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Accumulation of Nicotinamide N-Methyltransferase (NNMT) in Cancer-associated Fibroblasts: A Potential Prognostic and Predictive Biomarker for Gastric Carcinoma

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Summary

Nicotinamide *N*-methyltransferase (NNMT), a major metabolic regulator, has been identified as a predictor of cancer prognosis in ovarian and colorectal cancers. The study aims to evaluate the significance of stromal NNMT in gastric cancer (GC). Expression of stromal NNMT in 612 GC and 92 non-malignant tissues specimens was investigated by immunohistochemistry (IHC). The association between NNMT expression and occurrence of cancer or patient outcome was further analyzed, and the factors contributing to disease prognosis were evaluated by multiple Cox models. Stromal NNMT expression was higher in the malignant tissue ($p<0.001$). NNMT expression was significantly associated with GC stage ($p=0.006$). Compared to stromal "NNMT-low" cases, "NNMT-high" cases has lower disease-specific survival (hazard ratio [HR], 2.356; 95% confidence interval [CI] = 1.591–3.488; $p<0.001$) and disease-free survival (HR = 2.265; 95% CI = 1.529–3.354; $p<0.001$), as observed by multivariate Cox analysis after adjusting for stromal NNMT expression with other factors such as tumor grade and size. Notably, patients with stage II NNMT-low GC might be negatively affected by adjuvant chemotherapy, but lower stromal NNMT expression predicted a more favorable prognosis for GC. Our study confirmed that stromal NNMT expression is significantly increased in GC, which predicts an unfavorable post-operative prognosis for GC. (J Histochem Cytochem 69: 165–176, 2021)

Keywords

cancer-associated fibroblast, chemotherapy, gastric cancer, immunohistochemistry, NNMT, prognosis

Introduction

Gastric cancer (GC) is the fourth most common reason of cancer-related deaths worldwide, with high incidence and mortality rates in Asia, especially China.[1–3] GC is usually diagnosed during later stages; most patients die due to metastasis or disease recurrence after surgical excision. Chemotherapy resistance also contributes to the high mortality associated with this disease.[4,5] However, the underlying molecular mechanism is unknown, just like the biomarkers for

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prognosis and prediction of chemotherapy resistance. Therefore, it is important to identify new markers to indicate the prognosis of GC.[6]

Stromal cells surrounding cancer cells have been identified as important contributors for cancer development and progression; they constitute a microenvironment composed of different cells and matrix components. Among all cells in the microenvironment, cancer-associated fibroblasts (CAFs) secrete many molecules and serve as an organization for the interactions among different cells,[7–9] which support the growth of malignant cells. CAFs also release cancer-related factors to directly induce the proliferation and invasion of GC cells.[5,10–13] Therefore, CAFs have become a critical target in cancer diagnosis and treatment. However, the heterogeneity of CAFs in cancer has been reported in several publications.[14] Different subpopulations of CAFs might have different functions, including suppression of cancer progression.[14] Therefore, it is necessary that new subpopulations need to be explored for potential applications.

Nicotinamide *N*-methyltransferase (NNMT) catalyzes the methylation of pyridine compounds by using S-5′-adenosyl-L-methionine (SAM), which results in attenuation of histone methylation in multiple tissues.[15,16] NNMT is involved in the depletion of methyl donors and production of active metabolites, which regulate various metabolic pathways in body tissues such as hepatic tissue as well as in malignant cells.[17,18] Abnormal expression of NNMT is associated with malignancy and prognosis for different types of cancers, including GC.[19–23] However, the previous study primarily emphasized on the significance of NNMT in malignant cells and neglected the stromal compartments. Recently, stromal NNMT has been reported as a key regulator of CAFs in ovarian cancer;[24] the clinical significance of stromal NNMT in ovarian and colorectal cancers was also investigated. Until date, the significance of stromal NNMT expression in GC is unknown, although NNMT expression in cancer cells has been evaluated.[25–27]

Based on these findings, we performed tissue microarray (TMA) for the immunohistochemical analysis of NNMT, the NNMT represented by CAFs in tissue samples from GC patients. To further analyze whether stromal NNMT expressed in GC tissue is associated with the clinicopathological features of patients and whether the prognosis of GC patients is concerned with stromal NNMT.

Materials and Methods

Patients

Malignant and non-malignant, formalin-fixed, paraffin-embedded (FFPE) tissue samples were collected from 612 patients with GC, who underwent surgical excision at the first Affiliated Hospital, Second Military Medical University from December 2006 to July 2011. We excluded patients with autoimmune diseases and adjuvant chemotherapy or radiation therapy before surgery, all tissue specimens collected were prior to adjuvant chemotherapy. The characteristics of these patients are listed in Table 1, including age, gender, tumor size, differentiation grade, TNM stage (according to the American Joint Committee on Cancer Staging Manual, 7th edition), adjuvant chemotherapy, serum carcinoembryonic antigen and CA199 levels, epithelial NNMT, and Lauren's histologic type. Outcome follow-up was performed annually or biannually. The period (months) from the surgery until tumor recurrence or metastasis was defined as "disease-free survival" (DFS). The period (months) from surgery until death due to GC was defined "disease-specific survival" (DSS). All patients provided written informed consent. This study was ratified by the Institutional Review Board of the first Affiliated Hospital, Second Military Medical University.

Immunohistochemistry (IHC)

TMAs from FFPE blocks containing 612 malignant, 92 non-malignant tissues including 23 cases that had undergone laparoscopic sleeve gastrectomy (LSG) for weight loss (Supplementary Fig. 2) were commercially constructed by Outdo Biotech Company, Shanghai, for IHC-based analysis of NNMT. Details of TMA construction were presented in a previous study.[28] Briefly, all array slides were dewaxed with xylene and rehydrated with graded ethanol. Antigen retrieval was performed by immersing the slides in sodium citrate (pH, 6.0) and boiling in a pressure cooker for 30 min. Then, these slides were immersed in 3% H_2O_2 for 5 min to inhibit endogenous peroxides, and then incubated with a rabbit anti-human polyclonal antibody to NNMT (1:800, HPA059180; Sigma-Aldrich Co., St Louis, MO), overnight at C, according to the manufacturer's specifications. Antibody's specificity and corresponding information were published at The Human Protein Atlas website (https://www.proteinatlas.org/). Subsequently, the slides were incubated with secondary antibodies from Elivision™ super HRP (mouse/rabbit) IHC Kit (Kit-9922; Maxvision, Foshan, People's Republic of China). After washing with phosphate-buffered saline (containing 0.1% Tween-80), each slide was incubated with 3-3′-diamino-benzidine (DAB) solution for 25 sec and counterstained with hematoxylin for another 25 sec. The experiment was performed according to the protocol.

Table 1. Association of Stromal NNMT Expression with the Demographic and Clinical Variables of GC patients (*N*=612).

	NNMT+ CAFs		
Variables	NNMT High	NNMT Low	*p*
Case no.	405	207	
Age, *n* (%)			
≤60	224 (55.3)	122 (58.9)	0.392*
>60	181 (44.7)	85 (41.1)	
Gender, *n* (%)			
Male	285 (70.4)	154 (74.4)	0.295*
Female	120 (29.6)	53 (25.6)	
Tumor size (cm), *n* (%)			
≤5.0	309 (76.3)	146 (70.5)	0.122*
>5.0	96 (23.7)	61 (29.5)	
Differentiation grade, *n* (%)			
Well	8 (2.0)	5 (2.4)	0.780**
Moderate	121 (29.9)	63 (30.4)	
Poor	276 (68.1)	139 (67.1)	
TNM stage, *n* (%)			
I	107 (26.4)	88 (42.5)	0.006**
II	120 (29.6)	38 (18.4)	
III	178 (44.0)	81 (39.1)	
Adjuvant chemotherapy, *n* (%)			
Yes	285 (70.4)	138 (66.7)	0.348*
No	120 (29.6)	69 (33.3)	
Serum CEA, *n* (%)			
<5 ng/mL	323 (84.6)	157 (79.7)	0.141*
≥5 ng/mL	59 (15.4)	40 (20.3)	
Serum CA199, *n* (%)			
<37 U/mL	313 (85.1)	158 (84.5)	0.487*
≥37 U/mL	55 (14.9)	29 (15.5)	
Epithelial NNMT, *n* (%)			
NNMT high	295 (72.8)	113 (54.6)	<0.001*
NNMT low	110 (27.2)	94 (45.4)	
Lauren's histologic type, *n* (%)			
Intestinal	134 (33.1)	87 (42.0)	0.360**
Diffuse	205 (50.6)	76 (36.7)	
Mixed	66 (16.3)	44 (21.3)	

Abbreviations: CA199, carbohydrate antigen 199; CEA, carcinoembryonic antigen; GC, gastric cancer; NNMT, nicotinamide N-methyltransferase.
*Chi square test or Fisher's exact test. Missing values are excluded for all statistical tests.
**Mann–Whitney U test (non-parametric).

IHC Scores of NNMT Immune Staining

The stained TMA slides were observed by bright-field microscopy (Servicebio; digital scanning via Pannoramic MIDI; 3Dhistech, Budapest, Hungary), and the software CaseViewer was applied to score brown staining. Stromal and epithelial NNMT expression was evaluated using H-score method,[29]and classified into intestinal-type, diffuse-type or mixed-type GC by Lauren Classification.[30] The intensity of cytoplasmic NNMT staining was classified as negative, weak, moderate, and strong. H-scores were calculated by multiplying the percentage of positively brown-stained fibroblasts by the corresponding staining intensity (H-scores ranged from 0 to 300). Two independent investigators (ZL and SM) carried out the assessment, without access to any patient details. Inter-observer differences were averaged.

Statistical Analysis

Patient characteristics were compared between the two groups (with high- or low-
stromal NNMT expression) by Pearson Chi-square test (for categorical variables); TNM stage and differentiation grade were analyzed by Mann-Whitney U test (non-parametric). Paired or unpaired *T*-test was employed to compare the differences in the H-scores of NNMT expression between malignant and non-malignant specimens or different Lauren's histologic type. The optimal cut-off value of the IHC score was identified using the Maxstat package in R 3.5.1 (www.r-project.org) to define risk subgroups.[31,32] Differences in survival outcomes were compared using Kaplan–Meier curves and log-rank test. Factors contributing to patient survival were analyzed with univariate or multiple Cox proportional hazards model. All statistical analyses were performed by SPSS V.19.0 for Windows (Chicago, IL). Statistical significance was set at $p<0.050$.

Results

Stromal NNMT is Elevated in GC

IHC analysis showed that NNMT is primarily expressed in the epithelial cells and stromal cells (Fig. 1A). The pattern of NNMT expression has been reported in the previous study;[26] therefore, we focused on the expression pattern of NNMT in the stromal compartment. The results obtained for 612 malignant and 92 non-malignant specimens (Fig. 1B) and 69 paired malignant and adjacent normal tissues (Fig. 1C) consistently showed that stromal NNMT expression in malignant tissues was higher compared to that in the non-malignant tissues (all $p<0.010$). Among the intestinal-type and the mixed-type GC, the expression of stromal NNMT in the diffuse-type was the maximum (Fig. 1D). The differential expression between malignant tissues and non-malignant tissues of epithelial NNMT was not seen (Supplementary Fig. 1A, $p<0.001$). However, the epithelial NNMT expression in diffuse-type GC was substantially lower than intestinal-type or mixed-type GC (Supplementary Fig. 1B, $p<0.001$). This suggested

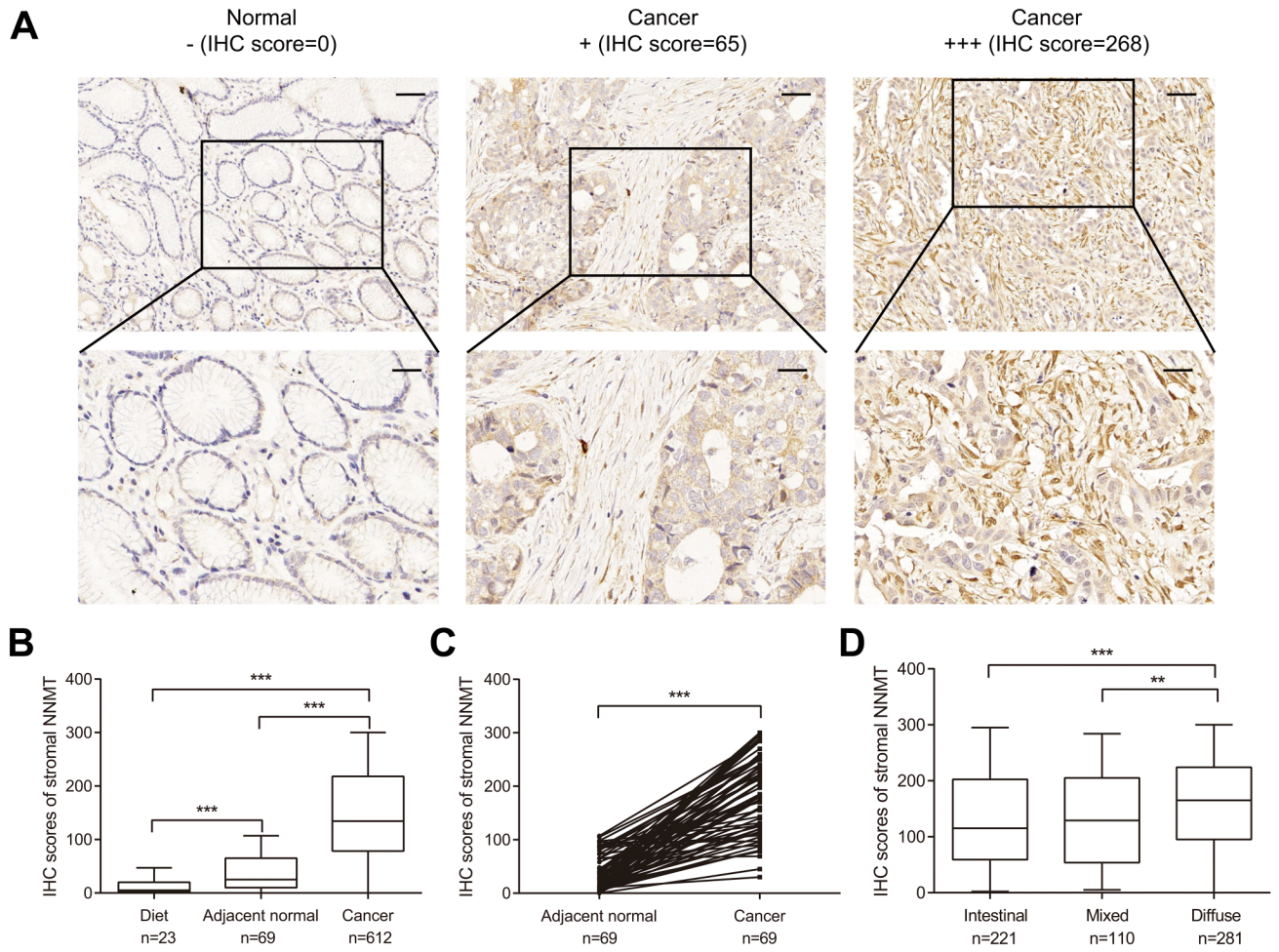

Figure 1. Associations between stromal NNMT expression and patient characteristics. Stromal nicotinamide *N*-methyltransferase (NNMT) expression is elevated in gastric cancer (GC). (A) Staining of representative stromal NNMT in gastric tissue. Tissues was scored as -, +, or +++ depending on the presence of negative, weak, and strong brown staining of fibroblasts, respectively. Bars, 100 or 50 μm. (B) Comparison of H-score of 612 malignant and 92 non-malignant specimens of GC tissue. (C) Comparison of H-score of 69 paired malignant and non-malignant specimens of GC tissue. (D) Comparison of H-score of Lauren's histologic type of GC tissue. ***$p<0.001$, **$p<0.01$.

that the stromal NNMT might be involved in the development of GC.

Association Between Stromal NNMT and Patient Characteristics

Using 95% quantile of the stromal NNMT IHC scores of 92 non-cancerous specimens as a cut-off value, we classified these 612 cases into 2 groups: high (IHC score $>$ 98) and low (IHC score $\leq$ 98) stromal NNMT tumors. Although no relationship was identified between the status of stromal NNMT expression and each characteristic, including gender, age, differentiation grade, serum CEA levels, serum CA199 levels and Lauren's histologic type (all $p>0.050$), as shown in Table 1, a significant association was noted between stromal NNMT expression and TNM stage ($p=0.006$), or epithelial NNMT ($p<0.001$), which indicated that stromal NNMT may be involved in the progression and prognosis of GC.

High Stromal NNMT Expression Predicted Unfavorable Survival

We randomly divided 612 patients into two groups: training set ($N=306$) and validation set ($N=306$), and confirmed the comparability between the two sets (Supplementary Table 1). In the training set, we first identified an IHC score of 213 as the optimal cut-off value, which was used to classify the patients into high (IHC score $\geq$ 213) or low (IHC score $<$ 213) stromal NNMT subgroups, with the most significant difference

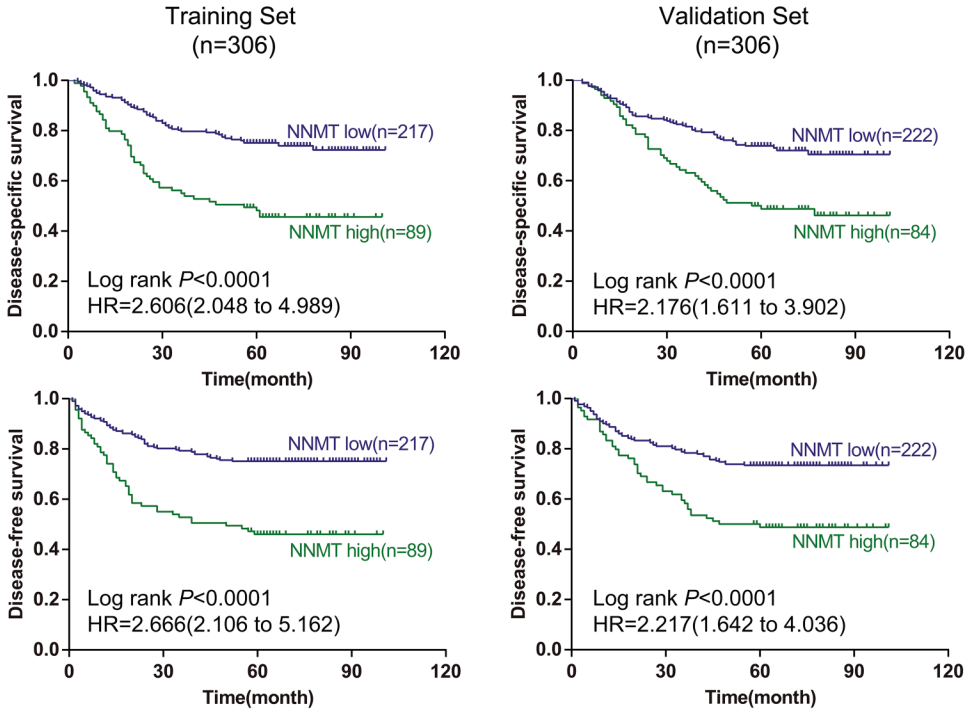

Figure 2. Associations between stromal NNMT expression and patient characteristics. Patients (*N*=612) were randomly divided into two groups: training set (*N*=306) and validation set (*N*=306), Kaplan-Meier survival curves provided the DFS and DSS periods of patients with high and low expression of NNMT in both sets (cut-off value = 213). *p* values were obtained by Kaplan-Meier analysis, with log-rank test. Green line: high expression of NNMT, that is, "NNMT-high," blue line: low expression of NNMT, that is, "NNMT-low." Abbreviations: NNMT, nicotinamide *N*-methyltransferase, DFS, disease-free survival; DSS, disease-specific survival.

being noted in DSS. As illustrated in Fig. 2, Kaplan–Meier curve analysis showed that patients with high stromal NNMT expression have a shorter DSS ($p<0.001$) and DFS ($p<0.001$) periods, compared to patients with low stromal NNMT expression in the training set. With the same cut-off value, the validation set was also classified into two subgroups. The prognostic significance between the subgroups was confirmed in the validation set (Fig. 2). Interestingly, in our cohort, the low epithelial NNMT group has shorter validity times of DSS ($p<0.010$) and DFS ($p<0.010$) than the same curve pattern in the validation group, while $p>0.050$ in the training group (Supplementary Fig. 1).

However, as shown in Table 2, stromal NNMT expression, variables such as TNM stage and tumor size, and chemotherapy were all significantly associated with patient survival (DFS and DSS), as indicated by univariate Cox analysis, for the training set (all $p<0.010$). This may confound the potential of stromal NNMT expression as a prognostic biomarker. With multiple Cox model analysis, high stromal NNMT expression was found to be an independent risk factor for the prognosis of GC, with a hazard ratio (HR) of 2.356 (95% confidence interval [CI] = 1.591–3.488; $p<0.001$) for DSS and an HR of 2.265 (95% CI = 1.529–3.354; $p<0.001$) for DFS. Next, as shown in Table 3, we evaluated the findings with the validation set and found that stromal NNMT expression still served as an independent factor with an HR of 2.087 (95% CI = 1.368–3.184; $p=0.001$) for DSS and an HR of 2.034 (95% CI = 1.327–3.119; $p=0.001$) for DFS. While the coincident result in Tables 2 and 3 with an HR of 0.541 (95 % CI = 0.355–0.824; $p=0.004$) for DSS and an HR of 0.531 (95% CI = 0.347–0.811; $p=0.003$) for DFS in Table 3 cannot be seen by the epithelial NNMT expression.

Stromal NNMT Predicts the Prognosis of Early GC

The relationship between stromal NNMT expression and patient outcome in patients with early stage GC (stages I and II) was further explored. Kaplan–Meier curve analysis showed that stromal NNMT expression can significantly predict DSS and DFS for early stage GC in both training and validation sets (Fig. 3). Especially, for stage I GC, high expression of stromal NNMT was found to be significantly associated with shorter DSS and DFS periods, compared to low expression of

Table 2. Cox Regression Analysis of IHC (stromal NNMT Expression) and Clinicopathological Covariates in the Training Set of GC Patients.

	Disease-Free Survival				Disease-Specific Survival			
	Univariate Analysis		Multivariate Analysis		Univariate Analysis		Multivariate Analysis	
Variables	HR (95% CI)	*p* Value	HR (95% CI)	*p* Value	HR (95% CI)	*p* Value	HR (95% CI)	*p* Value
Stromal NNMT								
High risk vs. low risk	2.679 (1.814−3.957)	<0.001	2.265 (1.529−3.354)	<0.001	2.616 (1.778−3.851)	<0.001	2.356 (1.591−3.488)	<0.001
Epithelial NNMT								
High risk vs. low risk	0.689 (0.464−1.022)	0.064			0.7155 (0.483−1.058)	0.094		
Age								
>60 vs. ≤60	1.487 (1.009−2.193)	0.046			1.434 (0.976−2.107)	0.066		
Gender								
Male vs. Female	0.740 (0.492−1.114)	0.149			0.760 (0.506−1.142)	0.187		
Tumor size(cm)								
>5 vs. ≤5	3.118 (2.107−4.614)	<0.001	1.930 (1.147−2.610)	0.009	3.031 (2.054−4.475)	0.012	1.691 (1.123−2.549)	0.009
Differentiation grade well + moderate vs. poor	0.600 (0.382−0.942)	0.027			0.582 (0.371−0.913)	0.018	0.630 (0398−0.996)	0.048
TNM stage								
III vs. II+I	5.510 (3.542−8.569)	<0.001	4.374 (2.751−6.955)	<0.001	5.364 (3.429−8.289)	<0.001	4.028 (2.534−6.403)	<0.001
Chemotherapy								
Yes vs. No	2.573 (1.546−4.282)	<0.001			2.630 (1.582−4.373)	<0.001		
Serum CEA (ng/mL)								
≥5 vs. <5	1.246 (0.752−2.064)	0.394			1.223 (0.739−2.024)	0.433		
Serum CA199 (U/mL)								
≥37 vs. <37	1.611 (0.981−2.647)	0.060			1.623 (0.988−2.664)	0.056		
Lauren's histologic type								
Diffuse + Mixed vs. Intestinal	1.846 (1.189−2.866)	0.006			1.881 (1.213−2.916)	0.005		

Abbreviations: IHC, immunohistochemistry; HR, hazard ratio; CI, confidence interval; CA199, carbohydrate antigen 199; CEA, carcinoembryonic antigen; GC, gastric cancer; NNMT, nicotinamide *N*-methyltransferase.

Table 3. Cox Regression Analysis of IHC (Stromal NNMT Expression) and Clinicopathological Covariates in the Validation Set of GC Patients.

	Disease-Free Survival				Disease-Specific Survival			
	Univariate Analysis		Multivariate Analysis		Univariate Analysis		Multivariate Analysis	
Variables	HR (95% CI)	*p* Value	HR (95% CI)	*p* Value	HR (95% CI)	*p* Value	HR (95% CI)	*p* Value
Stromal NNMT								
High risk vs. low risk	2.223 (1.499−3.296)	<0.001	2.034 (1.327−3.119)	0.001	2.183 (1.480−3.219)	<0.001	2.087 (1.368−3.184)	0.001
Epithelial NNMT								
High risk vs. low risk	0.544 (0.368−0.804)	0.002	0.531 (0.347−0.811)	0.003	0.544 (0.370−0.800)	0.002	0.541 (0.355−0.824)	0.004
Age								
>60 vs. ≤60	1.135 (0.769−1.674)	0.524			1.216 (0.829−1.783)	0.317		
Gender								
Male vs. Female	1.166 (0.743−1.831)	0.505			1.184 (0756−1.856)	0.460		
Tumor size(cm)								
>5 vs. ≤5	3.778 (2.558−5.581)	<0.001	2.225 (1.286−3.105)	0.001	3.678 (2.502−5.407)	<0.001	2.018 (1.304−3.123)	0.002
Differentiation grade well + moderate vs. poor	0.767 (0.496−1.184)	0.231			0.791 (0.517−1.209)	0.278		
TNM stage								
III vs. II+I	4.416 (2.881−6.769)	<0.001	1.998 (1.286−3.105)	0.002	4.125 (2.727−6.239)	<0.001	2.167 (1.347−3.486)	0.001
Chemotherapy								
Yes vs. No	5.418 (2.732−10.746)	<0.001	3.232 (1.437−7.270)	0.005	4.551 (2.435−8.505)	<0.001	3.011 (1.404−6.456)	0.005
Serum CEA (ng/mL)								
≥5 vs. < 5	2.565 (1.658−3.968)	<0.001			2.475 (1.604−3.821)	<0.001		
Serum CA199 (U/mL)								
≥37 vs. <37	2.857 (1.794−4.552)	<0.001			2.796 (1.758−4.447)	<0.001		
Lauren's histologic type								
Diffuse + Mixed vs. Intestinal	1.584 (1.025−2.447)	0.038			1.569 (1.023−2.408)	0.039		

Abbreviations: IHC, immunohistochemistry; HR, hazard ratio; CI, confidence interval; CA199, carbohydrate antigen 199; CEA, carcinoembryonic antigen; GC, gastric cancer; NNMT, nicotinamide *N*-methyltransferase.

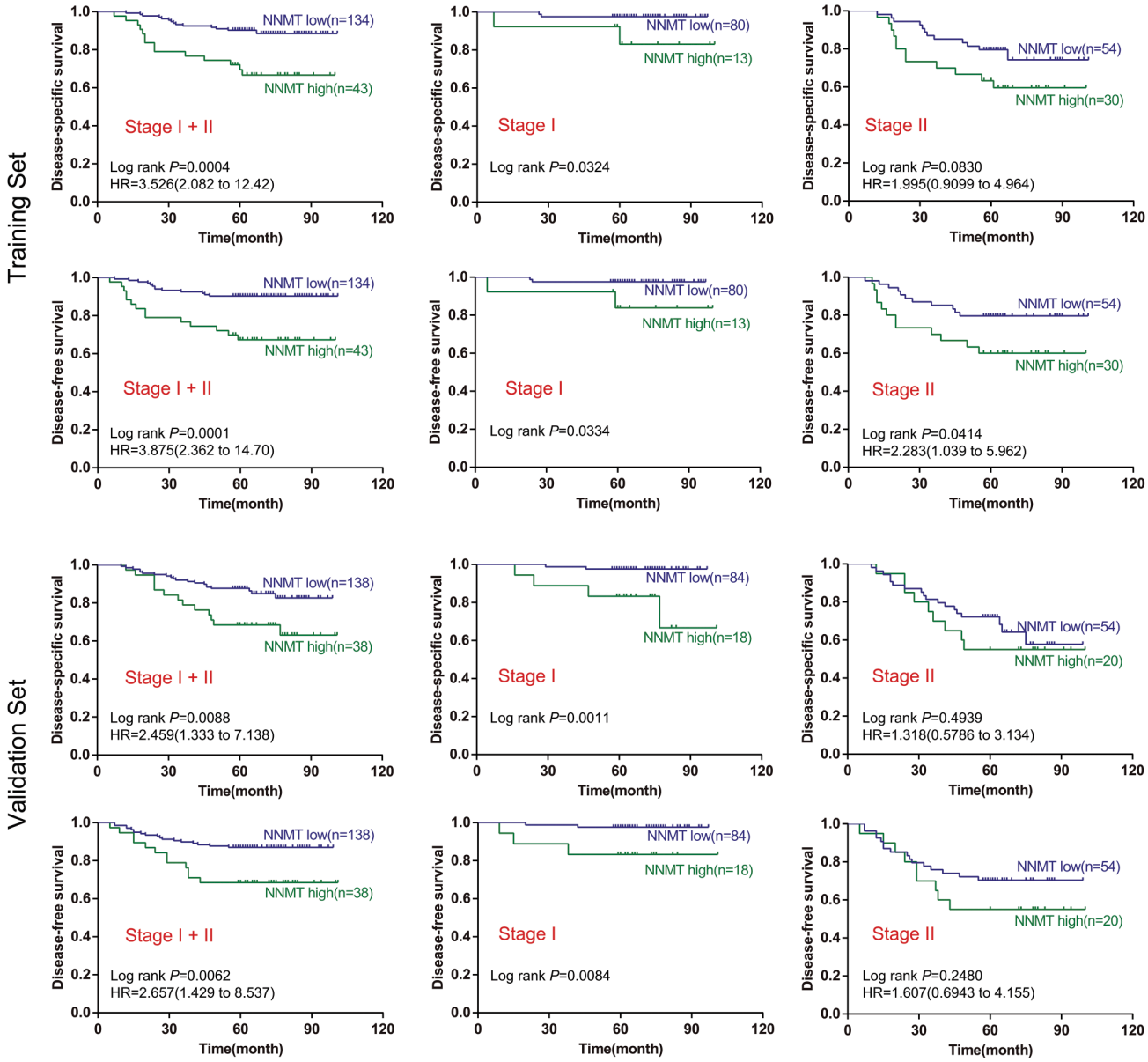

Figure 3. High stromal NNMT expression predicts unfavorable survival. Kaplan-Meier survival curves provided the DFS and DSS periods of patients showing high and low expression of NNMT during early TNM stage GC in the training and validation sets. *p* values were obtained by Kaplan-Meier analysis, with log-rank test. Abbreviations: NNMT, nicotinamide *N*-methyltransferase, DFS, disease-free survival; DSS, disease-specific survival.

stromal NNMT in both training and validation sets (Fig. 3). However, for stage II GC, only marginal significance was noted in the training set for stromal NNMT expression as a prognostic factor (Fig. 3).

Stromal NNMT Expression and Benefit from Adjuvant Chemotherapy

Patients with stage II and stage III tumors usually receive adjuvant chemotherapy; however, it is unknown whether patients with high or low stromal NNMT tumors will be protected from adjuvant chemotherapy. Next, we investigated the relationship between stromal NNMT expression and survival of patients with or without exposure to adjuvant chemotherapy. Preliminary analysis in our study (both training and validation sets) showed no difference in the survival of patients with stage II or III disease irrespective of whether they had received chemotherapy (Fig. 4). Among patients with stage II disease, adjuvant chemotherapy in patients

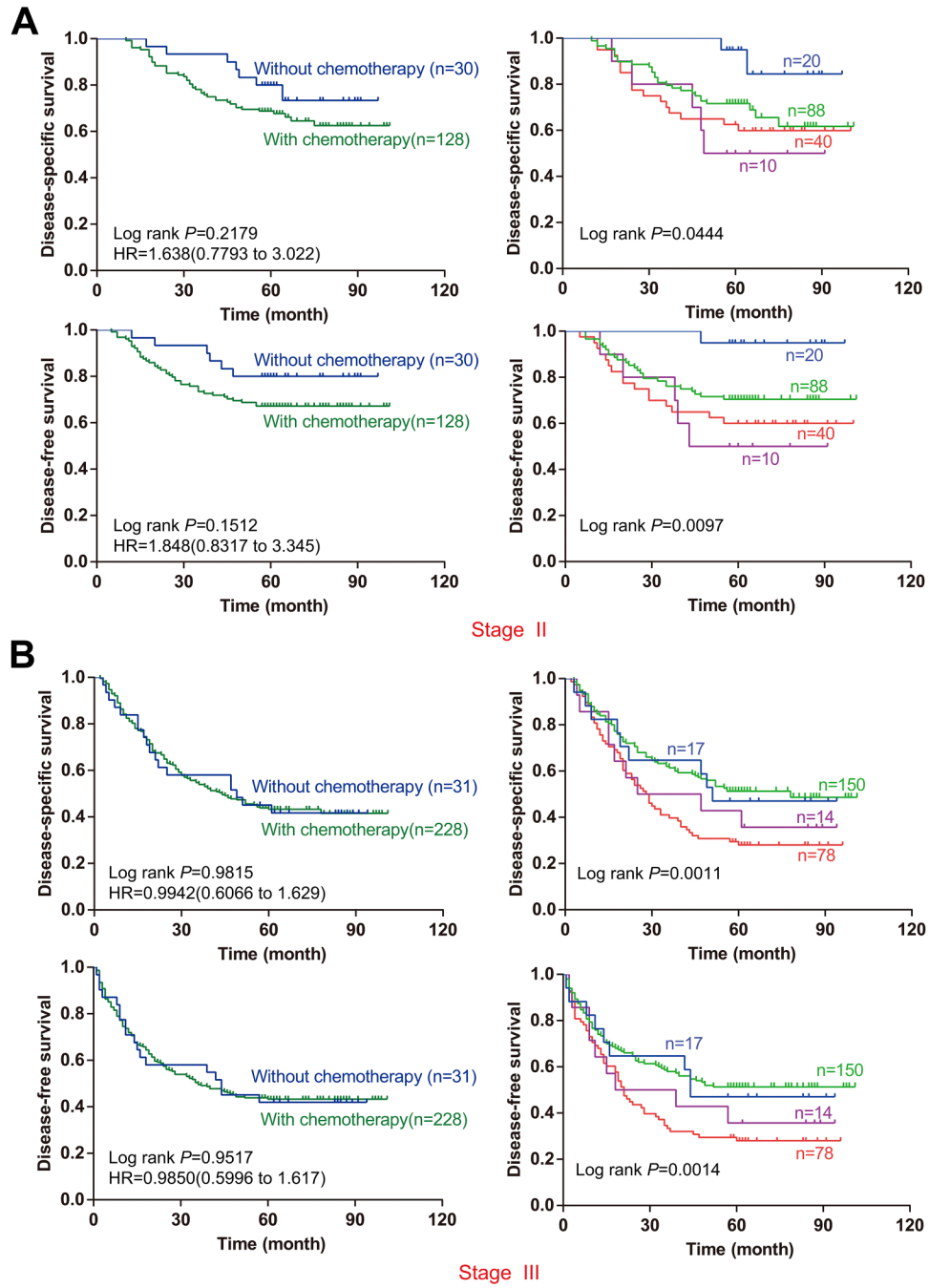

Figure 4. High expression of NNMT protein can be predictive of an unfavorable outcome of adjuvant chemotherapy in GC patients. (A) Association between NNMT expression and patient outcome in stage II patients with or without exposure to chemotherapy. (B) Association between NNMT expression and patient outcome in stage III patients with or without exposure to chemotherapy. Kaplan-Meier survival curves of patients with stage II and stage III GC with or without exposure to chemotherapy with low and high expression of NNMT are shown. *p* values were obtained by Kaplan-Meier analysis, with log-rank test. The right survival curve lines highlighted for the subgroups with NNMT low without chemotherapy (blue line), NNMT low with chemotherapy (green line), NNMT high without chemotherapy (purple line), and NNMT high with chemotherapy (red line). Abbreviations: NNMT, nicotinamide *N*-methyltransferase, GC, gastric cancer.

with low stromal NNMT expression resulted in shorter DFS and DSS periods (Fig. 4), which indicates that such treatment might harm these patients. However, for patients with high stromal NNMT expression, chemotherapy did not generate any survival benefit (Fig. 4). In case of stage III disease, none of the

patients with high or low stromal NNMT expression benefited from adjuvant chemotherapy (Fig. 4). We also examined the prognosis with divided the groups as Fig. 4 but adjusted the cut-off value into 98 (IHC score 98 = 95% quantile IHC score of non-cancerous specimens), although the findings failed to demonstrate the differential in DSS or DFS between the 4 groups (Supplementary Fig. 3). Therefore, our results indicated that although patients with high stromal NNMT expression might not benefit from adjuvant chemotherapy, some patients with low stromal NNMT expression might, in fact, be harmed by the same.

Discussion

CAFs are known to play a dual role in the development of GC. Not only do GC fibroblasts promote an inflammatory environment that supports tumor development via activation of CXCL12/CXCR4 signaling,[33] but they also reduce tumor development by secreting heterogeneous cytokines to inhibit MMP11 signaling.[14] It is interesting to note that fibroblasts are more frequently accumulated in malignant tissues compared to normal tissues. It is suggested that fibroblasts might play a non-negligible role in the development and invasion of GC. As a master metabolic regulator of CAFs, high stromal NNMT expression predicts poor prognosis in ovarian cancer or hepatocellular carcinoma.[22,24] Previous studies have reported that high NNMT expression is associated with worse prognosis of GC.[25,26] However, little is known about the accumulation of NNMT in the CAFs in GC.

To the best of our knowledge, the present study elucidated that NNMT protein is primarily distributed in the gastric stromal fibroblasts and epithelial cells. Furthermore, increased expression of stromal NNMT is found in the GC tissues, included in this study (*N*=612), compared to the non-malignant specimens and 69 paired malignant and adjacent normal tissues, and the expression levels were discrepant in diffuse-type GC according to Lauren Classifications with the highest for stromal NNMT and the lowest for epithelial NNMT.

NNMT expression in GC is known to be related to primary tumor size, lymph node metastasis, and TNM stage.[26] In our study, elevated stromal NNMT expression was also related to the TNM stage, and there was also a relation between stromal NNMT and epithelial NNMT. Thus, this TMA-based study strongly suggested that stromal NNMT is a potential marker candidate for TNM staging of GC, especially for early-stage cases.

GC produces noticeable symptoms during the pre-cancer and early stages, and therefore, early diagnosis of GC is very important to avoid peritoneal recurrence and distant metastasis, and to improve patient prognosis.[6,34] To identify a prognostic biomarker for early stage GC, we divided 612 clinical GC specimens into 2 subgroups, namely, high and low expression, by using an optimal cut-off value. In both training and validation sets, stromal NNMT expression levels were found to be related to DSS and DFS recorded for GC patients. Compared to the low expression group, the high expression group usually presented with shorter DFS and DSS periods. We further showed that high expression of stromal NNMT could be used to predict poor outcome in early stage GC (stage II + I) as well as advanced cancer (stage III), by adjusting the TNM stage and other factors, by univariate and multivariate Cox analyses. The results indicated that high expression of stromal NNMT might be an independent potential prognostic marker, in both early and advanced GC.

Compared to the stromal NNMT, the expression of epithelial NNMT appears to have an opposite trend. The epithelial NNMT level has no difference between non-malignant specimens and malignant tissues. The high expression of epithelial NNMT presented with longer DFS and DSS periods, which demonstrate that the NNMT expressed in stroma or epithelium has different relations to prognosis of GC patients in our cohort. While the previous study reported that NNMT high expression is related to shorter DSS and DFS,[26] our study shows the heterogeneous nature of NNMT expressed in different locations.

Different variants of adjuvant chemotherapy have been shown to improve patient prognosis after surgery in GC.[34] Considering the adverse effects induced and resistance imparted by chemotherapy, it is necessary to explore a biomarker to predict the success of chemotherapy. In this study, stromal NNMT expression was found to be related to poor survival outcome with adjuvant chemotherapy. However, stage II patients with low NNMT expression showed favorable survival. In our cohort, the effect of chemotherapy is shown in Fig. 4. Because of the distinct results, we liberalized the cut-off score to 98, while there was still no benefit for DSS or DFS.

It has been reported that the GC patients with better prognosis after chemotherapy according ACTS-GC trial and CLASSIC trial.[35,36] However, there has been also reported that the adequate surgery for homogeneous stomach cancer patients' populations have higher survival rates than those reported after neoadjuvant chemotherapy followed by incomplete surgery.[37] We speculated that the discrepancy of our cohort may be because of the difference of the region, diet, and other habits of the patient, or the chemotherapy

protocol preference of the doctor may be an important contributor. Thus, accumulation of NNMT protein in CAFs might positively contribute to drug resistance in GC of our cohort.

Previous studies have shown that serum NNMT has good sensitivity as a diagnostic marker in lung cancer and a prognostic marker in GC.[26,38] Based on our study population, stromal fibroblasts with increased NNMT expression indicated worse survival in GC, in both training and validation sets, by univariate and multivariate Cox analysis. Thus, accumulation of stromal NNMT might be a potential prognostic indicator for GC.

In conclusion and to summarize, our study has comprehensively evaluated the clinical significance of NNMT-positive fibroblasts in GC. It also shown that stromal NNMT is a favorable prognostic marker and a potential predictive biomarker to determine the effectiveness of chemotherapy in GC. However, the biological function or molecular mechanism of stromal NNMT as a diagnostic and prognostic biomarker in GC warrants detailed investigation.

Competing Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

LZ performed the experiments. GYY and WJC were responsible for the follow-up of GC patients; LZ, FZ and MMS were responsible for pathological analysis. GYY, HY, and WJC involved in the pathological diagnosis and recruitment of the patients in the hospital. LZ, YDN, MMS and WJC were responsible for statistical analysis. LZ, WJC and YDN designed and organized the study and wrote the manuscript.

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