Abstract. Previous studies have demonstrated that nicotinamide N-methyltransferase (NNMT) is aberrantly expressed in a number of tumors. In the present study, it was demonstrated that the gene and protein levels of NNMT were significantly increased in gastric cancer cells. Furthermore, upregulation of NNMT significantly increased the expression of mesenchymal markers, including α-smooth muscle actin (SMA), vimentin and fibronectin, but decreased the levels of epithelial cadherin. Since transforming growth factor (TGF)-β1 may serve a key function in epithelial-mesenchymal transition (EMT), the effects of NNMT on the expression of TGF-β1 were investigated in BGC-823 cells. The results demonstrated that overexpression of NNMT significantly induced the expression of TGF-β1. However, knockdown of NNMT inhibited the expression of TGF-β1, mothers against decapentaplegic homolog (Smad)2 and α-SMA. Additionally, pre-incubation with TGF-β1 partially eliminated NNMT-mediated changes in EMT. Collectively, the results demonstrated that upregulation of NNMT in gastric cancer cells may increase the expression of TGF-β1, therefore activating TGF-β1/Smad signaling, which in turn promotes EMT.

Introduction

Gastric cancer is one of the most common types of malignant tumor globally. In China, the incidence of gastric cancer is the second highest of all the malignant tumors (1,2). Early symptoms in the majority of patients with gastric cancer are not detected (3). The prognosis remains poor in patients with intermediate- and advanced-stage gastric cancer and the total 5-year survival rate is ~20% (3).

The poor prognosis of patients with gastric cancer is associated with increased invasiveness and metastasis (4,5). Epithelial-mesenchymal transition (EMT) is a biological process in which epithelial cells are transformed into mesenchymal-like cells under certain physiological or pathological conditions (6,7). It has been suggested that EMT serves a key function in tumor invasion and metastasis (8). Future studies investigating the molecular mechanism underlying EMT in tumor invasion and metastasis are required.

Nicotinamide N-methyltransferase (NNMT) is an S-adenosyl-L-methionine-dependent cytoplasmic enzyme that is mainly expressed in the human liver (9,10). NNMT is mainly involved in the biotransformation of drugs and other xenobiotic chemicals (11). Recent studies have demonstrated that NNMT is aberrantly expressed in a number of tumors, suggesting that NNMT serves an important function in tumor development (12,13). Additionally, the serum levels of NNMT were significantly increased in patients with colorectal, lung and renal cell carcinoma compared with those in the control group (10,14,15). Previous studies have also demonstrated that the upregulation of NNMT is associated with poor prognosis, advanced tumor stage, tumor cell migration and invasion (9,16,17). Although, overexpression of NNMT has been identified in gastric cancer (18), the underlying molecular mechanism remains unclear.

The present study aims to evaluate the expression of NNMT in gastric cancer cell lines and the underlying mechanism by which NNMT is involved in the progression of gastric cancer, thereby exploring novel therapeutic targets for gastric cancer patients.

Materials and methods

Cell culture. Gastric cancer MKN45, MGC-803, AGS and BGC-823 cell lines, and normal gastric epithelial GES-1 cells were obtained from the China Center for Type Culture Collection (Beijing, China). All cell lines were cultured in RPMI-1640 (GE Healthcare, Chicago, IL, USA) supplemented with 10% fetal bovine serum (FBS) (GE Healthcare), streptomycin (100 mg/ml) (Invitrogen; Thermo Fisher Scientific, Inc.,
Transient transfection. In brief, BGC-823 cells were seeded at the density of 10^5 cells/well in a 6-well plate. BGC-823 cells were transfected with small interfering RNA (siRNA) targeting NNMT (GCT  CAA GAG CAG CTA CTA CAT) or a non-specific siRNA (TTC  TCC GAA CGT GTC ACG T) (GenChem, Jiangsu, China), which was used as a negative control (NC), at a final concentration at 20 nM. Cells were pre-incubated at room temperature for 10 min with HiPerfect transfection reagent (Qiagen GmbH, Hilden, Germany). For subsequent experiments, BGC-823 cells were used at 48 h post-transfection.

Adenoviral vector construction. Recombinant adenoviruses expressing NNMT (Ad-NNMT) or a NC-adenovirus vector containing green fluorescent protein (Ad-Con) were obtained from Shanghai GeneChem Co., Ltd. (Shanghai, China). Briefly, BGC-823 cells were seeded at 10x10^5 cells/well in a 6-well plate. At 80% confluence, Ad-NNMT or Ad-Con was transfected into BGC-823 cells at a multiplicity of infection of 30.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from MKN45, MGC-803, AGS and BGC-823 cells using TRIzol reagent (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. RNA was reversed-transcribed into cDNA using the TaqMan RNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed using SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the iCycleriQ Real-Time PCR system (Bio-Rad Laboratories, Inc.) as described previously (19). The thermocycling conditions were as followed: at 95˚C for 10 min followed by 50 cycles of 95˚C for 10 sec, 55˚C for 10 sec and 72˚C for 5 sec; 99˚C for 1 sec; 59˚C for 15 sec; 95˚C for 1 sec; then cooling to 40˚C. Relative mRNA expression was normalized against the endogenous control, GAPDH, using the 2^(-ΔΔCt) method (20). The primers used in the current study was listed as follows: NNMT, forward, CTG  CCT AGA CGG TGT GAA GG, and reverse, CTT GAC CGC CTG TCT CAA CT, and GAPDH, forward, GAG AAG GCT GGG GCT CAT TT, and reverse, AGTGATGGCATGGACTGG.

Western blot analysis. Proteins samples were isolated from BGC-823 cells using radioimmunoprecipitation assay buffer [1% Triton X-100, 15 mmol/l NaCl, 5 mmol/l EDTA and 10 mmol/l Tris/HCl (pH 7.0); Beijing Solarbio Science & Technology Co., Ltd., Beijing, China] supplemented with a protease and phosphatase inhibitor cocktail (Merck KGaA, Darmstadt, Germany). A bicinchoninic protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to determine the protein concentration. Equal quantities of protein (15 µg) were separated by 12% SDS-PAGE. Electrophoresed proteins were then transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 8% skimmed milk in Tris-buffered saline with Tween-20 (TBST; pH 7.5) for 2 h at room temperature and were incubated with the following primary antibodies at 4˚C overnight: Anti-NNMT (1:1,000; cat no., ab19758; Abcam, Cambridge, UK), anti-TGFβ (1:1,000; cat no., ab92486; Abcam), anti-Smad2, (1:1,000; cat no., ab40855; Abcam), anti-epithelial (E)-cadherin (1:1,000; cat no., ab76055; Abcam), anti-SMA (1:1,000; cat no., ab7817; Abcam), anti-fibronectin (1:1,000; cat no., ab2413; Abcam), anti-vimentin (1:1,000; cat no., ab8978; Abcam) and anti-GAPDH (1:4,000; cat no., 5174; Cell Signaling Technology, Inc., Danvers, MA, USA). Following multiple washes with tris-buffered saline with Tween, the membranes were incubated with horseradish-peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse IgG or HRP-conjugated mouse anti-goat IgG (all 1:5,000; Origene Technologies, Inc.) for 2 h at room temperature prior to another wash in TBST. Protein
bands were visualized using enhanced chemiluminescence (EMD Millipore, Billerica, MA, USA) according to the manufacturer’s protocol. GAPDH was used as an internal control.

Cell migration and invasion assays. Cell migration assays were performed using Boyden chambers (8-µm pore filter; Corning Inc., Corning, NY, USA). For the cell invasion assay, the filter surfaces were precoated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). In brief, BGC-823 cells were seeded at a density of 1x10^6 cells/well for 24 h and then transfected with Ad-NC or Ad-NNMT were plated in the upper chamber in RPMI-1640 medium without FBS. RPMI-1640 medium (600 µl) with 20% FBS was plated in the lower chamber. After 48 h of incubation, non-migratory and non-invading cells were removed with cotton swabs. The migratory or invasive cells located on the lower side of the chamber were fixed in methanol for 30 min at 37°C and stained with 0.5% crystal violet for 1 h at 37°C. Stained cells were counted in 5 random fields using fluorescence microscopy (magnification, x40). All experiments were performed in triplicate.

Wound healing assay. In brief, BGC-823 cells were seeded at a density of 1x10^6 cells/well for 24 h. The confluent monolayer of cells was then wounded using a p200 pipette tip. The existing media was replaced and fresh RPMI-1640 (GE Healthcare) supplemented with 10% fetal bovine serum (FBS; GE Healthcare), streptomycin (100 mg/ml) (Invitrogen; Thermo Fisher Scientific, Inc.) and penicillin (100 IU/ml) were then added. The BGC-823 cells were then transfected with Ad-NC or Ad-NNMT for 24 and 48 h immediately following wounding, as per the aforementioned methodology. For each
well, 3 images were captured with a microscope at 0 and 24 h after wounding.

Treatment with TGF-β1. In order to further validate the effects of NNMT in EMT, in brief, BGC-823 cells were seeded at a density of 1x10^6 cells/well for 24 h. BGC-823 cells were pre-incubated with 20 nM TGF-β1 (cat no. SRP0300; Sigma-Aldrich; Merck KGaA) or distilled water for 24 h. si-NNMT or NC was transfected into BGC-823 cells following TGF-β1 treatment in the presence or absence of TGF-β1. BGC-823 cells were treated with si-NNMT or NC, as per the aforementioned methodology.

Statistical analysis. Data were analyzed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard error of the mean. Results were analyzed using Student’s t-test or one-way analysis of variance followed by Tukey’s honest significant difference test. P<0.05 was considered to indicate a statistically significant difference.

Results

Increased expression of NNMT in gastric cancer cells. First, the expression of NNMT was investigated in gastric cancer cells. As presented in Fig. 1A, the mRNA level of NNMT was significantly increased in MKN45, MGC-803, AGS and BGC-823 cells compared with that in normal GES-1 cells. Additionally, the protein expression levels of NNMT were also significantly increased in gastric cancer MKN45, MGC-803, AGS and BGC-823 cells compared with that in the GES-1 cells (Fig. 1B). The expression of NNMT was highest in the BGC-823 cells. Hence, BGC-823 cells were selected for further study.

NNMT promotes EMT in BGC-823 cells. The present study investigated whether NNMT may induce EMT in BGC-823 cells. The results demonstrated that overexpression of NNMT in BGC-823 cells (achieved using Ad-NNMT) led to significant changes in the morphology of the BGC-823 cells, which acquired an elongated and spindle-shaped phenotype (Fig. 2A). Additionally, upregulation of NNMT significantly increased the expression of mesenchymal markers, including α-SMA, vimentin and fibronectin, but decreased the levels of the epithelial marker E-cadherin (Fig. 2B). Furthermore, upregulation of NNMT significantly increased the migration of the BGC-823 cells at 24 and 48 h, as assessed using an in vitro wound healing assay (Fig. 2C). Treatment with Ad-NNMT increased the migratory and invasive abilities of the BGC-823 cells compared with that of the Ad-NC group, as
assessed using Boyden chamber assays (Fig. 2D). Collectively, these results suggest that NNMT may exert an oncogenic function in gastric cancer cells.

**NNMT increases the expression level of TGF-β1 in BGC-823 cells.** Since TGF-β1 serves a key function in EMT, the present study investigated whether NNMT may increase the expression levels of TGF-β1 in BGC-823 cells. The results demonstrated that overexpression of NNMT (achieved using Ad-NNMT) increased the expression of TGF-β1 in BGC-823 cells, as assessed using western blot analysis (Fig. 3A). Additionally, treatment with Ad-NNMT increased the expression of Smad2 and α-SMA in the BGC-823 cells compared with treatment with Ad-NC group (Fig. 3A). In the present study, the effects of downregulating the expression of NNMT using siRNAs were assessed. The results demonstrated that treatment with si-NNMT significantly inhibited the expression of TGF-β1, Smad2 and α-SMA compared with that in the NC group in the BGC-823 cells (Fig. 3B). These results suggest that NNMT may upregulate the expression of TGF-β1, therefore promoting EMT.

**TGF-β1-induced EMT is partially reversed in response to treatment with si-NNMT.** In order to further validate the

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**Figure 4.** TGF-β1-mediated EMT is partially reversed by silencing of NNMT in BGC-823 cells. (A) TGF-β1 significantly increased the expression of Smad2 and α-SMA. Treatment with si-NNMT partially reversed the changes in the expression of EMT markers, which were induced in response to treatment with TGF-β1. (B) Silencing of NNMT partially reversed TGF-β1-mediated cellular morphological changes (magnification, x40, x100 and x400). *P<0.05 and **P<0.01 vs. control. NNMT, nicotinamide N-methyltransferase; TGF, transforming growth factor; Smad, mothers against decapentaplegic homolog; Vi, vimentin; FN, fibronectin; E-cad, E-cadherin/epithelial cadherin; α-SMA, α-smooth muscle actin; Ad, adenovirus; NC, control; si, siRNA/small interfering RNA.
effects of NNMT in EMT, BGC-823 cells were treated with si-NNMT, with or without TGF-β1. As presented in Fig. 4A, treatment with TGF-β1 significantly increased the expression of Smad2, vimentin, fibronectin and α-SMA, but decreased the expression of E-cadherin in the BGC-823 cells. However, treatment with si-NNMT decreased the expression levels of Smad2, fibronectin and α-SMA (Fig. 4A). Combined treatment with si-NNMT and TGF-β1 significantly increased the expression levels of Smad2, α-SMA and fibronectin but decreased the expression levels of E-cadherin compared with that in the si-NNMT group (Fig. 4A). Furthermore, TGF-β1-mediated cell morphological changes were partially eliminated by the silencing of NNMT (Fig. 4B). These results demonstrate that NNMT induces EMT partially by mediating TGF-β1 signaling.

Discussion

NNMT catalyzes the methylation of nicotinamide and pyridine, therefore regulating the metabolism of drugs and other xenobiotics (21). The abnormal expression of NNMT has been widely identified in several types of tumor (22,23). It has been demonstrated that NNMT is involved in the malignant migration of tumor cells and that it may be used as a potential biomarker for predicting tumor invasion (24,25). In the present study, it was demonstrated that the expression of NNMT was significantly increased in gastric cancer cell lines compared with that in normal control cell lines. However, the precise molecular mechanism underlying the NNMT-mediated progression of gastric cancer remains unclear.

EMT is an important process in carcinogenesis since it increases the invasive ability of cancer cells (26,27). As a result of EMT, epithelial-derived tumor cells lose their features and obtain mesenchymal-like characteristics (28,29), and tumor cells invade the surrounding tissue and break through the capillary into the circulatory system, leading to distant metastasis (30). During EMT, the expression level of molecular markers for epithelial cells, including E-cadherin, are decreased, but the expression of molecular markers of mesenchymal cells, including vimentin, neuronal cadherin and α-SMA, are increased (31,32).

In the present study, the function of NNMT in the EMT-mediated changes in gastric cancer cells was evaluated. The results demonstrated that upregulation of NNMT led to morphological changes in BGC-823 cells, which acquired a spindle-shaped phenotype, suggesting that NNMT may promote EMT in cancer cells. To further investigate how NNMT regulated EMT, the effects of NNMT on the TGF-β1 signaling pathway were investigated. The results revealed that the overexpression of NNMT (achieved using Ad-NNMT) induced the expression of TGF-β1 in gastric cancer cells. Additionally, TGF-β1-induced EMT was partially reversed by the silencing of NNMT, as demonstrated by the morphological changes observed under light microscopy and the changes in the expression of EMT markers detected using western blot analysis. These results indicated that NNMT promoted EMT in gastric cancer cells mainly by upregulating TGF-β1 expression. It has been demonstrated that NNMT-mediated methylation is an important conjugation reaction in the biotransformation of a number of drugs and xenobiotics, including pyridine and other structurally-associated compounds (18). The epigenetic silencing of TGF-β1 genes is associated with the development of pituitary adenoma (33). In the present study, it was hypothesized that NNMT-mediated changes in DNA methylation may be involved in TGF-β1-induced EMT in gastric cancer cells. A previous study demonstrated that the phosphoinositide 3-kinase/protein kinase B signaling pathway exerts a critical function in the NNMT-mediated invasion of renal carcinoma cells (34). It has been revealed that NNMT increased the resistance to 5-fluorouracil partially through suppressing the signal-regulating kinase 1/p38 mitogen-activated protein kinase signaling pathway in colorectal cancer cells (17). Therefore, additional signaling pathways may be involved in the NNMT-mediated migration and invasion of gastric cancer cells and further studies are required to confirm these conclusions.

The results of the present study demonstrated that the overexpression of NNMT increased the expression of TGF-β1 in gastric cancer cells, suggesting that NNMT may activate the TGF-β1/Smad signaling, which in turn promotes EMT. These results suggest that NNMT may be associated with the occurrence and development of gastric tumors. Future studies investigating the clinical and pathological characteristics of NNMT may provide novel predictive markers for the pathological classification and prognosis of cancer.

References


