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Thrombomodulin regulates doxorubicin sensitivity through epithelial-mesenchymal transition in non-small cell lung cancer

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Abstract. – OBJECTIVE: Lung cancer has remained the highest about cancer-related mortality and drug resistance is involved in the recurrence of the disease. Thrombomodulin (TM) is down-regulated in several malignant tumors, but its role in drug resistance has not been elucidated in lung cancer. We aimed to investigate the role of TM in drug resistance to lung cancer.

MATERIALS AND METHODS: The mRNA and protein expression of TM were determined by real-time PCR and Western blot, respectively. TM expression was manipulated using siRNA or an overexpression system. The expression of epithelial-mesenchymal transition (EMT)-related markers (E-cadherin and vimentin) was detected by real-time PCR and Western blot.

RESULTS: We found that A549 and HCC827 cells with higher TM expression were more sensitive to doxorubicin than SPC-A-1 cells with lower TM. Also, downregulation of TM reduced the doxorubicin sensitivity in A549 and HCC827 cells. On the contrary, up-regulated TM increased the doxorubicin cytotoxicity in SPC-A-1 cells. Mechanically, ectopic expression of TM elevated the expression of E-cadherin, an epithelial marker. Conversely, overexpression of TM led to reduced expression of vimentin, a mesenchymal marker, leading to the reversal of EMT in lung cancer cells. As a result, SPC-A-1 cells overexpressing TM become more sensitive to doxorubicin treatment.

CONCLUSIONS: These findings showed that TM regulated drug sensitivity through EMT in lung cancer cells, suggesting that TM might be developed into a novel target for lung cancer patients resistant to conventional therapeutics.

Key Words:
Lung cancer, Thrombomodulin, Epithelial-mesenchymal transition, Drug resistance.

Introduction

Although great efforts have been made for lung cancer therapy, it is still the leading cause of cancer-related deaths worldwide in the past two or three decades, and the 5-year relative survival rate of lung cancer is only 11-15%[1,2]. Of note, 80% of lung cancers are non-small cell lung cancer (NSCLC) with poor therapeutic efficacy and low 5-year survival rate when diagnosed[3]. Accumulating evidences[4-6] have shown that the activation of oncogenes and inactivation of tumor suppressor genes are critically important for the proliferation, metastasis, invasion and drug resistance of NSCLC.

Thrombomodulin (TM) is a transmembrane protein, which was initially identified in vascular endothelial cells and characterized as an anticoagulant factor[7]. It is consisted of 5 domains, a N-terminal lectin-like domain, an epidermal growth factor (EGF)-like domain, a serine and threonine-rich domain, a transmembrane domain and a cytoplasmic domain[8,9]. TM is a multifunctional protein that exhibits anti-inflammation, anti-thrombosis, and protective function against wound healing and endothelial injury[9-11]. Also, TM has been found to be implicated in cancer initiation, progression and clinical prognosis[12,13]. For example, down-regulation of TM in tumor cells predicts the poor prognosis in several cancers, including breast cancer, lung cancer, leukemia and colorectal cancer[12,14,15]. Moreover, decreased expression of TM is also involved in the biological activities of tumor cells including cell invasion, migration and metastasis[16]. However, the molecular mechanism of TM in the modulation of drug resistance has not been reported in NSCLC cells. In the current study, we aimed to investigate the biological role of TM in the chemo-resistance in NSCLC cells as well as its underlying mechanism.

Materials and Methods

Cell Lines and Cell Culture

NSCLC cell lines, including A549, HCC827 and SPC-A-1, were purchased from the American

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Type Culture Collection (Manassas, VA, USA). These cell lines were cultured in DMEM supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA), 100 IU/ml penicillin and 100 IU/ml streptomycin. Cells were grown at 37°C in a humidified atmosphere with 5% CO_2. This study was approved by the Ethics Committee of Shanghai Chest Hospital.

Cell Transfection

For transfection, cells were grown to 70% confluence and transfected with the indicated siRNA duplex or plasmids using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. After 48h post-transfection, cells were harvested for further investigation.

CCK-8 Assay

For measurement of cell viability, a cell counting kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) was used according to the manufacture's instruction. Briefly, tumor cells were seeded in 96-well plates and incubated indicated drugs. Then 10 μL/well CCK8 solution was added, and absorbance was measured at 450 nm using an MRX II microplate reader (Dynex, Chantilly, VA, USA).

Real-time PCR

Total RNA was extracted using a RNeasy® Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. cDNA was reversely transcribed from 1 mg of RNA using a SuperScript® VILO™ cDNA Synthesis Kit (Thermo Fisher, Hanover, MD, USA). Real-time PCR was performed using an Applied Biosystems 7300 real-time PCR System. The PCR program started at 94°C for 15 s (denaturation), followed by 40 cycles of 57°C for 15 s (annealing) and 72°C for 30 s (extension). The relative mRNA expression of each gene was calculated by 2(-ΔΔCT) method.

Western Blot Analysis

NSCLC cells were collected and lysed with ice-cold lysis buffer supplemented with protease inhibitors. The protein extracts were subject to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane. Following blocking with 5% non-fat milk in tris buffered saline-tween (TBS-T) buffer, the membrane was incubated with the primary antibodies at 4°C overnight. The membrane was washed in PBST buffer and then incubated with the peroxidase-conjugated secondary antibody at room temperature for 2h. The protein bands were detected using an enhanced chemiluminescence kit (Pierce, Thermo Scientific, Waltham, MA, USA).

Statistical Analysis

Each experiment was repeated at least three times. Data were presented as means ± SD and analyzed by SPSS program (SPSS Inc. Chicago, IL, USA). The comparison between groups was made using analysis of variance (ANOVA). $p < 0.05$ was considered as statistically significant.

Results

Different TM Expression and Doxorubicin Sensitivity in NSCLC cells

To elucidate the possibility whether TM expression was involved in the doxorubicin sensitivity in lung cancer cells, we incubated tumor cells with doxorubicin for 24h and 48h, and then detect the cell viability changes of tumor cells. As a result, we observed different cellular responses to doxorubicin in the selected lung cancer cell lines including A549, HCC827 and SPC-A-1 (Figure 1A-1C). Particularly, we found that A549 and HCC827 cells were more sensitive to doxorubicin than SPC-A-1 cells (Figure 1A-1C). Moreover, the mRNA and protein expression of TM was detected in A549, HCC827 and SPC-A-1 cells using real-time PCR and Western blot, respectively. Consequently, TM expression was significantly higher in A549 and HCC827 cells compared with SPC-A-1 cells, both at the mRNA and protein levels (Figure 2A and 2B). These findings revealed that TM expression may be implicated in the drug resistance to doxorubicin in lung cancer cells.

Down-Regulation of TM Increased the Drug Sensitivity in lung Cancer Cells

To validate that TM was involved in the drug resistance of lung cancer cells, we silenced the expression of TM by transfecting TM siRNA into A549 and HCC827 cells. Real-time PCR and Western blot revealed that TM expression was successfully down-regulated in A549 and HCC827 cells (Figure 3A and 3B). As a result, CCK-8 assay showed that down-regulation of TM decreased the doxorubicin sensitivity in A549 and HCC827 cells (Figure 3C and 3D).

A previous study[17] reported that TM expression could be induced by atorvastatin in human aortic endothelial cells. Therefore, we incubated SPC-A-1 cells with different concentration of

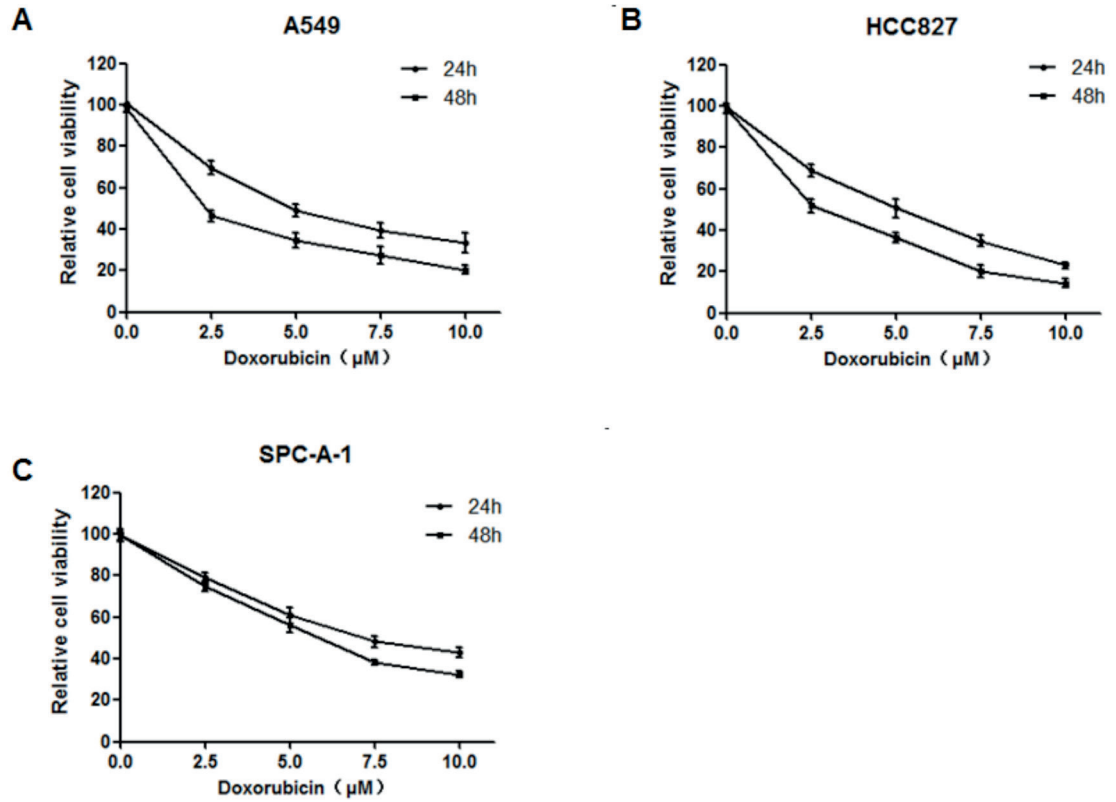

Figure 1. Different doxorubicin sensitivity in NSCLC cells. NSCLC cells, including A549, HCC827 and SPC-A-1, were incubated with doxorubicin at different concentrations (0, 2.5, 5, 7.5, and 10 μM) for 24h and 48h. The cell viability of A549, ***(A)*** HCC827, ***(B)*** and SPC-A-1 ***(C)*** was determined using CCK-8 assay.

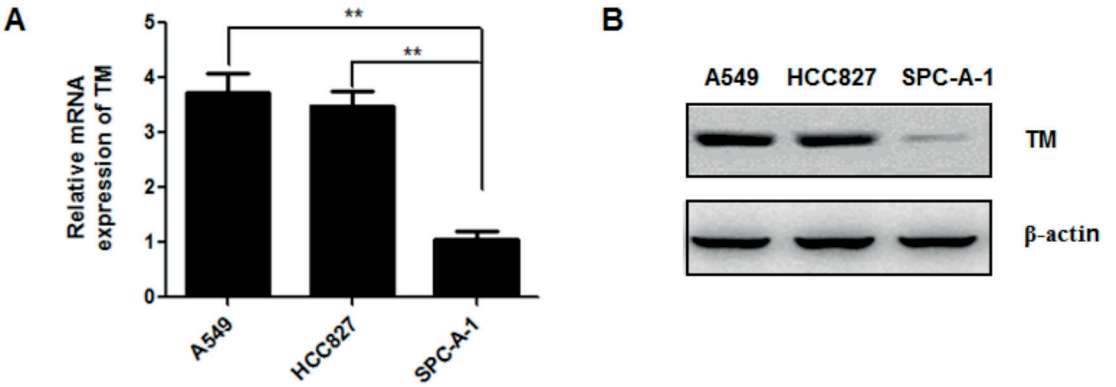

Figure 2. Different expression of TM in lung cancer cells. For measurement of TM expression, real-time PCR *(A)* and Western blot ***(B)*** were applied to determine the mRNA and protein expression of TM in A549, HCC827 and SPC-A-1 cells. $^{**}p$ <0.01, compared with SPC-A-1 cells.

atorvastatin (0, 3, 6, 9 μM) for 24h. Consistently, atorvastatin treatment significantly elevated the mRNA expression of TM in a dose-dependent manner (Figure 4A). Consequently, up-regulation of TM in atorvastatin-treated SPC-A-1 cells resulted in decreased cell viability in the presence of doxorubicin (Figure 4B). Taken together, these results demonstrated that TM could serve as a tumor suppressive protein in lung cancer cells.

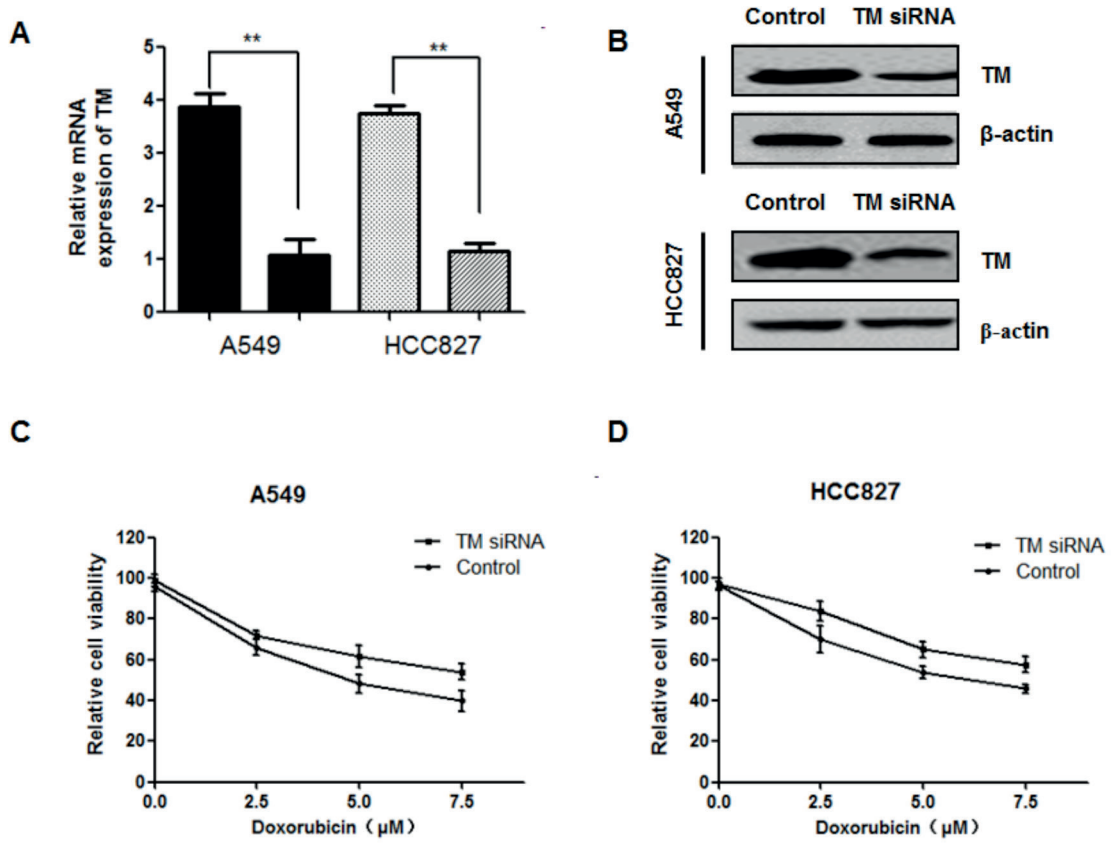

Figure 3. Effects of down-regulation of TM on drug sensitivity in lung cancer cells. For down-regulation of TM, TM siRNA was transfected into A549 and HCC827 cells. The efficacy was confirmed by real-time PCR ***(A)*** and Western blot *(B)*. Also, doxorubicin sensitivity was measured in A549 ***(C)*** and HCC827 ***(D)*** cells transfected with TM siRNA. $^{**}p$ <0.01, compared with control group.

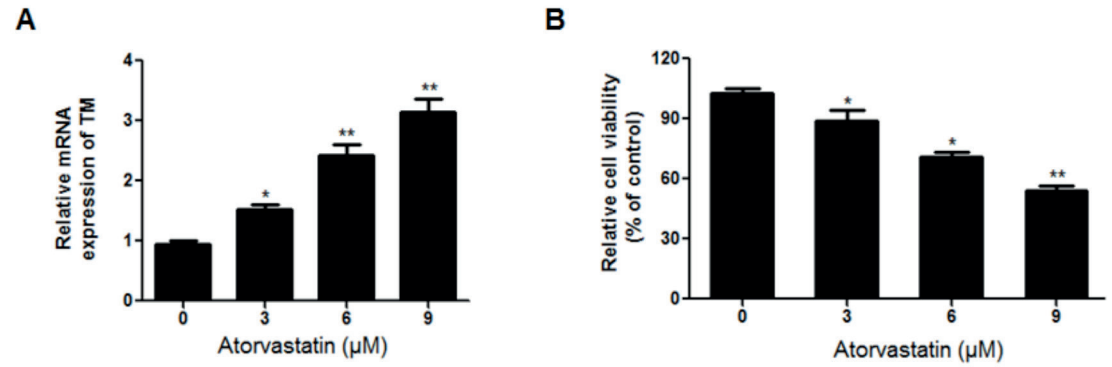

Figure 4. Atorvastatin treatment increased doxorubicin sensitivity. A549 cells were incubated with different concentration of atorvastatin (0, 3, 6, 9 μM) for 24h. The mRNA expression of TM was detected using real-time PCR ***(A)***, and cell viability ***(B)*** was measured by CCK-8 assay. $^{*}p$ <0.05; $^{**}p$ <0.01, compared with control group.

Down-Regulation of TM Promoted EMT in Lung Cancer Cells

To reveal the molecular mechanism of TM-mediated anti-cancer ability, we examined the expression of EMT-related markers by real-time PCR and Western blot. As a result, we found that the expression of E-cadherin (an epithelial marker) was increased (Figure 5A-5C), whereas the vimentin expression (a mesenchymal marker) was reduced in SPC-A-1 cells after

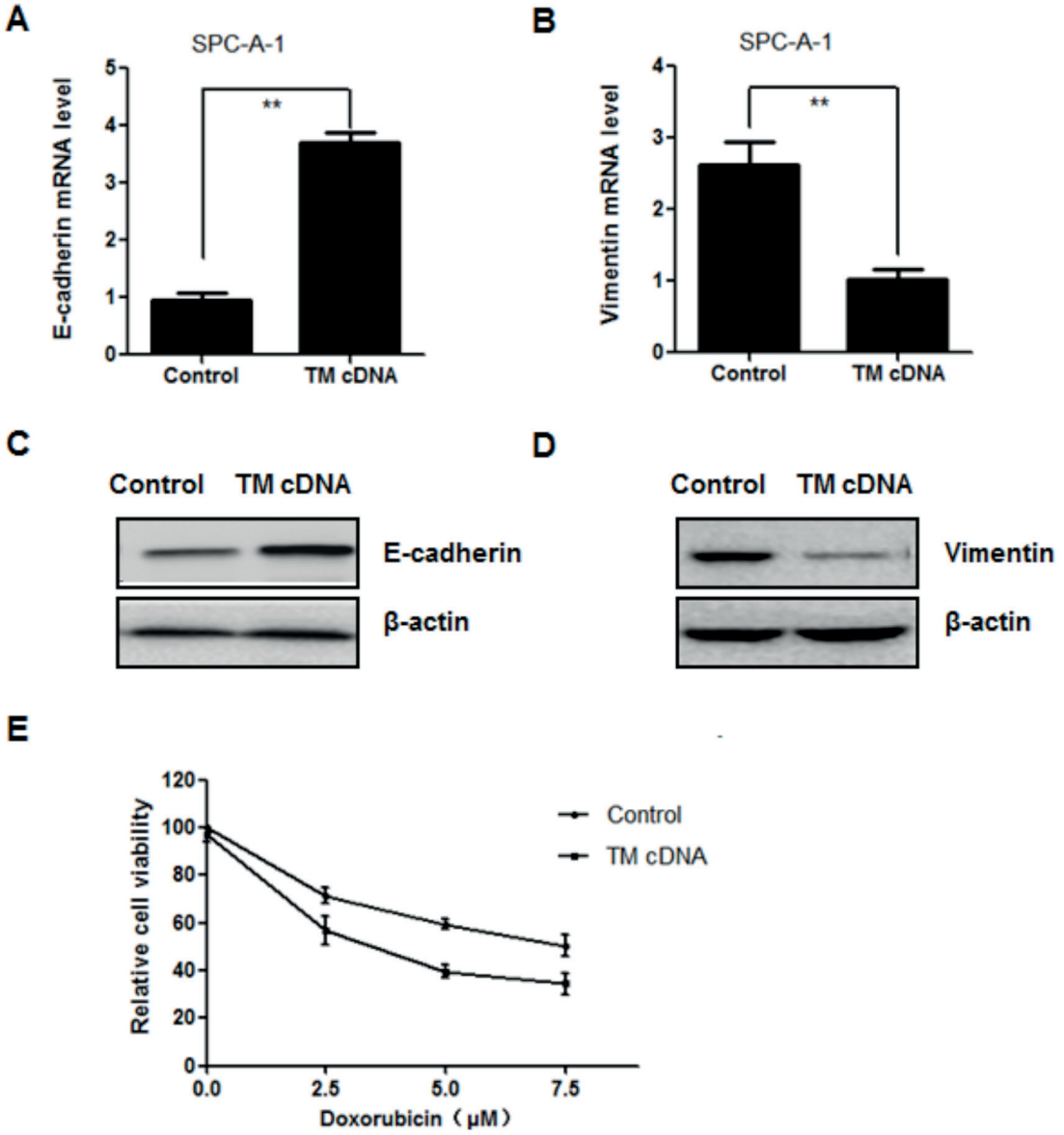

Figure 5. TM reversed EMT progression in lung cancer cells. SPC-A-1 cells were transfected with plasmids carrying TM cDNA sequence. The expression of E-cadherin (an epithelial marker) and vimentin (a mesenchymal marker) was measured by real-time PCR ***(A*** and ***B)*** and Western blot ***(C*** and ***D)***. Moreover, the cellular responses to doxorubicin were measured in SPC-A-1 cells transfected with or without plasmids encoding TM (E). $^{**}p$ <0.01, compared with control group.

transfection with TM cDNA (Figure 5B-5D). These data suggested that ectopic expression of TM reversed the progression of EMT in SPC-A-1 cells. As a result, we found that up-regulation of TM in SPC-A-1 cells elevated the chemosensitivity to doxorubicin (Figure 5E). These results demonstrated that EMT was responsible for TM-mediated drug sensitivity in lung cancer cells.

Discussion

Currently, pre-operative or post-operative combined chemotherapy has been regarded as an indispensable adjuvant treatment for patients diagnosed with lung cancer [18]. Nevertheless, drug resistance has become a great obstacle to successful treatment of chemotherapy[19]. Thus, our study aimed to identify novel potential targets that are involved in the che-

moresistance of lung cancer cells. In this study, we found that TM played a critical role in the doxorubicin resistance, which was mediated by EMT progression in lung cancer cells.

TM is a naturally occurring anticoagulant protein, which is constitutively expressed on the cell membrane of vascular endothelium[20]. Structurally, TM is composed of an N-terminal lectin-like domain, a hydrophobic region, six EGF-like domains, a serine/threonine-rich region, a transmembrane module and a cytoplasmic tail[8,9]. It has been shown that TM is expressed in lymphovascular tumors and thus could serve as a potential marker in diagnosis and treatment of such tumors[21]. Also, expression of TM has been identified in a variety of human cancers, including bladder cancer, esophageal squamous cell carcinoma, cervical cancer, lung cancer and hepatocellular carcinoma (HCC)[22-25]. In esophageal carcinoma, 69% of patients presented decreased expression of TM in the metastatic lesions[22]. In HCC, the recurrence-free survival was higher in TM-positive patients than that in the TM-negative patients[25]. Furthermore, TM plays an important role in the cellular behaviors in human cancers. Specifically, Zhang et al[26] found that up-regulation of TM inhibited melanoma cell growth both *in vitro* and *in vivo*. Consistently, down-regulation of TM enhanced tumor growth and invasive potential of bladder cancer cells[16]. However, the role of TM in drug resistance in lung cancer cells has not been reported. In our study, lung cancer cell lines, A549, HCC827 and SPC-A-1, were incubated with doxorubicin and their viabilities were determined. We found that A549 and HCC827 cells were more sensitive to doxorubicin than SPC-A-1 cells. Interestingly, the mRNA and protein levels of TM were obviously higher in A549 and HCC827 cells than those in the SPC-A-1 cells, suggesting that TM expression may be involved in the chemoresistance of lung cancer cells. Furthermore, we found that down-regulation of TM in A549 and HCC827 cells reduced cellular responses to doxorubicin. On the contrary, up-regulated expression of TM in atorvastatin-treated SPC-A-1 cells led to increase doxorubicin sensitivity. Taken together, these findings demonstrated that TM could regulate the chemoresistance in lung cancer cells.

Epithelial-mesenchymal transition (EMT) is a critical step ensuring tissue remodeling during the morphogenesis of multi-cellular organisms[27]. Accumulating evidence has suggested key roles of EMT in cancer initiation and progression[28]. During EMT, the expression of epithelial markers (such as E-cadherin) is reduced, while the levels of mesenchymal markers (including Twist1, Snail and vimentin) are up-regulated[29]. Several studies have reported that TM is implicated in the regulation of EMT. For instance, Kao et al[30] reported that TM was a novel target of snail and its down-regulation promoted tumorigenesis through EMT. Another study[23,31] revealed that knockdown of TM increased the migration, and up-regulation of TM inhibited the metastatic potential in prostate and cervical cancer via regulating EMT biomarkers. Consistently, our study found that ectopic expression of TM elevated E-cadherin levels (an epithelial marker) in SPC-A-1 cells. Moreover, overexpression of TM led to reduced expression of vimentin, a mesenchymal marker. Consequently, the doxorubicin sensitivity was obviously increased in SPC-A-1 cells overexpressing TM. These results showed that EMT was involved in the TM-mediated drug sensitivity in lung cancer cells.

Conclusions

We demonstrated that TM could regulate drug resistance in lung cancer cells through reversal of EMT. These findings proved that TM may act as a novel therapeutic target in the chemoresistance lung cancer cells.

Acknowledgements

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Conflict of Interest

The authors declare no conflicts of interest.

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