS-BRAF-ERK1/2 signaling[15]. Although KRAS expression was unaffected by *MIR375* overexpression, BRAF-ERK1/2 signaling was regulated on *MIR375* overexpression in CRC cells[15]. These results guided us to investigate a novel *MIR375* target gene that mediates the BRAF-ERK1/2 signaling pathway in CRC cells. In this study, we found that *MTDH* is a direct target gene of *MIR375* in CRC cells (Figure 1). Further, we confirmed that *MIR375* regulates the MTDH-mediated BRAF-ERK1/2 (MAPK3/1) signaling pathway (Figure 2A and 2B), which controls proliferation in CRC cells (Figure 2C). It is well known that MTDH contributes to the carcinogenic process of different tissues and organs by regulating multiple signaling pathways such as PI3K/AKT, NF-kB, and MAPK, which subsequently promotes tumorigenesis and metastasis[32,33].

MTDH promotes an invasive phenotype and angiogenesis via the PIK3CA-AKT signaling pathway. In addition, *PIK3CA* has been proven as a direct target of *MIR375* in CRC cells[34]. MTDH expression is upregulated in many types of cancers, and is crucial in oncogenic transformation and angiogenesis[35-37]. The potential role of MTDH in angiogenesis has been correlated with VEGFA expression *via* the PIK3CA-AKT pathway in head and neck squamous cells[38]. Thus, PIK3CA-AKT-VEGFA signaling is affected on *MIR375* overexpression or on *VEGFA* silencing (*siVEGFA*). We showed that PIK3CA-AKT-VEGFA signaling is downregulated on *MIR375* overexpression and *siVEGFA* treatment in CRC cells (Figure 3). Our previous study using xenograft mouse model showed that the expression level of the angiogenic marker, CD31 was significantly decreased in xenograft tumors on *MIR375* overexpression[15]. Consequently, these results suggest that *MIR375* regulates angiogenesis *via* the MTDH-mediated PIK3CA-AKT-VEGFA signaling pathway in CRC progression.

Furthermore, MTDH has been shown to regulate the anchorage independent

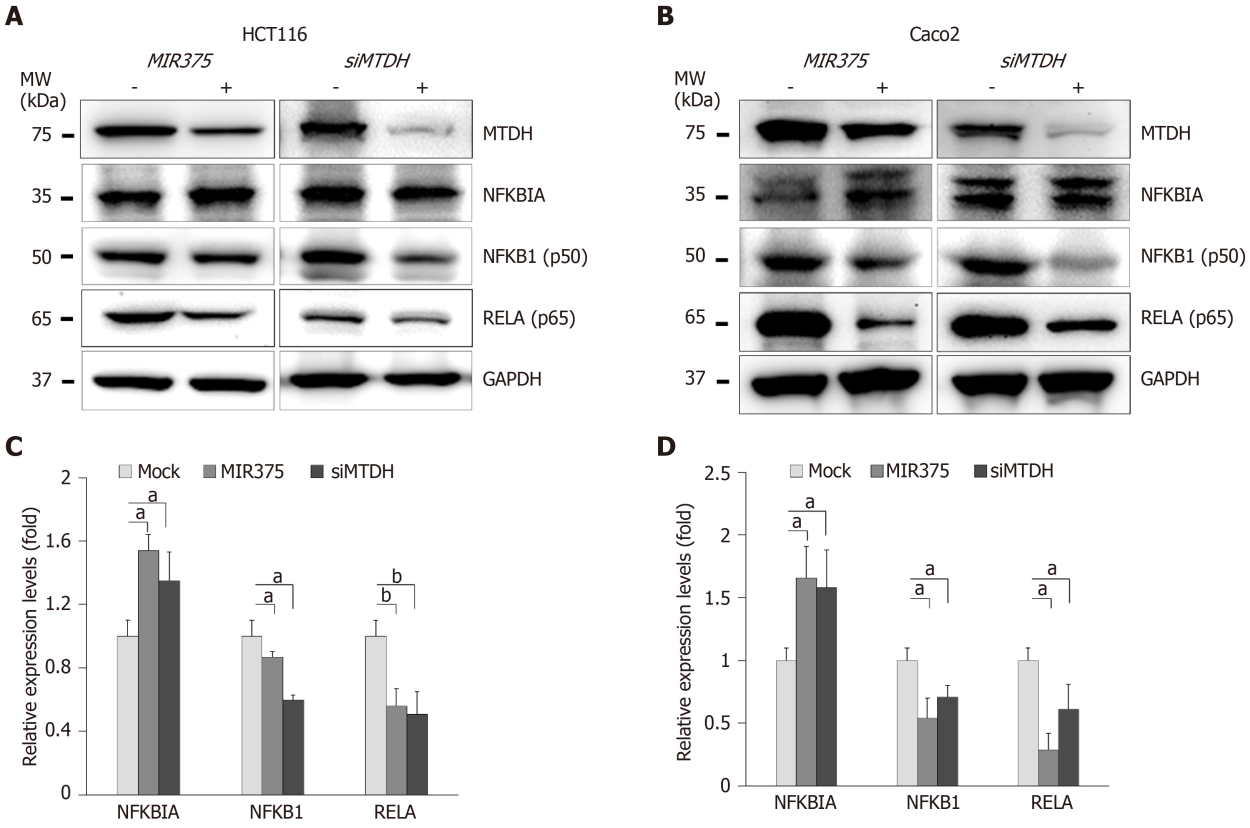

Figure 4 ***MicroRNA 375*** **regulates metadherin-mediated NFKB1 signaling in colorectal cancer cell lines.** Western blot analysis of NF-κB inhibitor alpha (NFKBIA), NFKB1 (p50), and RELA (p65) expression levels in colorectal cancer (CRC) cells. A: NFKBIA expression levels were upregulated on microRNA 375 (*MIR375)* mimic and *siMTDH* transfection in HCT116 cells; B: NFKBIA expression levels were upregulated on *MIR375* mimic and *siMTDH* transfection in Caco2 cells; C: NFKB1 and RELA levels were downregulated on *MIR375* mimic and *siMTDH* transfection in HCT116 cells; D: NFKB1 and RELA levels were downregulated on *MIR375* mimic and *siMTDH* transfection in Caco2 cells. Four independent experiments were performed with duplicates and *P* values were calculated using Student's *t*-test ($^{a}P < 0.05$, $^{b}P < 0.01$). *MTDH:* Metadherin; *MIR375:* MicroRNA 375; NFKBIA: NF-κB inhibitor alpha; CRC: Colorectal cancer.

growth and invasion of HeLa cells via activation of the NF-κB pathway[35]. MTDH has also been shown to be upregulated during CRC development and liver metastasis through the NF-κB signaling pathway[39]. Similarly, in malignant glioma cells, MTDH has been found to mediate invasion and migration through activation of the NF-κB signaling pathway[40]. In this study, we showed that *MIR375* regulates MTDH-mediated NFKB1 and RELA signaling by inhibiting NFKBIA expression in CRC cells (Figure 4).

In this study, *MIR375* expression levels were downregulated in CRC tissues (Supplementary Figure 1A). Inversely, MTDH, NFKB1, and RELA expression levels were predominantly upregulated in CRC tumor tissues compared to their expression levels in matched healthy colon tissues (Figure 5A). Additionally, immunohistochemistry staining of the CRC tissues showed that MTDH and RELA expression were upregulated in CRC tumor tissues. Overall, these results suggested that MTDH expression levels were negatively correlated with *MIR375* expression in CRC tissues.

In summary, our study found that *MIR375* expression is suppressed in tissues of patients with CRC and that *MTDH* is a direct target of *MIR375*. Furthermore, MTDH expression was upregulated in the tumors of CRC tissues on inhibiting *MIR375* expression. Overall, our results suggest that *MIR375* regulates MTDH-mediated signaling pathways such as MTDH-BRAF-MAPK, MTDH-PIK3CA-AKT, and MTDH-NFKBIA-NFKB1/RELA in CRC progression. Although we did not show MIR375-mediated VEGFA-VEGFR signaling in this study, our previous and present studies suggest that the generated VEGFA by MIR375-mediated PIK3CA-AKT or MTDH-PIK3CA-AKT signaling might effect to endothelial cell's angiogenesis. Subsequently, *MIR375* regulates cell proliferation, cell migration, and angiogenesis in CRC progression (Figure 6). Thus, we propose *MIR375* to be a promising therapeutic target in inhibiting CRC tumorigenesis. However, this needs to be further investigated.

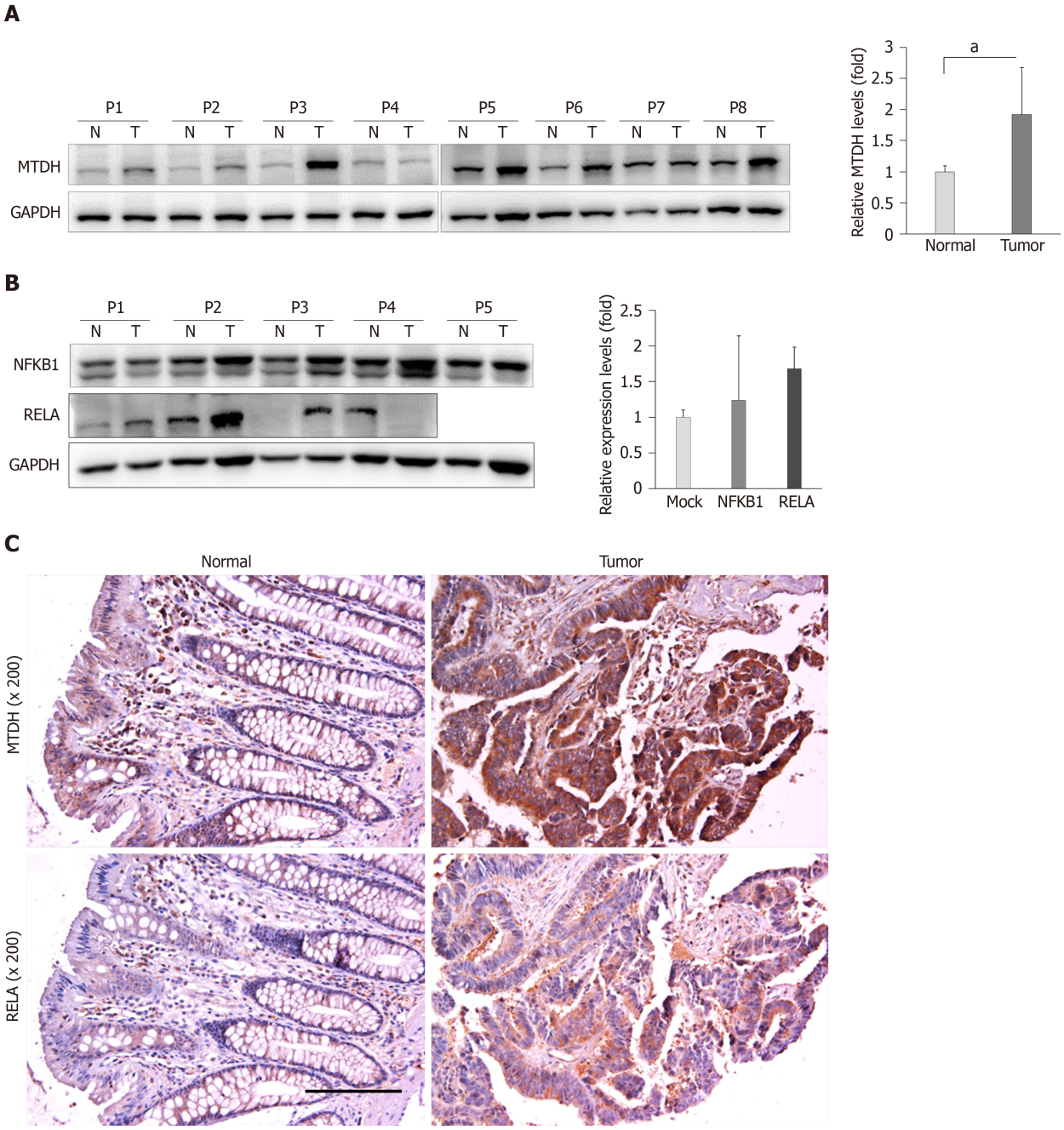

Figure 5 Endogenous metadherin expression in colorectal cancer tissues. A: metadherin (MTDH) expression levels were investigated in 15 colorectal cancer (CRC) tissue samples and matching healthy colorectal tissue samples. Results are shown for 8 colon cancer tissues in pairs. P1 to P8 indicate patients with colon cancer. Data are presented as fold change in the expression in tumor tissues relative to expression in matching healthy colon tissues. *P* values were calculated using Student's *t*-test ($^{a}P < 0.05$); B: Relative endogenous NFKB1 ($n = 5$) and RELA ($n = 4$) expression levels in colon cancer tissue samples and matching healthy colon tissue samples. Data are presented as fold change in the expression in tumor tissues relative to expression in matching healthy colon tissues; C: Immunostaining of MTDH in human CRC and adjacent healthy colorectal samples (200 × magnification). Experiments were independently performed three times in duplicates. *MTDH:* Metadherin.

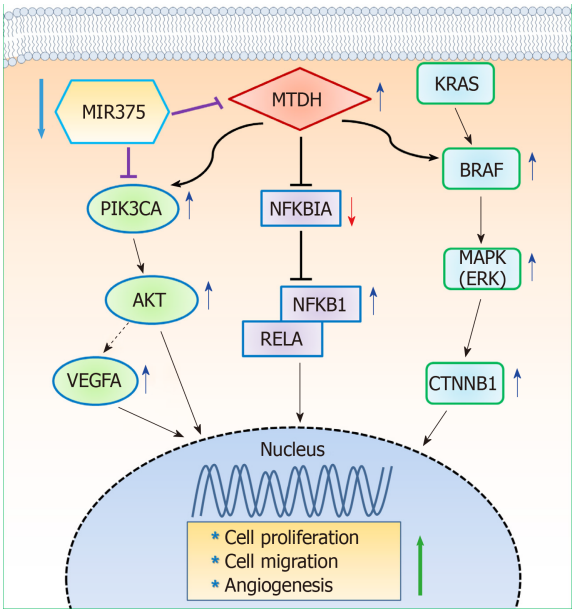

Figure 6 Diagrammatic representations of putative mechanisms of *microRNA 375* in regulating metadherin-induced cell proliferation, cell migration, and angiogenesis in human colorectal cancer. Decreased microRNA 375 (*MIR375)* expression in colorectal cancer (CRC) cells leads to upregulation of cellular metadherin (MTDH) levels. Subsequently, upregulated expression of MTDH promotes inhibition of NFKBIA and thus, NFKB1 and RELA expression is upregulated in CRC tissues and CRC cells. Upregulated MTDH expression level stimulates the BRAF-MAPK and PIK3CA-AKT signaling pathway. However, KRAS expression is unaltered by upregulation of MTDH. Consequently, downregulated *MIR375* expression levels in CRC leads to upregulation of cell proliferation, cell migration, and angiogenesis. This simple hypothetical mechanism of *MIR375*-mediated upregulation of angiogenesis is based on the results of previous studies and our current study. *MTDH:* Metadherin; *MIR375:* MicroRNA 375; CRC: Colorectal cancer.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) is the third most prevalent type of cancer worldwide. The cause of CRC is multifactorial including genetic variation and epigenetic and environmental factors. However, the precise molecular mechanism underlying the development and progression of CRC remains largely unknown. We previously found that microRNA 375 (*MIR375*) is significantly downregulated in CRC, and identified metadherin (*MTDH*) as a candidate target gene of *MIR375*.

Research motivation

MIR375 and their target *MTDH* will provide a new therapeutic information for human CRC.

Research objectives

To study the interaction and signaling between *MIR375* and *MTDH* in human CRC pathogenesis.

Research methods

We constructed luciferase reporter plasmids to confirm the effect of *MIR375* on *MTDH* gene expression. The expression levels of the *MIR375* and *MTDH* were measured by qRT-PCR, Western blot, or immunohistochemistry. The effects of *MIR375* on cell growth and angiogenesis were conducted by functional experiments in CRC cells. Assays were performed to explore functional correlation between *MTDH* and *MIR375* in human CRC cells and tissues.

Research results

In the present study, we found that the expression levels of MTDH were significantly downregulated in CRC cells by *MIR375* mimic or *siMTDH* transfection. MTDH expression was upregulated in human CRC tissues in comparing to match normal colon tissues. Upregulated MTDH expression levels were found to inhibit NF-κB inhibitor alpha (NFKBIA) expression, which further upregulated NFKB1 and RELA expression. We found that *MIR375* regulate the expression levels of molecules in MTDH-mediated BRAF-MAPK and PIK3CA-AKT signal pathways in CRC cells.

Research conclusions

MIR375 regulates cell proliferation and angiogenesis by regulation of MTDH-mediated signaling pathways such as MTDH-BRAF-MAPK, MTDH-PIK3CA-AKT, and MTDH-NFKBIA-NFKB1/RELA in CRC progression.

Research perspectives

This study provides insight into the role of *MIR375* in CRC pathogenesis by targeting MTDH. *MIR375* might be a new therapeutic target for CRC.

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