Metadherin Promotes Hepatocellular Carcinoma Metastasis through Induction of Epithelial–Mesenchymal Transition

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Abstract

**Purpose:** To investigate the expression of metadherin (MTDH) for its prognostic value in hepatocellular carcinoma (HCC) and its role in promoting HCC metastasis.

**Experimental Design:** This study employed a tissue microarray containing samples from 323 HCC patients to examine the expression of MTDH and its correlation with other clinicopathologic characteristics. The role of MTDH in the regulation of HCC metastasis was investigated both in vitro and in vivo using short hairpin RNA (shRNA)–mediated downregulation of MTDH in HCC cell lines with various metastatic potentials.

**Results:** The expression of MTDH was markedly higher in HCC tumors than in normal liver tissue. Particularly high MTDH expression was observed in tumors with microvascular invasion, pathologic satellites, poor differentiation, or tumor-node-metastasis stages II to III. Furthermore, the clinical outcome was consistently poorer for the MTDH high group than for the MTDH low group in the 1-, 3-, and 5-year overall survival (OS) rates and in the 1-, 3-, 5-year cumulative recurrence rates. In a nude mice model, the shRNA-mediated downregulation of MTDH resulted in a reduced migratory capacity in HCC cell lines, as well as a reduction in pulmonary and abdominal metastasis. Furthermore, we found that the expression level of MTDH correlated with four epithelial–mesenchymal transition (EMT) markers. Knockdown of MTDH expression in HCC cell lines resulted in downregulation of N-cadherin and snail, upregulation of E-cadherin, and translocation of β-catenin.

**Conclusions:** MTDH may promote HCC metastasis through the induction of EMT process and may be a candidate biomarker for prognosis as well as a target for therapy.

Introduction

Hepatocellular carcinoma (HCC) is the most common liver malignancy and a major health problem globally. Current standard practices for treatment of HCC, surgical resection, and liver transplantation are less than satisfactory due to high recurrence rates (1). Recently, several proteins and signaling pathways have been found to correlate with the prognosis of HCC patients. Because the mechanism underlying metastasis and recurrence is not thoroughly understood, the continued search for molecular markers which predict recurrence is essential.

Metadherin (MTDH) is a single-pass transmembrane protein with its gene located at chromosome 8q22 (2), the abnormalities of which have been identified in several tumor types (3, 4). MTDH is attracting increasing attention from oncologists because it is overexpressed in a number of malignancies, such as breast cancer, malignant glioma, and neuroblastoma (3, 5, 6), and its overexpression is associated with poor clinical outcome. MTDH plays an important role in the regulation of carcinogenesis and tumor progression (3, 6, 7). For instance, it can interact with NF-κB and promotes NF-κB–induced tumor progression and metastasis (8); it also enhances the chemoresistance of cancer through activation of the aldehyde dehydrogenase 3 family, member A1 (ALDH3A1), and the hepatocyte growth factor receptor (3).

A recent study reported that MTDH promotes HCC carcinogenesis through activation of the Wnt/β-catenin signaling pathway via activation of RK42/44 and upregulation of lymphoid-enhancing factor 1/T cell factor 1 (7). Nonetheless, the prognostic value of MTDH in HCC as well as its function in regulating HCC metastasis has yet to be
Recurrence and metastasis remain the most common lethal outcomes of hepatocellular carcinoma (HCC) after curative resection. Thus, it is critical to reveal the mechanism underlying HCC metastasis. Metadherin (MTDH) has been shown to promote carcinogenesis and tumor metastasis in a number of malignancies. This study explores the role of MTDH in HCC and verifies that MTDH is an independent predictor for outcome of HCC patients. In addition, we show that MTDH promotes HCC metastasis through the epithelial–mesenchymal transition process. Our results suggest that MTDH may be a biomarker for prognosis as well as a potential target for therapy in HCC.

Materials and Methods

Cells and animals

MHCC97-L (97L), MHCC97-H (97H), HCCLM3 (LM3) (human HCC cell lines with different metastatic potentials established at the Liver Cancer Institute, Zhong Shan Hospital, Fudan University, Shanghai, China), and HepG2 cells (a low metastatic human HCC cell line; American Type Culture Collection) were used in this study. Male athymic BALB/c nude mice were purchased from Shanghai Institute of Material Medicine, Chinese Academy of Science, and were raised in specific pathogen-free conditions. Animal care and experimental protocols were conducted in accordance with guidelines established by the Shanghai Medical Experimental Animal Care Commission.

Patients and follow-up

A tissue microarray (TMA) composed of samples from 323 Chinese HCC patients was used in this study. These patients underwent curative liver resection for primary tumors between January 2003 and March 2004 in the Liver Cancer Institute, Zhong Shan Hospital, Fudan University. The detailed clinicopathologic characteristics of the patients are listed in Table 1.

The inclusion criteria of the patient cohort include (i) having a distinctive pathologic diagnosis of HCC, (ii) surgical resection, defined as complete resection of all tumor nodules with the cut surface being free of cancer by histologic examination, and (iii) having a complete tumor nodules with the cut surface being free of cancer by histologic examination (9), and (iii) having a complete clinicopathologic and follow-up data. The exclusion criteria include (i) having macrovascular invasion or extrahepatic spread and (ii) having prior anticancer treatment. The detailed clinicopathologic characteristics of the patients are listed in Table 1.

The inclusion criteria of the patient cohort include (i) having macrovascular invasion or extrahepatic spread and (ii) having prior anticancer treatment. The detailed clinicopathologic characteristics of the patients are listed in Table 1.

Preoperative liver function of all patients was Child-Pugh A, 278 (86.1%) patients had been infected with hepatitis B and 7 (2.2%) with hepatitis C. Tumor stage was determined according to the 2010 International Union Against Cancer tumor–node–metastasis (TNM) classification and the Barcelona Clinic Liver Cancer (BCLC) staging classification. Tumor differentiation was graded by the Edmondson grading system. Ethical approval was obtained from the Zhong Shan Hospital research ethics committee and informed consent was obtained from each patient. After surgery, the patients were monitored until March 15, 2009, with a median follow-up of 60 months (range, 2.0–77.0 months). The follow-up procedures were carried out as described in our previous study (10).

TMA, immunohistochemistry, and immunofluorescence microscopy

The resected specimens were paraffin embedded and stored at 4°C. The construction of the TMA and the protocol for immunohistochemistry were described previously (11). The expression of MTDH was detected through an immunohistochemistry assay of the TMA, and the intensity of positive staining was measured with integrated optical density (IOD) as previously described (12). The intensity of MTDH was classified as either high or low expression [median value of IOD (mIOD) was used as the cutoff value; MTDH^High: IOD > mIOD, and MTDH^Low: IOD ≤ mIOD] The immunohistochemistry assay of CD151 was conducted according to the same protocol. Immunofluorescence microscopy was carried out as previously described (10) and images were obtained using a fluorescence microscope (BX51; Olympus).

Real-time PCR and Western blot

Total RNA was isolated using the Trizol reagent (15596-026; Invitrogen) and reverse transcribed to cDNA with PrimeScript RT reagent kit (RR037A; Takara). For real-time PCR (RT-PCR), SYBR Premix Ex Taq (DRR081; Takara) was used according to the manufacturer’s instructions. Cytoplasmic and nuclear protein were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (78833; Pierce). Western blots were carried out as described previously (13), the images were captured through Gel Doc XR system (Bio-Rad) and analyzed using Image Lab software (version 2.0). The primers and antibodies used in this study are listed in the Supplementary Table S1.

Cell transfection and clone selection

The functional role of MTDH in HCC cells was assessed by the short hairpin RNA (shRNA)–mediated stable silencing method without MTDH mutations. Three sequences of shRNA-targeting MTDH were designed and cloned into the pLKO.1 TRC cloning vector (Supplementary Table S1). The efficacy was evaluated by Western blot and the second sequence was used. The sequence of scrambled RNA (SCR) was used to generate the negative control plasmid pLKO.1-Scr (Supplementary Table S1). Viral particles were produced by cotransfection of the shRNA plasmid and the lentiviral enveloping and packaging plasmid, pMD2.G and psPAX2, into 293T cells. HCC cells transfected with the lentiviral
particles were selected with 3 μg/mL puromycin (P8833; Sigma-Aldrich).

**Cell migration and scratch assay**

A Transwell insert (3422; Corning) with an 8 μm pore size was used to carry out the 2-chamber migration assay. Cells in serum-free medium were seeded into the upper chamber in a 24-well plate. Serum-containing medium was used in the lower chamber as the attractant. After 24 hours of culturing, cells that had migrated to the bottom surface were fixed and counted after staining with Giemsa. The capacity of migration was assessed according to the quantity of cells. For the scratch assay, cells were grown to confluence in a 24-well plate, and a “wounding” line was scratched into the cell monolayer with a sterile 200-μL pipette tip. The width of the wound was measured under a microscope at 0 and 72 hours after the scratch to assess the migration ability of the cells. Results were analyzed with the Student t-test.

**Metastasis assays in vivo**

The metastasis assay in vivo was conducted as previously described (10). RFP-expressing LM3 cells (3.0 × 10^6) infected with lentivirus (LM3-SCR and LM3-shRNA) were implanted in the liver parenchyma of nude mice under anesthesia. After 6 weeks, the mice were sacrificed and the pulmonary and abdominal metastases were visualized with fluorescence stereomicroscopy (Leica Microsystems Imaging Solutions, Ltd.). Serial sections were made from the lung, and the total number of lung metastases was counted under the microscope. The metastases were classified into 4 grades on the basis of the number of tumor cells present at the maximal section for each metastatic lesion: grade I, 20 or more tumor cells; grade II, 20 to 50 tumor cells; grade III, 50 to 100 tumor cells; and grade IV, 100 or more tumor cells.

**Statistical analysis**

Statistical analyses were conducted with SPSS 17.0 software (SPSS). The correlation between MTDH expression and other clinicopathologic characteristics was evaluated using the Pearson x^2 test. Overall survival (OS) was defined as the interval between HCC resection and death; patients alive at the end of follow-up were censored. The time to recurrence was calculated from the HCC resection to the first radiological evidence of recurrence. Patients experiencing death in the absence of recurrence were censored in determining recurrence (14). The cumulative recurrence and survival rates were carried out by the Kaplan–Meier method and analyzed by the log-rank test. Univariate and multivariate analyses were based on the Cox proportional hazards regression model. Two-tailed value of P < 0.05 was used to indicate a significant result.

**Results**

MTDH promotes metastatic ability of HCC cells

To investigate the role of MTDH in HCC metastasis, we treated LM3 and 97H cells with a lentivirus-targeting MTDH to create LM3-shRNA and 97H-shRNA cell lines that

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**Table 1. Correlation between MTDH staining and clinicopathologic characteristics in 323 HCC patients**

<table>
<thead>
<tr>
<th>Variables</th>
<th>MTDH staining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High (n = 175)</td>
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<tr>
<td></td>
<td>n (%)</td>
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<tr>
<td>Age, y</td>
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</tr>
<tr>
<td>≤50</td>
<td>97 (55.4)</td>
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<td>&gt;50</td>
<td>78 (44.6)</td>
</tr>
<tr>
<td>Gender</td>
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<tr>
<td>Male</td>
<td>155 (88.6)</td>
</tr>
<tr>
<td>Female</td>
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<tr>
<td>HBsAg</td>
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<tr>
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<td>21 (12.0)</td>
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<tr>
<td>Positive</td>
<td>154 (88.0)</td>
</tr>
<tr>
<td>Anti-HCV</td>
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<tr>
<td>Negative</td>
<td>170 (97.1)</td>
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<tr>
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<td>5 (2.9)</td>
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<tr>
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<tr>
<td>No</td>
<td>20 (11.4)</td>
</tr>
<tr>
<td>Yes</td>
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</tr>
<tr>
<td>Serum AFP, ng/mL</td>
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<td>48 (27.4)</td>
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<td>Tumor diameter (cm)</td>
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<td>82 (46.9)</td>
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<tr>
<td>&gt;5</td>
<td>93 (53.1)</td>
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<td>Tumor number</td>
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<tr>
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<tr>
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<tr>
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<td>Yes</td>
<td>97 (55.4)</td>
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<tr>
<td>Pathologic satellites</td>
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<tr>
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</tr>
<tr>
<td>B/C</td>
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</table>

Abbreviations: AFP, alpha-fetoprotein; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus.
exhibited stably downregulated MTDH compared with their parental cell lines (Fig. 1A). We conducted an in vitro motility assay between parental and interfered HCC cells. Both the scratch assay and the 2-chamber migration assay showed that inhibition of MTDH in LM3 and 97H cells reduced the motility of these cells (Fig. 1B and C).

We then implanted RFP-expressing LM3-SCR and LM3-shRNA cells in the liver parenchyma of nude mice (6 for each group). After 6 weeks, both groups formed liver tumors. The number of pulmonary and abdominal metastases was counted according to the red fluorescence (Fig. 2A and B). LM3-SCR cells formed a greater number of metastases than LM3-shRNA cells in both the lung (53 ± 3.7 vs. 20 ± 2.6, \( P < 0.001 \), respectively) and abdomen (13 ± 2.1 vs. 6 ± 2.6, \( P = 0.03 \), respectively). The number of metastatic nodules of each grade was also greater in the LM3-SCR group (Fig. 2C and D). These in vivo experiments confirmed the role of MTDH in the promotion of HCC metastasis.

**Overexpression of MTDH predicts poorer prognosis in HCC patients**

To explore whether MTDH could be an important factor in determining clinical outcomes of HCC patients, we assessed its expression in a TMA of 323 HCC patients. The immunohistochemistry results showed that MTDH was primarily located in the membrane and the cytoplasm. Most of the tumor tissues expressed significantly higher levels of MTDH than adjacent nontumorous tissues, with MTDH\(^{\text{High}}\) accounting for 54.2% (175 of 323) of all the patients (Supplementary Fig. S1A–D). The Pearson \( \chi^2 \) test indicated that MTDH expression was closely associated with microvascular invasion (\( P < 0.001 \)), pathologic satellites (\( P = 0.007 \)), tumor differentiation (\( P = 0.002 \)), and TNM stage (\( P = 0.001 \); Table 1). These results suggest that tumors with more microvascular invasion or pathologic satellites, poorer differentiation, and TNM stages II to III are prone to exhibit higher MTDH expression. Furthermore, we found that the expression of MTDH did not correlate with other clinicopathologic characteristics such as age, gender, liver cirrhosis, serum alpha-fetoprotein, tumor diameter, tumor encapsulation, or BCLC stage.

As of the last follow-up in March 2009, 54.2% (175 of 323) of the patients had suffered from recurrence and 51.1% (165 of 323) had died with local or distant recurrence. The 1-, 3-, and 5-year OS and cumulative recurrence rates in the whole cohort were 85.4% and 25.4%, 62.4% and 40.7%, and 46.8% and 52.3%, respectively.
and 50.2%, 50.7% and 59.7%, respectively. Furthermore, the 1-, 3-, 5-year OS rates in the MTDH<sup>High</sup> group were significantly lower than those in the MTDH<sup>Low</sup> group (83.0% vs. 89.7%, 52.0% vs. 75.3%, 37.4% vs. 66.9%, respectively); and the 1-, 3-, 5-year cumulative recurrence rates were markedly higher in the MTDH<sup>High</sup> group than those in the MTDH<sup>Low</sup> group (32.4% vs. 16.8%, 61.2% vs. 38.2%, 70.7% vs. 47.8%, respectively; Fig. 3A and B). Univariate and multivariate analysis revealed that along with tumor diameter, encapsulation, microvascular invasion, and TNM stage, MTDH was an independent prognostic factor for both OS (HR = 1.870, \( P < 0.001 \)) and recurrence (HR = 1.695, \( P < 0.001 \); Supplementary Table S2 and S3).

Overexpression of MTDH is associated with EMT in HCC

The TMA images revealed an interesting phenomenon. At the margins of the tumor, several dispersed or clusters of tumor cells were observed to exhibit mesenchymal or amoeboid morphology and to express high levels of N-cadherin and low levels of E-cadherin (Fig. 3C). 2 key markers for tumor metastasis, indicating that these cells were undergoing the EMT process (15, 16). Moreover, the fact that these cells also expressed high levels of MTDH suggests that MTDH might be associated with the EMT process.

To confirm our hypothesis, we compared the expression of MTDH and 5 key markers and regulators of EMT.

Figure 2. Metastatic ability of LM3-SCR and LM3-shRNA cells. A, representative images of metastasis in lung (a and b) and abdomen (c and d) of the 2 cell lines are shown through fluorescence of the tumor cells. B, number of either pulmonary or abdominal metastases is significantly larger in the LM3-SCR group than the LM3-shRNA group. C, hematoxylin and eosin staining images of metastatic nodules in lung of the 2 cell lines (a and b) with magnification of the selected areas (c and d) showing the number of tumor cells in a single nodule. Yellow arrows point to the metastatic nodules. D, all grades of metastatic nodules in lungs of the LM3-SCR group are much more than the LM3-shRNA group.
(E-cadherin, N-cadherin, β-catenin, snail, and twist) in 4 HCC cell lines with different metastatic potentials (HepG2, 97L, 97H, and LM3) by RT-PCR (Fig. 4A) and Western blot (Fig. 4B). The results showed that MTDH expression was low in HepG2 (low metastatic), and the expression of N-cadherin and snail was also low in HepG2, but the expression of E-cadherin and β-catenin was high. On the contrary, high metastatic cell lines (97L, 97H, and LM3) expressed high level of MTDH, N-cadherin, and snail, but low level of E-cadherin and β-catenin. These results indicate that MTDH might play an important role in promoting the EMT process.

Inhibition of MTDH disrupts EMT

To further confirm the relationship between MTDH and the EMT process, we investigated the changes of EMT markers between MTDH-shRNA and parental cells using RT-PCR (data not shown) and Western blot (Fig. 4C). Both of these analyses revealed that HCC cells with inhibited MTDH expression displayed downregulated N-cadherin and snail and upregulated E-cadherin. The changes of total β-catenin were not significant. Because translocation of β-catenin from membrane to nucleus is a characteristic change during EMT (17), we assessed the cytoplasmic and nuclear expression of β-catenin in the cells. As evidenced by Western blot (Fig. 4D), inhibition of MTDH in HCC cells increased cytoplasmic β-catenin expression and reduced nuclear β-catenin, which implies that translocation of β-catenin was induced by MTDH. The changes of β-catenin were also confirmed by immunofluorescence staining (Supplementary Fig. S2A). These changes of EMT markers following MTDH knockdown were still evident in transplanted tumors in mice as shown by immunohistochemistry assays (Supplementary Fig. S2B).

Discussion

MTDH has emerged as an important molecule to modulate cell proliferation, angiogenesis, drug resistance, and stem cell transformation (18). This study used an extensive collection of HCC tumors to show that MTDH was highly expressed in a substantial proportion of HCC tumors, and the MTDH expression was clearly associated with the differentiation and stage of the tumor. These observations were reminiscent of previous reports in other malignancies such as breast cancer (3) and malignant glioma (5). This study also showed that MTDH was associated with TNM stage (I vs. II/III, \( P = 0.001 \)), but did not correlate with BCLC stage (A vs. B/C, \( P = 0.113 \)), which may be due to the fact that these staging systems use different variables related to the severity of liver disease, number and size of tumor nodules, and cancer spread (19). Moreover, our survival analysis revealed that overexpression of MTDH in HCC predicted shorter OS rate (A) and higher cumulative recurrence rate (B). C, two representative cases of serial sections from the same TMA. Case 1, arrows point to the cells undergoing EMT; case 2, a group of cells undergoing EMT is indicated. These are confirmed by hematoxylin and eosin staining (a and e) through morphologic changes. These cells express high levels of MTDH (b and f) and N-cadherin (d and h), but low levels of E-cadherin (c and g).
evaluated the expression of CD151 on the same TMA (Supplementary Fig. S1E and F). Consistent with our previous reports (10), univariate analysis showed that CD151 was a good predictor for OS and recurrence (Supplementary Table S2). Multivariate analysis (Supplementary Table S4) including MTDH and CD151 revealed that MTDH is comparable with CD151 in predicting both OS (HR = 1.830 vs. 1.686) and recurrence (HR = 1.628 vs. 1.523). These findings further proved that MTDH is an important prognostic factor for HCC patients after curative resection.

Several pieces of evidence in this study showed a close association between MTDH expression and HCC metastasis. First, the protein and mRNA expression levels of MTDH correlated with the metastatic potentials of the HCC cell lines (Fig. 4A and B). Second, functional assays showed that inhibition of MTDH significantly reduced the motility of HCC cells (Fig. 1B and C). Third, using an orthotopic xenograft model, we found that knockdown of MTDH in the implanted HCC cells markedly reduced the number of pulmonary and abdominal metastases (Fig. 2A–D). These results lend further credence to the notion that MTDH plays a crucial role in regulating HCC progression and metastasis.

The mechanisms for the functional roles of MTDH in enhancing different aspects of tumor malignancy remain poorly defined. One novel finding in this study is that MTDH expression was closely associated with key markers of EMT, such as E-cadherin, β-catenin, snail, and N-cadherin. EMT is a process whereby cells change from cobblestone shapes that exhibit tight cell–cell contact into spindle-shaped fibroblast-like shapes that lose cell–cell contact and cell polarity (21). In tumor cells, this process can result in an increase in mesenchymal-like cells and a decrease in epithelial-like cells, thus enhancing the invasive and metastatic potential of the tumors. Because EMT also plays an important role in HCC invasiveness and metastasis (20, 22, 23), a positive correlation between MTDH expression and EMT process may provide an explanation for the action of MTDH in HCC malignancy.

Figure 4. Expression of EMT markers in HCC cell lines. A, relative mRNA expression of MTDH, E-cadherin, N-cadherin, snail, twist, and β-catenin in HepG2, 97L, 97H, and LM3 cell lines, with β-actin as the control. B, Western blot analysis of these proteins in HCC cell lines. C, Western blot analysis of MTDH and EMT markers in parental, control, and knockdown cells. D, Western blot analysis of β-catenin in cytoplasm (β-actin as control) and nucleus (histone H4 as control) of the cultured cells showed that inhibition of MTDH resulted in increased cytoplasmic β-catenin and reduced nuclear β-catenin.
Our results showed that when MTDH was downregulated, E-cadherin and membranous β-catenin increased, whereas nuclear β-catenin decreased. Both E-cadherin and β-catenin are components of the adherens junctions of cell membrane, and the adherens junctions function by linking to the cortical actin cytoskeleton and mediating cell–cell adhesion (24). When EMT takes place, E-cadherin is cleaved, and β-catenin in the adherens junctions dissociates from E-cadherin and translocates into the nucleus to function in the Wnt/β-catenin signaling pathway, thereby enhancing the motility of the cells. Furthermore, our study showed that suppression of MTDH decreased snail expression. Snail acts as an important transcriptional repressor of E-cadherin by binding to 3 E-boxes in the E-cadherin promoter and inducing the EMT process (25, 26). Thus, we speculate that overexpression of MTDH in HCC might inhibit E-cadherin through direct contact or via a snail-mediated pathway, followed by the activation of the Wnt/β-catenin signaling pathway and enhanced cell mobility. In support of this working model, we found that downregulation of MTDH decreased the expression of N-cadherin, a cell adhesion molecule that mediates cell–cell adhesion and modulates cell migration and tumor invasiveness (15, 27). On the basis of these findings, we believe that MTDH may induce EMT in HCC.

In conclusion, this study show that overexpression of MTDH in HCC is a strong indicator for more aggressive tumors and poorer clinical outcome. MTDH promotes HCC metastasis through the induction of EMT process. The evidence presented strongly suggests that MTDH could be a candidate biomarker for HCC prognosis and a target for therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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