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Original Article

Elevated expression of metadherin in ovarian cancer and its tumor progressive roles in cell proliferation and survival

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Abstract: Objectives: We aimed to examine the expression of metadherin (MTDH) in human ovarian cancers and to evaluate its correlation to clinicopathological parameters. Methods: Its mRNA expression, DNA copy number alternation in ovarian serous cystadenocarcinoma provided by TCGA database were analyzed. The mRNA and protein expression of MTDH were examined by qRT-PCR and western blot, respectively in human ovarian cancer tissues. Additionally, the association between MTDH expression and a series of clinicopathologic features was statistically assessed. After knockdown of metadherin in skov3 cells, the cell proliferation and survival ability were assessed by MTT assay and colony formation assay, respectively. The upstream regulator of MTDH was predicted by using online bioinformatic tools, and downstream molecules regulating cell proliferation were analyzed by western blot. Results: In ovarian serous cystadenocarcinoma provided by TCGA database, we found consistent MTDH mRNA upregulation, and its DNA copy number obviously rised in cancerous samples. MTDH mRNA and protein were both significantly highly expressed in ovarian cancer tissues. We further found positive correlation of MTDH level with FIGO stage and histological grade of ovarian cancer patients. Cellular functional assays confirmed that knockdown of metadherin inhibited cell proliferation and survival. MiR-153 may function as an upstream regulator of MTDH by directly targeting its 3'-UTR. Downstream biomarkers regulating cell proliferation, such as CCND1 and MYC, were also altered consistently with MTDH. Conclusion: Abnormal high level of MTDH was correlated with both FIGO stage and pathological stage. It might function as an oncogene, and be used as an effective therapy target for ovarian cancer.

Keywords: Metadherin, ovarian cancer, clinical significance, cell proliferation and survival

Introduction

Ovarian cancer is the most lethal type of gynecologic malignancy, and the fifth leading cause of cancer-related deaths in females [1]. Because of lacking distinct symptoms, the majority of ovarian cancer patients are usually diagnosed at the advanced stages, left a 5-year survival rate of only 30% or less [2]. Relapse due to metastasis and chemoresistance are two major reasons for its poor prognosis [2]. Also, difficulty in early diagnosis of this disease makes it worse. Despite the encouraging improvements, the pathogenesis of ovarian cancer, especially the high-grade serous ovarian carcinoma, remains largely unknown. Therefore, identifying more biomarkers for the diagnosis and therapy of ovarian cancer is deemed as a great challenge, and will certainly benefit the health of patients.

Human metadherin (MTDH), also known as LYRIC, 3D3 or astrocyte elevated gene-1 (AEG-1), is a single-pass transmembrane protein coding by *MTDH* gene located at chromosome 8q22.5 [3]. It was originally identifed as a neuropathology-associated gene in primary human fetal astrocytes [4]. Gradually along with the increasing knowledge, however, MTDH has recently emerged as a crucial mediator of cancer cell transformation and a key converging point of several oncogenic signaling pathways [5, 6]. For instance, overexpression of MTDH has been observed in lung, breast, and hepatocellular carcinoma and correlated with poor clinical outcomes [7], and it exerts oncogenic

roles in these cancers [8-10]. Although it was known that higher MTDH expression was associated with the progression and prognosis of ovarian cancer [11, 12], the importance of MTDH in genetics of ovarian cancer remains to be further recognized.

Our present study had three main objectives. Firstly, we aimed to determine the expression of MTDH in human ovarian cancer tissues. Secondly, we also intend to analyze the association of MTDH expression with multiple clinicopathologic features, thus to evaluate its clinical significance. Finally, we try to explore the biofunctions of MTDH in ovarian cancer cells and find the upstream regulators and downstream molecules regulating ovarian cancer cell proliferation.

Materials and methods

Patients and tissues

Ovarian carcinoma tissues (OCT) and non-cancerous ovarian tissues (NOT) in this study were collected in Tianjin Hospital from January 2013 to December 2015. None of the patients received radiation therapy or chemotherapy before surgery. The clinical stage of all carcinoma was determined according to the International Federation of Gynecology and Obstetrics (FIGO) standards. The study was approved by the Ethics Committee of Tianjin Hospital. All the fresh samples were obtained at surgery, frozen in liquid nitrogen and stored at -80°C.

RNA extraction and quantitative real-time PCR

RNA was extracted from fresh tissues with Trizol reagents as per the manufacturer's protocol. The cDNA was synthesized with M-MLV Reverse Transcriptase (Promega). Quantitative real-time PCR (qRT-PCR) was performed with the SYBR green Premix Ex Taq II (Takara) with Step One Plus Real-Time PCR System (Applied Biosystems 7900). The expression of GAPDH was used as the endogenous control.

SiRNAs, MiRNAs and transfections

Control siRNAs and MTDH siRNAs were purchased from Santa Cruz. MiR-153 mimics, inhibitors and controls were purchased from Shanghai GenePharma (Shanghai, China). Transfections were performed by using lipofectamine 2000 according to the manufacturer's instructions.

Western blot analysis

Proteins were collected and lysed with RIPA buffer (Beyotime) with protease inhibitors. 30 μg proteins were separated on 9% SDS-PAGE gels and then transferred to PVDF membrane (Millipore). After blocking with 5% non-fat milk, the membrane was incubated overnight at 4°C with the primary MTDH, GAPDH, CCND1 and MYC antibody (Santa Cruz; 1:1000) then with HRP-coupled secondary antibody (Beyotime). Signal was detected with enhanced chemiluminescence (Millipore). Bands were acquired in scanner and analyzed using QUANTITY ONE software (Bio-Rad).

MTT assay

The cell proliferation rate in this study was detected by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, SIGMA) according to the manufacturer's instructions. The assay was performed in 96-well plates with 2×10^3 in each well for four continuous days. The absorbance at 490 nm was detected by a microplate reader.

Colony formation assay

The cell survival was evaluated by the colony formation assay. In briefly, cells were seeded at a density of 2000 cells per well in 6-well plates, and cultured in full medium for 10 days. After that, cells were fixed with a 4% paraformaldehyde solution, followed by staining with 0.5% crystal violet and counted under an inverted microscope.

Online database analysis

The DNA copy number and oncoprints of MDTH gene in ovarian serous cystadenocarcinoma were analyzed by using online TCGA database. The website is as follows: http://www.cbioportal.org/index.do.

Bioinformatic analysis

The information of human miR-153 was obtained in miRBase (http://www.miRBase.org/). The prediction of miR-153 targets was acquired from TargetScan program (http://www.targetscan.org/).

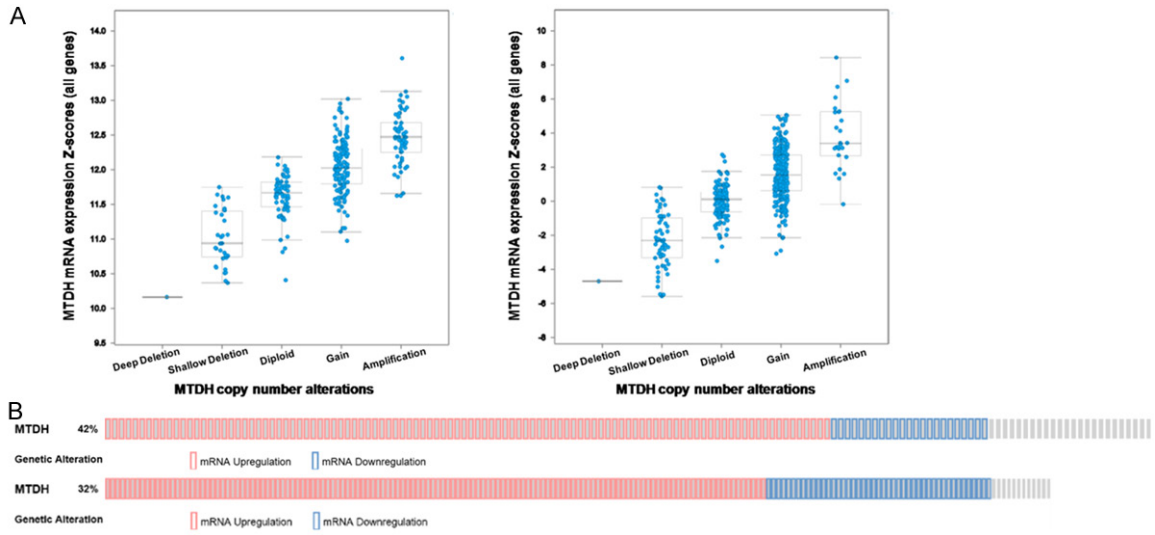

Figure 1. MTDH gene is amplified in ovarian cancer. The overviews of DNA Copy number alteration (A) and oncoprints of MTDH gene (B) in two cohorts of ovarian serous cystadenocarcinomas provided by TCGA database.

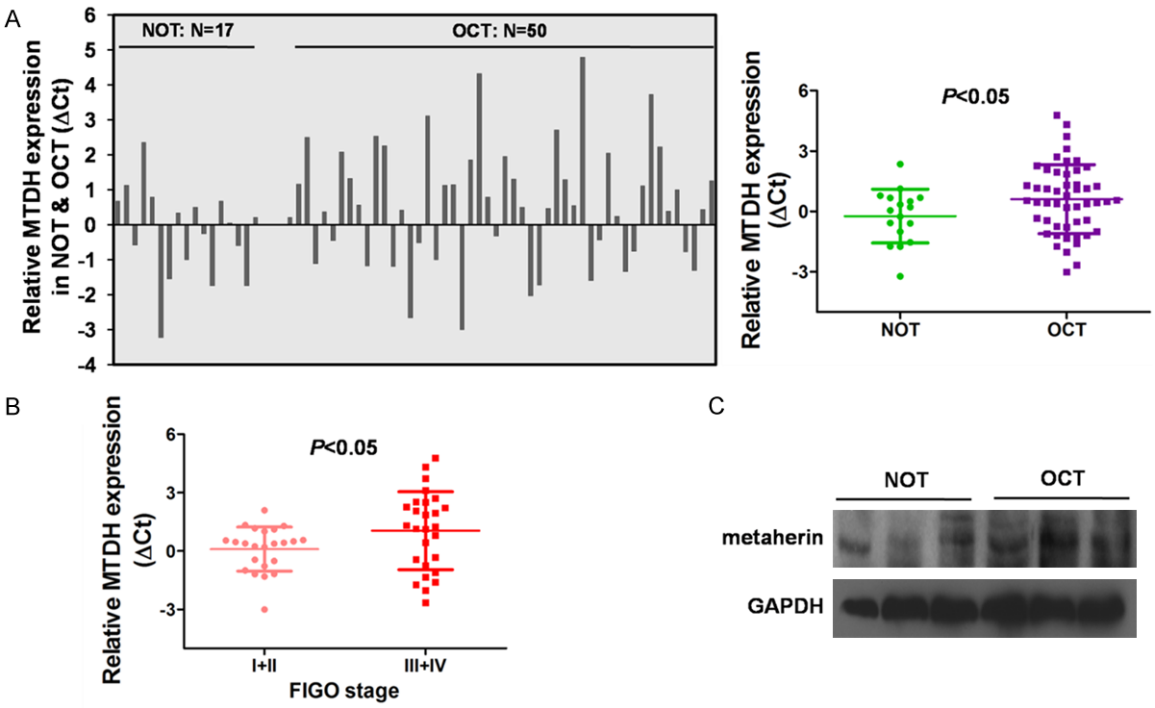

Figure 2. MTDH was upregulated in ovarian cancer tissues. A. qRT-PCR analysis of MTDH expression in 17 non-cancerous ovarian tissues (NOT) and ovarian carcinoma tissues (OCT) from patients. B. QRT-PCR analysis of MTDH expression between 23 ovarian cancer tissues of FIGO stage I+II and 27 ovarian cancer tissues of FIGO stage III+IV from patients. C. Western blot analysis of MTDH expression in 3 NOT and 3 OCT from patients. The right panel showed the quantitative results.

Statistical analysis

GraphPad Prism (v5.0, GraphPad Software) was used for statistical analysis. Student t-tests were used for comparing values between different groups. Chi-squared tests were used for analyzing the correlation between gene expression and clinicopathologic features. All tests

Table 1. Correlation analysis between the expression of *MTDH* gene and clinicopathological features of 50 ovarian cancers

Clinicopathological Features	*MTDH* gene expression		Chi-square	*P* value
	Low level N=25	High level N=25		
Age				
≤50	8	9		
>50	17	16	0.0891	0.765
Differentiation				
Low	15	13		
High	10	12	0.3247	0.5688
FIGO stage				
I+II	15	8		
III+IV	10	17	3.9452	0.0470*
Histological Grade				
I	11	5		
II	9	7		
III	5	13	6.0556	0.0484*
Lymph Node Metastasis				
Positive	5	8		
Negative	20	17	0.9356	0.3334

*P<0.05, compared between high level and low level groups.

were two-sided. The level of statistical significance was set at *P* value <0.05.

Results

MTDH gene is amplified in ovarian cancer

To investigate the expression of MTDH in ovarian cancer, we first analyzed its DNA copy number alteration in two cohorts of ovarian serous cystadenocarcinomas provided by TCGA database accessed by RNA seq method. The results shown in **Figure 1A** indicated that most of these ovarian cancer tissues exhibited increased DNA copy number, reflecting its consistent increased mRNA expression. In addition, the overall oncoprint of MTDH gene expression in these two cohorts of ovarian serous cystadenocarcinomas showed that MTDH mRNA expression was upregulated in 42% and 32% of all patients, respectively (**Figure 1B**). Only minor parts of the two population seen MTDH gene downregulation. Therefore, our online prediction supported that MTDH was increased in ovarian cancer, which might mainly origin from its increased DNA copy number.

Upregulation of MTDH in ovarian cancer tissues

Next, we collected 17 non-cancerous ovarian tissues (NOT) and 50 ovarian carcinoma tissues (OCT). The mRNA levels of MTDH were examined by qRT-PCR. As shown in **Figure 2A**, MTDH was significantly upregulated in ovarian cancers. Specifically, higher MTDH mRNA expression was observed in cancerous tissues with FIGO stages III-IV than in those FIGO stages I-II patients (**Figure 2B**, *P*<0.05). We also determined the protein expression of MTDH in selected OCTs by western blot. As shown in **Figure 2C**, MTDH protein was upregulated in OCTs compared with NOTs. Thus, our results collectively confirmed that both mRNA and protein expression of MTDH were significantly upregulated in ovarian cancer tissues.

Correlation analysis of MTDH expression with clinicopathologic features

To further understand the clinical significance of MTDH in ovarian cancer, we examined the relationship between MTDH expression and clinical features. A total of 50 patients were divided into two groups according to the median MTDH mRNA expression level: high and low MTDH groups. As shown in **Table 1**, we found positive correlation of MTDH level with FIGO stage (*P*=0.0470) and histological grade (*P*= 0.0484) of ovarian cancer patients. However, we found no significant association between MTDH expression and other features such as age, differentiation and lymph node metastasis (*P*>0.05). These results verified the importance of MTDH in ovarian cancer pathogenesis.

Knockdown of MTDH inhibits cell proliferation and cell survival

Based on the above results, ovarian cancer cells were selected for further study. The upregulation of MTDH gave a strong hint that it may function as an oncogene. To test this hypothesis, we assessed the bio-functions of MTDH by siRNA experiments. Either control siRNA or MTDH siRNA were transfected into the SKOV3 cells. Western blot assay confirmed that exogenously transducing siRNA and the knockdown effect was successful (**Figure 3A**). According to the data of the cell proliferation MTT assay, we plotted the absorbency curves at the absor-

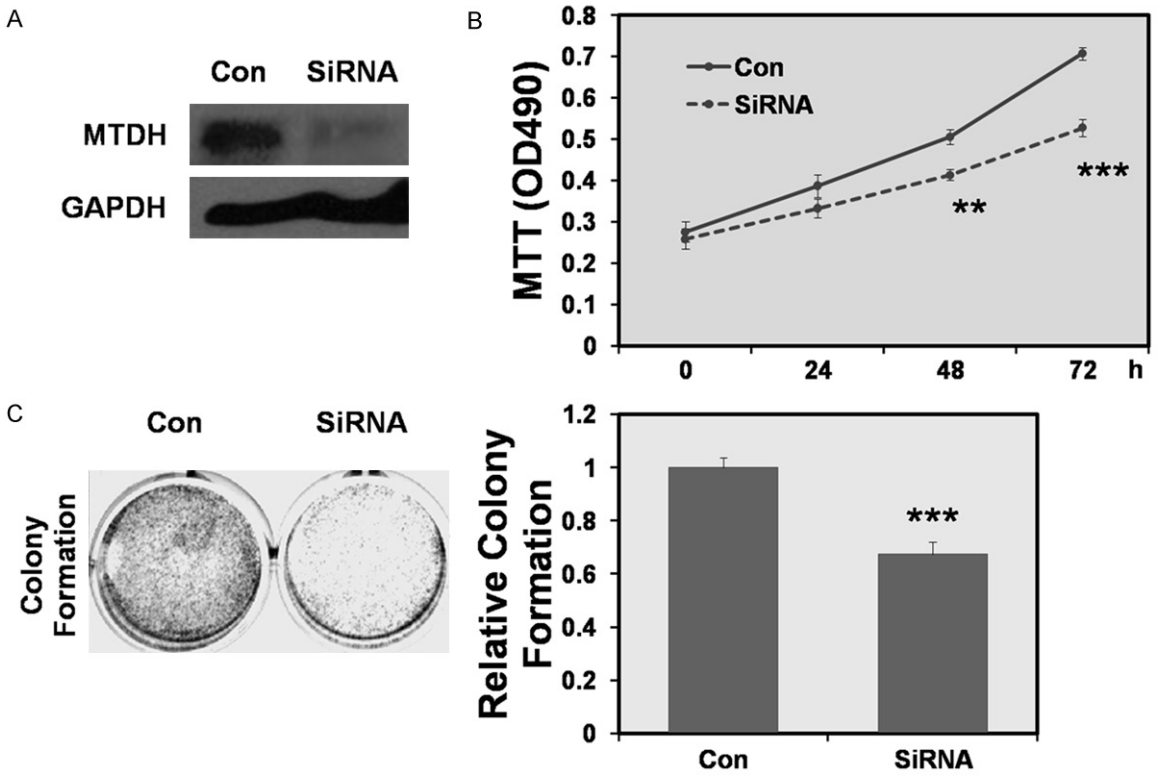

Figure 3. MTDH regulated ovarian cancer cell proliferation and survival. A. SKOV3 cells were transfected with control siRNA or MTDH siRNA. After 48 h, cells were harvested for western blot analysis. The right panel showed the quantitative results. B. MTT assay of the MTDH function on cell proliferation. C. Colony formation assay of the MTDH function on cell survival. Left panel showed the photographs of crystal violet staining, and the right panel showed the quantitative results. **: $P<0.01$, ***: $P<0.001$.

bance of 490 nm at different time points after transfections. We found that knockdown of MTDH significantly inhibited cell proliferation (**Figure 3B**). Moreover, colony formation assays were performed to evaluate the effect of MTDH in ovarian cancer cell survival. As shown in **Figure 3C**, when compared with the control group, the result demonstrated that knockdown of MTDH reduced the cell survival activity of ovarian cancer cells, and the quantitative results showed the significant difference ($P<0.001$). Taken together, these data indicated that knockdown of MTDH inhibited cell proliferation and survival capacity of ovarian cancer cells, and it may function as an oncoprotein.

The upstream and downstream molecules of MTDH

We then analyzed the upstream molecules of MTDH in regulating cell proliferation of ovarian cancer cells. By using the free online TargetScan program (http://www.targetscan.org/), we predicted that miR-153 may directly target MTDH gene in 3'-UTR region (**Figure 4A**). Then miR-153 mimics or inhibitor were enforced expressed in SKOV3 cells. The overexpression or knockdown effect of miR-153 was detected by quantitative real-time PCR, as shown in **Figure 4B**. Moreover, we analyzed the protein expression levels of MTDH and downstream cell proliferation markers including CCND1 and MYC after artificially changing the miR-153 level in our system. Western blot results in **Figure 4C** showed altered expression of MTDH as well as these markers after miR-153 overexpression or knockdown, which indicated inhibition of cell proliferation. These results suggested that miR-153 was an upstream regulator of MTDH. And MTDH contributed to cell proliferation inhibition through the downstream molecules CCND1 and MYC in ovarian cancer cells. Therefore, we summarized that miR-153 could directly target MTDH, which was an important

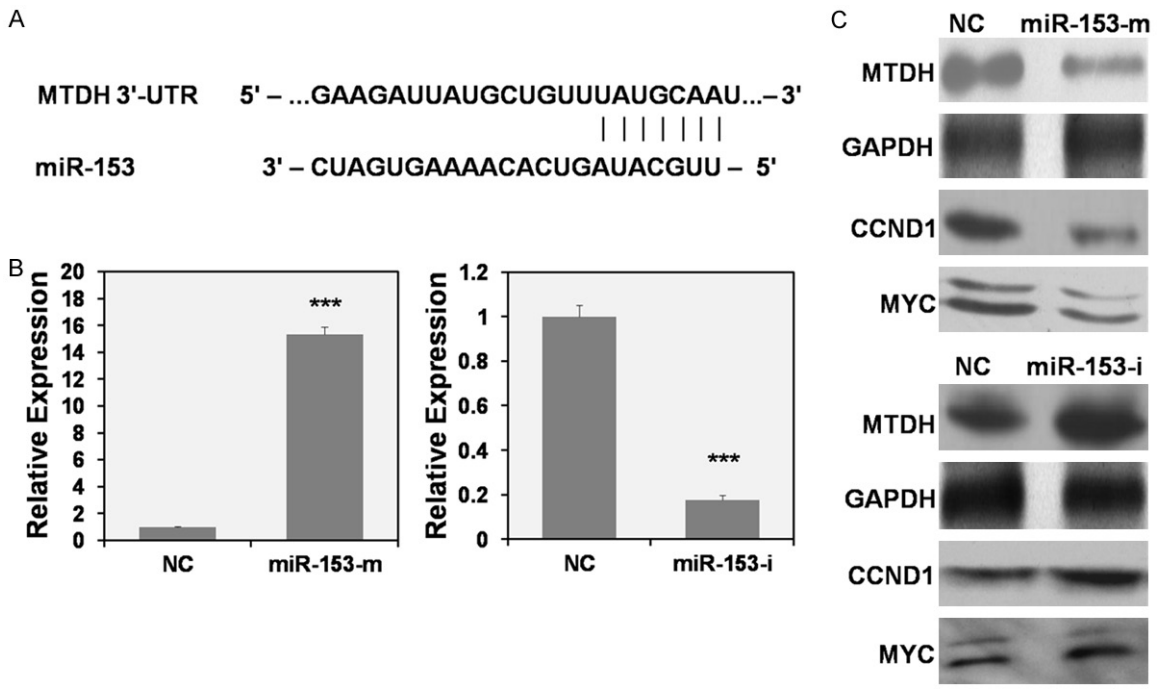

Figure 4. MTDH was negatively regulated by miR-153 in SKOV3 cells. A. The schematic diagram demonstrated the miR-153 binding site on the wild-type MTDH 3'-UTR. B. SKOV3 cells were transfected with miR-153 mimics or inhibitor. The expression level of miR-153 was detected by quantitative real-time PCR. C. Protein levels of MTDH and cell proliferation markers, CCND1 and MYC, were detected by western blot in SKOV3 cells transfected with miR-153 mimics or inhibitor. ***: P<0.001.

upstream regulator of MTDH involved in cell proliferation.

Discussion

In agreement with the former literature [11, 12], we were able to show the upregulation of MTDH mRNA and protein levels in ovarian cancer tissues. More importantly, we found its upregulation might result from its increased copy number and DNA amplification, which finally leads to its enhanced mRNA transcription and protein translation. Although we did not observe any significant association between MTDH expression and the patient's age at diagnosis, there were truly positive correlations between MTDH expression and tumor FIGO stage and histological grade.

Recent studies have revealed that MTDH is upregulated and functions as an oncogene in various cancers, including hepatocellular carcinoma [8], glioma [11], gastric cancer [12], and colorectal cancer [13]. Indeed, the level of MTDH overexpression significantly correlated with almost every aspect of tumor biology, including cancer cell proliferation, apoptosis, invasion, migration, epithelial mesenchymal transition (EMT), chemoresistance and radioresistance in indicated cancer types [3, 14-17]. Mechanistically, MTDH might function through regulating different signaling pathways that are closely related to cancer, such as nuclear factor-kappaB, PI3K/AKT, MAPK/ERK, AP-1 and Wnt/β-catenin [18].

In ovarian cancer, two reports had demonstrated that MTDH overexpression is associated with peritoneal dissemination, lymph node metastasis, International Federation of Gynecology and Obstetrics stage, histological grade, presence of residual tumor and tumor recurrence [11, 12]. They also concluded that higher MTDH level correlated with the progression and prognosis of ovarian cancer [11, 12]. Although we added the knowledge that MTDH upregulation might origin from its altered DNA copy number, we still did not disclosure more about the internal mechanisms governing its transcription regulation. In functional studies, we found that knockdown of MTDH in SKOV3 cells reduced the cell proliferation rate and cell sur-

vival. Though we have found that miR-153 might function as an upstream regulator of MTDH by directly targeting its 3'-UTR region, also downstream biomarker regulating cell proliferation were altered with MTDH, the exact mechanisms are largely unknown. Future studies are imminently and importantly needed to characterize the above problems, and will facilitate the development of novel cancer therapy options through molecular targeting of MTDH.

Taken together, our present study provided more evidence about the expression, clinical significance and functions of MTDH in ovarian cancer. We confirmed that higher MTDH expression resulting from increased DNA copy number was closely associated with FIGO stage and histological grade. Also, we proved that MTDH was directly targeted by miR-153 and it regulated cell proliferation through altering downstream biomarkers. Therefore, MTDH may serve as a biomarker for ovarian cancer patients who are in their advanced stages. These findings support that MTDH holds promise as a therapeutic target for ovarian cancer.

Disclosure of conflict of interest

None.

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