

Original Article

Nicotinamide N-methyltransferase: a potential biomarker for worse prognosis in gastric carcinoma

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Received January 26, 2016; Accepted February 4, 2016; Epub February 15, 2016; Published March 1, 2016

Abstract: In clinical practice, cancer stage (or grade) and some biomarkers, such as carcinoembryonic antigen (CEA) and CA199, are widely used to predict the prognosis of gastric carcinoma patients. Due to the limited role of prognostic indicators for gastric carcinoma, this condition remains one of the most fatal human malignancies with a dismal prognosis. Nicotinamide N-methyltransferase (NNMT, EC.2.1.1.1), a metabolizing enzyme, is involved in the development and progression of various carcinomas. However, the prognostic and biological functions of NNMT in gastric carcinoma are not yet clear. In the present study, NNMT was found to be overexpressed at the mRNA and protein levels in gastric carcinoma tissues compared with adjacent tissues. Importantly, the survival analysis verified that NNMT expression is an independent prognostic factor for overall survival of gastric cancer patients. Moreover, NNMT expression was related to primary tumor size, lymph node metastasis, distant metastasis, and TNM (tumor, node, and metastasis) stage. We also demonstrated that knockdown of NNMT inhibits cellular proliferation, invasion and migration *in vitro* and *in vivo*. Overall, the results of this study suggest that NNMT is a promising prognostic predictor for gastric cancer patients and could be used as a new target for gastric cancer therapy.

Keywords: Nicotinamide N-methyltransferase, prognosis, gastric carcinoma, tissue microarray

Introduction

Gastric cancer is the second leading cause of cancer-related mortality and the fourth most common cancer globally after lung, breast and colorectal cancers [1]. In East Asia, especially China, gastric cancer accounts for approximately half of all cancers [2]. Delayed detection of the primary tumor, progressive growth, and late detection of metastases hinder the search for a cure for gastric cancer [3]. Currently, reliable predictors for gastric carcinoma are lacking. Douglas Hanahan and Robert A. Weinberg emphasized in their eminent review *Hallmarks of cancer: the next generation* that “Reprogramming energy metabolism could be an emerging hallmark of cancer” [4]. The relationship between metabolism-related enzymes and cancer has become a popular topic of cancer research.

Nicotinamide N-methyltransferase (NNMT), a phase II metabolizing enzyme, mainly catalyzes

the methylation of nicotinamide and other pyridines into pyridinium ions [5]. Most studies of this metabolizing enzyme mainly examined its roles in metabolic diseases, such as diabetes [6] and obesity [7]. In 1984, Seifert, R was the first to prove that alteration of NNMT activity was involved in the development and progression of carcinoma *in vivo* [8]. Numerous subsequent studies demonstrated that NNMT was overexpressed in various cancers and was correlated with poor prognosis [9-16]. Studies have also indicated that NNMT influences the proliferative, migratory, invasive and differentiation profiles of various cancers [17-19]. For example, a previous study suggested that the phosphoinositide 3-kinase (PI3K)/Akt pathway plays a vital role in NNMT-dependent cellular invasion and matrix metalloproteinase 2 (MMP-2) activation in renal carcinoma [20]. Although NNMT expression was reported to be elevated in gastric carcinoma by a former study, the biological function and prognostic role of NNMT in gastric carcinoma still remain unclear [21].

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Table 1. NNMT expression in gastric benign and malignant tissues

Characteristic	NO. NNMT		Pearson χ^2	P-value
	High no.	%		
Gastric tissues			36.490	<0.001*
Chronic gastritis	35	10	28.6	
Intestinal Metaplasia	31	11	35.5	
Low-grade intraepithelial neoplasia	32	12	37.5	
High-grade intraepithelial neoplasia	42	17	40.5	
Carcinoma	641	364	56.8	
Adjacent tissue	94	30	31.9	

*P<0.05.

Table 2. Correlation of high NNMT expression with clinical attributes in gastric cancer

Groups	NO.	NNMT		Pearson χ^2	P-value
		High no.	%		
Gender				3.832	0.061
Male	464	246	53.0		
Female	153	95	62.1		
Age				1.978	0.161
≤60 years	247	128	51.8		
>60 years	370	213	57.6		
Histological type				4.431	0.351
Tubular	512	300	57.6		
Hybrid	7	3	42.9		
Mucinous	28	11	39.3		
Signet ring cell	21	11	52.4		
Others ^a	13	8	61.5		
Differentiation				5.066	0.079
High	32	12	37.5		
Moderate	157	87	55.4		
Low	383	222	58		
Others ^b					
Primary tumor size				33.468	<0.001*
T _{is}	33	17	21.2		
T ₁	62	28	45.2		
T ₂	120	55	45.8		
T ₃	345	214	62		
T ₄	48	34	70.8		
Lymph node metastasis				33.486	<0.001*
N ₀	238	101	42.4		
N ₁	108	60	55.6		
N ₂	117	74	63.2		
N ₃	145	103	71		
Distant metastasis				9.130	0.002*
M ₀	580	312	53.8		

The present study evaluated the role of NNMT as a prognostic determinant by analyzing NNMT expression in a large cohort of well-characterized gastric samples. To further explore the biological role of NNMT in gastric carcinoma, siRNA-mediated silencing was also performed *in vitro* and *in vivo*. Examining whether NNMT inhibition is a possible molecular approach for the treatment of gastric carcinoma has value.

Material and methods

Patient samples

For IHC analysis, 875 formalin-fixed paraffin-embedded (FFPE) tissue samples (35 chronic gastritis, 31 intestinal metaplasia, 32 low-grade intraepithelial neoplasia, 42 high-grade intraepithelial neoplasia, 641 carcinomas, and 94 adjacent tissue samples) from 781 patients were collected. These tissues were obtained from the Department of Pathology, Affiliated Hospital of Nantong University from 2003 to 2010. The details of these tissues and the IHC analysis are presented in **Tables 1-3**. In addition, 38 paired tissues (freshly frozen gastric carcinoma and adjacent tissues from Nanjing Medical University Affiliated Cancer Hospital, Second Affiliated Hospital of Nanjing Medical University and Nanjing Drum Tower Hospital) were also examined by qRT-PCR. Each patient provided written informed consent for the publication of this study, and the research protocol was approved by the Human Research Ethics Committee.

TMA construction and IHC analysis

A tissue microarray system (Quick-Ray, UT06, UNITMA, and

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M ₁	38	30	78.9		
TNM stage				46.134	<0.001*
0	34	9	26.5		
I	119	44	37.0		
II	200	112	56.0		
III	217	143	65.9		
IV	38	30	78.9		
Preoperative CEA, ng/ml				2.517	0.284
≤5	263	136	51.7		
>5	74	44	59.5		
Unknown	281	162	57.7		
Preoperative CA199, U/ml				2.590	0.274
≤37	276	143	51.8		
>37	50	30	60		
Unknown	292	169	57.9		

* $P < 0.05$; ^aothers: papillary adenocarcinoma, 4 cases; adeno-squamous carcinoma, 4 cases; squamous cell carcinoma, 2 cases; undifferentiated carcinoma, 2 cases; neuroendocrine carcinoma, 1 case. ^bothers: tumors other than tubular or papillary adenocarcinomas

Korea) was used as described previously in the Department of Clinical Pathology, Nantong University Hospital, Jiangsu, China [22]. The immunohistochemical analysis was performed as previously described [23]. The IHC analysis to evaluate the protein expression of NNMT in gastric tissues was conducted using anti-NNMT mouse monoclonal antibody (3D8) [NB-P2-00537].

NNMT immunostaining was scored by blinded observers according to the intensity and percentage of positive cells. The staining intensity was scored according to 4 grades: 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (intense staining). The product of the percentage of positive cells and the respective intensity scores was used as the final staining score, with a minimum value of 0 and a maximum value of 300. A cutoff value of 120 was found to be statistically significant using the X-tile software program (the Rimm Lab at Yale University; <http://www.tissuearray.org/rimmlab/>) [24].

RNA preparation, reverse transcription, and real-time quantitative PCR

Total RNA was isolated from tissue samples and cultured cells using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. RNA was reverse transcribed into cDNA using the Prime Script RT reagent Kit (Takara, China). Quantitative real-time PCR was

performed using the SYBR Prime-Script RT-PCR kit (Takara, Japan) and QuantStudio™ 6 Flex Real-Time PCR System. Relative NNMT expression was calculated and was normalized to β -actin using the $2^{-\Delta\Delta Ct}$ method. The primers used in the quantitative real-time PCR analysis are presented in **Table 4**. All experiments were conducted at least three times.

Cell culture and transfection

The gastric carcinoma cell lines MKN28, SGC7901, MGC803 and BGC823 were purchased from American Type Culture Collection (ATCC, Netherlands) and the Cell Bank of the Chinese Academy of Sciences

(Shanghai, China). The cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) medium containing 10% fetal bovine serum (FBS, Gibco) at 37°C in humidified air containing 5% CO₂. Small interfering RNA (siRNA) transfection. NNMT siRNA (si1 and si2) and negative control siRNA were purchased from Gene Pharma (Shanghai, China). Cells (5×10^4 cells/well) were seeded into 6-well plates and were transfected with 10 nM siRNA in phosphate-buffered saline (PBS) using Lipofectamine-2000 (Invitrogen) according to the manufacturer's instructions. The siRNA sequences are presented in **Table 5**.

Western blot assay

The cells were harvested for protein extraction, and the extracted proteins were quantified as previously described using a 12% or 4~20% polyacrylamide gradient sodium dodecyl sulfate (SDS) gel [23]. After the proteins were separated in the gel and transferred to a polyvinylidene difluoride (PVDF) membrane, the membrane was blocked in 2% BSA in TBST for 1 h. Then, the membranes were incubated overnight (4°C) with anti-NNMT mouse monoclonal antibody (3D8) [NB-P2-00537]. After incubation with Alexa Fluor 488-labeled Goat Anti-Mouse IgG (Beyotime Biotechnology, China) at room temperature for 2 h, the relative protein levels were calculated using GAPDH (Abcam, ab18-1602) as a loading control. Each assay was repeated three times.

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Table 3. Univariate and multivariate analyses of prognostic factors for overall survival in gastric cancer

	Univariate analysis			Multivariate analysis		
	HR	p> z	95% CI	HR	p> z	95% CI
NNMT expression						
High vs Low	1.894	<0.001*	1.509-2.378	1.446	0.028*	1.041-2.065
Gender						
Male vs Female	0.930	0.559	0.730-1.185			
Age						
>60 years vs ≤60 years	1.463	0.001*	1.169-1.831	0.434	0.878	0.633-1.217
Histological type						
Tubular vs Hybrid vs Mucinous vs Signet ring cell vs others ^a	0.929	0.257	0.817-1.055			
Differentiation						
High vs moderate vs low vs others ^b	1.397	<0.001*	1.137-1.716	1.106	0.575	0.778-1.572
Primary tumor size						
T ₄ vs T ₃ vs T ₂ and T ₁ vs T _{is}	2.017	<0.001*	1.747-2.329	1.436	0.014*	1.075-1.918
Lymph node metastasis						
N ₃ vs N ₂ vs N ₁ vs N ₀	1.702	<0.001*	1.554-1.865	1.288	0.004*	1.082-1.532
Distant metastasis						
M ₁ vs M ₀	3.745	<0.001*	2.641-5.312	3.195	<0.001*	1.697-6.013
TNM stage						
IV vs III vs II vs I	2.244	<0.001*	1.979-2.545	1.400	0.054	0.995-1.969
Preoperative CEA, ng/ml						
>5 versus ≤5	2.403	<0.001*	1.763-3.274	1.772	0.003*	1.216-2.582
Preoperative CA199, U/ml						
>37 versus ≤37	2.570	<0.001*	1.806-3.649	1.524	0.051	0.998-2.326

*P<0.05; ^aothers: papillary adenocarcinoma, 4 cases; adeno-squamous carcinoma, 4 cases; squamous cell carcinoma, 2 cases; undifferentiated carcinoma, 2 cases; neuroendocrine carcinoma, 1 case. ^bothers: tumors other than tubular or papillary adenocarcinomas.

Table 4. Primer series of NNMT and β-actin gene

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')
NNMT	GAGCAGAAGTTCTCCAGCCT	ACCATTCGATTGTGTAGCCA
β-actin	TGGAGAAAATCTGGCACCAC	GAGGCGTACAGGGATAGCAC

Cell proliferation

The cell proliferation assay was performed using Cell Counting Kit-8 (CCK-8; Dojindo, Tokyo, Japan) at 48 h after transfection. Briefly, 2000 cells were plated per well in 96-well plates and were cultured for 24 h. Then, 10 μl of CCK-8 reagent was added to each well, and the plates were incubated at 37°C for 2 h. The absorbances at 450 nm (A450) and 650 nm (A650) were measured using a Synergy 2 microplate reader (BioTek, Winooski, VT, USA) and were detected in quadruplicate at various time points for 6 days. The final absorbance was calculated as A650 minus A450, and cell viability was normalized using the following formula: (final absorbance treated/final absorbance control) × 100%. All experiments were performed at least three times.

Flow cytometry

The cells were collected and washed three times with PBS and maintained in ethanol for at least 24 h at -20°C. Then, the cells were incubated in propidium iodide (PI) staining solution (RNase A 100 μg/ml and PI 500 μg/ml) for 30 min at 4°C. Next, the cells were analyzed using a FACScan flow cytometer (BD Biosciences), and the cell cycle distributions were calculated using the Cell Quest software package (BD Biosciences) according to the manufacturer's protocol. The percentage of cells in G1, S, and G2 phase were counted and compared. Each assay was repeated three times.

Migration and invasion

The migration and invasion assays were performed using 24-well Transwell chambers (8 mm pores, Millipore, Billerica, MA). For the migration assay, tumor cells were resuspended in serum-free RPMI-1640 medium; 2 × 10⁵ cells were seeded into the upper chambers, and 0.5

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Table 5. Sequences of siRNA for NNMT expression

No.	Gene name	Sequence	
		Sense (5'-3')	Antisense (5'-3')
1	siRNA-homo-791	CCUCGGGAUUACCUAGAAATT	UUUCUAGGUAAUCCCGAGGTT
2	siRNA-homo-846	GCCAGAUUCUUAAGCACCUTT	AGGUGCUUAAGAAUCUGGCTT
3	NC-siRNA	UUCUCGGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

mL RPMI-1640 containing 10% FBS was added to the bottom chambers. Following a 24 h-incubation, the cells on the upper surface of the membrane were scrubbed off, and the migrated cells were fixed with 95% ethanol, stained with 0.1% crystal violet, and counted under a light microscope. The invasion assay protocol was similar to the migration assay protocol, except that the upper chambers were first covered with 1 mg/ml Matrigel. All experiments were conducted at least three times.

Xenograft experiment

MGC803 cells were transfected with sh-NNMT or negative control siRNAs (si1 and siNC, respectively) using Lipofectamine 2000 (Invitrogen). NNMT mRNA and protein levels were determined by qRT-PCR and Western blot, respectively. The cells were collected and injected into either side of the posterior flank of the same BALB/c nude mouse after 48 h. Tumor volume and weight were measured every 2 days. Tumor volume was measured as length \times width² \times 0.5. At 25 days after injection, the mice were sacrificed, and the final tumor weights were measured. All experimental manipulations were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Statistical analysis

All statistical analyses were performed using SPSS 21.0 statistical software (SPSS Inc. Chicago, IL) [25]. The relationships between NNMT expression and clinicopathological attributes were analyzed using the χ^2 test. Survival rates were calculated using the Kaplan-Meier method and were compared by the log rank test. The univariate and multivariate analyses followed the Cox proportional hazards regression model. A *P*-value of less than 0.05 was considered to indicate a statistically significant difference.

Bioinformatics

Level 3 TCGA data (TCGA_STAD_exp_HiSeq-2015-02-24) were used to evaluate the differential expression of NNMT between gastric carcinoma tissues (n=384) and normal tissues (n=37). Data were downloaded from the website of UCSC cancer browser: <http://genome-cancer.ucsc.edu> and were generated using an IlluminaHiSeq platform to examine pathologically diagnosed cancer tissue samples and relative normal tissue samples. The mRNA expression levels of various genes were measured and normalized. NNMT mRNA expression was obtained from the “genomic Matrix” file (using Editplus). Then, the data were grouped into a tumor tissue group and normal tissue group, and the Fisher’s t-test was used to compare the two groups.

The TCGA data was also used for KEGG Pathway enrichment analysis. The biological functions of the genes being examined were determined using the Database for Annotation, Visualization, and Integrated Discovery (DAVID). The pathway analysis using the KEGG Pathway database revealed that the differentially regulated genes were associated with critical biological functions. Analyses were performed to identify the biological functions and pathways of the identified differentially expressed genes (DEGs) based on the hypergeometric distribution algorithm. *P*<0.05 was chosen as the cut-off value for enriched functions and pathways.

Results

Expression of NNMT in gastric tissues

The Cancer Genome Atlas (TCGA) data analysis revealed that NNMT mRNA expression was upregulated in gastric carcinoma tissues compared with normal tissues (*P*<0.001) (**Figure 1A**). To verify those results, NNMT expression was analyzed in 38 paired primary cancerous

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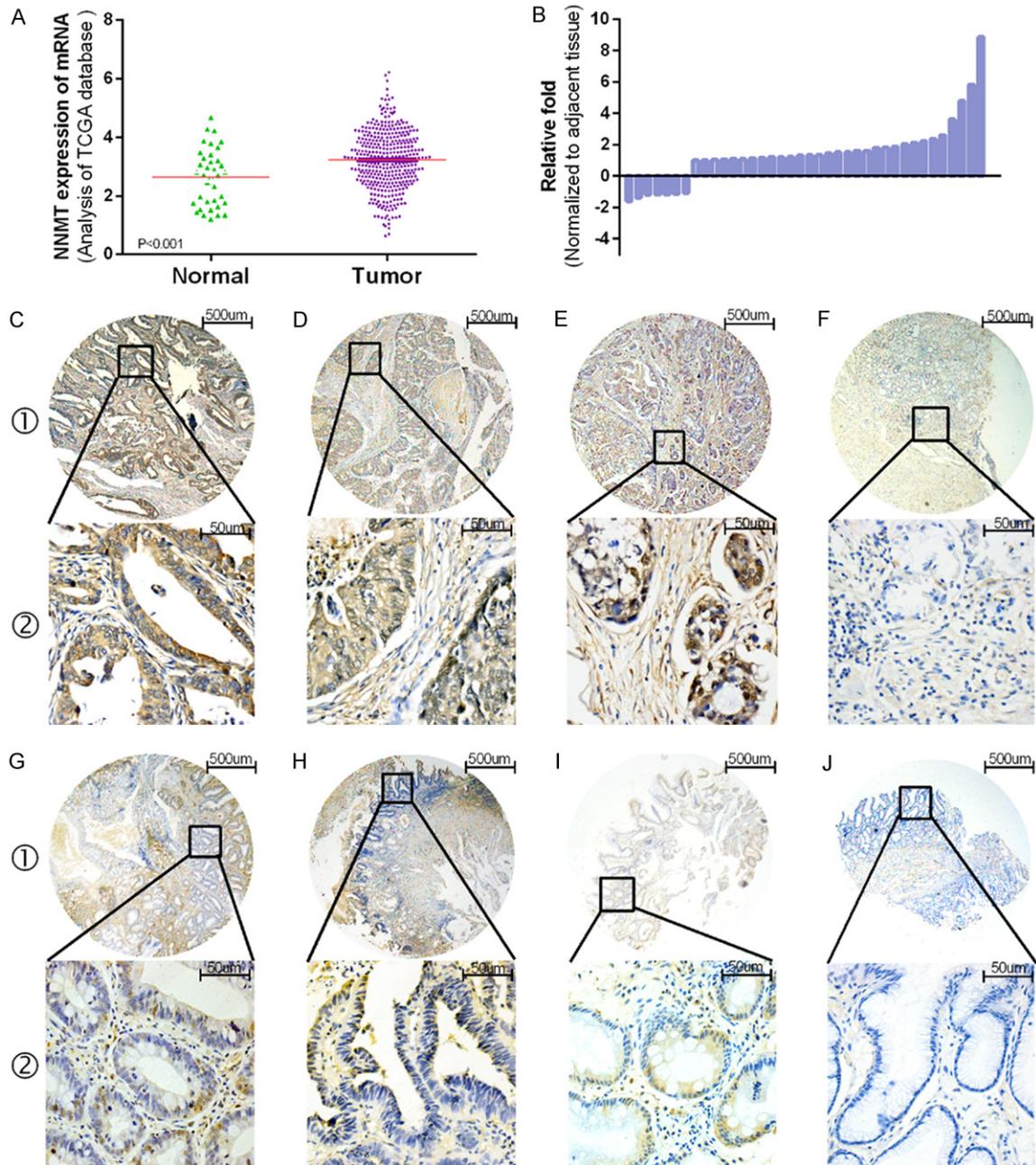


Figure 1. NNMT mRNA and protein expression levels in gastric tissues. (A) TCGA data analysis indicates that the NNMT mRNA level in 384 gastric carcinoma tissues is significantly increased compared with 37 non-cancerous tissues ($P < 0.001$). (B) NNMT was detected in 38 pairs of gastric tissues by qRT-PCR. The level of NNMT in gastric carcinoma tissues was significantly increased compared with non-tumorous tissues ($p = 0.003$). (C) Representative pattern of NNMT protein expression in gastric carcinoma tissues. Strongly positive tumor cytoplasmic immunohistochemical staining of NNMT in gastric carcinoma samples. (D, E) NNMT was expressed in both the cytoplasm and stroma, as indicated by strong IHC staining. (F) Representative non-cancerous tissue sample with low NNMT expression. (G, H, J) Precancerous lesions with low NNMT expression, such as low-grade intraepithelial neoplasia (G1 and G2), high-grade intraepithelial neoplasia (H1 and H2), chronic gastritis (I1 and I2), and intestinal metaplasia (J1 and J2).

and adjacent noncancerous tissues of gastric carcinoma by quantitative real-time polymerase

chain reaction (qRT-PCR). NNMT mRNA was overexpressed in gastric carcinoma tissues,

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with an average upregulation of 1.833-fold ($P=0.003$) (**Figure 1B**), which is consistent with the results of the TCGA data analysis.

Then, we used immunohistochemical staining to examine NNMT protein expression in gastric carcinoma tissues, benign gastric disease tissues and corresponding noncancerous tissues. As presented in **Table 1**, NNMT protein expression was increased in gastric carcinoma tissues compared with benign disease tissues and noncancerous tissues (Pearson $\chi^2=36.490$, $P<0.001$). Also, the progression of precancerous lesions was positively correlated with NNMT expression. **Figure 1C-J** demonstrates that NNMT expression was localized to the tumor cytoplasm in a multitude of immunohistochemistry (IHC) specimens (**Figure 1C**), whereas NNMT was expressed in both the cytoplasm and stroma in the other groups (**Figure 1D, 1E**).

Association of NNMT expression with clinicopathological characteristics in gastric cancers

The relationship between NNMT expression and the clinicopathological attributes of gastric cancer are presented in **Table 2**. High NNMT positive staining within the cytoplasm and interstitium was significantly associated with primary tumor size (Pearson $\chi^2=33.468$, $P<0.001$), lymph node metastasis (Pearson $\chi^2=33.486$, $P<0.001$), distant metastasis (Pearson $\chi^2=9.130$, $P=0.002$) and TNM stage (Pearson $\chi^2=46.134$, $P<0.001$). NNMT expression was not associated with any other clinical parameters, including gender, age, histological type, differentiation, carcinoembryonic antigen (CEA) status and CA199 status.

Prognostic value of NNMT protein expression in gastric cancer

We examined possible prognostic factors for gastric cancer using both univariate and multivariate Cox regression analyses. The univariate analyses showed that increased expression of NNMT, age, differentiation, primary tumor size, lymph node metastasis, distant metastasis, TNM stage, CEA status and CA199 status were associated with the prognoses of gastric carcinoma patients in terms of overall survival rate. The prognostic indicators identified in the univariate analyses were included in the multivariate analyses, which further indicated that NNMT expression was associated with poor

overall survival (hazard ratio (HR), 1.446; $P=0.028$; 95% confidence interval (CI), 1.041-2.065) as was primary tumor size, lymph node metastasis, distant metastasis and CEA status. The detailed findings are presented in **Table 3**. Kaplan-Meier survival curves revealed that low expression levels of NNMT and CEA and early cancer stage were associated with an enhanced rate of survival and that patients positive for the two markers NNMT and CEA had a significantly worse prognosis (**Figure 2**). These Kaplan-Meier survival curves will help us analyze the five-year survival rate of patients with gastric cancer (**Figure 2A, 2B, 2F**).

Knockdown of NNMT inhibits cellular proliferation, invasion and migration in vitro

To better demonstrate the role of NNMT in gastric carcinoma, we first measured the expression of NNMT in four gastric cancer cell lines (MKN28, SGC7901, MGC803 and BGC823). The MGC803 and BGC823 cell lines exhibited increased NNMT expression levels compared with the other two cell lines (**Figure 3A**), and these cell lines were used for NNMT silencing experiments conducted using two siRNA molecules (**Table 5**). The NNMT mRNA and protein levels were analyzed to determine the specific effects of siRNA treatment on NNMT expression. Compared with the negative control, both NNMT mRNA and protein levels were significantly reduced in the siRNA groups. NNMT mRNA expression was approximately 4-fold reduced in the siRNA groups compared with the negative control group (**Figure 3B**).

Induction of cell cycle arrest is an important mechanism for controlling tumor growth. We assessed the role of NNMT in regulating gastric carcinoma cell cycle progression to determine the effect of NNMT on gastric carcinoma cell growth. In MGC803 cells, NNMT downregulation caused a significant increase in the percentage of G2 phase cells and a concomitant decrease in the percentage of S phase cells, thus indicating the occurrence of G2 cell cycle arrest (**Figure 3D**). CCK-8 assays in MGC803 and BGC823 cell lines revealed that knockdown of NNMT reduced cell proliferation compared with the negative controls (**Figure 3C**).

To investigate the effect of NNMT on migration and invasion, two specific small interference RNAs, si1 and si2, and a negative control (si-NC) were transfected into MGC803 and

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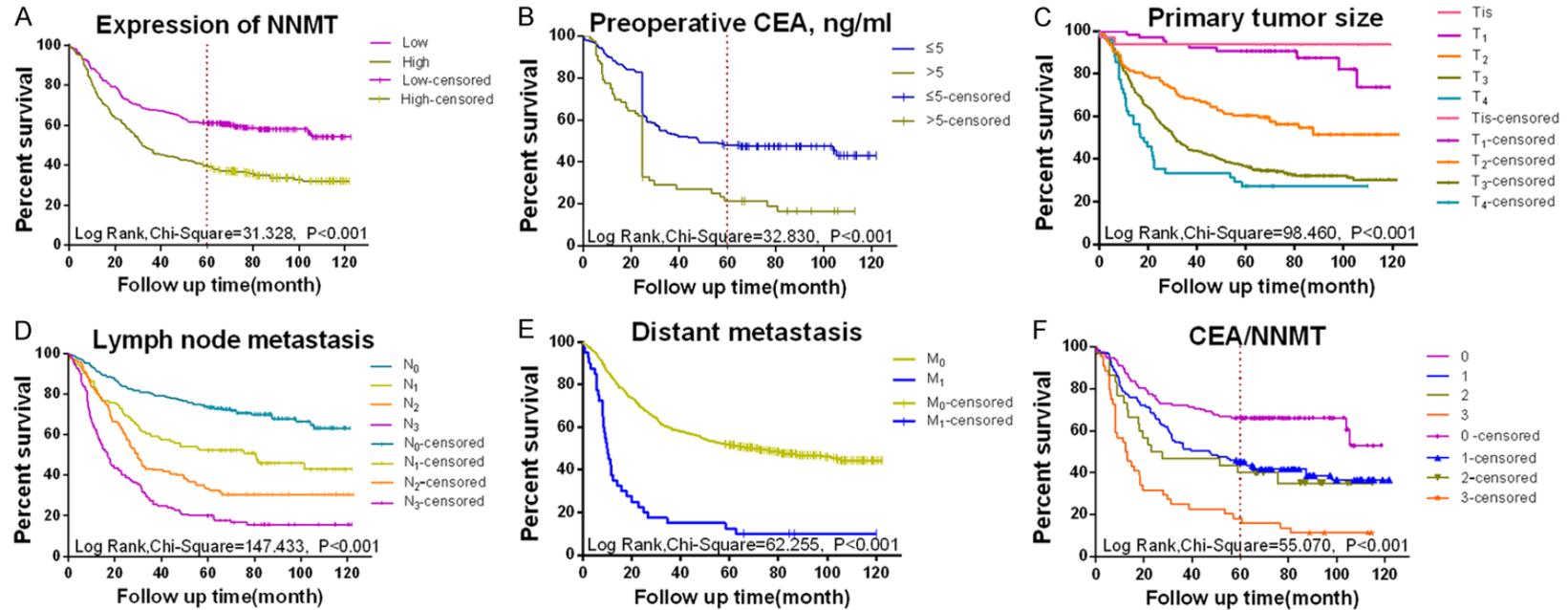


Figure 2. Survival curves of gastric carcinoma generated by the Kaplan-Meier method and the log-rank test. A. Overall survival curves of NNMT+ (green line, High) and NNMT- tissue samples (purple line, Low). B. Overall survival curves by primary tumor size: Tis (pink line, Tis), T1 (purple line, T1), T2 (orange line, T2), T3 (green line, T3) and T4 (blue line, T4). C. Overall survival curves by stage of lymph node metastasis: node-negative (blue line, N0), L1 (green line, L1), L2 (orange line, L2), and L3 (purple line, L3). D. Overall survival curves by distant metastasis: negative (green line, M0) and positive (light blue line, M1). E. Overall survival curves by preoperative CEA status: high (green line, ≤ 5) and low (blue line, >5). F. Overall survival curves by CEA and NNMT status: CEA-/NNMT- (purple line, 0), CEA-/NNMT+ (blue line, 2), CEA+/NNMT- (green line, 3) and CEA+/NNMT+ (orange line, 4).

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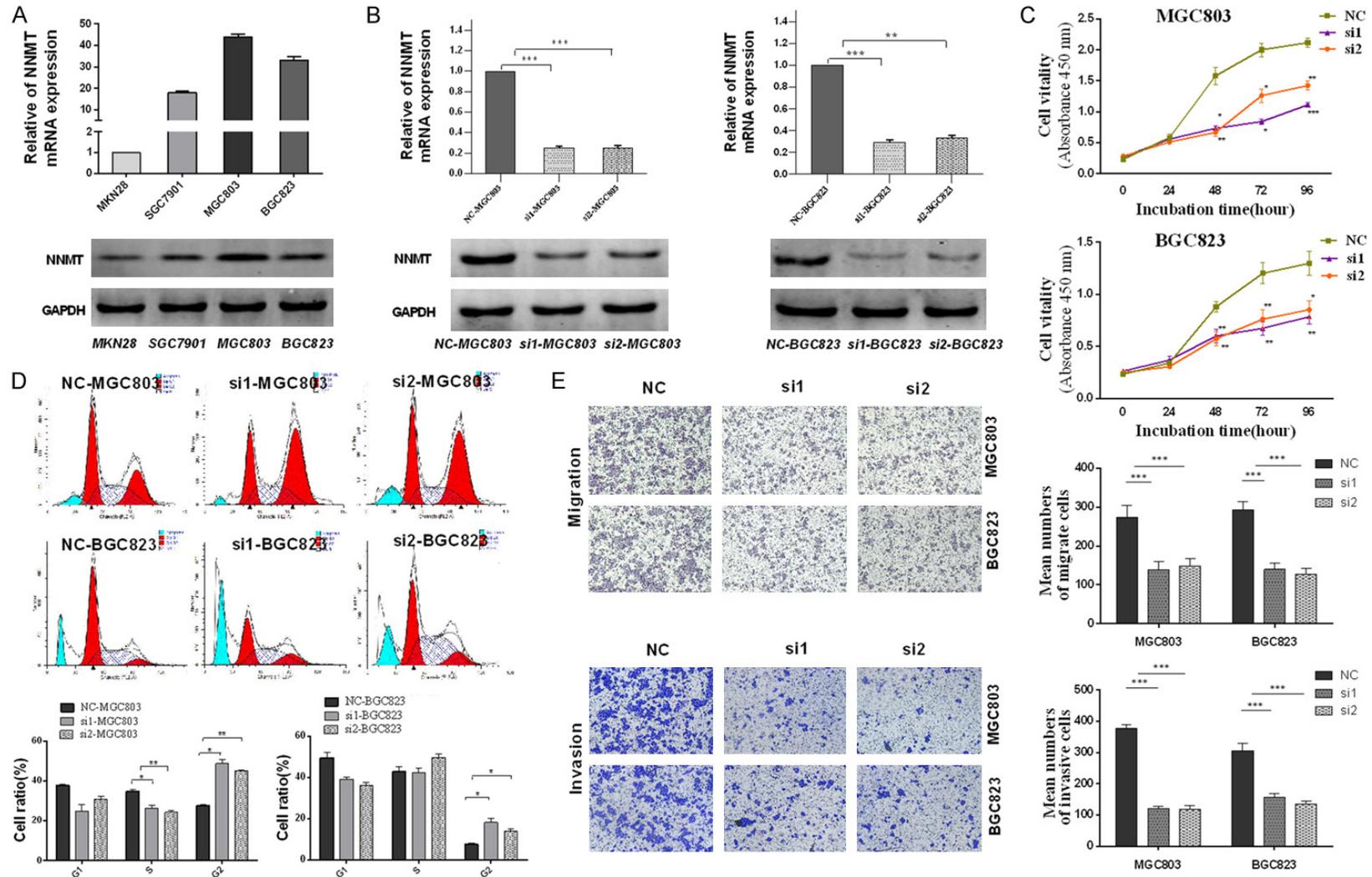


Figure 3. Knockdown of NNMT inhibited the proliferation, migration and invasion of gastric carcinoma cell lines in vitro. A. NNMT expression in MKN28, SGC7901, MGC803, and BGC823 cell lines. β -actin was used as the internal control in qRT-PCR, and GAPDH was used as the internal control in Western blot. B. In MGC803 and BGC823 cells, NNMT mRNA and protein levels were significantly reduced after transfection with NNMT siRNA (si1 and si2). C. NNMT promotes proliferation of gastric carcinoma cells. Cell proliferation assay using the CCK-8 kit: compared with negative control (NC), siRNA-mediated silencing of NNMT significantly inhibited cell proliferation in MGC803 and BGC823 cell lines. D. NNMT alters the cell cycle of gastric carcinoma cell lines. MGC803 and BGC823 cells transfected with NC (siRNA negative control) or NNMT siRNA (si1 and si2). Thirty-six hours after transfection, cells were stained and analyzed by flow cytometry. The cell cycle was arrested at the G2 phase. E. NNMT promotes migration of gastric carcinoma cell lines (MGC803 and BGC823). Compared with negative control (NC), cells transfected with NNMT siRNA (si1 and si2) showed weaker migratory and invasive abilities.

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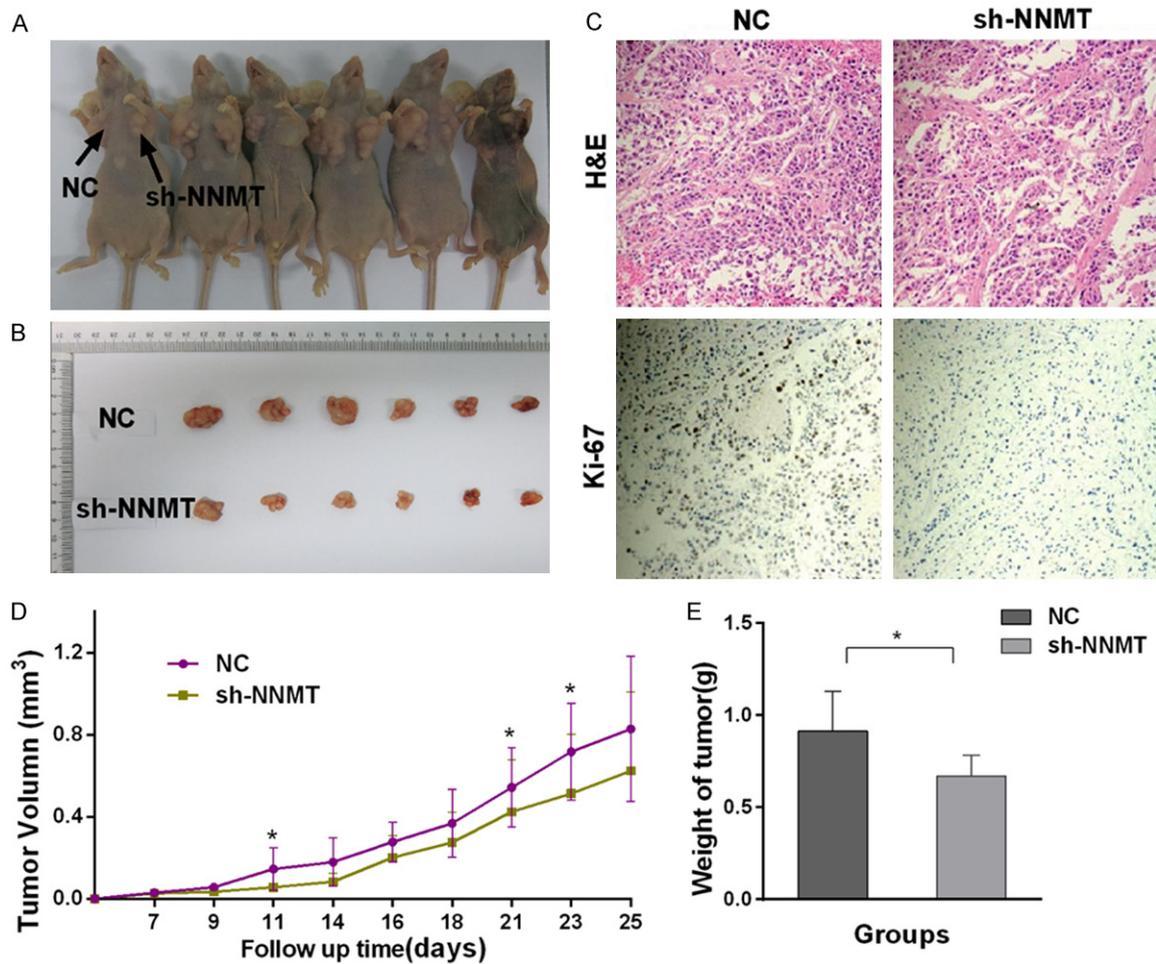


Figure 4. Knockdown of NNMT inhibited the proliferation of gastric carcinoma cell line in vivo. A, B. MGC803 cells were transfected with either Si-NNMT or sh-NC. The injection sites are indicated by arrows. D, E. The xenograft tumor weight and volume in the sh-NNMT group were significantly reduced compared with the sh-NC group. C. IHC staining was performed on xenograft tumors, and the Ki-67 staining signal was weaker in the sh-NNMT group compared with the sh-NC group. (Single asterisk indicates $P < 0.05$; double asterisks indicate $P < 0.01$; triple asterisks indicate $p < 0.001$; error bars indicate means \pm SEM).

BGC823 cells. Transwell migration assays indicated that NNMT knockdown led to a significant decrease in cell migration in MGC803 and BGC823 cell lines. To further assess the role of NNMT in the pathogenesis of gastric carcinoma, we also assessed whether NNMT knockdown inhibits invasion in MGC803 and BGC823 cell lines. Consistent with the previous results, knockdown of NNMT inhibited invasion in both cell lines (Figure 3E).

Knockdown of NNMT inhibited cell proliferation in vivo

To test whether NNMT regulates cell proliferation *in vivo*, we established xenograft tumor models in nude mice using MGC803 cells transfected with sh-NC or sh-NNMT. All nude

mice developed xenograft tumors at the injection site, and the xenograft tumors were harvested 7 days after injection. The average tumor volume and weight in the sh-NNMT group was significantly reduced compared with the negative control group (Figure 4D, 4E). And the IHC staining was performed on xenograft tumors, and the Ki-67 staining signal was weaker in the sh-NNMT group compared with the sh-NC group (Figure 4C). These data demonstrate that NNMT may inhibit tumor growth *in vivo*.

KEGG pathway enrichment analysis

A Kyoto Encyclopedia of Genes and Genome (KEGG) Pathway enrichment analysis of the resulting gene signature was performed. The

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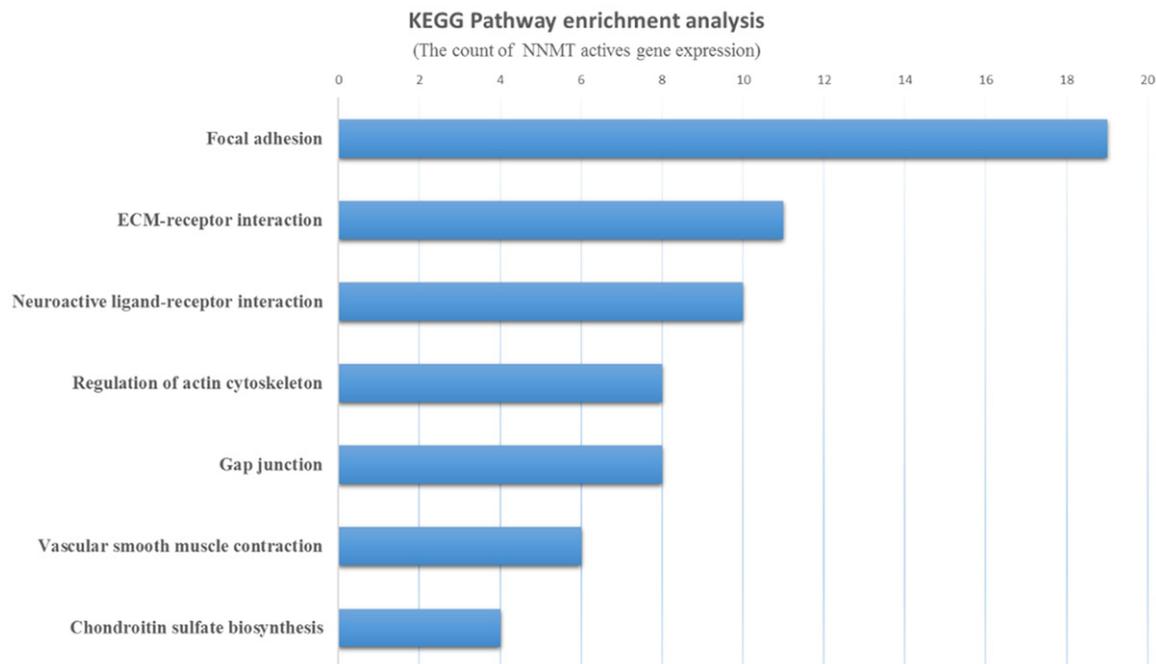


Figure 5. Kyoto Encyclopedia of Genes and Genome (KEGG) Pathway enrichment analysis. The vertical axis displays the KEGG annotation corresponding to the seven categories. The horizontal axis displays the number of significant genes corresponding to each functional type.

most significant five KEGG terms for NNMT are presented in **Figure 5** and include focal adhesion, ECM-receptor interaction, neuroactive ligand-receptor interaction, regulation of actin cytoskeleton, gap junction, vascular smooth muscle contraction and chondroitin sulfate biosynthesis.

Discussion

“Altered energy metabolism is proving to be as widespread in cancer cells as many of the other cancer-associated traits that have been accepted as hallmarks of cancer” [4]. NNMT, a metabolic enzyme, has attracted great attention. NNMT is normally expressed in adipose and liver tissue and participates in biotransformation of many drugs and xenobiotic compounds [7]. Recent studies have reported that NNMT is involved in the progress of cancers as an oncogene. Among the literature we reviewed, one of the most thoroughly explored relationships was that between NNMT and colorectal cancer. Researchers have reported that NNMT plays an important role in colorectal cell proliferation, differentiation, and apoptosis and that the diagnostic value of NNMT appears to be at least comparable to that of the typical biomarker CEA [5, 26]. Both gastric and colorectal car-

cinoma originate from the digestive system, and they share many similar characteristics, such as a similar histological structure and route of metastasis. Based on these similarities, similar diagnostic and even treatment strategies, such as CEA, may be utilized for these two cancer types.

To develop rational and specific approaches for early diagnosis and treatment, this study explored the relationship between NNMT and gastric carcinoma. Cancer stage (or grade) and some biomarkers, such as CEA and CA199, are used to predict gastric carcinoma prognosis clinically [27]. To facilitate prognostic studies, we previously collected a considerable amount of specimens with relevant clinical parameters (including survival parameters) [28].

In our study, similar to a previous report [21], NNMT mRNA and protein levels were both up-regulated in gastric carcinoma compared with normal tissues, and the TCGA database indicated a similar tendency. Previous studies reported that NNMT was overexpressed in several cancer types. Using serum tests, the sensitivity of NNMT was increased compared with CEA (ROC=0.84 vs. ROC=0.79) in colorectal carcinoma patients [26]. A recent study also

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found that NNMT was elevated in urine [29]. Consistent with the aforementioned results, we not only found that NNMT was overexpressed in gastric carcinoma tissues but also that increased NNMT level was associated with the aggravation of precancerous lesions. This finding has rarely been reported previously. Considering our relatively large sample size, this finding may help clinicians make better treatment decisions and eventually improve patient prognosis.

Similar to CEA, to a certain extent, the prognostic value of NNMT was very significant. In recent decades, studies have suggested that NNMT overexpression predicts poor prognosis in various carcinomas. For example, a previous study reported that NNMT mRNA upregulation was correlated with worse OS in bladder carcinoma [30]. Another study reported that NNMT protein expression was a prognostic factor in renal carcinoma, but the sample size of that study was relatively small (only 46) [11]. In our study, gastric carcinoma patients with increased NNMT expression had worse survival in both univariate and multivariate analyses, indicating that NNMT is a possible prognostic indicator for gastric carcinoma. A more specific analysis revealed that patients positive for two markers, NNMT and CEA, had a significantly worse prognosis than those who were positive for one or neither of the two markers. The 5-year survival rates for patients with different NNMT and CEA expression levels ranged from 75% (NNMT+/CEA+) to 15% (NNMT-/CEA-), suggesting that a more accurate clinical diagnosis can be obtained by combining these two markers.

Our tissue microarray (TMA) data showed that elevated NNMT expression was associated with primary tumor size, lymph node metastasis, and distant metastasis and TNM stage. However, due to the specificity of the tumors, NNMT expression and the clinical profiles of the gastric cancer patients examined in this study differed from those of other cancers. For example, NNMT expression is associated with cancer cell differentiation but not with clinical stage in colorectal carcinoma. Also, an oral tongue squamous cell carcinoma study indicated that overexpression of NNMT was associated with smaller tumor size and lower TNM stage [31].

To further understand the biological role of NNMT in gastric carcinoma, we also performed

NNMT knockdown studies and found that NNMT knockdown inhibits cell proliferation, invasion and migration both *in vitro* and *in vivo*.

NNMT knockdown caused cell cycle arrest at the G2-phase. NNMT may promote proliferation of gastric carcinoma cells by altering the cell cycle. Other studies also reported that NNMT expression was correlated with cancer cell proliferation [16, 32]. However, not all previous research findings are consistent with our data. For example, a previous study found that overexpression of NNMT reduced the proportion of cells in the G1 phase and simultaneously increased the proportion of cells in the G2 and S phases [5]. We found that NNMT depletion can also inhibit cell migration and invasion. We also observed abnormal NNMT expression in mesenchymal tissues, indicating that NNMT may be involved in the epithelial-mesenchymal transition (EMT). NNMT was closely associated with other important members of molecular pathways, such as SHH, PI3k/Akt and stat3 [33-36]. Moreover, KEGG pathway analysis indicated that the genes that are co-expressed with NNMT are mainly involved in focal adhesion and extracellular matrix (ECM)-receptor interaction pathways, which are important for mechanical strength and participate in EMT [37]. Based on those findings, we conclude that NNMT plays a vital role in gastric cancer progression.

NNMT overexpression is associated with chemotherapy and radiotherapy resistance. Previous studies showed that NNMT may serve as a drug-resistance gene for Taxol, Erlotinib, Everolimus and Dasatinib chemotherapies [38-40]. Thus, targeting this drug-resistant gene will help obtain a better chemotherapy outcome, and this technique serves as a novel chemotherapy approach. Previous studies previously demonstrated that anti-NNMT agents potentially have therapeutic effects in cancer [15]. However, given that NNMT is not a membrane-bound protein, the therapeutic effect of anti-NNMT antibodies is limited. However, with the development of siRNA *in vivo* transfection, NNMT may have a great impact on cancer therapy in the future.

Overall, NNMT is a promising molecular biomarker that may predict the progression of precancerous lesions and patient survival in gastric carcinoma. Furthermore, NNMT may serve as a therapeutic target in the future. Further

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experiments are necessary to confirm whether NNMT does indeed act as tumor suppressor in gastric carcinoma. Studies of the mechanisms of the involvement of NNMT in gastric carcinoma will need to be conducted.

Acknowledgements

This work was supported by the Major Scientific and Technological Special Project for “significant new drugs creation” (No. 2012ZX0910-3301-040), the Innovative Team of Jiangsu Province (No. LJ201126) and the National Natural Science Foundation (No. 81572390).

Disclosure of conflict of interest

None.

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References

- [1] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2015. *CA Cancer J Clin* 2015; 65: 5-29.
- [2] Jiang L, Wang C, Sun C, Xu Y, Ding Z, Zhang X, Huang J and Yu H. The impact of pri-miR-218 rs11134527 on the risk and prognosis of patients with esophageal squamous cell carcinoma. *Int J Clin Exp Pathol* 2014; 7: 6206-6212.
- [3] Yamamoto M, Rashid OM and Wong J. Surgical management of gastric cancer: the East vs. West perspective. *J Gastrointest Oncol* 2015; 6: 79-88.
- [4] Hanahan D and Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; 144: 646-674.
- [5] Xie X, Yu H, Wang Y, Zhou Y, Li G, Ruan Z, Li F, Wang X, Liu H and Zhang J. Nicotinamide N-methyltransferase enhances the capacity of tumorigenesis associated with the promotion of cell cycle progression in human colorectal cancer cells. *Arch Biochem Biophys* 2014; 564: 52-66.
- [6] Kannt A, Pfenninger A, Teichert L, Tonjes A, Dietrich A, Schon MR, Kloting N and Bluher M. Association of nicotinamide-N-methyltransferase mRNA expression in human adipose tissue and the plasma concentration of its product, 1-methylnicotinamide, with insulin resistance. *Diabetologia* 2015; 58: 799-808.
- [7] Kraus D, Yang Q, Kong D, Banks AS, Zhang L, Rodgers JT, Pirinen E, Pulitilkunnit TC, Gong F, Wang YC, Cen Y, Sauve AA, Asara JM, Peroni OD, Monia BP, Bhanot S, Alhonen L, Puigserver P and Kahn BB. Nicotinamide N-methyltransferase knockdown protects against diet-induced obesity. *Nature* 2014; 508: 258-262.
- [8] Seifert R, Hoshino J and Kroger H. Nicotinamide methylation. Tissue distribution, developmental and neoplastic changes. *Biochim Biophys Acta* 1984; 801: 259-264.
- [9] Xu J, Capezzone M, Xu X and Hershman JM. Activation of nicotinamide N-methyltransferase gene promoter by hepatocyte nuclear factor-1beta in human papillary thyroid cancer cells. *Mol Endocrinol* 2005; 19: 527-539.
- [10] Xu J and Hershman JM. Histone deacetylase inhibitor depsipeptide represses nicotinamide N-methyltransferase and hepatocyte nuclear factor-1beta gene expression in human papillary thyroid cancer cells. *Thyroid* 2006; 16: 151-160.
- [11] Yao M, Tabuchi H, Nagashima Y, Baba M, Nakaigawa N, Ishiguro H, Hamada K, Inayama Y, Kishida T, Hattori K, Yamada-Okabe H and Kubota Y. Gene expression analysis of renal carcinoma: adipose differentiation-related protein as a potential diagnostic and prognostic biomarker for clear-cell renal carcinoma. *J Pathol* 2005; 205: 377-387.
- [12] Zhang J, Xie XY, Yang SW, Wang J and He C. Nicotinamide N-methyltransferase protein expression in renal cell cancer. *J Zhejiang Univ Sci B* 2010; 11: 136-143.
- [13] Sartini D, Pozzi V, Renzi E, Morganti S, Rocchetti R, Rubini C, Santarelli A, Lo Muzio L and Emanuelli M. Analysis of tissue and salivary nicotinamide N-methyltransferase in oral squamous cell carcinoma: basis for the development of a noninvasive diagnostic test for early-stage disease. *Biol Chem* 2012; 393: 505-511.
- [14] Jiang Q, Yu YC, Ding XJ, Luo Y and Ruan H. Bioinformatics analysis reveals significant genes and pathways to target for oral squamous cell carcinoma. *Asian Pac J Cancer Prev* 2014; 15: 2273-2278.
- [15] Sartini D, Morganti S, Guidi E, Rubini C, Zizzi A, Giuliani R, Pozzi V and Emanuelli M. Nicotinamide N-methyltransferase in non-small cell lung cancer: promising results for targeted anti-cancer therapy. *Cell Biochem Biophys* 2013; 67: 865-873.
- [16] Sartini D, Seta R, Pozzi V, Morganti S, Rubini C, Zizzi A, Tomasetti M, Santarelli L and Emanuelli M. Role of nicotinamide N-methyltransferase in non-small cell lung cancer: in vitro effect

Prognosis values of NNMT in gastric carcinoma

- of shRNA-mediated gene silencing on tumorigenicity. *Biol Chem* 2015; 396: 225-234.
- [17] Bi HC, Pan YZ, Qiu JX, Krausz KW, Li F, Johnson CH, Jiang CT, Gonzalez FJ and Yu AM. N-methylnicotinamide and nicotinamide N-methyltransferase are associated with microRNA-1291-altered pancreatic carcinoma cell metabolome and suppressed tumorigenesis. *Carcinogenesis* 2014; 35: 2264-2272.
- [18] Yu T, Wang YT, Chen P, Li YH, Chen YX, Zeng H, Yu AM, Huang M and Bi HC. Effects of nicotinamide N-methyltransferase on PANC-1 cells proliferation, metastatic potential and survival under metabolic stress. *Cell Physiol Biochem* 2015; 35: 710-721.
- [19] Thomas MG, Saldanha M, Mistry RJ, Dexter DT, Ramsden DB and Parsons RB. Nicotinamide N-methyltransferase expression in SH-SY5Y neuroblastoma and N27 mesencephalic neurones induces changes in cell morphology via ephrin-B2 and Akt signalling. *Cell Death Dis* 2013; 4: e669.
- [20] Tang SW, Yang TC, Lin WC, Chang WH, Wang CC, Lai MK and Lin JY. Nicotinamide N-methyltransferase induces cellular invasion through activating matrix metalloproteinase-2 expression in clear cell renal cell carcinoma cells. *Carcinogenesis* 2011; 32: 138-145.
- [21] Lim BH, Cho BI, Kim YN, Kim JW, Park ST and Lee CW. Overexpression of nicotinamide N-methyltransferase in gastric cancer tissues and its potential post-translational modification. *Exp Mol Med* 2006; 38: 455-465.
- [22] Sun R, Wang X, Zhu H, Mei H, Wang W, Zhang S and Huang J. Prognostic value of LAMP3 and TP53 overexpression in benign and malignant gastrointestinal tissues. *Oncotarget* 2014; 5: 12398-12409.
- [23] Huang J, Zhang X, Tang Q, Zhang F, Li Y, Feng Z and Zhu J. Prognostic significance and potential therapeutic target of VEGFR2 in hepatocellular carcinoma. *J Clin Pathol* 2011; 64: 343-348.
- [24] Lu C, Wang X, Zhu H, Feng J, Ni S and Huang J. Over-expression of ROR2 and Wnt5a cooperatively correlates with unfavorable prognosis in patients with non-small cell lung cancer. *Oncotarget* 2015; 6: 24912-21.
- [25] Tafisiri E, Darbouy M, Shadmehr MB, Zagryazhskaya A, Alizadeh J and Karimipour M. Expression of miRNAs in non-small-cell lung carcinomas and their association with clinicopathological features. *Tumour Biol* 2015; 36: 1603-1612.
- [26] Roessler M, Rollinger W, Palme S, Hagmann ML, Berndt P, Engel AM, Schneidinger B, Pfeiffer M, Andres H, Karl J, Bodenmuller H, Ruschoff J, Henkel T, Rohr G, Rossol S, Rosch W, Langen H, Zolg W and Tacke M. Identification of nicotinamide N-methyltransferase as a novel serum tumor marker for colorectal cancer. *Clin Cancer Res* 2005; 11: 6550-6557.
- [27] Jing JX, Wang Y, Xu XQ, Sun T, Tian BG, Du LL, Zhao XW and Han CZ. Tumor markers for diagnosis, monitoring of recurrence and prognosis in patients with upper gastrointestinal tract cancer. *Asian Pac J Cancer Prev* 2014; 15: 10267-10272.
- [28] Zhao H, Zhu H, Jin Q, Zhang S, Wang W, Wang D and Huang J. Association of high expression of Grobeta with clinical and pathological characteristics of unfavorable prognosis in gastrointestinal stromal tumors. *Dis Markers* 2015; 2015: 171035.
- [29] Sartini D, Muzzonigro G, Milanese G, Pozzi V, Vici A, Morganti S, Rossi V, Mazzucchelli R, Montironi R and Emanuelli M. Upregulation of tissue and urinary nicotinamide N-methyltransferase in bladder cancer: potential for the development of a urine-based diagnostic test. *Cell Biochem Biophys* 2013; 65: 473-483.
- [30] Riestler M, Taylor JM, Feifer A, Koppie T, Rosenberg JE, Downey RJ, Bochner BH and Michor F. Combination of a novel gene expression signature with a clinical nomogram improves the prediction of survival in high-risk bladder cancer. *Clin Cancer Res* 2012; 18: 1323-1333.
- [31] Emanuelli M, Santarelli A, Sartini D, Ciavarella D, Rossi V, Pozzi V, Rubini C and Lo Muzio L. Nicotinamide N-Methyltransferase upregulation correlates with tumour differentiation in oral squamous cell carcinoma. *Histol Histopathol* 2010; 25: 15-20.
- [32] Chakrabarti S, Multani S, Dabholkar J and Saranath D. Whole genome expression profiling in chewing-tobacco-associated oral cancers: a pilot study. *Med Oncol* 2015; 32: 60.
- [33] Feldmann G, Habbe N, Dhara S, Bisht S, Alvarez H, Fendrich V, Beaty R, Mullendore M, Kari-kari C, Bardeesy N, Ouellette MM, Yu W and Maitra A. Hedgehog inhibition prolongs survival in a genetically engineered mouse model of pancreatic cancer. *Gut* 2008; 57: 1420-1430.
- [34] Teng PN, Hood BL, Sun M, Dhir R and Conrads TP. Differential proteomic analysis of renal cell carcinoma tissue interstitial fluid. *J Proteome Res* 2011; 10: 1333-1342.
- [35] Su Kim D, Choi YD, Moon M, Kang S, Lim JB, Kim KM, Park KM and Cho NH. Composite three-marker assay for early detection of kidney cancer. *Cancer Epidemiol Biomarkers Prev* 2013; 22: 390-398.
- [36] Zhang J, Wang Y, Li G, Yu H and Xie X. Downregulation of nicotinamide N-methyltransferase induces apoptosis in human breast cancer cells via the mitochondria-mediated pathway. *PLoS One* 2014; 9: e89202.
- [37] Liu CY, Lin HH, Tang MJ and Wang YK. Vimentin contributes to epithelial-mesenchymal transition cancer cell mechanics by mediating cyto-

Prognosis values of NNMT in gastric carcinoma

- skeletal organization and focal adhesion maturation. *Oncotarget* 2015; 6: 15966-15983.
- [38] Hsu YC, Chen HY, Yuan S, Yu SL, Lin CH, Wu G, Yang PC and Li KC. Genome-wide analysis of three-way interplay among gene expression, cancer cell invasion and anti-cancer compound sensitivity. *BMC Med* 2013; 11: 106.
- [39] Ujiiie H, Tomida M, Akiyama H, Nakajima Y, Okada D, Yoshino N, Takiguchi Y and Tanzawa H. Serum hepatocyte growth factor and interleukin-6 are effective prognostic markers for non-small cell lung cancer. *Anticancer Res* 2012; 32: 3251-3258.
- [40] D'Andrea FP. Intrinsic radiation resistance of mesenchymal cancer stem cells and implications for treatment response in a murine sarcoma model. *Dan Med J* 2012; 59: B4388.