RESEARCH COMMUNICATION

Establishment and Characterization of MTDH Knockdown by Artificial MicroRNA Interference - Functions as a Potential Tumor Suppressor in Breast Cancer

Song Wang[1], Jie-Zhi Shu[1] , Yi Cai[1], Zheng Bao[1], Qing-Mo Liang[1,2]*

Abstract

Background: Considerable evidence suggests that metadherin (MTDH) is a potentially crucial mediator of tumor malignancy and an important therapeutic target for simultaneously enhancing chemotherapy efficacy and reducing metastasis risk. Inhibition of MTDH expression by RNA interference has been shown in several previous research, but silencing MTDH expression by microRNA (miRNA) interference in breast cancer has not been established. In the present study, we investigated the role of MTDH-miRNA in down-regulation of proliferation, motility and migration of breast carcinoma cells. Methods: Expression vectors of recombinant plasmids expressing artificial MTDH miRNA were constructed and transfected to knockdown MTDH expression in MDA-MB-231 breast cancer cells. Expression of MTDH mRNA and protein was detected by RT-PCR and Western blot, respectively. MTT assays were conducted to determine proliferation, and wound healing assays and transwell migration experiments for cell motility and migration. Results: Transfection of recombinant a plasmid of pcDNA-MTDH-miR-4 significantly suppressed the MTDH mRNA and protein levels more than 69% in MDA-MB-231 breast cancer cells. This knockdown significantly inhibited proliferation, motility and migration as compared with controls. Conclusions: MTDH-miRNA may play an important role in down-regulating proliferation, motility and migration in breast cancer cells, and should be considered as a potential small molecule inhibitor therapeutic targeting strategy for the future.

Keywords: MTDH - microRNA - breast cancer - proliferation - motility - migration

Asian Pacific J Cancer Prev, **13**, 2813-2818

Introduction

Based on the GLOBOCAN 2008 estimates, breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths (Jemal et al., 2011). Tremendous problems with metastatic breast cancer thetapy is the development of tumor progression, metastasis, and resistance to chemotherapies. And although much has been learned about its genetic and biochemical basis, it is a heterogeneous disease and is stratified by race, stage, grade,and estrogen/progesterone receptor status, it is far more difficult to validate a therapeutic target gene for devolopment of new anticancer strategies that could largely decrease both growth, invasion, metastatis , and drug resistance.

Metadherin (MTDH, known as AEG-1 or LYRIC) was originally cloned as an HIV-1 and TNF-α–inducible gene in primary human fetal astrocytes (Kang et al., 2005), however the subsequent research demostrated that it expression is elevated in some solid tumors, including those of gynecologic tumors (breast, endometrial, and ovarian tumors) (Li et al., 2008; Song et al., 2010; Li et al., 2011), Gastrointestinal carcinoma (hepatocellular and gallbladder, colon and gastric carcinomas) (Song et al., 2010; Chen et al., 2011; Jianbo et al., 2011), head and neck cancers (Hui et al., 2011; Nohata et al., 2011), etc. These findings establish MTDH as an important therapeutic target for simultaneously enhancing chemotherapy efficacy and reducing metastasis risk. Dramatically, Brown et al. (2004) showed that strong MTDH staining was observed in 17 out of 31 samples of breast carcinoma patients while MTDH staining was absent in 18 out of 20 samples of normal breast tissue , this experiment disclosed that MTDH protein is overexpressed in metastatic breast cancer tissue ,and selectively targeted phage as well as cells to the lung, indicating that MTDH protein mediates localization at the metastatic site in breast cancer. Further more, Li et al. (2008) analyzing 225 breast cancer patients showed increased expression of the MTHD protein in 44.5% of cases that correlated with the progression of the disease and the patient survival. Interestingly, in this study, the metastatic tumors showed more nuclear staining of MTDH. Similarly, Hu et al. (2009) analyzing 170 breast

*[1]Department of Oncosurgery, Affiliated Nanhua Hospital, [2]Department of Oncosurgery, First Affiliated Hospital of South China University, University of South China, Hengyang, China *For correspondence: hylqm@vip.sina.com, wangmomo1983@sohu.com*

cancer patients revealed that overexpression of MTDH occurs in up to 47% of breast cancer patients and promotes metastatic seeding cancer cells to the lung as well as chemoresistance of breast tumors. However, in tissue sections of human breast cancer used in this study, MTDH protein was localized predominantly in the cytoplasm. Recently, Yang Q and his coworks (Li et al., 2011; Zhao et al., 2011) demonstrated that MTDH could promote epithelial-mesenchymal transition (EMT) and activation of Lipopolysaccharide (LPS) -induced cell migration and invasion. Conversely, inhibition of MTDH by RNAi (MTDH-siRNA and MTDH-shRNA) significantly inhibits metastatsis, chemoresisitance, migration and invasion of breast cancer (Brown et al., 2004; Hu et al., 2009; Li et al., 2011; Zhao et al., 2011). However, the ability of MTDH knockdown by microRNA (MTDH-miRNA) to targeted breast cancer therapies, which have come to symbolize the future of breast cancer therapeutics, has not yet been explored.

RNA interference (RNAi) strategies include small interfering RNA (siRNA), short hairpin RNA (shRNA), and microRNA (miRNA), due to its specificity ,adaptability and breadth of targeting capability, has great potential to serve as a personalized gene therapy for cancer. MicroRNAs (miRNAs) are 22 nt small RNAs that function as post transcriptional negative regulators of gene expression , are useful for the design of new therapies against cancer polymorphism (Singh et al., 2001; Wang et al., 2011). In comparison, Unlike siRNA and shRNA ,which requier a perfect match with the target mRNA, miRNA typically exerts translational inhibition by binding to partially complementary mRNA (most effectively to multiple miRNA binding sites in the 3'-UTR), so it could affect hundreds of different genes since a perfect match is not a prerquisite for miRNA to function effectively (Wang et al., 2011). And for shRNA ,the intracellular trafficking is a noteworthy barrier as the plasmid has to traverse through the gel-like cytoplasm in order to translocate to the nucleus, while miRNA is generated in the nucleus and transported to the cytoplasm as mature (Singh et al., 2001). When comparing shRNA versus miRNA-mediated silencing, the latter seems to confer more efficient silencing with less toxic effects, and ues of miRNA backbone makes shRNA less cytotoxic (McBride et al., 2008; Boudreau et al., 2009; Singh et al., 2011). In addition, it was demonstrated that more than 50% of miRNA genes are located in fragile sites and cancer-associated genomic regions (Calin et al., 2004). To conclude, these results suggested that miRNA is a potent, non-toxic silencing vehicle to abolish the expression of the mutated gene with or without allelic discrimination, therefore it plays an important role in the pathogenesis of human cancers.

In the current study, we conducted proliferation assay as well as cell motility and migration assay to determine the role and possible mechanism of MTHD in the infiltrative growth of the breast cancer cells (MDA-MB-231 cells), with the assistant of RNA interference (RNAi) method mediated by recombinant plasmids expressing the artificial miRNA for the silencing MTHD, shows the potential of miRNA mimetics in RNAi applications for the treatment of dominantly metastatic breast cancer.

Materials and Methods

Cell culture

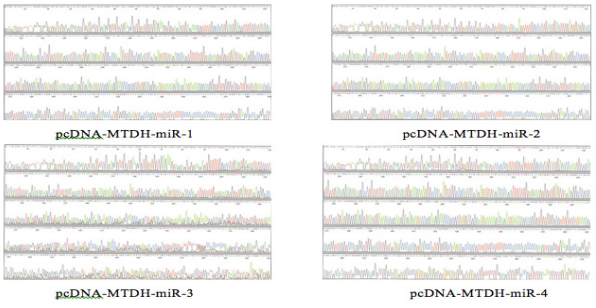
The human breast cancer cell line MDA-MB-231 was purchased from the Institute of Auragene Bioscience (Changsha, P.R. China) and cultured in DMEM supplemented with 10% fetal bovine serum (containing 100 U/ml pf penicillin and streptomycin, respectively) at 37 °C in a humidified atmosphere of 5% CO_2. Cells at logarithmic phase were were used for the experiment.

Construction of expressing plasmids and screening of target sites

Four precursor microRNAs (pre-miRNA) sequences targeting to MTDH (GenBank accession number 92140) were designed using BLOCK-iTTM RNAi Designer (https://rnaidesigner.invitrogen.com/rnaiexpress/setOption.do?designOption=mirna). The pre-miRNA sequencing primers were showed in Figure 1A. Double-stranded oligonucleotides encoding pre-miRNA sequence were annealed and inserted into pcDNATM6.2-GW/EmGFP-miR expression vector (Invitrogen) to construct recombinant plasmid pcDNA-MTDH-miR -1, -2, -3, and -4, respectively. pcDNA-MTDH-miR-neg, including no insert targeting to MTDH, was constructed as a control. Then, DH5α-competent E.coli cells were transformed. To

Oligo name	Oligomeric single-stranded DNA sequence 5'to 3 '
micRNA-1F	TGCTGAGAAGTGGTAGACTGAGAAACGTTTTG
	GCCACTGACTGACGTTTCTCACTACCACTTCT
micRNA -1R	CCTGAGAAGTGGTAGTGAGAAACGTCAGTCAG
	TGGCCAAAACGTTTCTCAGTCTACCACTTCTC
micRNA -2F	TGCTGTGCTGGTGCATTCCAATCAGAGTTTTGG
	CCACTGACTGACTCTGATTGATGCACCAGCA
micRNA -2R	CCTGTGCTGGTGCATCAATCAGAGTCAGTCAG
	TGGCCAAAACTCTGATTGGAATGCACCAGCAC
micRNA -3F	TGCTGAGAAGTAGCAGGTGGAAGAGTGTTTT
	GGCCACTGACTGACACTCTTCCCTGCTACTTCT
micRNA -3R	CCTGAGAAGTAGCAGGGAAGAGTGTCAGTCA
	GTGGCCAAAACACTCTTCCACCTGCTACTTCTC
micRNA -4F	TGCTGTCAACAGTCCGCCCATTTGGTGTTTTGG
	CCACTGACTGACACCAAATGCGGACTGTTGA
micRNA -4R	CCTGTCAACAGTCCGCATTTGGTGTCAGTCAGT
	GGCCAAAACACCAAATGGGCGGACTGTTGAC
	Negative control
Negative-F	tgctgAAATGTACTGCGCGTGGAGACGTTTTGGCC
	ACTGACTGACGTCTCCACGCAGTACATTT
Negative-R	cctgAAATGTACTGCGTGGAGACGTCAGTCAGTG

(A) Pre-miRNA Sequences



(B) pcDNA-MTDH-miRs

Figure 1. Construction of Recombinant Plasmids Containing the miRNA Insert Fragments Against MTDH. (A) Predicted secondary structure of the pre-miRNA targeting MTDH (-1, -2, -3, and -4) and a control pre-miRNA (Neg). (B) The DNA sequencing results showed that the inserts were correct, and no mutant was found in the recombinants

verify the recombinants, the recombinant plasmids were exracted for sequence detection.

Using the instructions for Lipofectamine 2000 (Invitrogen), we transiently transfected the recombinant plasmids in cultured MDA-MB-231 cells. After 24 h, we observed the transfection effect under a fluorescent microscope. After 48 h, real-time RT-PCR and Western blot testing were used to identify the target site with the highest interfering efficiency.

Establishment of MDA-MB-231 cells with stable expression of pcDNA-MTDH-miR-4B

RT-PCR and Western blot results showed that pcDNA-MTDH-miR-4 had the highest efficiency of MDA-MB-231 knockdown among four recombinant plasmids (Figure 2) . So we stable transfected the recombinant plasmids for MDA-MB-231 RNAi with pcDNA-MTDH-miR-4. pcDNA-MTDH-miR-neg and two pcDNA-MTDH-miR-4 (numbered C and D) were transfected into MDA-MB-231 respectively by Lipofectamine 2000 (Invitrogen), and screened for resistance with 1-9 ug/ml of blasticidin (Invitrogen) for 2 weeks ,and stable cell lines after selection were obtained with 4ug/ml of blasticidin. The antibiotic-resistant cell clones were harvested and further screened by dilution titer. To obtain the high expression cell clones, MDA-MB-231 cells, pcDNA-MTDH-miR-neg, pcDNA-MTDH-miR-4A (5^{th} generation of stable transfected cells , numbered A) and pcDNA-MTDH-miR-4B (5^{th} generation of stable transfected cells, numbered D) expression was evaluated by luciferase assay, the cells with strong fluorescence were picked out using a dental explorer under an inverted fluorescence microscope, then maintained and amplified in the selection medium. The selected cells was collected for RT-PCR.

Reverse transcription ploymerase chain reaction

Total RNA was extracted with TRIzol (Invitrogen) ,quantified it spectrophotometrically, and then generate single-stranded cDNA synthesis with a Superscript II kit (Invitrogen) according to the manufacturer's instructions. RT-PCR analysis was performed for amplification of MTDH genes and β-actin gene, β-actin was set as an internal loading control. The primer sequences used in this study were as follows: MTDH, forward, 5'-GGACTGTTGAAGTGGCTGAG-3', reverse, 5'-TCGGTGGTAACTGTGATGGT-3'. β-actin forward, 5'- ATCATGTTTGAGACCTTCAACA -3', reverse, 5'- CATCTCTTGCTCGAAGTCCA -3'. The PCR cycle consisted of 94 °C for 5 minute, 94 °C for 30 seconds, 52 °C for 30 seconds (β-actin was 60 °C for 30 seconds), and 30 cycles of 72 °C for 30 seconds followed by final extension at 72 °C for 8 minutes. Experiments were performed in triplicate in the same reaction.

Western blot

Western blot was performed to detect the protein levels of MTDH in each group of cells . Cells were lysed in RIPA buffer and centrifuged at 12,000-16,000 rpm for 10 minutes. After separation by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen). For MTDH detection, membranes were incubated with anti-MTDH antibodies (1:1000; Abcam, Hong Kong) at 4 °C overnight and with HRP-conjugated goat anti-rabbit IgG for 1h at 37 °C. After washing, the bands were detected by LumiGLO Reagent and Peroxide detection system (Cell Signaling Technology) and β-actin was used as an endogenous protein for normalization. Blots were scanned and analyzed using the AlphaImager 2200 (Alpha Innotech) for the measurement of the band intensities.

Cell proliferation by MTT

Cells were seeded to 96-well microtiter plate at 5×10^4 cells/ml and incubated for 24, 48, 72, 96 hours, respectively. Ten-microliter MTT reagent [3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide, 5 mg/ml inphosphate-buffered saline (PBS); Sigma] was added to each well, and after 4 hours of incubation at 37 °C. The resulting formazan crystals were dissolved in 100 μl 10% SDS-HCl. The optical absorbance at 570 nm was read with a microplate reader (Molecular Devices SpectraMax M2, USA) according to the manufacturer's protocol. Triplicate wells (MDA-MB-231 cells, pcDNA-MTDH-miR-neg group, pcDNA-MTDH-miR-4D group) were analyzed for each experiment and the count values are given as means of triplicate wells.

Cell motility and migration assay

Wound healing assay: Cells were seeded at 5×10^4 cells/ml in six-well tissue culture plates and starved overnight, later wounds were incised by scratching the cell monolayers using pipette tips, keep the scratch width limited to 0.5 cm. Images of the scratch were captured with a microscope immediately after incision (0 hour) and 24 and 48 hours after scratching. The distances between the two edges of the scratched cells in three fields were measured and the average distance was used to calculate the healing rate using the following formula: Healing rate = (the distance before healing – the distance after healing/ the distacne before healing) ×100% .

Transwell migration assay: 1×10^5 cells/ml suspension was added into the upper chamber of the transwell chambers (8 μm pore size, BD, Franklin Lakes, NJ, USA) and 500 μL RPMI 1640 containing 10% newborn calf serum (as a chemokine) was added into the lower chamber of the transwell. The transwell was then cultured at 37 °C in a incubator supplemented with 5% CO_2 24 h later, cells on the upper surface of polycarbonate membrane of the transwell were removed with a cotton swab and the cells that migrated onto the lower surface of the membrane were fixed with 4% paraformaldehyde for 15 min, washed three times with PBS for 5 min each and stained with crystallization violet for 3 min. After further wash with PBS, the membrane was air dried and cell number on the membrane was counted under microscope at 400 magnification. The number of migrated cells was expressed as the average of five randomly selected fields.

Statistical ananlysis

Each experiment was performed at least three times, and data were shown as the mean ± standard where

applicable, and analyzed using SPSS13.0 statistical software package. Statistical analyses of the data were performed using ANOVA followed by post-hoc Dunnett's t-tests. Values. A P value less than 0.05 was considered as statistically significant.

Results

Recombinant plasmid pcDNA-MTDH-miRs was verified by DNA sequencing

Identification of specific and efficient miRNA sequence against MTDH. The DNA sequencing results identified that the inserts of four pre-miRNA sequences targeting to MTDH were correct and no mutant was found in the recombinant plasmid expression vectors pcDNA-MTDH-miRs (Figure 1B).

Transient transfection of the recombinant plasmids and transient knockdown of MTDH expression in MDA-MB-231 cells

Successful transient transfection of recombinant plasmid pcDNA-MTDH-miRs into MDA-MB-231 cell line was confirmed by detecting EmGFP expression using fluorescence microscope. The transfection efficiencies of the four plasmids were measured using flow cytometry at 48 hours after transfection. The results showed that transfection rate of pcDNA-MTDH-miRs into MDA-MB-231 cells was 80% (Figure 2A). To determine the efficiency of MTDH-miRNA-mediated transient gene silencing, total protein and mRNA were extracted from plasmid-transfected cells and parental MDA-MB-231 cells for Western blot and RT-PCR assays at 48 hours after transfection, respectively (Figure 2 B, C). The expressions of MTDH were compared among parental, neg-transfected control, and miRNA-knockdown cells. Both RT-PCR and Western blot results showed that pcDNA-MTDH-miR-4 had the highest efficiency of MTDH knockdown among four recombinant plasmids. So we selected the recombinant plasmid pcDNA-MTDH-miR-4 for stable transfection into MDA-MB-231.

Selection and propagation of MDA-MB-231 cells with stable transfection of recombinant pcDNA-MTDH-miR4

After drug selection procedure, we obtain fifth generation of stable transfected MDA-MB-231 cell monoclone with high miRNA expression named pcDNA-MTDH-miR4 –B. The expression of MTDH miRNA-knockdown cells were compared among parental and neg-transfected control cells. The fluorescence microscopy results indicated the expression of EmGFP in almost all cells transfected with miRNA-expressing plasmid. Flow cytometry assay showed the pcDNA-MTDH-miR4–B and neg-transfected control plasmid transfection rate was more than 90% (Figure 3A). RT-PCR were employed to assess the knockdown efficiency of the cells, selected and propagated pcDNA-MTDH-miR4–B-infected cells exhibited a dramatically high efficiency of MTDH expression knockdown compared with the neg-transfected control cells and MDA-MB-231 cells, while there was no significanct difference in MTDH expression levels between MDA-MB-231 cells and neg-transfected

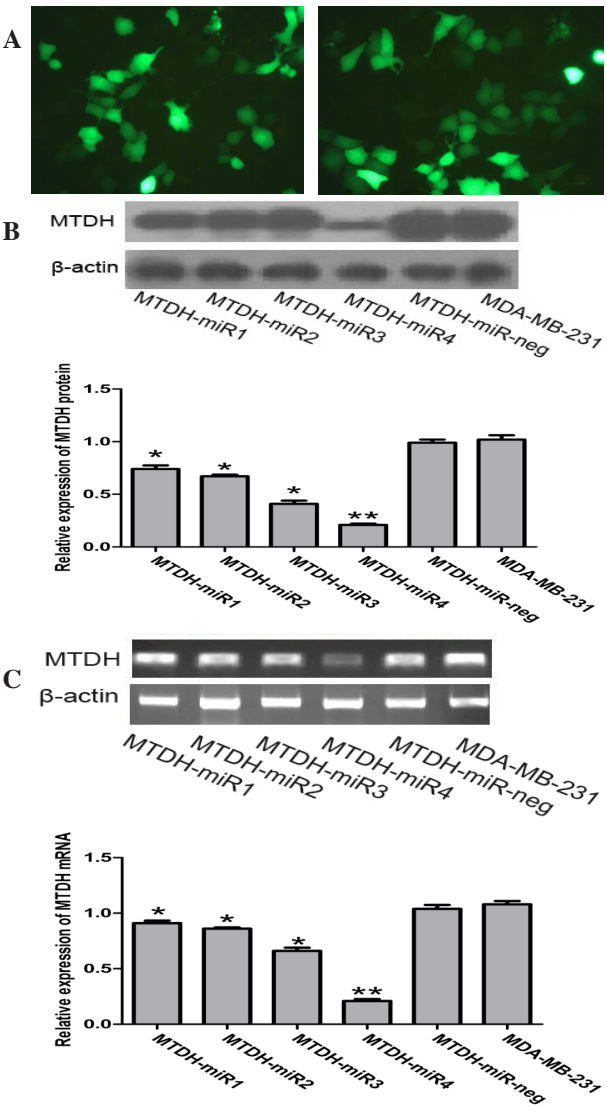


Figure 2. Impact of Recombinant Plasmid Expression Vectors pcDNA-MTDH-miRs on MTDH Expression Levels. (A) MDA-MB-231 cells successfully transient transfected with recombinant pcDNA-MTDH-miRs expressed EmGFP (Fluorescence microscopy), transfection rate was 80%. Western blot and RT-PCR results showed MTDH expression was significant reduced in MDA-MB-231 cells transfected with pcDNA-MTDH-miR4. (B) Western blot results showed MTDH protein levels were down-regulated by 19.35%, 13.46%, 37.85%, and 69.23%, respectively, in MDA-MB-231 cells transfected with pcDNA-MTDH-miR1, pcDNA-MTDH-miR2, pcDNA-MTDH-miR3, and pcDNA-MTDH-miR4. (C) RT-PCR results showed MTDH mRNA levels were down-regulated by 6.94%, 11.27%, 30.43%, and 69.39%, respectively, in MDA-MB-231 cells transfected with pcDNA-MTDH-miR1, pcDNA-MTDH-miR2, pcDNA-MTDH-miR3, and pcDNA-MTDH-miR4

control cells (Figure 3B). So we selected and propagated the MDA-MB-231 cells with stable transfection of recombinant pcDNA-MTDH-miR4 –B for further experiments.

Knockdown of MTDH gene decreased the proliferation of MDA-MB-231 cells

To further investigate the potential functions of MTDH-miRNA, we assessed the proliferation of each group of cells with MTT assay. We generated two stable transfection cells, which expressed an empty vector

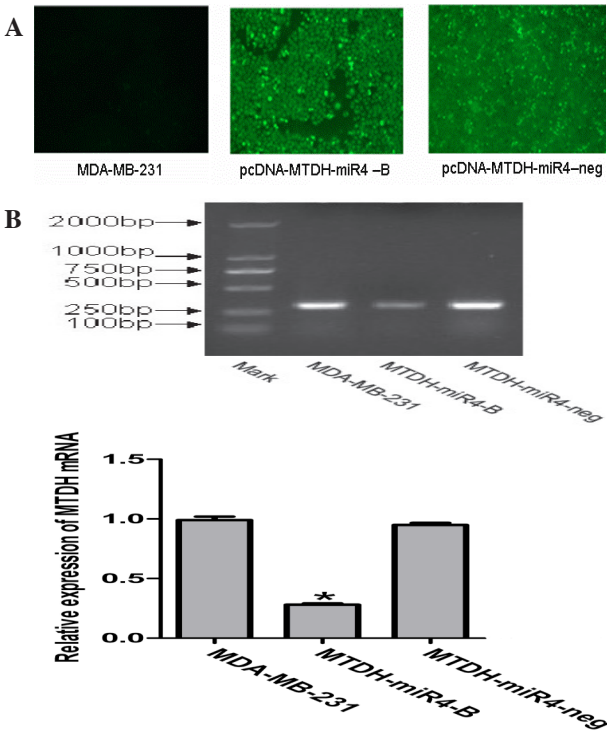


Figure 3. Transfection of Recombinant Plasmids pcDNA-MTDH-miR-4 into MDA-MB-231 Cells and Obtain Fifth Generation of Stable MTDH Silencing Cell. (A) Stable MTDH silencing cell line transfection of recombinant pcDNA-MTDH-miR4–B and miR4–neg control were shown by fluorescence microscopy with high EmGFP expression. (B) RT-PCR demonstrated the highest down-regulation of MTDH mRNA in MDA-MB-231 cells transfected by pcDNA-MTDH-miR4–B compared to the MDA-MB-231 cells (the control group), MTDH-miR4–neg cells (the negative control group)

(pcDNA-MTDH-miR4-neg), MTDH miRNA (pcDNA-MTDH-miR4-B). MDA-MB-231 cells were transduced with these two recombinant plasmid, and the total number of cells remaining in culture were assessed with MTT assay every 24 hours. pcDNA-MTDH-miR4-B -transduced cells exhibited significantly decreased in proliferation, whereas the proliferation of pcDNA-MTDH-miR4-neg -transduced cells was unaffected when compared to the parental MDA-MB-231 cells. The growth curve of pcDNA-MTDH-miR4-B -transduced cells was much gentle. MTDH-miRNA can greatly diminish the proliferation rate of the MDA-MB-231 cells.

Knockdown of MTDH gene could significantly decrease the motility and migration of MDA-MB-231 cells in vitro

Impact of MTDH knockdown on the motility and migration of MDA-MB-231 cells was ananlyzed by wound healing and transwell migration assay in vitro. Wound healing assay: Factorial design analysis of variance showed that knockdown of MTDH by transfection of pcDNA-MTDH-miR4-B significantly attenuated the mean healing rate of MDA-MB-231cells from 41.2% to 62.6%, compared with the parental MDA-MB-231 cells, and the healing rates at 24h , 48h. By contrast, the healing rate of pcDNA-MTDH-miR4-neg-transduced cells was not affected when compared to the parental MDA-MB-231 cells. Transwell migration assay: pcDNA-MTDH-miR4–B-infected cells significantly decreased migration compared with parental MDA-MB-231 cells. By contrast, transfection of pcDNA-MTDH-miR4-neg did not affect MDA-MB-231 cell migration. In conclusion, the cells in knockdown group had significantly decreased motility and migration potency compared with the cells in control groups.

Discussion

MTDH is located at chromosome 8q22, it was found to encode a single-pass transmembrane protein with a calculated molecular mass of 64 KDa, containing 12 exons and 11 introns with a full-length of 86,082 bp, and a cDNA of 3611 bp (excluding the poly-A tail). It is known to be a potent mediator in the development of malignancies and component of oncogenic signaling pathways,such as NF-kappaB, Ha-ras, PI3K/AKT, MAPK, WNT pathways. For example, previous studies showed that MTDH knockdown induced prostate cancer cell apoptosis through upregulation of forkhead box (FOXO) 3a activity, and depletion in the expression of NF-kappaB and AKT signaling pathway (Kikuno et al., 2007). Moreover, the expression of MTDH increases in malignant glioma and MTDH regulates in vitro invasion and migration of malignant glioma cells by activating the nuclear NF-kappaB signaling pathway (Sarkar et al., 2008). In addition, MTDH was also found to activate Wnt/beta-catenin signaling pathway via ERK42/44 activation and upregulated lymphoid-enhancing factor 1/T cell factor 1 (LEF1/TCF1), the ultimate executor of the Wnt pathway, important for Hepatocellular carcinoma progression (Yoo et al., 2009). Further more, Microarray analysis of breast cancer cells revealed that MTDH knockdown led to decreased expression of chemoresistance genes ALDH3A1, MET, HSP90, AND HMOX1, and increased expression of pro-apoptotic genes BNIP3 and TRAIL (Hu et al., 2009). Following these initial dentification, MTDH was thought to be a potential focus for targeted therapy based on its multi-facet roles in regulating cancer apoptosis, invasion, metastasis, and chemoresistance.

Previous article demonstrated several possible avenues to develop breast cancer treatments on the basis of molecular targeting of MTDH. Brown et al. (2004) showed that anti-MTDH (Polyclonal antibodies) inhibited lung metastasis by about 40% compared to 4T1 cells treated with rabbit lgG, but did not observe any difference between the growth of mammary fat pad tumors formed from 4T1 cells pretreated with the anti-MTDH or rabbit lgG; Similarly, they suggested MTDH-reactive siRNA formed about 80% fewer experimental lung metastases than cells expressing scrambled-siRNA, but did not affect 4T1 cells viability and cell growth. And Hu et al. (2009) revealed that MTDH knockdown by MTDH-shRNA ruduced the lung metastasis burden of LM2 (an MDA-MB-231 subline) by 3- to 5-fold ,but did not affect the growth, migration, or invasiveness of various breast cance cell lines, including MDA-MB-231 sublines, MCF7, and T47D. Conversely, Li J and coworks (Lee et al., 2008) reported that ectopic expression of MTDH in MCF-7 and MDA-MB-435 breast cancer cells dramatically

enhanced cell proliferation and their ability of anchorage-independent growth, whereas silencing endogenous MTDH with shRNAs inhibited cell proliferation and colony-forming ability of the cells on soft agar. Our results were consistent with theirs, we conducted proliferation assay , wound healing assay, and transwell migration assay, demonstrated that MTDH-miRNA could not only decrease motility and migration of MDA-MB-231 cells, but also to inhibit the proliferation of MDA-MB-231cells.

These powerful effect were associated with a strong decrease in the expression of MTDH in cells induced by artificial miRNA. In comparison, MTDH protein expression in MTDH-siRNA cells was reduced by about 40% relative to the scrambled-siRNA cells ,and MTDH-siRNA cells expressed about 40% less MTDH mRNA than the scrambled-siRNA cells (Brown et al., 2004), while our data showed that infection with the recombinant plasmids expressing pcDNA-MTDH-miR-4 dramatically reduced the MTDH protein and mRNA expression by about 69% relative to the MDA-MB-231cells (Figure 2 and 3). Although we have not demonstrated the mechanisms underlined the inhibition of MTDH gene silencing, it has been evidenced that knockdown of MTDH could downregulate the transcriptional activity of FOXO1 by inducing its phosphorylation through the PI3K/Akt signaling pathway (Li et al., 2009), and MTDH is an oncogene cooperating with Ha-ras as well as functioning as a downstream target gene of Ha-ras and may perform a central role in Ha-ras-mediated carcinogenesis (Lee wt al., 2008).

Taken together, our recent studies have established a functional role of MTDH in several crucial aspects of breast cancer progression, including motility, migration and proliferation. Furthermore, we suggest that miRNA might be a powerful small molecule inhibitors can be used to block the intracellular function of MTDH. Therefore, further experiment in vivo is urgently needed to realize MTDH-miRNA breast cancer therapeutic potential.

Acknowledgements

This work was supported by grants from the Health Department of Hunan Province (No. B2009-061 and No. B2010-054) and the Foundation of HengYang Science and Technology Bureau (No. 2011KS34) .

References

Brown DM, Ruoslahti E (2004). Metadherin, a cell surface protein in breast tumors that mediates lung metastasis. *Cancer Cell*, **5**, 365-74.

Boudreau RL, Martins I, Davidson BL et al (2009). Artificial microRNAs as siRNA shuttles: improved safety as compared to shRNAs in vitro and in vivo. *Mol Ther*, **17**, 169-75.

Calin GA, Sevignani C, Dumitru CD, et al (2004). Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci U S A*, **101**, 2999-3004.

Chen D, Yoo BK, Santhekadur PK, et al (2011). Insulin-like growth factor-binding protein-7 functions as a potential tumor suppressor in hepatocellular carcinoma. *Clin Cancer Res*, **17**, 6693-701.

Hui AB, Bruce JP, Alajez NM, et al (2011). Significance of dysregulated metadherin and microRNA-375 in head and neck cancer. *Clin Cancer Res*, **17**, 7539-50.

Hu G, Chong RA, Yang Q, et al (2009). MTDH activation by 8q22 genomic gain promotes chemoresistance and metastasis of poor-prognosis breast cancer. *Cancer Cell*, **15**, 9-20.

Jemal A, Bray F, Center MM, et al (2011). Global cancer statistics. *CA Cancer J Clin*, **61**, 134.

Jian-bo X, Hui W, Yu-long H, et al (2011). Astrocyte-elevated gene-1 overexpression is associated with poor prognosis in gastric cancer. *Med Oncol*, **28**, 455-62.

Kang DC, Su ZZ, Sarkar D, et al (2005). Cloning and characterization of HIV-1-inducible astrocyte elevated gene-1, AEG-1. *Gene,* **353**, 8-15.

Kikuno N, Shiina H, Urakami S, et al (2007). Knockdown of astrocyte-elevated gene-1 inhibits prostate cancer progression through upregulation of FOXO3a activity. *Oncogene*, **26**, 7647-55.

Lee SG, Su ZZ, Emdad L, et al (2008). Astrocyte elevated gene-1 activates cell survival pathways through PI3K-Akt signaling. *Oncogene*, **27**, 1114-21.

Li C, Liu J, Lu R, et al (2011). AEG -1 overexpression: a novel indicator for peritoneal dissemination and lymph node metastasis in epithelial ovarian cancers. *Int J Gynecol Cancer*, **21**, 602-8.

Li J, Yang L, Song L, et al (2009). Astrocyte elevated gene-1 is a proliferation promoter in breast cancer via suppressing transcriptional factor FOXO1. *Oncogene*, **28**, 3188-96.

Li J, Zhang N, Song LB, et al (2008). Astrocyte elevated gene-1 is a novel prognostic marker for breast cancer progression and overall patient survival. *Clin Cancer Res*, **14**, 3319-26.

Li X, Kong X, Huo Q, et al (2011). Metadherin enhances the invasiveness of breast cancer cells by inducing epithelial to mesenchymal transition. *Cancer Sci*, **102**, 1151-7.

McBride JL, Boudreau RL, Harper SQ, et al (2008). Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: implications for the therapeutic development of RNAi. *Proc Natl Acad Sci U S A*, **105**, 5868-73.

Nohata N, Hanazawa T, Kikkawa N, et al (2011). Tumor suppressive microRNA-375 regulates oncogene AEG-1/MTDH in head and neck squamous cell carcinoma (HNSCC). *J Hum Genet*, **56**, 595-601.

Sarkar D, Park ES, Emdad L, et al (2008). Molecular basis of nuclear factor-kappaB activation by astrocyte elevated gene-1. *Cancer Res*, **68**, 1478-84.

SinghS, NarangAS, MahatoRI (2011). Subcellular fate and off-target effects of siRNA, shRNA, and miRNA. *Pharm Res*, **28**, 2996-3015.

Song H, Li C, Li R, Geng J (2010). Prognostic significance of AEG-1 expression in colorectal carcinoma. *Int J Colorectal Dis*, **25**, 1201-9.

Song H, Li C, Lu R, Zhang Y, Geng J (2010). Expression of astrocyte elevated gene-1: a novel marker of the pathogenesis, progression, and poor prognosis for endometrial cancer. *Int J Gynecol Cancer*, **20**, 1188-96.

Wang Z, Rao DD, Senzer N, Nemunaitis J (2011). RNA interference and cancer therapy. *Pharm Res*, **28**, 2983-95.

Yoo BK, Emdad L, Su ZZ, et al (2009). Astrocyte elevated gene-1 regulates hepatocellular carcinoma development and progression. *J Clin Invest*, **119**, 465-77.

Zhao Y, Kong X, Li X, et al (2011). Metadherin mediates lipopolysaccharide-induced migration and invasion of breast cancer cells. *PLoS One*, **6**, e29363.