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From Cell Biology to Tissue Engineering

Nephronectin is a prognostic biomarker and promotes gastric cancer cell proliferation, migration and invasion

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Summary. Gastric cancer (GC) is a malignant disease with high incidence and mortality rates worldwide. Nephronectin (NPNT) was found to be dysregulated in some kinds of cancer. The goal of our study was to explore the expression profile of NPNT based on large numbers of GC samples with detailed clinicopathological and prognostic data from our institution and the data from a public database. A total of 117 GC samples and 73 corresponding non-tumorous adjacent tissues (NATs) were obtained from GC patients and used to detect expression of NPNT through immunohistochemistry. Western blot and qRT-PCR were performed to examine expression of NPNT in GC cell lines. Our results found that the positive expression ratio of NPNT in GC tissues is significantly higher than that in NATs ($p<0.001$). Chi-squared analysis results showed positive expression ratio of NPNT was significantly associated with depth of tumor invasion ($p=0.049$) and TNM stage ($p=0.017$). Kaplan-Meier survival and cox analysis results showed that patients with positive NPNT protein expression tend to have poorer prognosis than those with negative NPNT expression ($p=0.0032$) and NPNT expression was independent prognostic factor. High expression level was seen in GC cell lines. Furthermore, through a series of cancer cell proliferation, invasion and migration associated experiments, we found that NPNT could evidently promote GC cell proliferation, invasion and migration, as well as epithelial-mesenthymal transition. In summary, NPNT was evidently overexpressed in GC and had an oncogenic role. In the future, NPNT could serve as a promising therapeutic target for treating GC patients.

Key words: Gastric cancer, Immunohistochemistry, Nephronectin, Prognosis, Proliferation, Invasion

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Introduction

Gastric cancer (GC) is a malignant disease with high incidence and mortality rates worldwide (Chen et al., 2016; Bray et al., 2018). The latest global cancer statistical data show there are approximately 1,033,701 new cases of GC and an estimated 782,685 new deaths all over the world in 2018, ranking it as the fifth most common cancer and the third main cause of cancer-related death (Bray et al., 2018). Among the different regions around the world, the highest rates of morbidity and mortality are still seen in east Asia including China, Japan and Korea. The symptoms of early stage GC are so latent that it is of great difficulty to be noticed (Li et al., 2015). Under such circumstance, many cases of GC are diagnosed at an advanced stage, which means these patients are difficult to cure and have poor prognosis (Bertuccio et al., 2009; Park et al., 2016). Therefore, it is extremely urgent to identify meaningful biomarkers which can contribute to diagnosing GC and helping predict the prognosis of GC patients.

Nephronectin (NPNT), an extracellular matrix

Abbreviations. GC, gastric cancer; NPNT, Nephronectin; NATs, non-tumorous adjacent tissues; IHC, Immunohistochemistry

protein, was first discovered in the developing mouse kidney, functioning as an integrin α8β1 ligand (Brandenberger et al., 2001). NPNT is a 62 kDa protein and consists of a signal peptide, five EGF-like domains, an MAM domain, and an arginylglycylaspartic acid (RGD) motif (Brandenberger et al., 2001; Zimmerman et al., 2018). As to physiological function, NPNT is involved in kidney development, osteoblast differentiation, osteogenic angiogenesis and injury repair etc. (Teo et al., 2017; Sun et al., 2018).

Recently, some researchers have reported the influence of NPNT on various types of cancer (Kuphal et al., 2008; Steigedal et al., 2018; Wang et al., 2018). Recent work by Tonje S. Steigedal has presented a comprehensive study of the expression profile and localization of NPNT in tissue microarrays from 842 cases of breast cancer and found that NPNT was significantly overexpressed in breast cancer tissues (Steigedal et al., 2018). Additionally, data from Borowsky's study indicated higher levels of NPNT in metastatic mammary tumor cells compared with non-metastatic cells in a mouse breast cancer model, suggesting that NPNT may act as a pro-metastatic factor (Borowsky et al., 2005). In contrast to the above findings, studies of NPNT in melanoma detected a reduced expression of NPNT in malignant melanoma compared with primary melanocytes and found overexpression of NPNT could inhibit invasive and migratory capacity (Kuphal et al., 2008). These studies suggested that the expression profile of NPNT presented tissue-specific features in different cancers. The variability of NPNT expression in cancers makes it interesting and meaningful to discover NPNT expression in GC. And up to now, far too little attention has been paid to discover the expression profile of NPNT in GC.

In the current study, we attempt to explore the expression profile of NPNT based on large numbers of GC samples and investigate the functional role of NPNT in GC. The findings in our study may increase the understanding of the important role of NPNT in GC as well as provide a relationship between NPNT expression and prognosis.

Materials and methods

Cell culture

The normal stomach epithelial cell GES-1 and gastric cancer cell lines (SGC-7901, MGC-803 and BGC-823) were purchased from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). AGS cells were acquired from ATCC (Manassas, USA). All these cells were cultured at 37°C in RPMI 1640 medium (Hyclone) containing 10% fetal bovine serum (Hyclone) in a humidified incubator in an atmosphere containing 5% CO_2 (Thermo, Waltham, MA, USA). Mycoplasma testing has been done for the cell lines used in the current study and all cell lines used were free of mycoplasma contamination.

Tissue specimens

A total of 117 formalin-fixed and paraffin-embedded (FFPE) tumor samples, together with 73 corresponding non-tumorous adjacent tissues (NATs) were obtained from GC patients who accepted curative radical gastrectomy at The First Hospital of China Medical University between 2009 and 2011. Before accepting operation therapy, patients did not receive any chemotherapy or radiotherapy. For all resected tissues, two pathologists evaluated and confirmed all the pathologic diagnostic results. Tumor stage was defined according to the 8th American Joint Committee on Cancer/International Union Against Cancer tumor-node-metastasis (TNM) classification system. All patients included agreed to our study and written informed consent was obtained. Our study was approved by the ethics committee of the China Medical University (No. AF-SOP-07-1).

RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA were measured using a nano-photometer UV/Vis spectrophotometer (Implen)

Table 1. Sequences of primers used for quantitativereal-time PCR.

Primer Name	Sequence
NPNT- forward	5'-AGTACTTGGTGGCCTCCGAAGAC-3'
NPNT- reverse	5'-GTGGTGGTGGTGGTGGAGTAGG-3'
GAPDH- forward	5'-CAGGAGGCATTGCTGATGAT-3'
GAPDH- reverse	5'-GAAGGCTGGGGCTCATTT-3'
Cytokeratin-forward	5'-GCCGTGGTTGTGAAGAAGATC-3'
Cytokeratin-reverse	5'-CCTGTTCCCAGTGCTACCCT-3'
E-cadherin-forward	5'-AAGTGCTGCAGCCAAAGACAGA-3'
E-cadherin-reverse	5'-AGGTAGACCCACCTCAATCATCCTC-3'
MMP2-forward	5'-CTTTGACGGTAAGGACGGACT-3'
MMP2-reverse	5'-CATACTTCACACGGACCACTTG-3'
MMP9-forward	5'-CCAACTACGACACCGACGAC-3'
MMP9-reverse	5'-TGGAAGATGAATGGAAACTGG-3'
N-cadherin-forward	5'-TGGACCATCACTCGGCTTA-3'
N-cadherin-reverse	5'-ACACTGGCAAACCTTCACG-3'
α-SMA-forward	5'-GGGACATCAAGGAGAAACTGTG-3'
α-SMA-reverse	5'-CCATCAGGCAACTCGTAACTC-3'
SNAI1-forward	5'-AGTGGTTCTTCTGCGCTACTG-3'
SNAI1-reverse	5'-TGCTGGAAGGTAAACTCTGGA-3'
Twist1-forward	5'-GTCCGCAGTCTTACGAGGAG-3'
Twist1-reverse	5'-TGGAGGACCTGGTAGAGGAA-3'
ZEB2-forward	5'-TGAGGATGACGGTATTGC-3'
ZEB2-reverse	5'-ATCTCGTTGTTGTGCCAG-3'
Vimentin-forward	5'-CCTGAACCTGAGGGAAACTAA-3'
Vimentin-reverse	5'-GCAGAAAGGCACTTGAAAGC-3'

and RNA with high purity (A260/A280 value between 1.9 and 2.0) was used. Before qRT-PCR, first-strand cDNA was synthesized from 1 μg of total RNA using a Reverse Transcription Kit (TaKaRa, Tokyo, Japan). qRT-PCR was performed in the LightCycler® 480II (Roche, Basel, Switzerland) using TB Green (TaKaRa, Tokyo, Japan). The comparative Ct method was used to calculate the relative expression of RNAs (Livak and Schmittgen, 2001). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as reference for mRNA, and sequences for the primer used in this study are listed in Table 1.

Immunohistochemistry (IHC)

Tumor tissues were fixed in formalin and embedded in paraffin. Then the FFEP tissues were cut and processed into 5 μm tissue slides to perform IHC. Tissue slides were incubated with anti-NPNT (1:1000 dilution, ab64419, Abcam, USA), and kept overnight at 4°C and were further stained with BOSTER SABC IHC kit (BOSTER, Wuhan, China). Immunostaining was conducted using the Envision System with diaminobenzidine (Dako, Glostrup, Denmark). The immunoreactivity for NPNT was scored in a semiquantitative method. Expression for NPNT was independently assessed by two pathologists who were blinded to the clinicopathological data of patients included in the current study.

Evaluation of IHC staining

The immunoreactivity was semi-quantitatively scored by scaling staining intensity and extent. Staining intensity was divided into 4 levels: negative, weak, moderate and strong staining. These four levels were respectively scored for 0, 1, 2, 3. Staining extent was graded into five levels: 0 (≤5%), 1 (6-25%), 2 (26-50%), 3 (51-75%) or 4 (76-100%), according to the percentage of positively stained cells. Finally, an immunoreactivity score (IS: 0-12) for each case was generated by multiplying the staining intensity score and the staining extent score. The IS score ≤4 for each case was regarded as negative expression, and an IS score >4 was considered as positive expression.

Western blotting

Primary cells were crushed using RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Waltham, MA, USA), and then separated by SDS-PAGE gel electrophoresis before transferring to PVDF membranes (Merck Millipore, Billerica, MA, USA). Then the PVDF membranes were incubated with primary antibody diluted by 5% milk overnight. TBST was used to wash the primary antibody, and membranes were then

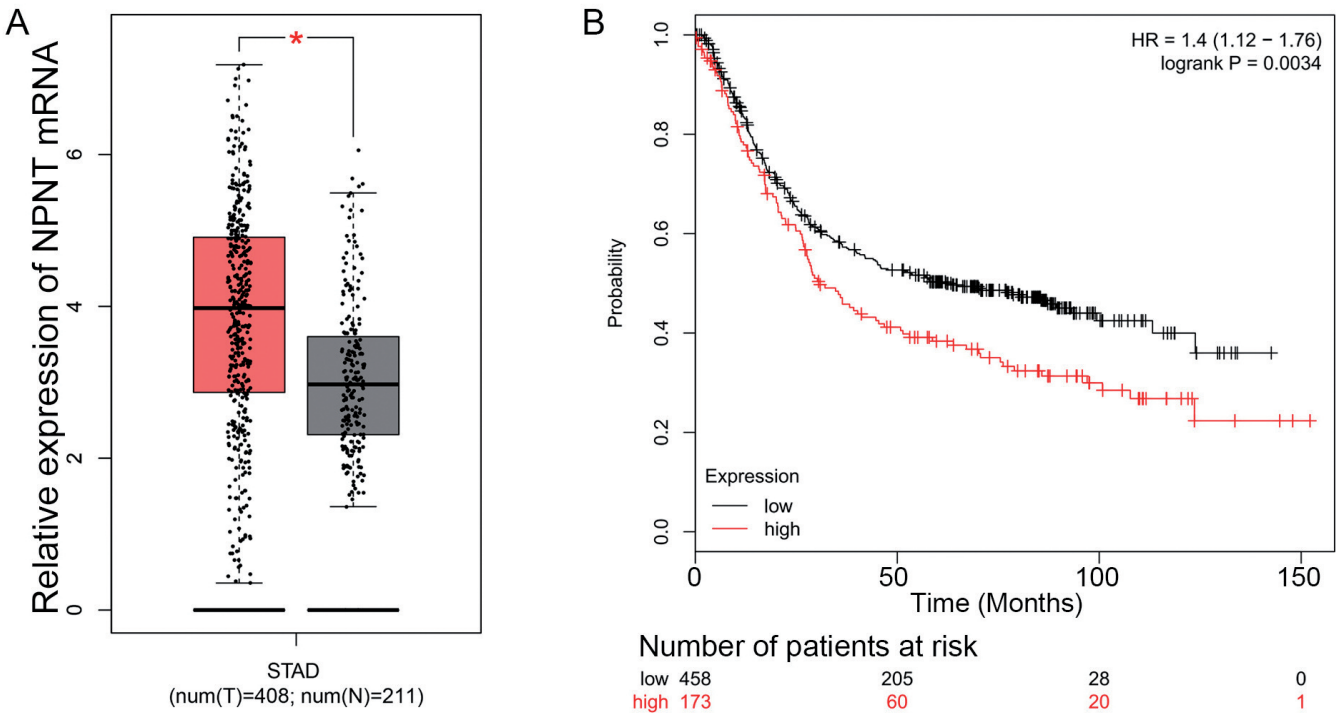

Fig. 1. Bioinformatic analysis of NPNT expression and its prognostic value in GC. **A.** The TCGA data showed NPNT was overexpressed in stomach adenocarcinoma tissues (n=408) compared with normal tissues (n=211) using GEPIA (http://gepia.cancer-pku.cn). **B.** Kaplan-Meier Plotter showed that NPNT mRNA expression was correlated to survival [HR=1.4 (1.12-1.76), P=0.0034].

incubated with secondary antibodies for visualization.

Lentivirus infection

To stably overexpress or knockdown NPNT expression in GC cell lines, full length NPNT or shRNA sequences specifically targeting NPNT mRNA were cloned into vectors. Viral particles were obtained through Lipofectamine 2000 (Invitrogen, USA) transfection in HEK293T cell lines, and with VSVG, RSV-Rev packaging plasmids. GC cells were infected by lentivirus combined with infection enhancer (Genechem, Shanghai, China) and selected by puromycin (Solarbio, Beijing, China) for 2 weeks to obtain stable expression cell lines.

Cell counting kit 8 (CCK8) assay

CCK8 assay was performed to measure the proliferation capacity of GC cells. The NPNT stable overexpressed or knockdown cells were seeded into 96-well culture plates (Corning, USA) for 0, 24, 48, 72 and 96 hours. During each time set, ten microliters of CCK8 (Dojindo, Rockville, MD, USA) cells were gently added into plates and incubated for 1 hour at 37°C incubator. The optical density was measured by a microplate reader (Bio-Rad, California, USA) at a wavelength of 450 nm.

Statistics

The correlation between NPNT expression and the clinicopathological features of GC patients was analyzed using the χ^2 test. The Kaplan Meier method was applied to analyze the relationship between NPNT expression and GC patients' survival. The univariate and multiple cox proportional hazards model was applied to evaluate the relationship between NPNT expression and survival outcomes. Data were represented by mean ±SD for 3 independent experiments and measurements were analyzed using an independent 2-tailed Student's t test. For all statistical tests, p value of less than 0.05 was considered statistically significant.

Results

Bioinformatic analysis of NPNT expression profile in GC tissues from public database

In order to explore the expression profile of NPNT in GC, we initially applied the public RNA sequencing data in TCGA to analyze NPNT mRNA expression via GEPIA (http://gepia.cancer-pku.cn) (Tang et al., 2017). There are 408 GC tissues and 211 normal stomach tissues included in GEPIA websites and the analysis results show NPNT mRNA was significantly upregulated in GC tissues ($p<0.05$, Fig. 1A). Next, we further analyzed the relationship between NPNT expression and GC patients' survival based on Kaplan-Meier Plotter (http://kmplot.com/analysis/) (Szasz et al., 2016). The results from Kaplan-Meier Plotter showed that NPNT mRNA expression was correlated to patient's survival (HR=1.4 (1.12-1.76), P=0.0034, Fig. 1B). GC patients with high NPNT expression showed high tendency to have poor survival outcome.

The expression of NPNT in GC tissues and its relationship with clinicopathological characteristics and survival

Subsequently, to further verify the expression profile of NPNT in GC, IHC was performed in a cohort of GC tissues from our own institution. Our cohort included 117 GC tissues and 73 non-tumorous adjacent tissues (NATs). As shown in Fig. 2, NPNT staining was mainly localized in cytoplasma. The positive expression ratio of NPNT in GC tissues (40.17%, 47/117) is significantly higher than that in NATs (10.96%, 8/73) ($p<0.001$, Table 2). Moreover, chi-squared analysis was applied to assess the relationship between NPNT expression and GC patients' clinicopathological characteristics. The results showed positive expression ratio of NPNT was significantly associated with depth of tumor invasion ($p=0.049$) and TNM stage ($p=0.017$). Higher NPNT positive expression ratio was evidently observed more in T4 stage GC cases (46.3%, 37/80) than in T1-T3 cases (27.0%, 10/37). Meanwhile, there was no significant correlation between NPNT expression and gender, age, ascites, tumor sites, tumor size, Borrmann types, histological grade, lymphatic invasion, lymphatic metastasis (Table 3).

In order to evaluate the prognostic value of NPNT expression, Kaplan-Meier survival analysis was performed. The results showed that patients with positive NPNT protein expression tend to have poorer prognosis than those with negative NPNT expression ($p=0.0032$, Fig. 3A). Furthermore, we performed multiple variate Cox analysis and our results showed NPNT expression ($p<0.05$) and TNM stage ($P<0.001$) were independent prognostic factor for predicting GC patients' survival (Table 4).

NPNT evidently promotes GC cell proliferation

Due to the high expression in GC tissues, real-time PCR and western blotting were performed to examine the expression profile in GC cell lines. As for the

Table 2. The expression profile of NPNT in GC tissues and NATs.

	cases	NPNT expression		Chi-square value	P value
		Negative (%)	Positive (%)		
GC tissues	117	70 (59.83%)	47 (40.17%)	18.650	<0.001
NATs	73	65 (89.04%)	8 (10.96%)		

GC tissues, gastric cancer tissues; NATs, nontumorous adjacent tissues.

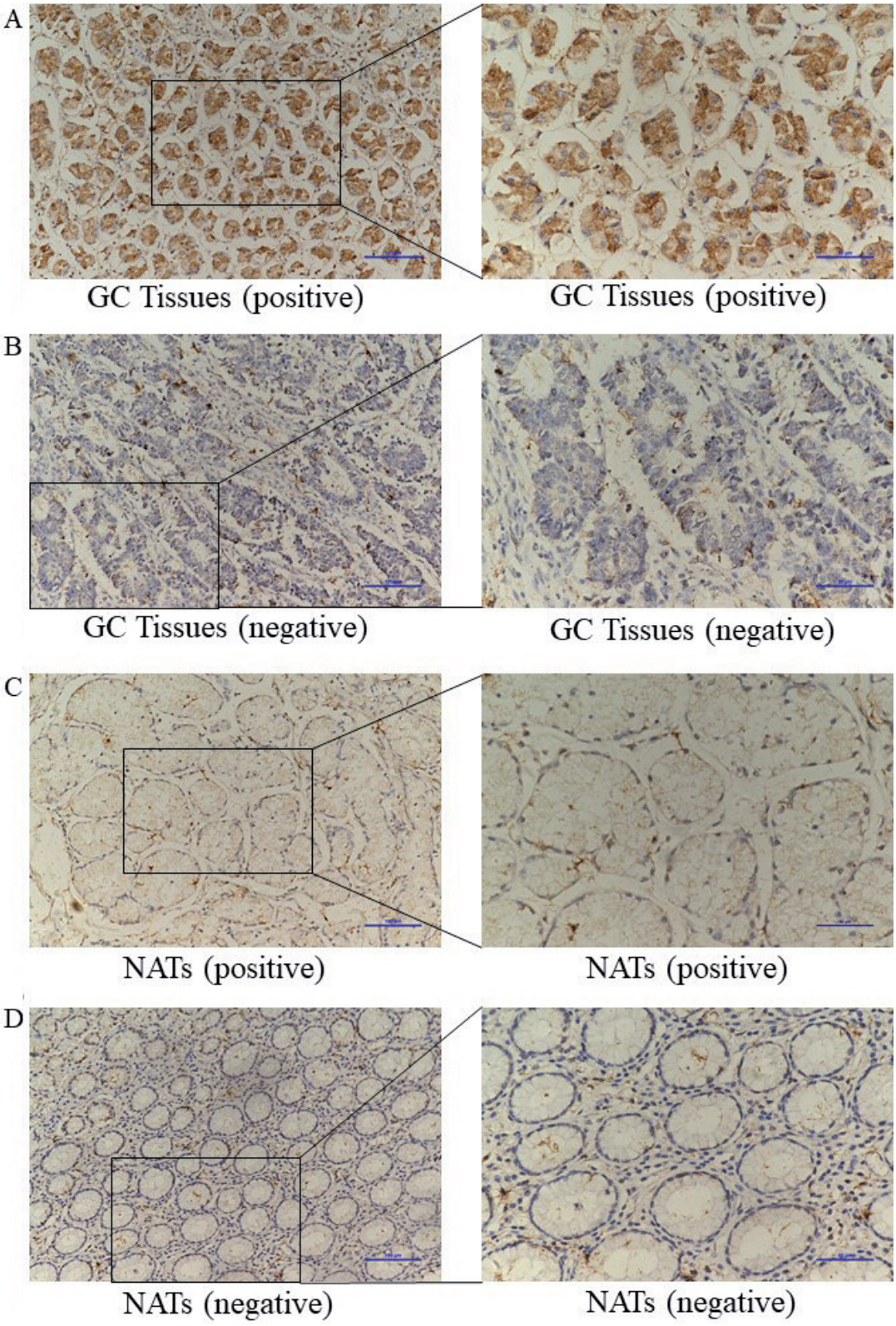

Fig. 2. Representative immunohistochemistry images of NPNT in GC tissues and NATs. **A.** The representative immunohistochemistry images of NPNT positive expression in gastric cancer tissues. NPNT staining was mainly localized in cytoplasma. **B.** The representative immunohistochemistry images of NPNT negative expression in gastric cancer tissues. **C.** The representative immunohistochemistry images of NPNT positive expression in non-tumorous adjacent tissues. NPNT staining was mainly localized in cytoplasm. **D.** The representative immunohistochemistry images of NPNT negative expression in non-tumorous adjacent tissues. Scale bars: 100 μm; boxed area, 50 μm.

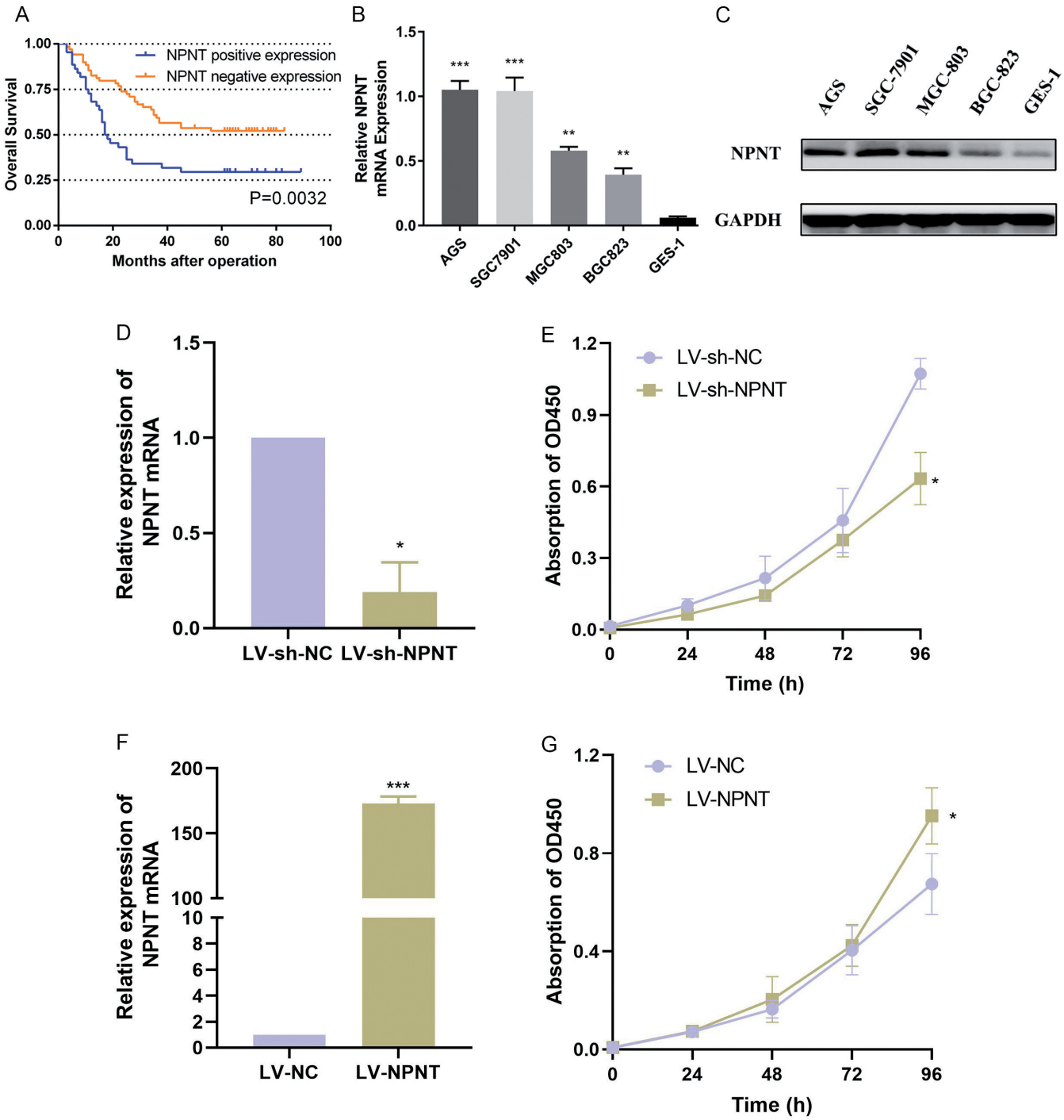

Fig. 3. NPNT promoted gastric cancer cell proliferation. **A.** Kaplan-Meier curves indicating that high level of NPNT protein expression correlates with poor prognosis of patients with gastric cancer. **B.** Realtime quantitative PCR analysis showing high NPNT mRNA expression in gastric cancer cell lines compared with normal stomach epithelial cell line GES-1. **C.** Western blot analysis showing high NPNT protein expression in gastric cancer cell lines compared with normal stomach epithelial cell line GES-1. **D.** Realtime quantitative PCR analysis showing that a NPNT stable knockdown cell line was constructed via lentivirus infection. **E.** the proliferation of NPNT knockdown cell was evaluated via CCK-8 assay. The proliferation was inhibited by knocking down NPNT expression. **F.** Realtime quantitative PCR analysis showing NPNT stably overexpressed cell line was constructed via lentivirus infection. **G.** the proliferation of NPNT overexpressed cell was evaluated via CCK-8 assay, showing that overexpression of NPNT promotes gastric cancer cell proliferation. All experiments were independently performed three times to obtain the presented data. For all figures, *P<0.01, **P<0.01; ***P<0.001 two-tailed paired Student's t-test.

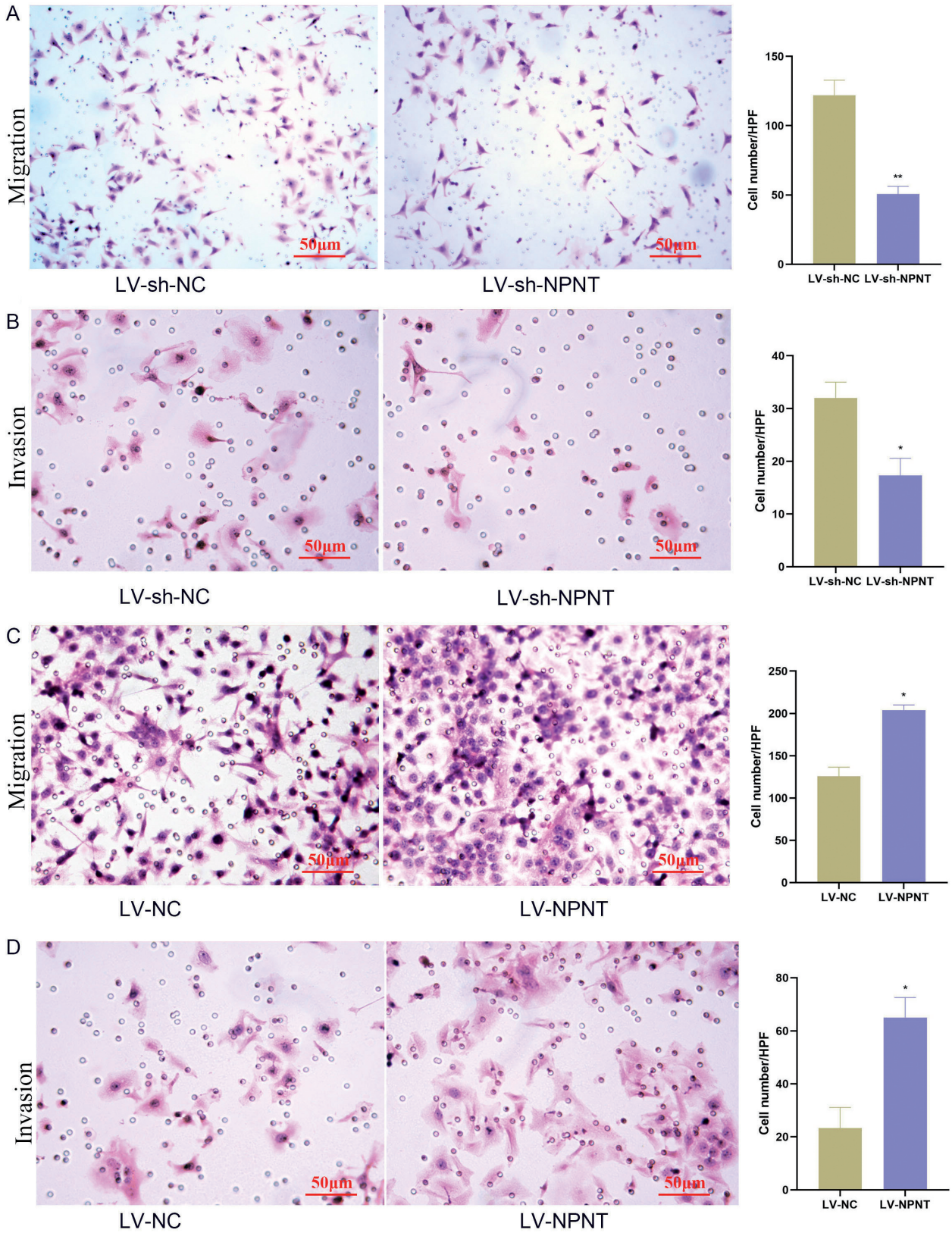

Fig. 4. NPNT increased GC cell migratory and invasive capacities. **A-B.** Transwell migration and invasion assays were used to investigate the migratory and invasive capacities in NPNT knockdown GC cells. Cells were incubated for 48 hours then counted under the microscope. **C-D.** Transwell migration and invasion assays were used to investigate the migratory and invasive capacities in GC cells with NPNT overexpression. Cells were incubated for 48 hours then counted under the microscope. Data are presented as mean ± SD. All experiments were independently performed three times to obtain the presented data. Scale bars: 50 μm.

transcriptional level, NPNT was significantly upregulated in GC cell lines (AGS, BGC-823, MGC-803, SGC-7901) compared with the normal stomach epithelial cell line GES-1 (Fig. 3B). Meanwhile, the results of western blotting also showed that NPNT was significantly overexpressed in GC cell lines at translational level (Fig. 3C).

To explore the function of NPNT in GC, we knocked down NPNT expression in GC cell lines by using lentivirus packaged short hairpin RNA. Quantitative RT-PCR results showed NPNT was obviously decreased after lentivirus infection (Fig. 3D). By performing CCK8 assay using these cells, we found a significant decrease of proliferation capacity in NPNT knockdown cells compared with the control group (Fig. 3E). On the contrary, NPNT overexpression obviously enhanced the proliferation of GC cells (Fig. 3F,G). Taken together, these results showed that NPNT functions as an oncogenic gene in GC and promotes GC cell proliferation.

NPNT increases GC cell invasion, migration as well as epithelial-mesenchymal transition

The significant relationship of NPNT expression to

Table 3. Comparison of expression levels of NPNT with clinicopathological characteristics in patients with gastric cancer.

Clinicopathological characteristics	cases	NPNT expression		P value
		Negative (%)	Positive (%)	
Gender				0.068
Male	81	44(54.3%)	37(45.7%)	
Female	36	26(72.2%)	10(27.8%)	
Age (years)				0.339
≤60	66	42(63.6%)	24(36.4%)	
>60	51	28(54.9%)	23(45.1%)	
Ascites				0.423
Negative	65	41(63.1%)	24(36.9%)	
Positive	52	29(55.8%)	23(44.2%)	
Tumor sites				0.517
upper	10	6(60.0%)	4(40.0%)	
Moderate	20	9(45.0%)	11(55.0%)	
lower	81	51(63.0%)	30(37.0%)	
total	6	4(66.7%)	2(33.3%)	
Tumor size				0.986
≤4cm	20	12(60.0%)	8(40.0%)	
>4cm	97	58(59.8%)	39(40.2%)	
Borrmann types				0.174
Borrmann I-II	10	8(80.0%)	2(20.0%)	
Borrmann III-IV	107	62(57.9%)	45(42.1%)	
T stage				0.049*
T1-3	37	27(73.0%)	10(27.0%)	
T4	80	43(53.8%)	37(46.3%)	
Histological grade				0.057
Well differentiated	79	52(65.8%)	27(34.2%)	
Poor differentiated	38	18(47.4%)	20(52.6%)	
Lymphatic invasion				0.386
Negative	80	50(62.5%)	30(37.5%)	
Positive	37	20(54.1%)	17(45.9%)	
Lymphatic metastasis				0.105
N0-2	75	49(65.3%)	26(34.7%)	
N3	42	21(50.0%)	21(50.0%)	
TNM stage				0.017*
I+II	37	28(75.7%)	9(24.3%)	
III+IV	80	42(52.5%)	38(47.5%)	

*: p<0.05.

Table 4. The result of univariate and multiple variate Cox analysis.

Clinicopathological characteristics	cases	Univariate		Multivariate	
		5 year(%)	P value	HR(95%CI)	P value
NPNT expression			<0.01*		<0.05*
Negative	70	50.0%		1	
Positive	47	27.7%		1.827(1.131-2.950)	
Gender			0.980		
male	81	40.7%			
female	36	41.7%			
Age (years)			0.328		
≤60	66	45.5%			
>60	51	35.3%			
Ascites			0.062		
Negative	65	47.7%			
Positive	52	32.7%			
Tumor sites			<0.05*		
upper	10	0.0%			
moderate	20	30.0%			
lower	81	40.0%			
total	6	45.7%			
Tumor size			0.278		
≤4cm	20	50.0%			
>4cm	97	39.2%			
Borrmann types			<0.01*		
Borrmann I-II	10	90.0%			
Borrmann III-IV	107	36.4%			
T stage			<0.01*		
T1-3	37	59.5%			
T4	80	32.5%			
Distant metastasis			<0.05*		
M0	111	42.3%			
M1	6	16.7%			
Resection			<0.05*		
R0	107	43.9%			
R1/2	10	10.0%			
Histological grade			0.857		
Well differentiated	79	40.5%			
Poor differentiated	38	42.1%			
Lymphatic invasion			0.087		
Negative	80	46.3%			
Positive	37	29.7%			
Lymphatic metastasis			<0.001*		
N0-2	75	53.3%			
N3	42	19.0%			
TNM stage			<0.001*		<0.001*
I+II	37	73.0%			1
III+IV	80	26.3%		4.027(2.043-7.939)	

*: p<0.05.

T stage and TNM stage motivated us to investigate whether NPNT enhanced GC cell invasion and migration. For this purpose, transwell migration and invasion assays were performed on NPNT knockdown and overexpressed GC cells. As shown by matrixgel-coated or uncoated transwell assays, depletion of NPNT expression significantly decreased migratory and invasive capacities of GC cells (Fig. 4A,B). In contrast, ectopic overexpression of NPNT presented a significant increase in GC cell migration and invasion (Fig. 4C,D).

As epithelial-mesenchymal transition (EMT) was closely associated with cancer invasion and metastasis, we further investigated the effects of NPNT on EMT. The results showed knockdown of NPNT can decrease the expression of mesenchymal markers, including N-cadherin, vimentin, ZEB2 and SNAI1, and the invasion-associated marker MMP9 (Fig. 5A). In addition, ectopic expression of NPNT in GC cells elevated the expression of mesenchymal markers including Twist1, vimentin, ZEB2, SNAI1 as well as invasion-associated marker MMP9 (Fig. 5B).

Discussion

GC is among the cancers of greater mortality and morbidity. GC carcinogenesis is a complex process of several steps. For years, studies have been carried out to elucidate the underlying mechanism of gastric tumorigenesis (Hu et al., 2019; Zuo et al., 2019). From these scientific explorations, many biomarkers, such as DNA, RNA or protein, have been introduced. These biomarkers are specifically dysregulated in cancers and

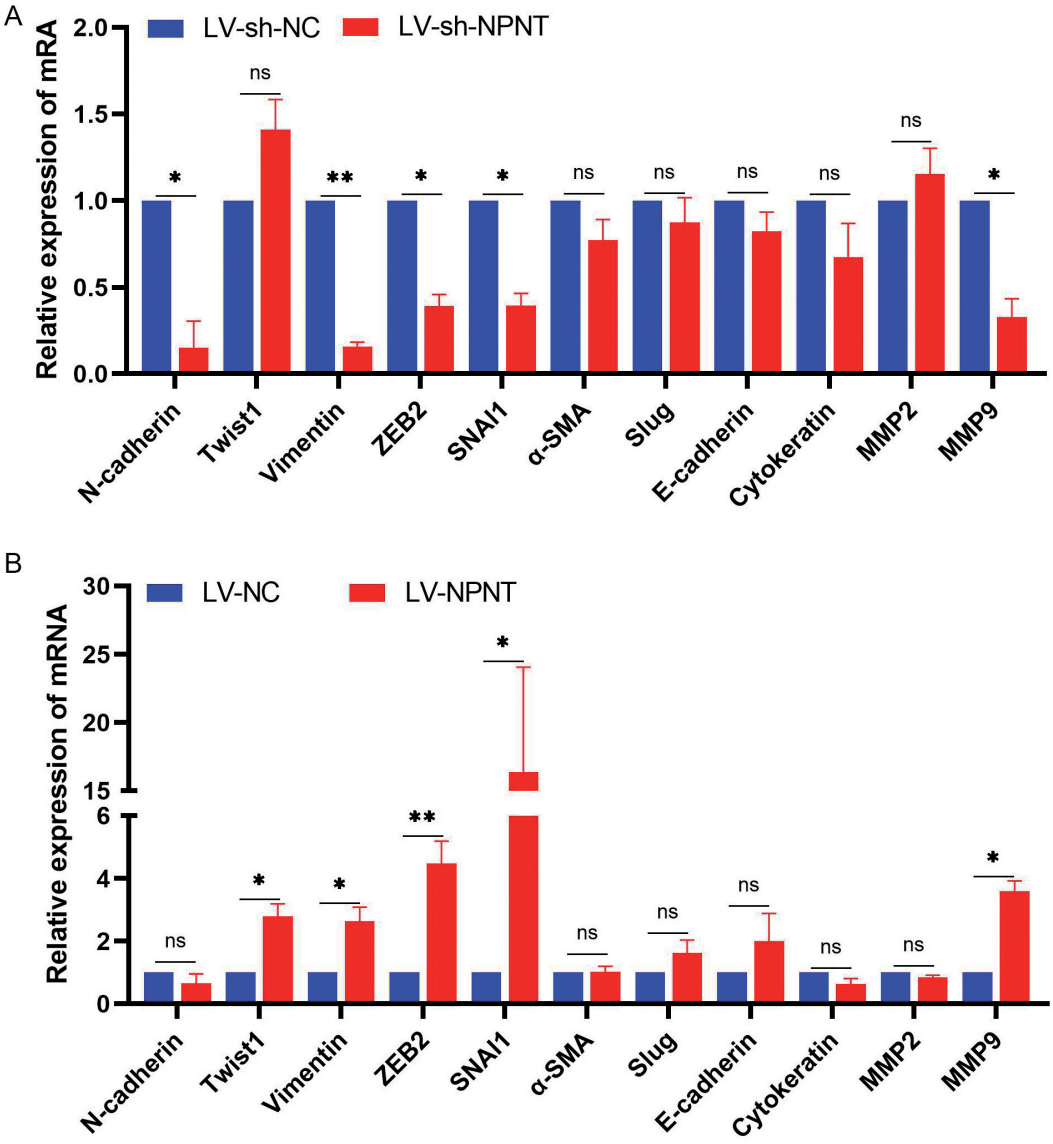

Fig. 5. A. the transcriptional levels of EMT related markers detected by realtime quantitative PCR in NPNT knockdown gastric cancer cells. **B.** the transcriptional levels of EMT related markers detected by realtime PCR in NPNT overexpression gastric cancer cells. Data are presented as mean ± SD. All experiments were independently performed three times to obtain the presented data. For all figures, *P<0.01, **P<0.01; ***P<0.001 two-tailed paired Student's t-test.

can be used as indicators for diagnosing cancers or predicting cancer patients' prognosis. In the present study, we found that NPNT was significantly upregulated in GC at both transcriptional and translational level. To our knowledge, it is the first time that the upregulated expression of NPNT in GC has been reported. Also, our findings provided clinical evidence that NPNT expression is negatively correlated with GC patients' survival, indicating that patients with positive NPNT expression have poorer survival outcomes.

NPNT is an extracellular matrix protein which was reported to be dysregulated in cancer (Sun et al., 2018). Prior studies have explored the expression profile of NPNT in breast cancer (Steigedal et al., 2018). As mentioned in these studies, NPNT was found to be upregulated in breast cancer and correlated with survival outcome. Our study set out with the aim of assessing the expression profile of NPNT in GC. The results of our study indicated that NPNT was significantly overexpressed in GC tissues compared with NAT tissues. We also confirmed that NPNT was upregulated in GC cell lines. These results are consistent with expression profiles of NPNT obtained from breast cancer. Furthermore, an important clinically relevant finding was that the NPNT expression profile was significantly correlated with patients' survival outcome and patients with high NPNT expression may have a poor prognosis. Therefore, it can be concluded that NPNT may be a conceivable prognostic predicting biomarker for GC.

As was revealed by our studies on GC and other research on breast cancer, NPNT was found to be upregulated in these cancers. However, the reasons for NPNT overexpression in cancer still remain unclear. The methylation level of gene promoter region is an important reason for abnormal expression of genes (Popadin et al., 2013; Dunn et al., 2014; Husquin et al., 2018). Upon analyzing TCGA data, results showed beta value for promoter methylation level of NPNT in GC was 0.08 (upper quantile: 0.065; lower quantile: 0.098) (beta value below 0.25 was considered hypomethylation) (Chandrashekar et al., 2017). The hypomethylation status of NPNT promoter may be a possible reason for its overexpression in GC. Other unknown factors may also contribute to the upregulation of NPNT in GC. Therefore, more high-quality studies on the current topic are recommended in the future.

In our current study, we have compared the relationship between NPNT expression and clinicopathological parameters. The results showed that there was obvious association between NPNT expression and T stage and TNM stage, which indicates that NPNT overexpression may have correlation with tumor progression. The T stage reflects the tumor invasion depth. The close relationship between NPNT expression and T stage reveals that NPNT may potentially regulate GC invasion. Furthermore, through a series of cancer cell proliferation, invasion and migration associated experiments, we found that NPNT promote GC cell proliferation and invasion, as well as migration, indicating that NPNT is an important oncogenic gene and may be a therapeutic target for treating GC.

We also found that knockdown of NPNT can decrease the expression of mesenchymal markers, including N-cadherin, vimentin, ZEB2 and SNAI1, and the invasion-associated marker MMP9 and ectopic expression of NPNT in GC cells elevated the expression of mesenchymal markers, including Twist1, vimentin, ZEB2, SNAI1 as well as invasion-associated marker MMP9. Overexpression of NPNT significantly upregulated the expression of Twist 1. Twist1 expression in NPNT knockdown cells also showed a small increasing tendency although there was no statistically significant difference. This may be explained by the low expression abundance of twist1 in GC cells observed during our experiment, which make it easily to be upregulated. Also, N-cadherin was significantly decreased in NPNT knockdown cells compared with control groups. The expression of N-cadherin seems decreased after overexpressing NPNT in GC cells although there is no statistically significant difference. This may be explained by the high expression abundance of N-cadherin in GC cells, which make it easily to be downregulated.

NPNT may also enhance the expression of EMT related genes including vimentin, ZEB2, SNAI1 and invasion associated gene MMP9. Vimentin is an intermediate filament protein that has been found to be upregulated in mesenchymal cells and is regarded as an important marker for EMT (Vuoriluoto et al., 2011; Zhou et al., 2019). Additionally, vimentin can also regulate mesenchymal cell migration, maintain mesenchymal shape, and increase the focal adhesion (Zhou et al., 2019). ZEB2 is a classical EMT-associated transcription factor which can regulate many gene expressions to promote cell polarity regulation, cell migration and anchorage-independent growth capacity etc (Zhang et al., 2019). Like ZEB2, SNAI1 is also an important EMT-associated transcription factor. SNAI1 belongs to the zinc finger transcription factor and may regulate induction of EMT. It can bind the promoter regions of many epithelial markers including occludin, claudin-1 and E-cadherin, and transcriptionally represses the expression of these genes (Goossens et al., 2017; Al-Hattab et al., 2018). In addition, NPNT can also enhance the expression invasion associated gene MMP9 which belongs to the matrix metalloproteinase family member. The MMP family members may degrade the extracellular matrix and facilitate cell migration. In our study, we reported that NPNT may increase the expression of the above EMT-associated genes including vimentin, ZEB2, SNAI1 and MMP9. Through regulating these genes, NPNT may promote EMT and GC cell migration and invasion.

In conclusion, this is the first study that has extensively characterized the NPNT expression profile in a large cohort of GC patients as well as from a public database. Our results found that NPNT was evidently overexpressed in both GC tissues and GC cell lines.

Also, significant association was found between NPNT expression and GC patients' survival. Moreover, NPNT can evidently promote GC cell proliferation, invasion as well as migration. Future investigations on the role of NPNT as a potential prognostic marker and a possible target for precise therapy in GC are required.

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