# Original Article Downregulation of metadherin inhibits the invasiveness of diffuse large B cell lymphoma

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Received February 2, 2016; Accepted April 26, 2016; Epub August 1, 2016; Published August 15, 2016

**Abstract:** Metadherin (MTDH), also known as astrocyte elevated gene-1 (AEG-1) or lysine-rich CEACAM1 co-isolated (LYRIC), a novel oncogene, is involved in the development and progression of malignant diseases, such as breast cancer, prostate cancer, non-small cell lung cancer and so on. MTDH has been found highly expressed and promoted the pathogenesis of diffuse large B cell lymphoma (DLBCL) mediated by activation of Wnt/β-catenin pathway. Our previous study has found that MTDH was correlated with DLBCL. We assume that MTDH could not only enhance cell proliferation and inhibit cell apoptosis, but also could regulate the invasion and migration in DLBCL. This study aimed to illuminate that MTDH could promote the invasion and migration of DLBCL. We found that the protein expression of MTDH was significantly upregulated in DLBCL cell lines (LY1 and LY8 cell lines) compared with peripheral blood mononuclear cells (PBMCs) from healthy samples. Specific small interfering RNA (siRNA) mediated downregulation of MTDH resulted in reducing abilities of migratory and invasion of LY8 cell line by using transwell assay. Moreover, we confirmed and extended the expression level of MTDH correlated with two epithelial-mesenchymal transition markers. Downregulation of MTDH expression in LY8 cells resulted in induction of the expression of ZEB1 and vimentin. These results indicated that there might be an EMT-like process regulated by MTDH in DLBCL.

Keywords: Diffuse large B cell lymphoma, epithelial-mesenchymal transition, metadherin, invasion, migration

#### Introduction

Diffuse large B cell lymphoma (DLBCL) is one of the highest incidences of non-Hodgkin's lymphoma (NHL) in the world. As a highly malignant B cell-derived tumor, DLBCL is an aggressive disease with heterogeneity. This heterogeneity is characterized by its different clinical manifestations, histological features, genetic abnormalities, response to therapy, as well as prognosis [1-3]. As an aggressive disease, the extranodal involvement is not rare in DLBCL patients. The most frequently affected sites are stomach, Waldeyer's ring, bone, liver, salivary gland, adrenal, kidney and thyroid [4]. Recently, remarkable progresses have been made in exploring the pathogenesis of DLBCL and finding the effective therapies. Although the survival outcomes of the majority of DLBCL patients can be significantly improved by using the combination of anti-lymphoma monoclonal antibody (rituximab) with chemotherapy, unfortunately, there are still a large number of DLBCL patients with a poor prognosis [5-7]. About 40% DLBCL patients die in relapsed or refractory disease [8].

Epithelial to mesenchymal transition (EMT) is an essential process in progression to malignancy [9-11]. In the process of EMT, cancer cells lose the adhesive ability along with acquiring the properties of invasion and migration. It was shown that tumor cells lost epithelial phenotype markers for example E-cadherin and acquired mesenchymal markers such as cytoskeletal proteins: vimentin and  $\beta$ -catenin, with the upregulation of nuclear expression of several transcription factors (ZincfingerE-boxbindingprotein1/ZEB1, Snail, Twis, et al) [12, 13]. EMT plays a vital role in promotinginvasion and migration of tumor cells, and pertains to tumor development [14-16].

Metadherin (MTDH), also known as astrocyte elevated gene-1 (AEG-1) or lysine-rich CEACAM1

co-isolated (LYRIC), is a novel oncogene, located at chromosome 8q22 [17]. It is associated with tumor cell progression, such as cell proliferation, apoptosis, invasion and metastasis [18]. Several studies have indicated that MTDH was highly expressed in a host of human malignancies, such as breast cancer, lymphoma and urologic neoplasms so on [19-23]. Abundant experimental findings indicate that MTDH could regulate EMT in avariety of malignant diseases [24-26]. We have previously shown that MTDH was overexpressed in DLBCL and demonstrated that MTDH could regulate the Wnt/ $\beta$ -catenin signal pathway to promote the development of DLBCL [21]. In this study, we hypothesized that MTDH might play a role in cells invasion and migration in DLBCL. In addition, we also investigated that the expression of MTDH was inhibited by small interfering RNA (siRNA) along with the decrease of ZEB1 and vimentin expression. This suggested there might be an EMT-like process in DLBCL.

# Materials and methods

# Patients and samples

Paraffin-embedded samples, including thirty cases of DLBCL and fifteen lymph node tissues with reactive lymphoid hyperplasia, were collected from the Affiliated Hospital of Qingdao University. All of the patients were histologically confirmed and newly diagnosed according to the WHO criteria between 2012 and 2014. This study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University, and prior consents were obtained from all the patients involved.

# Cells lines and cell culture

LY1 and LY8 cell lines, Human DLBCL cell lines, were cultured in Iscove's modified Dulbecco's medium (IMDM; Gibco). Both of them were supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA). All cell lines were cultured at 37°C in a humidified 5%  $CO_2$  incubator.

# Antibodies

Rabbit anti-MTDH-specific polyclonal antibody was purchased from Proteintech Group (Chicago, IL). Mouse anti-GAPDH monoclonal antibody was purchased from (Zhongshan Golden Bridge, Beijing, China). Rabbit anti-vimentin antibody was purchased from Abcam (Cambridge, MA). Mouse anti-ZEB1 antibody was purchased from Abcam (Cambridge, MA).

### Immunohistochemistry

To examine whether the MTDH protein is overexpressed in DLBCL tissues, 30 paraffinembedded, archived DLBCL tissues and 15 reactive hyperplasia of lymphoidtissues were analyzed by immunohistochemistry with an antibody against human MTDH.

Briefly, paraffin-embedded specimens were cut into 3 µm sections and baked at 65°C for 30 min before being deparaffinized in turpentine, and rehydrated in a descending series of alcohols (100, 95, 85 and 75%). High-pressure antigen retrieval was performed using EDTA-Tris solution (pH 9). The slides were cooled at room temperature and then incubated with 3% H\_O\_ for 30 minutes to inhibit endogenous peroxidase, followed by incubation with normal serum for 30 minutes to block non-specific staining. After that, rabbit anti-MTDH (1:200) antibody was incubated with the slides overnight at 4°C in a humidified chamber. After washed with phosphate-buffered saline (PBS), the tissue sections were treated with biotinylated antirabbit secondary antibody (ZhongshanGolden-Bridge Biotechnology Company, Beijing, China), followed by further incubation with streptavidin-horseradish peroxidase complex. Between all steps of the immunostaining procedures, the slides should be washed with PBS, except incubating primary antibody. Tissue sections were then stained with diaminobenzidine Kit (DAB, ZhongshanGoldenBridge Biotechnology Company, Beijing, China) and counterstained with hematoxylin, dehydrated and mounted. In negative controls, the primary antibody was replaced with PBS, and immunostaining of samples and negative controls occurred simultaneously.

The immunohistochemically stained slides were evaluated and scored separately by two independent pathologists blind to all clinical data. Five high-power fields (at 10x40 magnification) were assessed randomly, and MTDH expression was determined by a combination of the percentages of nuclear and cytoplasmic staining and the intensity of staining in tumor cells [27]. The percentages of staining were scored as 1 (<10%), 2 (10-34%), 3 (35-70%), 4

Table	1. Prime	er Sequences
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Name	Sequence
MTDH-F	5'-TTACCACCGAGCAACTTACAAC-3'
MTDH-R	5'-ATTCCAGCCTCCTCCATTGAC -3'
Vimentin-F	5'-CTCTTCCAAACTTTTCCTCCC-3'
Vimentin-R	5'-AGTTTCGTTGATAACCTGTCC-3'
ZEB1-F	5'-GATGATGAATCGAGTCAGATC-3'
ZEB1-R	5'-ACAGCAGTGTCTTGTTGTTGT-3'
β-actin-F	5'-TGGCACCCAGCACAATGAA-3'
β-actin-R	5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'

(>70%), and the intensity of staining was evaluated with grading from 0 to 3 (0, negative; 1, weak; 2, moderate; and 3, strong). The intensity and percentage scores were multiplied to generate the final staining score ranged from 0 to 12, and the tumors were categorized into two groups based on this score: the low expression group (scores 0-5) and the high expression group (scores 6-12).

#### Downregulation of human MTDH by RNA interference (RNAi)

MTDH siRNA lentivirus and negative control siRNA lentivirus were synthesized by GeneChem (Shanghai, China). The sequence of MTDH siRNA was 5'-AACAGAAGAAGAAGAACCGGA-3' according to the previous studies [21]. To decrease MTDH expression, MTDH siRNA lentivirus was transfected into LY8 cells with the multiplicity of infection (MOI) 150 according to the manufacturer's instructions. To determine infection efficiency of the MTDH siRNA lentivirus transfection, we used fluorescence microscopy and flmi cytometry on the third day after transfection. Stable cell lines which were tranfected with MTDH siRNA lentivirus were selected for 3 days with 5  $\mu$ g/ml puromycin 72 hours later after transfection. Then we collected stable transfected MTDH siRNA cells in exponential growth period to extract protein. MTDH mRNA and protein levels were analyzed by realtime PCR and western blotting, respectively.

#### RNA extraction, reverse transcription and realtime PCR

Total RNA was isolated from cells using the RNAiso Plus reagent (Takara, Dalian, China) and reverse transcribed to complementary DNA (cDNA) with PrimeScript RT reagent kit with gDNA eraser (Takara, Dalian, China) according to the manufacturer's instructions. Real-time quantitative polymerase chain reaction (PCR) analysis was performed on a LightCycler480 real-time PCR instrument (Roche Diagnostics, Mannheim, Germany) by using a SYBR Premix Ex Taq II kit (Takara, Dalian, China). Each reaction was performed in triplicate. The  $2^{-\Delta\Delta CT}$  method with Light Cycler-480 Gene Scanning Version 1.5 Software (Roche Diagnostics) was used to analyzed data. The primers used in this study are listed in the supplementary **Table 1**.

#### Transwell invasion and migration assay

Invasion experiment in transwell inserts (3422, Corning, Acton, MA, USA) with an 8 µm pore size coated with matrigel (BD Biosciences, Bedford, MA, USA) in a 24-well plate. After dry and rehydration, the upper chambers were plated 4×10^5 cells per well in IMDM medium without FBS and the lower well were added to 600 µI IMDM medium with 10% FBS. After 24 hours of culturing at 37°C in a humidified 5% CO incubator, cells that had invaded to the bottom surface were fixed by 4% paraformaldehyde solution and counted after staining with hematoxylin. The cells invaded to the bottom surface were counted under a light microscope (200x) in at least 5 random visual fields. The migration assay was using a similar approach to the invasion assay, except that the transwell inserts were not coated with the matrigel. For this assay, 2×10^5 cells per well were seeded into the upper chambers. Cells that had migrated to the low chambers were manually counted with a hemocytometer after 24 hours of incubation. Results were analyzed by the Student t test. Each experiment was performed in tripartite.

# Protein extraction and western blot analyses

Peripheral blood mononuclear cells (PBMCs) were extracted from peripheral blood of healthy samples by using lymphocyte separation medium (TBD science, Tianjin, China). The protocol was approved by the Shandong Provincial Hospital Ethics Committee and obtained informed consent from all participants. Cellular protein was extracted from LY1, LY8 cells and PBMCs using RIPA lysis buffer and 1% PMSF incubation on ice for 30 minutes. Then the cell lysate was centrifugated at 12000 g for 30 minutes at 4°C. The total protein concentrations were analyzed by using the BCA assay kit



**Figure 1.** MTDH is overexpressed in DLBCL samples and DLBCL cell lines. A. MTDH negative expression in reactive hyperplasia of lymph node specimens (x400). B. MTDH low expression in DLBCL (x400). C. MTDH high expression in DLBCL (x400). D. The protein expression of MTDH, Vimentin and ZEB1 in LY8 cells and PBMC.

(ShenergyBiocolor). Equal amount (40 µg) of total protein was immunoblotted and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After incubated with 5% nonfat dry milk in Tris-buffered saline Tween-20 (TBST) for 1 hour, PVDF membranes onto which transferred were incubated with antibodies against MTDH, vimentin, ZEB1 and GAPDH overnight at 4°C. Antibody against MTDH, vimentin and GAPDH were respectively in recommended dilutions of ratio of 1:1000 dissolved inprimary antibody dilution buffer (Beyotime, China). Antibody against ZEB1 was used at 1:2000 dilution. Anti-rabbit and antimouse antibodies (HRP; ZSGB-BIO) were incubated in recommended dilutions of 1: 10000 in secondary antibody dilution buffer (Beyotime, China). All experiments were performed in triplicate. The expression level of GAPDH presented as normalized control for the relative density of protein bands. System LAS4000 mini (Fuji fi C, Tokyo, Japan) was used to observe the membranes incubated with chemiluminescent HRP substrate (Millipore). Then data was analyzed by Multi Gauge Version 3.0 software (Fuji film).

#### Statistical analysis

All statistical analyses were performed using the statistics software SPSS 20.0 for Windows (version 20.0, SPSS, Chicago, IL, USA). Fisher's exact test was used to evaluate the relationship between the MTDH expression level and clinicopathological parameters. Mean  $\pm$  standard deviation (SD) was used to express the protein level of MTDH, vimentin and ZEB1. Statistical significance was defined as \*, *P*<0.05 and \*\*, *P*<0.001 in a two-tailed test.

#### Results

#### Overexpression of MTDH in DLBCL

In this present study, significantly elevated levels of MTDH were observed in DLBCL lymph node tissues, whereas the protein expression of MTDH was barely detectable in the reactive hyperplasia of lymph node tissues (**Figure 1**). MTDH expression was detected prominently in the cytoplasm and occasionally in the nucleus. In addition, MTDH protein level in DLBCL cell lines (LY1 and LY8 cells), is also higher compared with the PBMCs (*P*<0.05, **Figure 2**).



**Figure 2.** Transfection efficiency of lentiviruses in LY8 cells. A. Light microscopy and fluorescence microscopy images showing cells infected with negative control siRNA (NC) lentiviruses LY8 cells; B. Expression of green fluorescent protein (GFP) in LY8 cells infected with NC lentiviruses as determined by flow cytometry; C. Light microscopy and fluorescence microscopy images showing cells infected with MTDH siRNA (si-MTDH) lentiviruses in LY8 cells; D. Expression of GFP in LY8 cells infected with si-MTDH lentiviruses as determined by flow cytometry.

#### Relationship between MTDH protein expression and the clinicopathological parameters of DLBCL

Chi-squared tests demonstrated that there were significant correlations between MTDH expression and the clinical characteristics of DLBCL. MTDH expression had no association with patient age and gender (P>0.05). Nevertheless, the expression of MTDH ana-

lyzed by immunohistochemical staining was remarkably correlated to the clinical staging and B symptoms of patients with DLBCL (*P*<0.05, **Table 2**). MTDH expression had noassociation with patient age and gender. These results suggest that the overexpression of MTDH is related with the clinical progression of DLBCL.

#### Downregulation MTDH inhibit invasion and migration potential of DLBCL cells

To determine the role of MTDH in DLBCL invasion and migration, we transfected LY8 cells with MTD-H siRNAlentivirus. Stably low-expressed MTDH LY8 cells were screened out by using 5 µg/ml puromycin 48 hours post transfection for 3 days. As shown in Figure 2D, the infection efficiency of the MTDH siRNA was 94.9%. We examine the level of MTDH expression in these cells by Western blotting. Compared with control cells, MTDH protein of stable transfected MTDH siRNA cells was decreased (P< 0.05, Figure 3). Upon revealing that downregulation of MTDH decreased the invasion and migration potential of DLBCL cells, we performed transwell invasion assays and

migration assays using 2-chamber system. Cells migrated and invaded to the IMDM medium with 10% FBS in the wells of 24-well plate. As indicated in **Figure 4**, the relative cell migration ratio of LY8 cells transfected with MTDH siRNA was  $21.67\% \pm 4.410\%$ , whereas the ratio of negative control was  $51.08\% \pm 8.816\%$  (*P*<0.05, **Figure 4**). Similarly, the number of invasive LY8 cells transfected with MTDH siRNA was  $39.33 \pm 7.446$ , while the number of negative control signates and the signates of the signa

Deremetere	MTDH expression			
Parameters	Low (%)	High (%)	P-value	
Age (years)				
<60	6 (50.0%)	6 (50.0%)	0.458	
≥60	6 (33.3%)	12 (66.7%)		
Gender				
Male	8 (50.0%)	8 (50.0%)	0.284	
Female	4 (28.6%)	10 (71.4%)		
Clinical stage				
I	6 (75.0%)	2 (25.0%)	0.04	
II	4 (50.0%)	4 (50.0%)		
III	1 (16.7%)	5 (83.3%)		
IV	1 (12.5%)	7 (87.5%)		
B symptoms				
Yes	0 (0%)	9 (100.0%)	0.004	
No	12 (57.1%)	9 (42.9%)		

**Table 2.** Correlations between MTDH expression and clinicopathological parameters ofDLBCL patients by immunochemistry analysis

# tive control was $153.0 \pm 9.452$ (P<0.001, Figure 4).

# Downregulation of MTDH inhibits EMT-like process

EMT plays an important role in invasion and migration of malignant cells [14], in addition, it is previously reported that MTDH promoted tumor cells invasion and migration by regulation EMT [18]. We therefore investigated whether EMT markers (ZEB1 and vimentin) expressed in LY8 cells. As expected, the protein levels of ZEB1 and vimentin werehigher compared with PBMCs by using Western blotting (P<0.05, Figure 1D). To further confirm whether the role of MTDH expression in invasion and migration was mediated by EMT-like process in DLBCL, we evaluated the protein level of ZEB1 and vimentin in the transfected LY8 cells by using western blotting analysis. As shown in the Figure 3, the protein levels of ZEB-1 and vimentin were downregulated (P < 0.05).

# Discussion

In this study, MTDH protein expression analysis from patient samples and DLBCL cell lines (LY1 and LY8 cells) confirmed that MTDH was overexpressed in DLBCL. Further statistical analysis of the correlation between MTDH and the clinical parameters of DLBCL patients indicated that the overexpression of MTDH correlated with advanced stage and the presence of B symptoms, whereas had no relevance to the age and gender. Interference the expression of MTDH could affect the ability of invasion and migration of DLBCL cells.

DLBCL is an aggressive lymphoma subtype with high invasiveness. Numerous experimental studies and clinical observations have reported that the development and progression of DLBCL is associated with aberrant gene expression. MTDH is a novel oncogene in carcinogenesis. Its abnormal expression is associated with diverse biological behaviors in tumor progression, including modulating cell proliferation and apoptosis, invasion and migration, drug resistance, and so on [18, 28, 29]. Li et al [30] have demonstrated that silencing MTDH expression could suppress cell growth and induces apoptosis in hepatocellular carcinoma (HCC) cells. Liu et al [31] confirmed that MTDH was connected with angiogenesis in breast cancer. Li et al [24] found that knockdown the expression of MTDH inhibited invasive and metastatic potential of HCC through regulation of EMT. And our previous studies had revealed that MTDH was overexpressed in DLBCL, andconfirmed that MTDH worked as an oncogene to promote lymphoma cells proliferation and inhibit their apoptosis through  $Wnt/\beta$ -catenin signal pathway, and enhances chemo-resistance to doxorubicin [21, 32].

Here, we further discovered that MTDH could affect the ability of invasion and migration in DLBCL cells. Interference by siRNA transfection to eliminate MTDH could reduce the invasion and migration ability of DLBCL cells. It is a further validation of MTDH works as an oncogene promoting the development of tumors. In addition, we found that ZEB1 and vimentin were overexpressed in DLBCL cells. This is in line with the phenomenon about overexpression of the transcription factors of EMT in solid tumors which are related with increased invasiveness. However, lymphomas lack features related with epithelial cancers, such as adherens junctions. Considering that we harbor the idea that there might be an EMT-like process in DLBCL.

EMT is an essential process in progression to malignancy. It is involved in the invasion and migration of various cancers, including cervical cancer, squamous cell carcinoma of the head, breast cancer and so on [14, 33, 34]. ZEB1, a transcription factor of EMT, is significant for

#### MTDH downregulation inhibits DLBCL cellular invasiveness



**Figure 3.** MTDH downregulation inhibits the expression of ZEB1 and Vimentin protein in DLBCL cells. Western blot analysis of MTDH, ZEB1 and Vimentin protein in LY8 cells transfected with negative control siRNA lentiviruses (NC) and MTDH siRNA lentiviruses (si-MTDH). Data expressed as mean ± SD. \*P<0.05 versus negative control.



**Figure 4.** MTDH downregulation reduces invasion and migration ability of LY8 cells. A. The ability of invasion and migration of LY8 transfection cells (si-MTDH) compared with negative control cells (NC). \*P<0.05. B. The ability of invasion of LY8 transfection cells (si-MTDH) compared with negative control cells (NC). \*\*P<0.001.

regulating the balance of epithelial and mesenchymal gene expression. The overexpression of ZEB1 has been thought to be an adverse prognostic factor in tumors, such as lung cancer, breast cancer [35, 36]. ZEB1 is a transcription factor that promotes tumor invasion and migration by inducing EMT in tumors [37]. Its expression correlates with loss of E-cadherin and is associated with advanced diseases or metastasis, which indicates the relevance of ZEB1 and tumor progression. Sanchez-Tillo et al [38] found that ZEB1 was overexpressed in mantle

cell lymphoma (MCL) and it promoted cell proliferation, and had a differential reaction to chemotherapy drugs. Lemma et al [39] investigated ZEB1 was highly expressed in DLBCL by using immunohistochemical staining. Vimentin, an intermediate filament protein, has been regarded as the main mesenchymal marker. It was one of the biomarkers of EMT. Upregulation of vimentin during EMT plays an important role in cell adhesion and motility [40-42]. Overexpression of vimentin detected in tumor cells and tissue normally represented the highly proliferation and invasion of malignancies [43, 44]. Maxwell et al [45] reported that vimentin were observed in primary DLBCL tissues. Interference of vimentin expression repressed the invasiveness of resistant cells to the CHOP chemotherapeutic regimen in DLBCL.

Based on the results of the experiment, we found interference of MTDH expression by using siRNA lentivirus in DLBCL cells led to changes of ZEB1 and vimentin, which was in line with data about MTDH could promote invasion and migration through regulating EMT in solid malignancies. Inhibition of the ability of invasion and migration reduces tumor cellactivities, which can be better recognized by the immune system. Our findings further confirmed that MTDH might be an expected therapeutic target for DLBCL. There might be likely present a potential mechanism to change the EMT-like process in DLBCL. These results provide novel theoretical foundation for MTDH function as a potential prognostic biomarker for DLBCL aggressiveness and a valuable therapeutic target for DLBCL.

We indicated that downregulation of MTDH by using small interfering RNA (siRNA) lentivirus in DLBCL cells could result in the reduction of ZEB1 and vimentin expression, accompanied with the decreased invasive and migratory abilities of DLBCL cells. This is in line with our finding that MTDH is associated with invasiveness of DLBCL and mediates the EMT-like process. In our study, the significance of these markers may be generally related to the activation of signaling cascades other than directly with epithelial cells. Protein changes of EMT markers following the decreased expression of MTDH indicate that down-regulation of MTDH could inhibit EMT-like process in LY8 cells.

In brief, this study demonstrated MTDH could modulate the ability of invasion and migration

in DLBCL. And we also find that there is an EMTlike process mediate by MTDH in DLBCL cells. The evidence presented suggests that MTDH extremely likely be a biomarker for DLBCL prognosis and a novel target fortherapy of DLBCL.

# Acknowledgements

This study was partly supported by: National Natural Science Foundation (No. 81473486 and No. 81270598), National Public Health Grand Research Foundation (No. 201202017), Natural Science Foundations of Shandong Province (No. ZR2012HZ003 and No. 2009-ZRB14176), Technology Development Projects of Shandong Province (No. 2014GSF118021, No. 2010GSF10250, and No. 2008GG2NS0-2018), Program of Shandong Medical Leading Talent, and Taishan Scholar Foundation of Shandong Province.

# Disclosure of conflict of interest

#### None.

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