Abstract. Metadherin (MTDH) is a multifunctional oncogene involved in tumor cell migration and metastasis through regulating a number of oncogenic signaling pathways in various human malignancies. Previous studies have demonstrated that MTDH is overexpressed in human colorectal cancer (CRC) and associated with cancer progression and a poor prognosis. However, the underlying mechanisms remain largely unknown. The present study investigated the expression and role of MTDH in CRC cells as well as the underlying mechanism of this. Western blot analysis and quantitative polymerase chain reaction were conducted to determine protein and mRNA expression of MTDH in three human CRC cell lines. A short hairpin RNA (shRNA) targeting MTDH was introduced into CRC HCT116 cells to stably inhibit MTDH expression. A Cell Counting Kit-8 assay, colony formation assay, Transwell assay and flow cytometry were used to investigate the effect of MTDH-knockdown on cell proliferation, migration, apoptosis and cell cycle arrest. Western blotting was performed to examine the protein expression levels of cell growth- and apoptosis-associated genes. The results demonstrated that MTDH was commonly expressed in CRC cell lines. MTDH silencing significantly suppressed cell growth, colony forming ability and migration while inducing the apoptosis of HCT116 cells. In addition, MTDH depletion induced S phase cell cycle arrest in HCT116 cells. Mechanistically, knockdown of MTDH markedly downregulated the expression of phosphorylated protein kinase B, c-Myc, proliferating cell nuclear antigen and B-cell lymphoma 2 (Bcl-2) protein in HCT116 cells, and the expression of p53 and Bcl-2-associated X protein was significantly increased compared with the negative control shRNA group (P<0.05), suggesting that MTDH may function through the expression of numerous types of apoptosis-associated and signaling channel proteins in CRC cells. Taken together, these data indicated that MTDH may serve as a biomarker and candidate therapeutic target for CRC.

Introduction

Colorectal cancer (CRC) is the third most common type of cancer worldwide, accounting for 8% of all cancer-related mortality (1). There is an urgent requirement to identify potential therapeutic targets to effectively inhibit CRC cell growth, invasion and metastasis (2,3). It is well-established that cancer progression is associated with genetic and epigenetic alterations along with the constructional changes in the CRC microenvironment. The activities of several signaling pathways, including Ras, Wnt and Myc signaling, have been correlated with CRC carcinogenesis, which provides potential biomarkers for early diagnosis and prognosis of CRC as well as therapeutic targets for CRC (4,5).

Metadherin (MTDH), also known as astrocyte elevated gene 1 (AEG-1) and lysine-rich CEACAM1 co-isolated (LYRIC), is located on chromosome 8q22 and encodes a single-pass transmembrane protein (6). MTDH has been reported to be overexpressed in solid tumors and promotes tumor cell proliferation, migration and invasion (7-10). Clinical studies have also demonstrated that MTDH overexpression is associated with tumorigenesis, tumor development and short survival times in hepatocellular carcinoma (HCC), gastric and breast cancer, and CRC (11-14). MTDH-mediated tumor progression is regulated by multiple signaling pathways, including nuclear factor-κB (NF-κB), phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and Wnt (15-18). The overexpression of MTDH in CRC is significantly correlated with
the Union for International Cancer Control stages, Ki-67 expression, histological differentiation and shorter survival times (19). However, the expression and role of MTDH in CRC as well as the underlying mechanism remain largely unknown.

The present study determined the protein and mRNA expression level of MTDH in human CRC cell lines and investigated its role in CRC cell behavior, including proliferation, colony-forming and migratory abilities, cell cycle arrest, and apoptosis in vitro. The present study also investigated the underlying molecular mechanism of MTDH-regulated CRC growth by detecting the protein expression of important cell growth- and apoptosis-associated genes in MTDH-deficient CRC cells.

Materials and methods

Cell lines. CRC SW480, HCT116 and LoVo cell lines (Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China), and the normal colonic mucosa epithelial NCM460 cell line (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Corning Incorporated, Corning, NY, USA), containing 10% fetal bovine serum (FBS; Shanghai VIAN-SAGA Biotech Ltd., Shanghai, China), streptomycin (100 µg/ml), and penicillin (100 IU/ml) at 37˚C in a humidified atmosphere of 5% CO₂.

Reverse transcription polymerase chain reaction (RT-PCR). Extraction of total RNA from cells was performed using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The following primer sequences: MTDH forward, 5'-AAGCAGTGCAAACACGGTACG-3' and reverse, 5'-CACCCCTGGTGCTGAGCAGA-3'; and GAPDH forward, 5'-AAGCAGTGCAAACACGGTACG-3' and reverse, 5'-GCACCTTATCACGTGCT-3', qPCR was performed using a SYBR Master Mix (Takara Biotechnology Co., Ltd.) on a Stratagene MX3000p Real-time PCR thermocycler (Agilent Technologies, Inc., Santa Clara, CA, USA) with an initial denaturation step at 95˚C for 10 min, followed by 45 cycles of 95˚C for 3 sec and 60˚C for 30 sec. The experiments were performed in triplicate. mRNA levels of MTDH were analyzed using the 2-ΔΔCq method (20).

Size-based simple and traditional western blot analyses. MTDH proteins following shRNA-mediated knockdown for simple western analysis of total receptor levels were diluted to a final protein concentration of 1X sample buffer. 1X fluorescent molecular weight markers and 40 mM DTT, prior to being heated at 95˚C for 5 min in a Master mix (ProteinSimple, San Jose, CA, USA) and processed at room temperature in a Sally Sue Simple Western instrument (ProteinSimple). Proteins were subsequently incubated with anti-MTDH (cat. no., Ab45338; dilution, 1:20; Abcam, Cambridge, MA, USA) and anti-β-actin (cat. no., SC-69879; dilution, 1:50; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) primary antibodies, and processed as indicated.

Lentivirus-mediated short hairpin RNA (Lenti-shRNA) against MTDH. The Lenti-shRNA vector system (pGCSIL-GFP-puro- mycin) was constructed, packaged and purified by Shanghai GeneChem Co., Ltd. (Shanghai, China). Cells were seeded in 6-well plates at 3x10⁵ cells/well using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The following 3 specific MTDH short hairpin RNAs (shRNAs): shMTDH-1, 5'AGGAAATAAAGGATCTTGATG-3'; shMTDH-2, 5'GAGGATTTAATACCAAGAACA-3'; and shMTDH-3, 5'AACTTAACCCGATCATATAT-3', as well as a negative control shRNA (shNC), 5'TTCTCCGAGCCGTTTGACG-3'. The cells were cultured for the next 48 h and were then harvested for RT-PCR and simple western blot analysis or prepared for the following experiments.

RT-quantitative PCR (RT-qPCR). Isolation of total RNA was conducted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The sequences of the primers (Shanghai GeneChem Co., Ltd.) used for qPCR were as follows: MTDH forward, 5'-AAGCAGTGCAAACACGGTACG-3' and reverse, 5'-CACCCCTGGTGCTGAGCAGA-3'; and GAPDH forward, 5'-AAGCAGTGCAAACACGGTACG-3' and reverse, 5'-GCACCTTATCACGTGCT-3', qPCR was performed using a SYBR Master Mix (Takara Biotechnology Co., Ltd.) on a Stratagene MX3000p Real-time PCR thermocycler (Agilent Technologies, Inc., Santa Clara, CA, USA) with an initial denaturation step at 95˚C for 10 min, followed by 45 cycles of 95˚C for 3 sec and 60˚C for 30 sec. The experiments were performed in triplicate. mRNA levels of MTDH were analyzed using the 2-ΔΔCq method (20).
(TBST) for 1 h at room temperature, prior to being incubated with primary antibodies against C-Myc (cat. no., 6341S; dilution, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), GAPDH (cat. no., SC-32233, dilution, 1:4,000; Santa Cruz Biotechnology, Inc.), B-cell lymphoma 2 (Bcl-2; dilution, 1:1,000; Abcam), Bcl-2-associated X protein (BAX; cat. no., AB7977; dilution, 1:1,000; Abcam), phosphorylated protein kinase B (p-Akt; cat. no., 13038; dilution, 1:1,000; Cell Signaling Technology, Inc.), Akt (cat. no., 9272, dilution, 1:1,000; Cell Signaling Technology, Inc.), p53 (cat. no., 2527, dilution, 1:1,000; Cell Signaling Technology, Inc.) and proliferating cell nuclear antigen (PCNA; cat. no., 2586S; dilution, 1:1,000; Cell Signaling Technology, Inc.) overnight at 4°C. Following three rinses with TBST, the membrane was incubated with a horseradish peroxidase-conjugated rabbit IgG secondary antibody (cat no., sc-2004; dilution, 1:5,000; Santa Cruz Biotechnology, Inc.) and mouse IgG (cat no., sc-2005; dilution, 1:5,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The protein bands were detected using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.) and analyzed using Bio-Rad 680 Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell proliferation assay. The proliferation rate of HCT116 cells was evaluated by a Cell Counting Kit-8 (CCK-8; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). A total of 5,000 stably-transfected cells were seeded and grown overnight in a 96-well plate in six replicates. A total of 10 μl CCK-8 reagent was added into each well at different time points. Following incubation at 37°C for 2 h, absorbance was measured at 450 nm with a Bio-Rad plate reader (Bio-Rad Laboratories, Inc.).

Colony formation assay. A total of 2,000 stably-transfected HCT116 cells were plated into each well of a 6-well plate in triplicate. Following incubation at 37°C for 10 days, the cells were stained with crystal violet for 10 min at room temperature. Colonies containing ≥50 cells were counted under an Olympus IX71 inverted light microscope (magnification, x100; Olympus Corporation, Tokyo, Japan). The images were captured using an Olympus digital camera (Olympus Corporation).

Cell apoptosis assay. Stably-transfected HCT116 cells were collected and resuspended in binding buffer (Invitrogen; Thermo Fisher Scientific, Inc.). On the seventh day after transfection, cell apoptosis was examined using the Annexin V-allophycocyanin (APC) apoptosis detection kit (cat. no., 88-8007-72; eBioscience; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocols. A FACS Calibur flow cytometer (cat. no., 557706; BD Biosciences, Franklin Lakes, NJ, USA) was used to analyze the percentage of apoptotic cells. The cells were stained with 40 μg/ml PI (cat. no., P4170; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 100 μg/ml RNase A (cat. no., EN0531; Fermentas; Thermo Fisher Scientific, Inc.) in the dark for an additional 30 min at room temperature. The results were analyzed using a FACSCalibur flow cytometer (cat. no., 557706; BD Biosciences). The results were analyzed using Multicycle software (version 300; Phoenix Flow System, San Diego, CA, USA).

Transwell migration assay. Transwell assays were conducted using Transwell chambers (Corning Incorporated, Corning, NY, USA) in a 24-well format. A total of 2x10^5 HCT116 cells in 0.1% FBS-containing serum-free RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) were loaded into the upper chamber and 30% FBS-containing medium was added into the lower chamber. Cells were incubated for 48 h at 37°C. The migrating cells stained with crystal violet at 37°C for 1 h were counted in random microscopic fields under an Olympus IX71 inverted light microscope (magnification, x100; Olympus Corporation). The experiments were performed in triplicate.

Statistical analysis. All experiments were independently repeated at least three times. Data are expressed as the mean ± standard deviation. Statistical significance was evaluated using Student’s t-test or one-way analysis of variance with
Results

MTDH mRNA is highly expressed in human CRC cells. The expression pattern of MTDH in human CRC SW480, HCT116 and LoVo cell lines, and normal colonic mucosa epithelial NCM460 cell line was examined by RT-qPCR and western blot analysis. The results demonstrated that the MTDH mRNA and protein expression levels were significantly higher in the human CRC cells than in the NCM460 cells (Fig. 1A and B). In addition, a higher level of MTDH mRNA was observed in HCT116 cells compared with that in the SW480 or LoVo cells.

Prism 4.0 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.
Therefore, HCT116 cells were used to examine the function of MTDH in CRC cell behavior in the subsequent experiments.

**MTDH is essential for HCT116 cell proliferation in vitro.** To investigate the role of MTDH in CRC cell proliferation, lentiviral vectors expressing shMTDH or shNC were introduced into HCT116 cells to stably silence MTDH expression. Fluorescent staining, simple western and qPCR assays were used to assess the knockdown efficacy of shMTDH. As demonstrated in Fig. 2A, cells were infected with a lentivirus containing MTDH shRNA (shMTDH) or an empty vector (shNC). The protein expression of MTDH was significantly inhibited by shMTDH in HCT116 cells compared with the negative control (Fig. 2B). Additionally, the mRNA expression of MTDH was also significantly decreased in shMTDH-transfected cells compared with that in shNC cells (Fig. 2C). These data suggested that MTDH expression was efficiently inhibited by shMTDH. The infection efficiency was 94.2% in the shMTDH-2 group relative to shNC. Since shMTDH-2 exhibited improved inhibition of MTDH expression in HCT116 cells, it was selected for subsequent experiments. To determine whether MTDH is essential for CRC cell growth, cell viability and colony formation assays were performed in a MTDH-deficient HCT116 cell line. As demonstrated in Fig. 3A, knockdown of MTDH notably inhibited...
Knockdown of MTDH in HCT116 cells inhibits cell proliferation and migration. In addition, a colony formation assay revealed that shMTDH markedly suppressed the colony-forming ability of HCT116 cells compared with shNC (Fig. 3B). Taken together, these data indicated that MTDH is essential for HCT116 cell growth in vitro and may serve a promotive role in CRC tumorigenesis.

Knockdown of MTDH significantly induces HCT116 cell apoptosis. Cell apoptosis is critically important in cell growth suppression (21). To investigate the mechanism underlying MTDH-mediated HCT116 cell growth, cell apoptosis analysis was performed using PI-APC-Annexin in HCT116 cells. As demonstrated in Fig. 3C, flow cytometry revealed that the percentage of apoptosis was significantly increased to 14.84% (SD=0.22%, P<0.01) in the MTDH-shRNA HCT116 group, from 3.05% (SD=0.05%) in the shNC group, suggesting that knockdown of MTDH may induce cell apoptosis, resulting in an inhibition of cell proliferation in CRC cells.

Knockdown of MTDH induces cell cycle arrest at the S phase. To further investigate the mechanism underlying MTDH-mediated growth and apoptosis of HCT116 cells, a cell cycle analysis was performed. As demonstrated in Fig. 4, compared with the negative control, MTDH deficiency in HCT116 cells induced a significant increase in the cell population in the S phase (48.97 vs. 26.61%, P<0.01), indicating that MTDH may serve an essential role in S phase arrest in CRC cells.

MTDH is required for CRC cell migration. To further investigate whether MTDH has an effect on CRC cell migration, a Transwell migration assay was performed to test the migratory ability of MTDH-deficient HCT116 cells. As demonstrated in Fig. 5, MTDH deficiency significantly suppressed the migratory ability of HCT116 cells compared with the negative control, suggesting that MTDH may promote CRC metastasis.

Knockdown of MTDH inhibits p-Akt and c-Myc, and increases apoptosis-related protein expression. To further investigate the underlying molecular mechanism of MTDH-mediated

Figure 4. Knockdown of MTDH induced S phase cell cycle arrest in the HCT116 cell lines. HCT116 cells were stably transfected with shNC- or shMTDH-treated cells, or a blank control. Flow cytometry was performed for cell cycle analysis. The percentages of cells in the cell cycle phases are shown. Data are expressed as the mean ± standard deviation. **P<0.01 vs. shNC and shMTDH groups. MTDH, metadherin; sh, short hairpin RNA; NC, negative control.

Figure 5. Knockdown of MTDH suppressed the migration of HCT116 cells. A Transwell chamber assay was performed to determine the migration of blank control, and shNC- and shMTDH-transfected HCT116 cells. Cells were allowed to migrate for 48 h and crystal violet was used to stain the migrating cells that were quantified under a microscope. Representative images of migrating cells are shown. Cell numbers are expressed as the fold-change relative to NC cells in the graph. Data are expressed as the mean ± standard deviation. **P<0.01 vs. shNC and shMTDH groups. Magnification, x100. MTDH, metadherin; sh, short hairpin RNA; NC, negative control.
CRC cell growth and migration, the present study examined the activity of Akt/c-Myc signaling and apoptosis-related protein in shMTDH-transfected HCT116 cells using western blot analysis. As demonstrated in Fig. 6, knockdown of MTDH markedly downregulated the expression of p-Akt, c-Myc and Bcl-2 protein in HCT116 cells, and the expression of p53 and Bax protein was significantly increased compared with the shNC group (P<0.05). Indicating that MTDH may function through the expression of multiple types of apoptosis-related and signaling channel protein in CRC cells.

Knockdown of MTDH downregulates the expression of PCNA. To determine whether the depletion of MTDH can regulate PCNA expression in CRC cells, the protein expression of PCNA in MTDH-deficient HCT116 cells was analyzed. As demonstrated in Fig. 6, the protein expression of PCNA in shMTDH-transfected cells was significantly downregulated compared with that in shNC-transfected cells, suggesting that PCNA may serve as a downstream effector of MTDH to regulate CRC cell growth, apoptosis, cell cycle and migration.

Discussion

The present study investigated the expression and role of MTDH in CRC cells in vitro and revealed that MTDH was highly expressed in CRC cell lines. In addition, shRNA-mediated knockdown of MTDH significantly inhibited CRC cell proliferative, colony-forming and migratory abilities while inducing cell apoptosis and S phase cell cycle arrest. Mechanistically, knockdown of MTDH markedly downregulated the protein expression of p-Akt, c-Myc, Bcl-2 and PCNA, while upregulating the protein of p53 and Bax expression of in HCT116 cells. These results suggested that MTDH-knockdown induces apoptosis of HCT116 cells, and its mechanism may be associated with upregulation of Bax protein expression and downregulation of Bcl-2 protein expression. MTDH is essential for CRC cell growth and migration in vitro possibly through the Akt/c-Myc signaling pathway.

MTDH can be induced by human immunodeficiency virus (HIV)-1 infection or recombinant HIV-1 envelope glycoprotein (gp120) treatment in primary human fetal astrocytes (22). Previous studies have reported that MTDH expression is positively correlated with high risks of cancer in humans (23,24). A recent study demonstrated that the knockdown of MTDH can inhibit ovarian cancer cell growth and invasion while inducing apoptosis and cell cycle arrest at the G0/G1 phase (25). In addition, MTDH was revealed to be highly abundant in HCC cells and tissues, and knockdown of MTDH can suppress HCC cell growth and colony formation in vitro, as well as inhibiting xenografted tumor growth in vivo (26). Furthermore, the present study also demonstrated...
that MTDH depletion significantly inhibits cell proliferation and colony formation while inducing apoptosis and cell cycle arrest at the S phase in CRC cells. Based on these findings, we hypothesized that MTDH may function as an oncogene and serve a critical role in CRC development and progression.

Metastasis is one of the most critical hallmarks of cancer. The upregulation of MTDH has been demonstrated to promote invasive and metastatic abilities of non-small cell lung cancer cells (23). MTDH overexpression in patients with primary CRC may be considered as a biomarker for lung-specific metastases (27). Consistent with these findings, the present study demonstrated that MTDH depletion can suppress CRC cell migration (Fig. 5), indicating that MTDH is possibly involved in CRC metastasis and may serve as a novel biomarker for CRC metastasis. Targeting MTDH may be a potential therapeutic strategy against CRC.

A previous study has demonstrated that MTDH regulates malignancy development through various cellular signaling cascades, including the MAPK, Ha-ras, NF-κB, PI3K/Akt and Wnt pathways (28). Akt signaling is important for regulating tumor cell proliferation, invasion, apoptosis, cell cycling and survival. Previous studies have demonstrated that the PI3K/Akt signaling pathway is aberrantly activated in human cancer (29,30). Although the overexpression of Akt frequently occurs during CRC carcinogenesis, this is not the case in CRC with microsatellite instability (31). c-Myc is a protooncogene (32) that regulates cell growth and proliferation through regulating a number of downstream target genes, serving an oncogenic role in multiple types of human cancer (33). The upregulation of c-Myc significantly enhances the invasive and metastatic abilities of cancer cells (34). Another previous study demonstrated that MTDH expression can be induced by Ha-ras via the PI3K-Akt signaling pathway (16). MTDH also affects Akt phosphorylation, which is involved in c-Myc-suppressed apoptosis (35). Therefore, as one of the downstream effectors of c-Myc and Ha-ras signaling, MTDH may serve a key role in tumor development and progression (16). The present study consistently indicated that knockdown of MTDH leads to a markedly reduced expression of c-Myc, p-Akt, Bcl-2 and PCNA. MTDH shRNA also upregulated the protein expression of p53 and Bax in HCT116 cells, suggesting that MTDH silencing inhibits CRC cell proliferation and migration. MTDH-knockdown induces apoptosis possibly by upregulation of Bax protein expression and downregulation of Bcl-2 protein expression, and the Akt and c-Myc signal channel may be involved in the changes in protein expression. Akt activation may inhibit its downstream target glycogen synthase kinase-3, which phosphorylates c-Myc at 58 asinine residue, resulting in the inhibition of c-Myc protein degradation (36,37). Therefore, MTDH-activated Akt signaling may cause an upregulation of the protein level of c-Myc, which further promotes CRC development.

In summary, the results of the present study demonstrated that MTDH expression is commonly expressed in CRC cell lines and shRNA-mediated knockdown of MTDH inhibits HCT116 cell proliferation, colony formation and migration while inducing cell cycle arrest at the S phase and apoptosis, which is not only associated with the downregulation of p-Akt, c-Myc, Bcl-2 and PCNA expression, but also associated with the upregulation of p53 and Bax protein. Further studies are required to elucidate the mechanisms underlying the regulatory activity of MTDH in CRC. Therefore, targeting MTDH may be a potential therapeutic strategy in CRC treatment. Further in vivo investigation is required to develop MTDH inhibitors for CRC therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

JWL and CZH performed the research, the data acquisition and drafted the manuscript; JHL, JHY, YPL, WFZ, MXZ, YL and QHC provided assistance for data acquisition, data analysis and statistical analysis; LGL designed the research, edited and reviewed the manuscript. ZSX provided assistance on the data acquisition, data analysis and statistical analysis. All authors have read and approved the content of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

References

18. Li PP, Feng LL, Chen N, Ge XL, Lv X, Lu K, Ding M, Yuan D

17. Wei J, Li Z, Chen W, Ma C, Zhan F, Wu W and Peng Y: AEG-1

16. Lee SG, Su ZZ, Emdad L, Sarkar D and Fisher PB: Astrocyte


