Prognostic potential of METTL3 expression in patients with gastric cancer

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Received August 13, 2021; Accepted October 27, 2022

DOI: 10.3892/ol.2022.13651

Abstract. Methyltransferase-like 3 (METTL3) is a crucial component of the m6A methyltransferase complex, which serves pivotal roles in tumor progression. The present study investigated the prognostic significance of METTL3 expression in gastric cancer (GC). The expression levels of METTL3 were assessed by immunohistochemistry in formalin-fixed paraffin-embedded (FFPE) tissue specimens from 158 patients with GC. Propensity score matching (PSM) analysis was performed to clarify its prognostic potential. METTL3 gene expression was also investigated in fresh frozen specimens from another independent cohort of 57 patients with GC to establish its clinical relevance. Knockdown of METTL3 by small interfering RNA transfection was performed to evaluate its function *in vitro*. METTL3 expression was significantly higher in cancerous tissues compared with in corresponding normal mucosa (P<0.0001), and high METTL3 expression was an independent prognostic factor for overall and disease-free survival in the FFPE cohort of patients with GC. PSM analysis revealed that elevated METTL3 expression was significantly associated with poor survival outcomes, which was subsequently validated in another cohort of fresh frozen specimens. Knockdown of METTL3 inhibited proliferation, invasion, migration and anoikis resistance in GC cells. In conclusion, METTL3 expression may be used as a clinically feasible prognostic marker and could serve as a potential therapeutic target in patients with GC.

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Key words: gastric cancer, prognosis, METTL3, RNA methyltransferases

Introduction

Gastric cancer (GC) is one of the most common cancers and the third leading cause of cancer-related deaths worldwide, primarily because of rapid disease progression to advanced stages and highly malignant potential (1,2). Despite recent progress in the diagnosis and treatment of GC patients, one-third of diagnosed patients have already an advanced stage disease characterized by extensive infiltration, lymph node metastasis, or distant metastasis (3,4). Therefore, the discovery of novel biomarkers to identify high-risk populations with regards to their survival outcomes is desperately needed to assist physicians in selecting GC patients who require intensive post-treatment surveillance for early recurrence.

Methyltransferase-like 3 (METTL3) is a representative RNA methyltransferase that maintains the homeostasis of m^6A methylation, and controls cell differentiation and proliferation through methylation of its target mRNAs (5). Accumulating evidence has revealed a pivotal role of METTL3 in the pathogeneses of various human malignancies, including breast and ovarian cancer (6-8). Interestingly, regardless of its m^6A catalytic activity, the oncogenic potential of METTL3 is mediated by the translation process of target oncogenes in lung cancer, suggesting that METTL3 promotes cancer cell growth, survival, and invasion (6). Although a growing number of studies have demonstrated the functions of METTL3 in experimental systems, the clinical impact of METTL3 expression in GC remains poorly understood.

Our previous studies have shown that several metastasis-associated genes are differentially expressed in advanced GC and can be used as biomarkers for prognosis of in this malignancy (9-15). In this study, we systematically investigated the prognostic impact and biomarker potential of METTL3 expression using multiple cohorts of clinical specimens including both FFPE and fresh frozen samples, and clarified the clinical significance of METTL3 expression in

GC patients. We also assessed the functional role of METTL3 in GC development by conducting a series of *in vitro* experiments.

Materials and methods

Tissue samples and patient characteristics. This study included analysis of a total of 158 patients (111 men and 47 women, median age: 69 years, age range: 18-88 years) who received surgery for primary GC from 2005 to 2011 at the Department of Gastrointestinal Surgery, Mie University Hospital, Japan for immunohistochemistry analysis. Immunohistochemical analysis of METTL3 protein expression was performed using Formalin-fixed, paraffin-embedded (FFPE) samples with primary GC (FFPE cohort). We used the tumor node metastasis (TNM) classification to assess clinicopathological findings. This cohort included 70 GC patients with a stage I disease, 24 with stage II, 26 with stage III and 38 with stage IV tumors. Histological findings indicated that 86 patients exhibited an intestinal type GC while 72 were diffuse type. Post-operative follow-up data was obtained from all enrolled patients, and the median follow-up duration was 24.5 months (range: 1-83 months).

In addition, we analyzed tissue specimens from a fresh frozen cohort of 57 GC patients, (44 men, 13 women, median age: 69 years, age range: 48-85 years) who received surgery for primary GC from 2011 to 2015 at the Department of Gastrointestinal Surgery, Mie University Hospital, Japan. To investigate METTL3 gene expression by quantitative polymerase chain reaction (qPCR). Fifty-seven gastric specimens were preserved immediately after surgical resection in *RNAlater* (Qiagen) and stored at -80˚C until RNA extraction to investigate METTL3 gene expression through qPCR. There were 10 patients with stage I, 11 with stage II, 23 with stage III, and 13 with stage IV GCs. Histological findings for these fresh frozen samples identified 29 patients had intestinal-type GC and 28 had the diffuse type. Post-operative follow-up data were obtained from all patients, and the median follow-up duration was 17.2 months (range: 1-98 months).

In both cohorts, patients who underwent endoscopic mucosal resection, neoadjuvant therapy, or had non-gastric carcinomas were excluded. All enrolled patients were followed up after their initial hospital discharge with physical examinations, tumor marker assays (carcinoembryonic antigen and carbohydrate antigen 19-9) performed every 1-3 months, and computed tomography every 6 months. Endoscopic examinations were performed when necessary. All participants provided written informed consent. The study protocol was approved by the Institutional Review Board of Mie University (approval no. H2019-197). This study was performed in accordance with The Declaration of Helsinki.

Immunohistochemistry. For the immunohistochemical measurement of METTL3 expression, we used FFPE sections (2-3 μm thick) from 158 GC patients. Following deparaffinization and dehydration of the cells, the specimens were boiled in 10 mM sodium citrate buffer to retrieve antigens, as described previously (13). These specimens were then blocked and incubated with the primary antibody overnight at 4˚C. The primary antibody against METTL3 (Abcam) was diluted at a 1:500 ratio. Antibody binding was detected by a horseradish peroxidase Envision kit (Dako Cytomation), and all sections were counterstained with hematoxylin.

Assessment of METTL3 expression using immunohistochemistry. The expression of METTL3 was first assessed by scanning the entire tissue specimen under low power magnification (x40), and then confirmed under high power (x200 and x400). A scoring system for immunoreactivity was used, as described previously (13,16): (A) fraction of positively stained cells: 0, ≤5%; 1, 6-25%; 2, 26-50%; 3, 51-75%; and 4, >75%; (B) intensity of staining: 0, colorless; 1, pale yellow; 2, yellow; and 3, brown. Scores of immunoreactivities were defined as (A) multiplied by (B) (the fraction of positive stained cells multiplied by the intensity of staining). Assessment of METTL3 expression in stained FFPE sections were separately conducted by two experts without knowledge of the clinicopathological or survival data of any of the patients, to ensure confidence in histopathological analysis. METTL3 expression in FFPE sections was re-evaluated if scores of immunoreactivities by the two experts differed by more than 3.

Total RNA extraction and cDNA synthesis. We used Mixer Mill MM 300 homogenizer (Qiagen) to homogenize the fresh frozen specimens. Total RNA from fresh frozen tissues and cell lines was isolated using a RNeasy mini kit (Qiagen), as previously described (13). UV absorbance at 260 and 280 nm were used for measurement of the concentration and quality of RNA, and $OD_{260/280}$ ratios of 1.8-2.1 were considered adequate. Five μg of total RNA with random hexamers and Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) were used for cDNA synthesis.

Reverse transcription-qPCR (RT-qPCR). StepOne™ Real Time PCR System (Applied Biosystems) was used for RT-qPCR analysis, as previously described (10,13). Primers for METTL3 and GAPDH were created by Primer 3 software (Biology Workbench version 3.2, San Diego Supercomputer Center, University of California). The designed sequences were as follows: METTL3: forward, ACATGCTGCCTCAGA TGTTG; reverse, GGATTGTTCCTTGGCTGTTG; GAPDH: forward, GGA AGGTGA AGGTCG GAGTC; reverse, AATGAAGGGGTCATTGATGG. qPCR was performed with Power SYBR Green PCR Master Mix (x2) (Applied Biosystems), and the following cycling conditions were used: 95˚C for 10 min, 40 cycles at 95˚C for 15 sec, and 60˚C for 1 min. Relative gene expression levels of METTL3 were determined by the standard curve method, and quantitative normalization was conducted using GAPDH gene expression as an internal control, as previously described (16).

Cell lines. Human GC cell lines MKN7 (intestinal type), MKN74 (intestinal type), KATO III (diffuse type), NUGC3 (diffuse type), and NUGC4 (diffuse type) were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan). The cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics, and cultured at 37˚C in a humidified atmosphere with 5% CO_2, as described previously (13).

METTL3 siRNA interference. METTL3-specific siRNA (Silencer® Select Validated siRNA (Assay ID: s32143, s32141), standard purity) and negative control siRNA (Silencer™ Negative Control siRNA: Catalog Number: 4390843) were purchased from Ambion (Austin, TX). The sequences used were as follows: METTL3-specific siRNA(Assay ID: s32143): Sense sequence: GCAGUUCCU GAAUUAGCUATT; Antisense sequence: UAGCUAAUU CAGGAACUGCTG; METTL3-specific siRNA(Assay ID: s32141): Sense sequence: GAACGGGUAGAUGAAAUU ATT; Antisense sequence: UAAUUUCAUCUACCCGUU CAT; negative control siRNA: Forward transfections were conducted by mixing siRNA oligonucleotides (50 nM) with Lipofectamine RNAiMAX (Invitrogen) and Opti-MEM I (Invitrogen) and applying the mixture to cells at 24 h after plating. A series of *in vitro* assays were conducted after 48 h of incubation.

Cell proliferation assay. A WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] colorimetric assay was performed to assess cell proliferation, as previously described (13). METTL3 siRNA-transfected and negative control siRNA-transfected cells (5,000 cells/well) were seeded in 96-well plates (Becton Dickinson Labware) in 100 μl culture medium. After 0-72 h of culture, the medium was discarded and replaced with 90 μl fresh medium, followed by the addition of 10 μl WST-8 reagent solution (Cell Counting Kit; Dojindo Laboratories). The cells were then incubated for 2 h at 37˚C. Each independent experiment was conducted three times. Cell proliferation was investigated by colorimetric comparison by reading OD values using a microplate reader (SoftMax; Molecular Devices) at an absorption wavelength of 450 nm.

Cell invasion and migration assay. Cell invasion and migration were assessed using Biocoat Matrigel invasion chambers and control inserts (Becton Dickinson Labware), as described previously (13,16). A total of $5x10^4$ transfected cells/well were seeded in both the invasion and control chambers. We used 10% fetal bovine serum as the chemoattractant, and seeding cells were incubated for 48 h at 37˚C. The incubation medium containing cells was removed from the top chamber using cotton swabs and serum-free medium. The membranes were fixed in methanol, stained with Mayer's hematoxylin, dehydrated in ethanol, and mounted on glass slides. The number of cells that invaded the underside of the membrane was then counted. Each independent experiment was conducted three times.

Anoikis assay. We used six-well Costar Ultra-Low Attachment Microplates for Anoikis assays, as described previously (9,13). A total of $1x10^6$ transfected cells/2ml/well were seeded in each well and incubated for 48 h in a humidified atmosphere (37˚C with 5% CO_2). After induction of anoikis, an MTT assay was performed with cells seeded at $5x10^3$ cells/well in microtiter plates (96 wells, flat bottom) in a final volume of 100 μl culture medium per well. The spectrophotometric absorbance of the samples was measured as described above. Each independent experiment was conducted three times.

Statistical methods. Statistical analysis was conducted using Medcalc version 19.3.1 (Broekstraat 52, 9030), as previously described (13). Results were expressed as the mean ± standard deviation (SD). Differences between groups were estimated by one-way ANOVA, Wilcoxon's signed rank test, the Mann-Whitney U-test, or Kruskal-Wallis test as appropriate based on the normality of distribution determined by using the Shapiro-Wilk test and equality of variance for comparable groups using Levene's test. When multiple hypothesis testing was performed, Tukey-Kramer method was used for post-hoc analysis after ANOVA, and Dunn method was used after the Kruskal-Wallis test. Differences between categorized groups were estimated by the χ^2 test. Cochran-Armitage test for trend was used in place of χ^2 test if the variable had two columns and three or more rows. For time-to-event analyses, survival estimates were calculated by Kaplan-Meier analysis, and groups were compared by the log-rank test. Receiver operating characteristic (ROC) curves were established to determine cutoff values for analysis of survival outcomes by Youden's index in each cohort. Overall survival (OS) was measured from the date the patient underwent surgery until the date of death from any cause, (cancer-unrelated deaths were not censored) or the last known follow-up for patients that were still alive. Disease-free survival (DFS) was measured from the date the patient underwent curative surgery to the date of disease recurrence, death from any cause (cancer unrelated deaths were not censored), or until the last contact with the patient. Cox's proportional hazards models were used to estimate hazard ratios (HR) for death or recurrence. Assumption of proportionality was confirmed for the Cox proportional hazards analyses by generating Kaplan-Meier survival curves (e.g., high vs. low METTL3 expression groups) and ensuring that the two curves did not intersect each other. Variables with $P<0.05$ in the univariate analysis were included in the multivariate analysis. Clinical variables that were considered for univariate and multivariate analyses, in addition to the target METTL3 expression status, were previously identified confounding factors that affected the prognosis of patients with gastric cancer: sex, age at diagnosis, histological type (intestinal or diffuse), T stage (T1/2 or T3/4), venous invasion (present or absent), lymphatic vessel invasion (present or absent), nerve invasion (present or absent), lymph node metastasis (present or absent), and distant metastasis (presence or absence).

To clarify the prognostic value of METTL3 expression in GC patients, we conducted propensity score matching (PSM) analysis (13). High or low expression of METTL3 protein in GC tissues was designated as the objective factor. By applying logistic regression analysis, a continuous propensity score ranging from 0 to 1 was generated. Matched covariates included gender (male or female), T classification (T1/2 or T3/4), venous invasion (presence or absence), lymphatic vessel invasion (presence or absence), lymph node metastasis (presence or absence), and distant metastasis (presence or absence), in accordance with the results of the univariate analysis for the risk of high METTL3 expression in GC tissues. Matching on the estimated propensity scores with the maximum allowable difference of 0.001 yielded 74 matched pairs with high or low METTL3 expression (37 patients in each group, 95% CI: -0.000039 to 0.000033, P=0.87). All

Table I. Clinicopathological variables and METTL3 protein expression in gastric cancer patients.

Characteristic	All patients	High[b] (n=61)	Low (n=97)	P-value
Sex				0.029[c,d]
Male	111	49	62	
Female	47	12	35	
Age, years				0.05[d]
<69[a]	88	28	60	
≧69	70	33	37	
Histological type				0.06[d]
Intestinal type	86	39	47	
Diffuse type	72	22	50	
Pathological T category				0.0002[c,d]
pT1/2	79	19	60	
pT3/4	79	42	37	
Vessel invasion				<0.0001[c,d]
Present	86	48	38	
Absent	72	13	59	
Lymphovascular invasion				0.011[c,d]
Present	117	52	65	
Absent	41	9	32	
Lymph node metastasis				0.004[c,d]
Present	73	37	36	
Absent	85	24	61	
Distant metastasis				0.0004[c,d]
Present	38	24	14	
Absent	120	37	83	
UICC TNM classification				0.0001[c,e]
Stage I	70	16	54	
Stage II	26	12	14	
Stage III	24	9	15	
Stage IV	38	24	14	

[a]Median age at surgery was 69 years in this cohort; [b]cutoff threshold of METTL3 expression was determined by ROC analysis with Youden's index for overall survival of GC patients; [c]P<0.05; [d]χ^2 test; [e]Cochran-Armitage test for trend. METTL3, methyltransferase-like 3.

P-values were two-sided, and values of <0.05 were considered statistically significant.

Results

METTL3 protein was mainly expressed in GC cells compared with cancer stroma and adjacent normal mucosa. At first, we evaluated the cellular distribution of METTL3 protein expression in GC tissues using immunohistochemical analysis. METTL3 protein expression was primarily expressed in the nuclei of tumor cells and significantly increased in GC cells compared with cancer stroma and adjacent normal gastric mucosa (P<0.0001; Figs. 1A, S1A and B).

METTL3 expression was associated with disease development in the FFPE cohort of GC patients. Next, we investigated associations between clinicopathological factors and METTL3 expression in the FFPE cohort. We defined a cutoff value of >7.5 as the high staining group (n=61) and ≤7.5 as the low staining group (n=97) based on ROC analyses with Youden's index correction for METTL3 expression. The high staining group was significantly associated with males (P=0.029), an advanced T stage (P=0.0002), the presence of venous invasion (P<0.0001), lymphatic vessel invasion (P=0.011), lymph node metastasis (P=0.004), distant metastasis (P=0.0004), and advanced TNM stage classification (P=0.0001) in the FFPE cohort of GC patients (Table I; Fig. 1B).

Increased expression of METTL3 protein is an independent prognostic factor for both OS and DFS in the FFPE cohort of GC patients. To investigate the potential use of METTL3 expression as a prognostic biomarker, we performed time-to-event analysis. GC Patients with increased expression of METTL3 showed poorer prognosis in terms of both OS and DFS compared to those with low expression (OS: P<0.0001, Fig. 1C; DFS: P=0.0005, Fig. 1D; log-rank test).

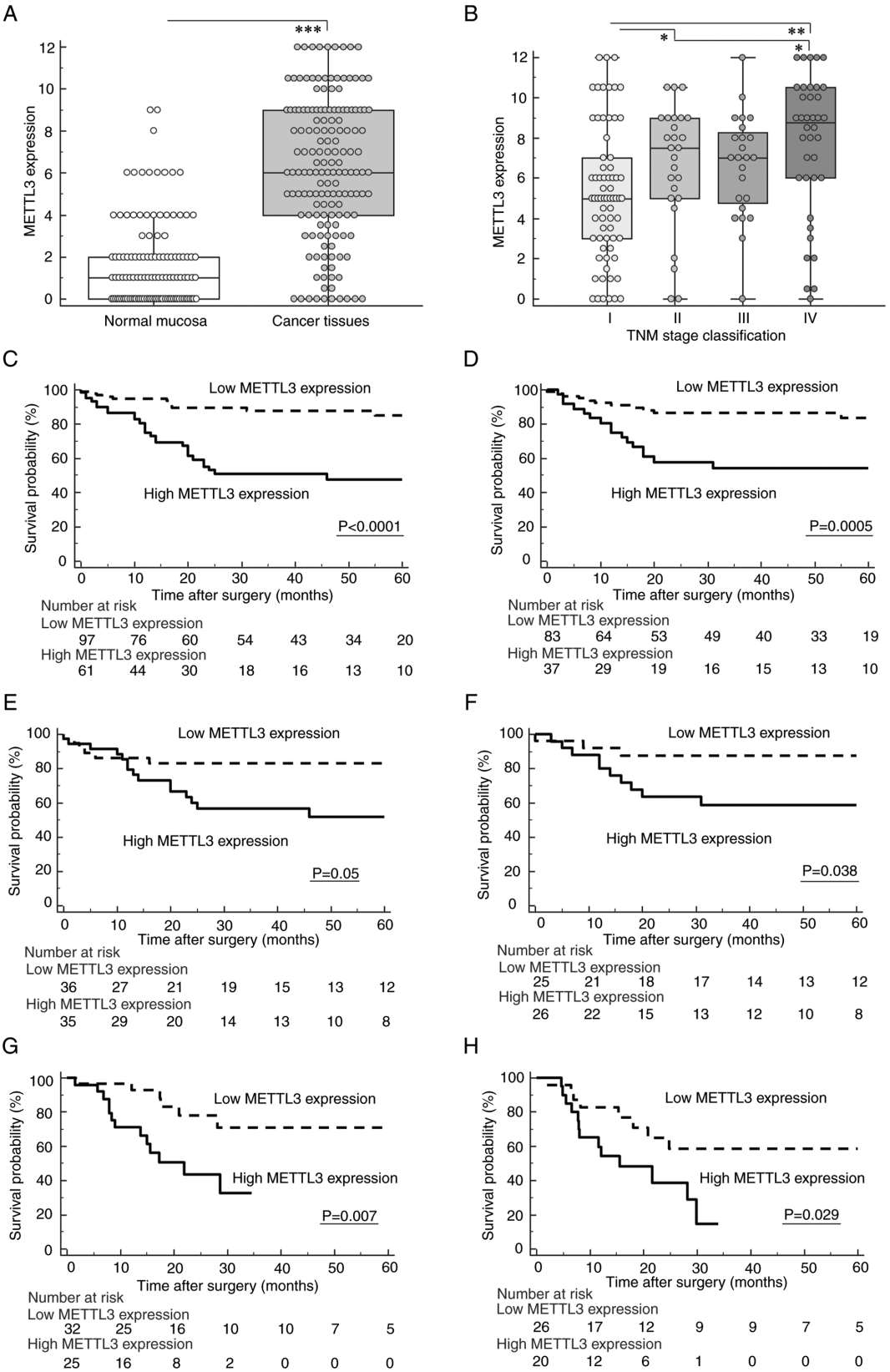

Figure 1. Dysregulation pattern of Methyltransferase-like 3 (METTL3) expression and its prognostic potential in overall survival (OS) and disease-free survival (DFS) of gastric cancer (GC) patients. (A) METTL3 expression was significantly increased in GC tissues compared with adjacent normal mucosa. (B) Scattergrams of METTL3 expression according to the UICC classification in GC patients. (C and D) Kaplan-Meier survival curves for OS (C) and DFS (D) of GC patients based on METTL3 expression in GC tissues of the formalin-fixed, paraffin-embedded cohort. Patients with high expression of METTL3 exhibited a significantly poorer survival compared with those in the low expression group (OS: P<0.0001; DFS: P=0.0005, log-rank test). (E and F) Survival curve analysis subdivided by METTL3 expression in GC tissues using PSM analysis demonstrated that high METTL3 expression was significantly correlated with poor OS (E) (P=0.05, log-rank test) and DFS (F) (P=0.038, log-rank test). (G, H) Kaplan-Meier survival curves for OS (G) and DFS (H) of GC patients based on METTL3 expression in the fresh frozen cohort. Elevated expression of METTL3 in GC tissues was significantly associated with poor oncological outcomes in the fresh frozen cohort (OS: P=0.007; DFS: P=0.029, log-rank test). All statistical tests were two-sided. ***P<0.001, **P<0.01, *P<0.05. METTL3, methyltransferase-like 3; OS, overall survival; DFS, disease-free survival; GC, gastric cancer.

Table II. Multivariate analysis for predictors of survival in the GC cohort using immunohistochemical analysis.

A, Overall survival

	Univariate			Multivariate		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
Gender (male)	1.75	0.83-3.68	0.14			
Age (≧69 years old)[a]	2.44	1.29-4.6	0.006[b]	2.57	1.33-4.98	0.005[b]
Histological type (intestinal type)	0.99	0.53-1.84	0.97			
T classification (pT3/4)	3.25	1.63-6.46	0.0008[b]	0.63	0.23-1.72	0.37
Vessel involvement (present)	2.89	1.38-6.09	0.005[b]	0.84	0.34-2.06	0.7
Lymphatic vessel involvement (present)	1.52	0.7-3.31	0.29			
Lymph node metastasis (present)	5.21	2.52-10.8	<0.0001[b]	3.95	1.55-10.1	0.004[b]
Distant metastasis (present)	5.96	3.06-11.6	<0.0001[b]	3.28	1.47-7.34	0.004[b]
High METTL3 expression[c]	4.39	2.23-8.65	<0.0001[b]	3.24	1.57-6.68	0.001[b]

B, Disease-free survival

	Univariate			Multivariate		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
Gender (male)	2.24	0.92-5.5	0.08			
Age (≧69 years old)[a]	2.41	1.16-5.02	0.019[b]	1.7	0.79-3.67	0.18
Histological type (intestinal type)	0.85	0.41-1.78	0.67			
T classification (pT3/4)	3.94	1.86-8.37	0.0003[b]	1.64	0.67-4.01	0.28
Vessel involvement (present)	1.98	0.93-4.19	0.08			
Lymphatic vessel involvement (present)	1.24	0.57-2.7	0.6			
Lymph node metastasis (present)	5	2.37-10.6	<0.0001[b]	3.36	1.39-8.12	0.007[b]
High METTL3 expression[c]	3.4	1.63-7.09	0.001[b]	2.4	1.12-5.16	0.025[b]

[a]Median age at surgery was 69 years for GC patients; [b]P<0.05; [c]Cutoff threshold of METTL3 expression was determined by ROC analysis with Youden's index for overall survival of GC patients. HR, hazard ratio; METTL3, methyltransferase-like 3; GC, gastric cancer.

Next, we determined the potential of the METTL3 expression status as a predictive biomarker for recurrence and prognosis in GC patients using multivariate Cox regression analysis. These analyses demonstrated that increased METTL3 expression in GC tissues was an independent prognostic factor for both OS [hazard ratio (HR), 3.24; 95% confidence interval (CI), 1.57-6.68; P=0.001, Table IIA) and DFS (HR, 2.4; 95% CI, 1.12-5.16; P=0.025, Table IIB) in the FFPE cohort of GC patients.

PSM analysis validated the prognostic impact of METTL3 expression in GC patients. PSM analysis has come into the limelight as a new statistical method to overcome selection bias and different patient characteristics to elevate the evidence level of a non-randomized observational study (17). To clarify the potential of METTL3 expression as a prognostic biomarker in GC patients, we performed PSM analysis using FFPE cohort and categorized 74 GC patients (37 patients in each group) for further analysis. No differences in patient characteristics between high- and low-staining group were found following PSM analysis (Table SI). Kaplan-Meier survival curve analysis demonstrated that high expression of METTL3 was significantly associated with poor prognosis in terms of both OS and DFS (OS: P=0.05, Fig. 1E; DFS: P=0.038, Fig. 1F; log-rank test) in the PSM cohort of GC patients. Collectively, these findings clearly indicated that METTL3 protein expression in GC tissues might be used as a prognostic biomarker in GC patients.

The prognostic impact of METTL3 expression was successfully validated in fresh frozen cohort of GC patients. We confirmed the biomarker potential of METTL3 expression using FFPE specimens to identify GC patients with high-risk for oncological outcomes. In a clinical setting, assessment of METTL3 expression using preoperative biopsy specimens could provide valuable information for physicians to decide the treatment course for GC patients. From the aspect that preoperative biopsy specimens are usually preserved in a fresh frozen specimen, we further evaluated METTL3 gene expression using fresh frozen specimens from an independent GC cohort to clarify the prognostic impact of METTL3 gene expression. We quantified expression levels of METTL3 in 57 GC tissues using RT-qPCR, and the cut-off threshold for

METTL3 expression were consistent method of the FFPE cohort and performed ROC analyses with Youden's index to determine prognosis of GC patients. Interestingly, in accordance with METTL3 protein expression in the FFPE cohort, the high expression group was significantly associated with the presence of venous invasion (P=0.001) in the fresh frozen cohort (Table III).

To further confirm the prognostic potential of METTL3 expression in GC patients, we investigated whether assessment of METTL3 gene expression in fresh frozen specimens could identify GC patients with high-risk for poor survival outcomes. Survival curve analysis showed that high METTL3 gene expression in GC tissues was significantly associated with poorer prognosis in terms of both OS and DFS in the fresh frozen cohort (OS: P=0.007, Fig. 1G; DFS: P=0.029, Fig. 1H). Surprisingly, multivariate Cox regression analysis also revealed that increased METTL3 expression was an independent prognostic factor, especially for OS (OS: HR, 3.38; 95% CI, 1.17-9.73; P=0.024, Table IVA; DFS: HR, 2.69; 95% CI, 0.97-7.45; P=0.058, Table IVB), in the fresh frozen cohort of GC patients. Collectively, our findings highlighted that quantification of METTL3 gene expression in fresh frozen specimens could also provide valuable information for oncologists to identify patients at high-risk for recurrence and poor prognosis in GC patients.

siRNA transfection led to significant inhibition of METTL3 expression in GC cell lines. Considering the prognostic burden of METTL3 expression in GC tissues as described above, we further evaluated the biological role of METTL3 in GC development. At first, we assessed METTL3 expression by RT-qPCR analysis in established GC cell lines (Fig. 2A) and demonstrated that METTL3 was highly expressed in MKN7 and NUGC3 cells compared to all other GC cell lines. Therefore, we decided to use MKN7 and NUGC3 cell lines for further knockdown experiments. Treatment of GC cell lines with METTL3 siRNA transfection (Silencer® Select Validated siRNA (Assay ID: s32143)) showed significant inhibition of this methyl transferase mRNA expression (up to 60%) compared to those with negative control siRNA at 48 h post-transfection (Fig. 2B).

METTL3 knockdown could inhibit proliferation, invasion, and migration of GC cells. To determine whether METTL3 siRNA transfection affected cell proliferation in human GC cell lines, we used MTT assays. METTL3 knockdown significantly suppressed tumor cell growth at 48 and 72 h after cell transfection in both MKN7 and NUGC3 cell lines (Fig. 2C and D). We further conducted *in vitro* invasion and migration assays to reveal that METTL3 siRNA transfection of MKN7 and NUGC3 GC cells resulted in significant inhibition of the invasive and migratory potentials compared with cells transfected with control siRNA (Fig. 3A and B).

METTL3 siRNA transfection suppressed anoikis resistance in GC cells. Anoikis is an apoptotic process induced by loss of cell adhesion (18), and resistance to anoikis is a pivotal metastatic process in malignancies (19). To clarify whether METTL3 knockdown promoted anoikis, we assessed the number of viable suspended MKN7 and NUGC3 cells in low attachment plates through MTT assays. Interestingly, both GC cell lines with METTL3 inhibition demonstrated significant decreases in the number of viable cells compared with cells transfected with control siRNA (Fig. 3C and D). Collectively, these findings suggest that METTL3 is involved in the pathogenesis of GC by enhancing cell growth, invasive and migratory potentials, and anoikis resistance in GC cells.

METTL3 inhibition using different METTL3-siRNAs suppressed proliferation in GC cells. To further clarify whether these results are siRNA-specific phenomena,

Table III. Clinicopathological variables and METTL3 mRNA expression in gastric cancer patients.

Variable	n	METTL3 expression		P-value
		High[b] (n=25)	Low (n=32)	
Sex				
Male	44	20	24	0.66[c]
Female	13	5	8	
Age, years				
<69[a]	25	10	15	0.61[c]
≧69	32	15	17	
Histological type				
Intestinal type	29	12	17	0.7[c]
Diffuse type	28	13	15	
Pathological T category				
pT1/2	12	6	6	0.63[c]
pT3/4	45	19	26	
Vessel invasion				
Present	35	19	16	0.047[c,d]
Absent	22	6	16	
Lymphovascular invasion				
Present	48	20	28	0.45[c]
Absent	9	5	4	
Lymph node metastasis				
Present	38	18	20	0.67[c]
Absent	17	7	10	
Distant metastasis				
Present	13	7	6	0.41[c]
Absent	44	18	26	
UICC TNM classification				
Stage I	10	5	5	0.62[e]
Stage II	11	3	8	
Stage III	23	10	13	
Stage IV	13	7	6	

[a]Median age at surgery was 69 years in this cohort; [b]Cutoff threshold of METTL3 expression was determined by ROC analysis with Youden's index for overall survival of GC patients; [c]χ^2 test; [d]P<0.05; [e]Cochran-Armitage test for trend. METTL3, methyltransferase-like 3.

Table IV. Multivariate analysis for predictors of survival in GC cohort using qPCR analysis.

A, Overall survival

	Univariate			Multivariate		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
Gender (male)	2.41	0.56-10.4	0.24			
Age (≧69 years old)[a]	2.98	1.07-8.31	0.036[b]	3.09	0.98-9.73	0.05
Histological type (diffuse type)	1.46	0.59-3.65	0.41			
T classification (pT3/4)	5.77	0.77-43.3	0.09			
Vessel involvement (present)	2.32	0.77-7.01	0.13			
Lymphatic vessel involvement (present)	3.83	0.51-28.8	0.19			
Lymph node metastasis (present)	10.1	1.34-76.6	0.025[b]	10.5	1.33-82.4	0.026[b]
Distant metastasis (present)	2.48	0.97-6.35	0.06			
High METTL3 expression[c]	3.54	1.34-9.37	0.011[b]	3.38	1.17-9.73	0.024[b]

B, Disease-free survival

	Univariate			Multivariate		
	HR	95% CI	P-value	HR	95% CI	P-value
Gender (male)	1.01	0.37-2.77	0.98			
Age (≧69 years old)[a]	3.06	1.22-7.68	0.017[b]	3.55	1.24-10.2	0.018[b]
Histological type (diffuse type)	1.69	0.71-4.01	0.24			
T classification (pT3/4)	2.74	0.81-9.33	0.11			
Vessel involvement (present)	2.88	0.97-8.56	0.06			
Lymphatic vessel involvement (present)	2.71	0.63-11.7	0.18			
Lymph node metastasis (present)	3.35	1.1-10.2	0.033[b]	4	1.25-12.8	0.02[b]
High METTL3 expression[c]	2.61	1.07-6.36	0.035[b]	2.69	0.97-7.45	0.058

[a]Median age at surgery was 69 years for GC patients; [b]P<0.05; [c]Cutoff threshold of METTL3 expression was determined by ROC analysis with Youden's index for overall survival of GC patients. HR, hazard ratio; METTL3, methyltransferase-like 3; GC, gastric cancer.

we performed additional *in vitro* analysis using different METTL3-siRNA transfection. Treatment of GC cell lines with METTL3 siRNA transfection [Silencer® Select Validated siRNA (Assay ID: s32141)] also showed significant inhibition of METTL3 expression (up to 60%) compared to those with negative control siRNA at 48 h post-transfection (Fig. S2A). We further conducted MTT assay after treatment of GC cell lines with METTL3 siRNA transfection, and successfully verified that inhibition of METTL3 expression significantly suppressed tumor cell growth at 48 and 72 h after cell transfection in both MKN7 and NUGC3 cell lines (Fig. S2B and C).

Discussion

In the last decade, treatment options for GC have progressed drastically to improve the prognosis of GC patients (2). However, GC is an aggressive cancer, and the survival rate of advanced GC remains poor (1). Currently, the pathological TNM stage classification is the best available prognostic indicator. However, the differences in oncological outcomes of GC patients largely depend on the underlying molecular heterogeneity, and the TNM stage classification is inadequate at predicting survival outcomes accurately for individual GC patients. Therefore, identification of high-risk populations may help physicians to decide the treatment course and improve the prognosis of GC. In this study, we systemically investigated the clinical value and potential role of METTL3 in GC and made several novel discoveries. First, METTL3 protein expression was significantly elevated in tumor cells of GC tissues compared with adjacent normal mucosa. Second, increased expression of METTL3 was significantly correlated with well-established disease development factors and was an independent prognostic factor for both OS and DFS in the FFPE cohort. Third, PSM analysis clearly demonstrated the biomarker potential of METTL3 expression in GC tissues to identify poor oncological outcomes of GC patients. Fourth, we confirmed the prognostic value of METTL3 expression using fresh frozen specimens from GC patients. Finally, knockdown of METTL3 expression inhibited various oncological functions of GC cells, including proliferation, invasion, migration and anoikis resistance.

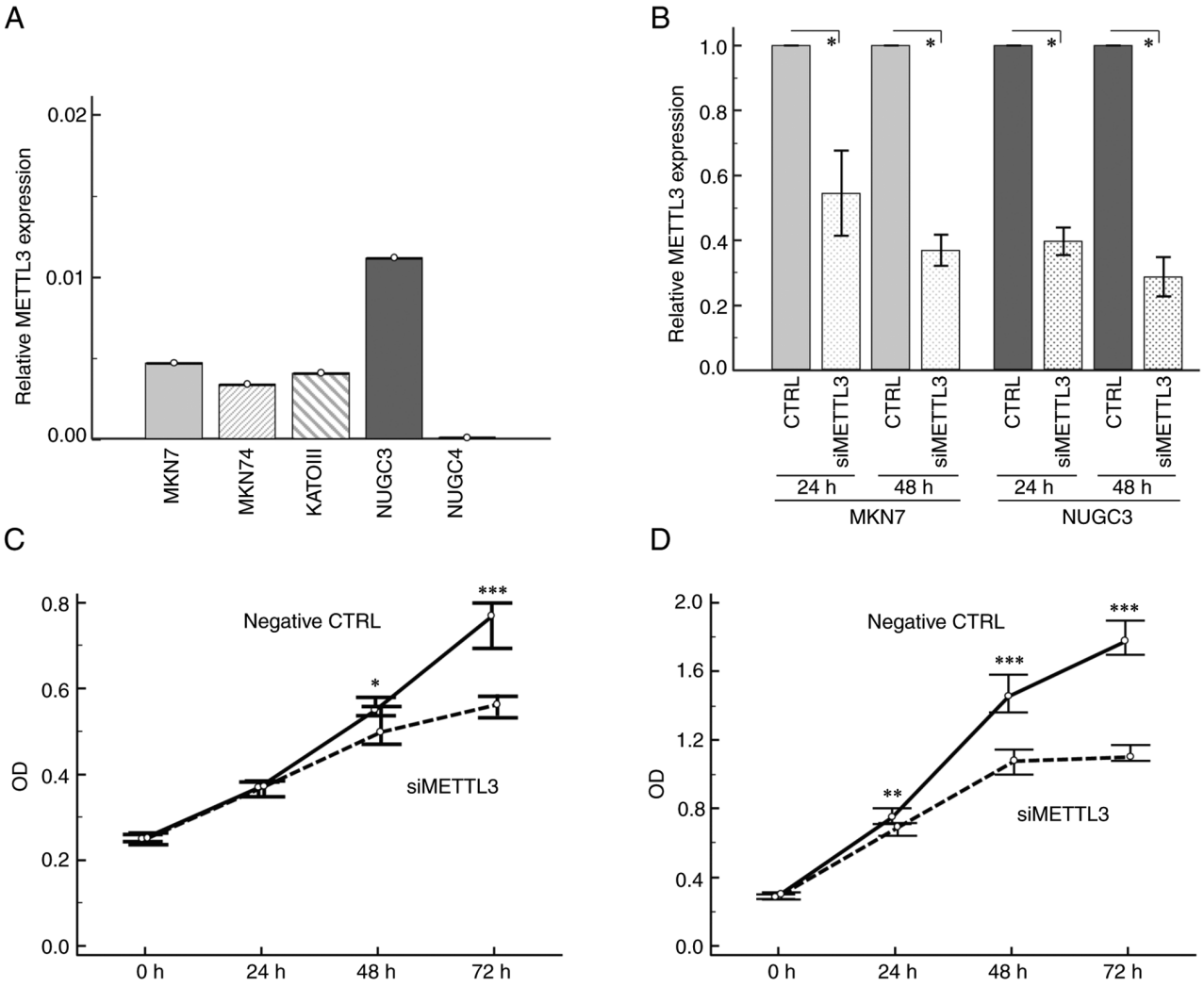

Figure 2. *In vitro* analyses of gastric cancer (GC) cell lines subjected to Methyltransferase-like 3 (METTL3) siRNA transfection. (A) Semi-quantitative RT-qPCR analysis to detect METTL3 gene expression in GC cell lines. (B) METTL3 expression was significantly suppressed by METTL3 siRNA transfection in both MKN7 and NUGC3 cell lines. GC cells were transfected with either METTL3-siRNA (siMETTL3) or negative control siRNA (Negative CTRL), and expression ratio of siMETTL3 to Negative CTRL cells was measured at 24 and 48 h after transfection by RT-qPCR. (C and D) Proliferative effects of METTL3 in GC cells at 48 and 72 h. The proliferation potential was significantly suppressed after METTL3 knockdown compared with Negative CTRL MKN7 (C) and NUGC3 (D) cells. RT-qPCR, reverse transcription-quantitative PCR. ***P<0.001, **P<0.01, *P<0.05 as indicated or vs. siMETTL3. METTL3, methyltransferase-like 3; GC, gastric cancer; siMETTL3, METTL3-siRNA; negative CTRL, negative control siRNA.

Medical treatments for GC patients have advanced tremendously over the last decade, and several clinical trials have revealed the therapeutic impact of various chemotherapies and molecular targeted therapies, including trastuzumab (20), ramucirumab (21,22), nivolumab (23), trifluridine/tipiracil (24), on oncological outcomes, especially in unresectable GC patients. In the adjuvant setting, a recent study demonstrated significant survival benefits of docetaxel combined with S-1 in post-operative patients with stage III GC (25). Furthermore, a recent randomized controlled trial showed no survival benefit of additional gastrectomy over chemotherapy alone in patients with non-curable advanced GC (26). Considering such evidence, identification of a high-risk population for recurrence and survival using pre-operative or post-operative specimens may provide feasible information for physicians to decide the best treatment course possible for GC patients with chemotherapeutic regimens. This could considerably improve survival rates of GC patients.

METTL3 plays a central functional role in oncogenesis (27,28), and several studies have corroborated its potential as a prognostic biomarker in various cancers (29,30). For instance, Lin and co-workers assessed METTL3 protein expression through immunohistochemical analysis in 100 patients with hepatocellular carcinoma (HCC) (29). They demonstrated that METTL3 protein was significantly upregulated in the nuclei of HCC cells compared with normal liver tissues, and that HCC patients with strong expression of METTL3 protein had poorer survival than those with weak expression, similar to our findings in GC cells. Another study conducted immunohistochemical analysis of surgical specimens from 162 ovarian carcinoma patients (30). METTL3 was frequently upregulated in ovarian carcinoma and high expression of METTL3 protein was significantly associated with a poor survival rate. Consistent with this evidence, a major finding of this study was the prognostic impact of METTL3 expression in FFPE specimens of GC patients. Increased expression of METTL3 was significantly

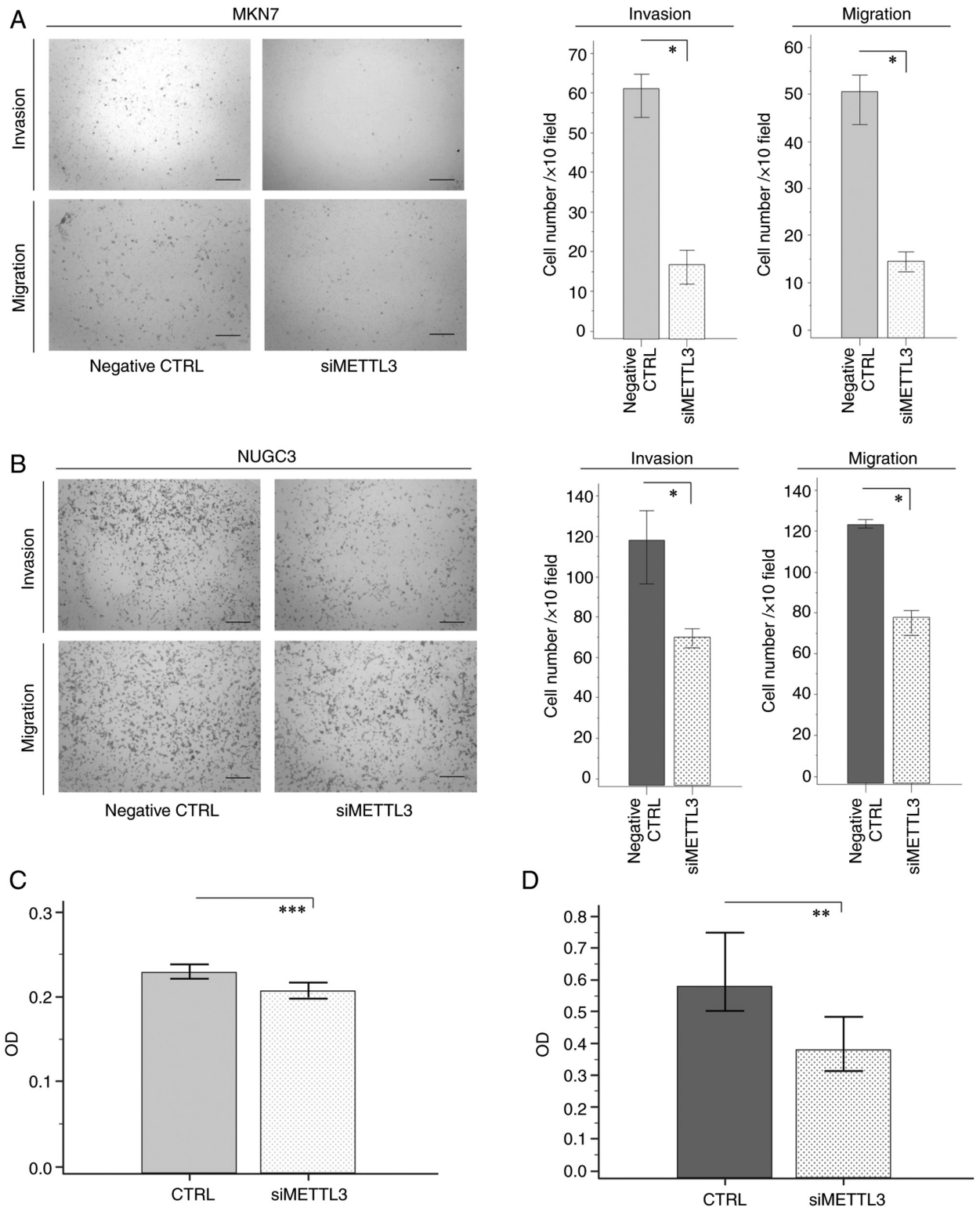

Figure 3. Invasion, Migration, and anoikis assay of gastric cancer (GC) cell lines subjected to Methyltransferase-like 3 (METTL3) siRNA transfection. (A and B) Invasion and migration assays demonstrated that knockdown of METTL3 expression significantly inhibited invasion and migration abilities in both MKN7 (A) and NUGC3 (B) cell lines. Scale Bar=500 um. (C and D) Anoikis resistance of GC cells after treatment with or without METTL3 siRNA. Both MKN7 (C) and NUGC3 (D) cell lines with METTL3 inhibition demonstrated significant decrease in the numbers of viable cells, which were significantly lower than those transfected with negative control siRNA. All statistical tests were two-sided. One-way analysis of variance. $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$ as indicated or vs. siMETTL3. METTL3, methyltransferase-like 3; GC, gastric cancer; siMETTL3, METTL3-siRNA; Negative CTRL, negative control siRNA.

associated with well-established disease development factors and an independent prognostic factor for both the DFS and OS of GC patients. Interestingly, the PSM analysis clearly revealed and confirmed the prognostic value of METTL3 expression even after adjustment of covariate factors based on the different GC patient characteristics. In addition, we quantified METTL3 gene expression in fresh frozen specimens, assuming pre-operative biopsy specimens, and

validated the prognostic value of METTL3 by RT-qPCR analysis. Collectively, these findings suggested that assessment of METTL3 expression in fresh frozen or surgical specimens can be used to decide the treatment course or chemotherapeutic regimens for GC patients.

Accumulating evidence has continued to reveal the pivotal role of epigenetic regulation, including DNA methylation, histone modification, and non-coding RNAs, in disease development of malignancies (31). RNA methylation was classically identified in the 1970s (32,33), and the biological function of this RNA modification was recently recognized as a pivotal epigenetic alteration that is deeply involved in the post-transcriptional regulation of major genes associated with human cancers (34). N6-methyladenosine (m^6A) modification is the most prevalent internal modification in eukaryotic cells (35), and several lines of evidence have demonstrated that dysregulation of m^6A methylation regulates cancer development (27,28). METTL3 is a representative methyltransferase responsible for m^6A modification, and its catalytic subunit forms the m^6A methyltransferase complex with METTL14, WTAP, and RBM15 (36). Furthermore, Lin and coworkers found that the N-terminal of METTL3 is sufficient to promote translation, while the catalytic domain containing the C-terminus of METTL3 has no effect on promoting translation, further confirming that METTL3 promotes translation of oncogenes independently of its catalytic activity and m^6A readers (6). Based on these findings, emerging studies have demonstrated the oncogenic role of METTL3 in various processes of disease development, including cell proliferation, invasion, and migration in malignancies including lung cancer, liver cancer, breast cancer, bladder cancer, and myeloid leukemia progression (6-8,37-39). Chen *et al* conducted a series of *in vitro* and *in vivo* analyses, and demonstrated that knockdown of METTL3 significantly inhibited cell proliferation, migration, and tumorigenicity via suppressor of cytokine signaling 2 mRNA m^6A modification in hepatocellular cell carcinoma (38). Another study showed that METTL3 knockdown reduced proliferation, invasion, and the anti-apoptotic potential via regulation of AKT phosphorylation in ovarian cancer cells (8). Not only is this evidence consistent with our findings, we went a step further by confirming that METTL3 knockdown inhibited various oncogenic phenotypes related to oncogenesis, including proliferation, invasion, migration, and anoikis resistance in GC cell lines. Collectively, these findings suggest that METTL3 plays critical roles in the malignant potential of GC, which may be a therapeutic target.

In conclusion, our study highlights that assessment of METTL3 expression in fresh frozen or FFPE specimens may provide pivotal information for the identification of high-risk GC patients. In addition, METTL3 might be a novel therapeutic target in GC patients.

Acknowledgements

The authors thank Mrs. Yuki Orito and Dr Amphone Okada (Gastrointestinal and Pediatric Surgery, Division of Reparative Medicine, Institute of Life Sciences, Mie University Graduate School of Medicine) for providing excellent technical assistance.

Funding

This work was supported in part by Grants-in-Aid for Scientific Research (grant nos. 18K08566, 18K08700, 18K08674, 18K08591 and 20K09004) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YO, YT, MO and KN conceived and designed the study. TI, HI, TK, TS, MK, HY, HF, TY and MO provided the samples. YO, YT, CY, MR, TI, HI, TK, TS, MK, TY, and IM acquired the data. YO, YT, AG, MR, HY, HF, IM, MO and KN analyzed and interpretated the data. YO and YT performed the statistical analysis. YO, YT, AG, MO and KN drafted the manuscript. YO and YT confirm the authenticity of raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of Mie University (approval no. H2019-197). The requirement for informed consent was waived, and an information disclosure statement was uploaded onto the homepage of our hospital website for opt-out.

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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