Original Article

Upregulated expression of NOP2 predicts worse prognosis of gastric adenocarcinoma by promoting tumor growth

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Abstract

Background: NOP2 nucleolar protein plays a crucial role in early embryo development and cell proliferation. The role of NOP2 in human gastric adenocarcinoma has not been elucidated. In the present study, we aimed to examine the expression levels of NOP2 and dissected whether NOP2 expression was associated with aggressive clinicopathological outcomes of patients with gastric adenocarcinoma.

Methods: Clinicopathological analysis was performed in patients with gastric adenocarcinoma. Expression of NOP2 was tested by immunohistochemistry staining and quantitative RT-PCR. The prognostic role of NOP2 in gastric adenocarcinoma patients was assessed by univariate and multivariate analysis. The effect of NOP2 on cell proliferation was examined through cellular experiments and mice models.

Results: NOP2 expression was elevated in gastric adenocarcinoma tissues compared to normal gastric tissues. High expression of NOP2 was significantly correlated with tumor size, invasion depth, and lymph node metastasis. Moreover, patients with high NOP2 expression had poorer overall survival, and NOP2 was identified as an independent prognosis factor. Using the gastric adenocarcinoma cells, we found that NOP2 can promote tumor cell proliferation both *in vitro* and *in vivo*.

Conclusions: Overexpression of NOP2 significantly correlates with a poorer prognosis of gastric adenocarcinoma patients and suggested the potential of NOP2, which may serve as a novel prognostic biomarker in gastric adenocarcinoma.

Keywords: Gastric adenocarcinoma, NOP2, prognosis

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INTRODUCTION

NOP2 nucleolar protein is also named as proliferation-associated nucleolar protein P120, which is involved in the ribosomal assembly. Recognized to play critical roles in regulating cell cycle and nucleolar activity, NOP2 is highly expressed during the proliferation of stem cells and in the adult brain.[1] NOP2 is reported to be negative or undetectable in most normal resting cells but increases remarkably in some actively proliferating cells.[2] Distinct expression of NOP2 has been observed in tumor cells and nontumorous cells. For example, NOP2 protein content can distinguish between non-neoplastic and

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malignant lesions in oral pathology.[3] Similarly, NOP2 is positively expressed in glioma tissues and cell lines, while its expression is not detected in adjacent brain tissues.[4] Another example is that NOP2 expression is undetectable in mild dysplasia adenomas but shows positive expression in colorectal cancers, which is also significantly related to the Ki-67 expression.[5] Interestingly, NOP2 was reported to be significantly positively correlated with age in colorectal cancer.[6] Considering the rapid proliferation of malignant cells, NOP2 expression may provide a reliable indication of proliferation rapidity regardless of tumor origin.[7] Indeed, expression of NOP2, measured either at the protein or the mRNA level, correlates with cell proliferation rate.[8] Overexpression of NOP2 results in malignant transformation of NIH/3T3 cells *in vitro* and produces rapidly growing tumors in nude mice.[9] In contrast, antisense-mediated inhibition of NOP2 expression prevents G1- to S-phase transition and thus inhibits cell proliferation.[10]

Moreover, dysregulated expression of NOP2 is correlated with the prognosis of tumor patients. Breast cancer patients with positive NOP2 expression exhibit worse prognosis than those with negative NOP2.[11] Different histological types of lung cancer possess distinct NOP2 expression patterns,[12] and lung adenocarcinoma patients with higher NOP2 expression experience early recurrence and shorter survival compared with those with lower NOP2[13,14] Consistently elevated NOP2 expression is associated with poor prognosis of renal clear cell carcinoma[15] and prostate adenocarcinoma.[16]

The mortality from gastric cancer (GC), whose treatment and prognostic prediction are unsatisfactory, ranks third among the malignant tumors worldwide, and its incidence is even higher in East Asia.[17-19] Till now, the expression and clinical significance of NOP2 in gastric cancer has not been elucidated. Here we explored its mRNA and protein levels in gastric adenocarcinoma and investigated its role in prognostic prediction. In addition, we conducted *in vitro* and *in vivo* experiments to validate its oncogenic effects and thus provided evidence for its therapeutic potential.

PATIENTS AND METHODS

Patient enrollment

Between January 2020 and September 2020, we identified a total of 31 patients with gastric adenocarcinoma that underwent surgical treatment at the Baoan District Hospital of Traditional Chinese Medicine. The fresh-resected tumor tissues and paired adjacent nontumorous samples were flash-frozen in liquid nitrogen for mRNA extraction. In addition, we enrolled another retrospective cohort containing 148 gastric adenocarcinoma patients. All diagnoses were confirmed by routine pathological examination, and the inclusion criteria were as follows: (1) complete and detailed clinicopathological data; (2) postoperative survival time more than 1 month; (3) no preoperative neoadjuvant chemotherapy or radiotherapy; (4) no history or signs of other malignancies. Follow-up data were recorded until March 2021. The median follow-up time was 22 months, ranging from 2 to 77 months. Tumor staging and histological classification were assessed according to the American Joint Committee on Cancer (AJCC) classification.

This study was approved by the Ethics Committee of the Baoan District Hospital of Traditional Chinese Medicine. Written informed consent was obtained from all patients.

Online database

The data from 408 gastric cancers and 211 nontumorous stomach tissues were retrieved from the Cancer Genome Atlas (TCGA, http://cancergenome-nih.gov/) for mRNA data re-analysis using the online website GEPIA (http://gepia.cancer-pku.cn/detail.php).

RNA extraction and qPCR

The mRNA levels of NOP2 and ACTB in the 31 pairs of gastric cancers and the corresponding tumor-adjacent normal tissues were detected with qPCR. First, TRIzol reagent (Thermo Fisher) and RNeasy protect mini kit (Qiagen, Hilden, Germany) were used to extract the total RNAs of these tissues. After that, Primescript RT reagent kit (Takara BIO Inc.) was used for reverse transcription PCR.[20] The quantification of qPCR was finally achieved using the Thermo Fisher 7500 PCR System. The results were analyzed using the ACTB as the internal control in a $2^{-\Delta\Delta Ct}$ method. The qPCR primers were designed as follow: NOP2: Forward 5'-AAGGGTGCCGAGACAGAACT-3'; Reverse 5'-GAGCACGACTAGACAGCCTC-3'ACTB: Forward 5'-CATGTACGTTGCTATCCAGGC-3'; Reverse 5'-CTCCTTAATGTCACGCACGAT-3'.

Tissue microarray (TMA) and immunohistochemistry

The 148 cases of formalin-fixed and paraffin-embedded gastric cancer tissues were used to test the protein expression of NOP2. In brief, the 4-μm sections were first deparaffinized with xylene and rehydrated with graded ethanol. Then, 3% hydrogen peroxide was applied to inactivate the endogenous peroxidase activity. Slides were boiled in citrate buffer (pH = 6.0) for 10 min for optimal antigen retrieval and then in 5% bovine serum albumin for 30 min, to eliminate unspecific antigen binding. The primary antibody of NOP2 was used to incubate the

specimen at 4°C overnight. The biotin-labeled secondary antibody and streptavidin-peroxidase were used to incubate the slides. Finally, the visualization of slides was achieved using incubation 3, 3'-diaminobenzidine substrate for 10 min.

Evaluation of IHC results

The results of IHC were semi-quantified through evaluation by two senior pathologists who were blinded to the clinical information. Briefly, the two pathologists independently observed more than 500 cancer cells in more than five randomly selected fields and counted the percentage of positively stained cancer cells that were predominantly stained in the cell nucleus. When the difference of the positive percentage was over 15% between the two pathologists, the section was re-evaluated. According to the median percentage, a cut-off value of 35% was used to define low NOP2 protein expression and high NOP2 protein expression.

Cell culture and shRNA

MKN28 and MKN45 cells were cultured in RPMI1640 medium supplemented with 10% FBS in the standard cell culture condition. The shRNAs targeting NOP2 and control shRNA hairpins were synthesized by Integrated DNA Technologies as reported,[21] and cloned into the lentiviral vector pAPM. The transduction of shRNAs was conducted according to the manufacturer's instructions.[22]

CCK-8 assay

Cell counting kit-8 (CCK-8) was purchased from Dojindo (Tokyo, Japan). Briefly, 5000 cells were seeded into 96-well-plates and cultured for designated time points (6, 24, 48, 72, and 96 h) for detection. At each time point, 10 μL of CCK-8 solution was added to each well and cultured for another 4 h before detection. The OD450 values were finally detected using a microplate reader.

Colony formation

Single-cell suspensions of gastric cancer cells were seeded into 6-well plates at 700 cells/well and incubated at 37°C for 14 days. Then, the cells were fixed with 4% formaldehyde for 30 min, followed by staining with crystal violet solution for another 30 min. The numbers of colonies were counted and compared.

Xenografts

Four-week nude mice were procured from Shanghai Animal Center (Shanghai, China). The mice were housed under standard conditions. Stable transduced cells were subcutaneously injected into the nude mice, and the tumor size was measured using vernier calipers every 5 days. After 25 days, the mice were sacrificed to isolate the xenografts. The animal study was reviewed and approved by the Ethics Committee of the Baoan District Hospital of Traditional Chinese Medicine.

Statistics

All the statistical analyses were performed using SPSS 22.0 software (SPSS, Chicago, IL, USA). The association between NOP2 expression and clinicopathological parameters was assessed by Chi-square test or Fisher's exact test. The overall survival rates were calculated using Kaplan–Meier method, and the statistical differences between subgroups were calculated using log-rank test. Independent prognostic factors were identified by multivariate analysis with Cox regression model. $P < 0.05$ was considered statistically significant.[23]

RESULTS

Patients' characteristics

Among the 148 enrolled patients, there were 34 females and 114 males. According to the conventional definition of "elderly patients,"[24,25] 65 cases were diagnosed at ages younger than 65 years, while the other 83 cases were at older ages. There were 41 cases with cardia tumor location, 21 cases with fundus location, and 6 cases with "cardia-fundus location." Therefore, we combined these patients into a "cardia or fundus location" group (n = 68). Similarly, due to a limited case number of pylorus tumor location (n = 3) and the existence of "body-antrum location" (n = 16), we combined these patients into "stomach body or antrum or pylorus" group (n = 80). The tumor size was less than 2.0 cm in 23 patients, 2.0–5.0 cm in 84 patients, and larger than 5.0 cm in 41 patients. Only 10 patients showed well differentiation (grade I), 54 cases showed moderate differentiation (grade II), and the other 84 cases showed poor tumor differentiation (grade III). According to the tumor invasion depth, 32 cases were diagnosed with stage T1, 23 cases with stage T2, 75 cases with stage T3, and 18 cases with stage T4. Based on the lymph node metastasis, 57 cases showed negative lymph node and were staged with N0 stage, 38 cases with N1 stage, 34 cases with N2 stage, and 19 cases with N3 stage. Only 33 patients underwent total or subtotal gastrectomy, while the other 115 cases underwent partial gastrectomy. As for the postoperative treatment, 93 cases accepted adjuvant chemotherapy, while the other 55 cases were absent from adjuvant chemotherapy.

NOP2 expression in gastric adenocarcinoma

First, we extracted the mRNA from 31 pairs of sample specimens and compared using the RT-qPCR method.

The mRNA level of NOP2 was found to be significantly higher in gastric cancer tissues compared to that in adjacent nontumorous tissues [Figure 1a, $P < 0.001$]. Considering the limited case number, we retrieved its mRNA level from the TCGA dataset based on the microarray data, which also demonstrated a higher NOP2 mRNA level in gastric cancer tissues [Figure 1b, $P < 0.001$].

Next, we tested the protein expression of NOP2 by immunohistochemistry staining. NOP2 showed detectable but different expression levels in gastric adenocarcinoma tissues [Figure 1c], while almost undetectable in adjacent nontumorous stomach tissues [Figure 1d]. By sub-grouping patients into the high-NOP2 group (n = 74) and low-NOP2 group (n = 74) based on the immunohistochemistry data, we found that large-sized tumors were more prevalent to exhibit higher NOP2 protein levels [Table 1, $P = 0.006$]. Moreover, the protein level of NOP2 was positively correlated with the T stage ($P = 0.011$) and N stage ($P = 0.001$) of gastric adenocarcinoma [Table 1]. The correlation test indicated that higher NOP2 expression may contribute to gastric cancer progression.

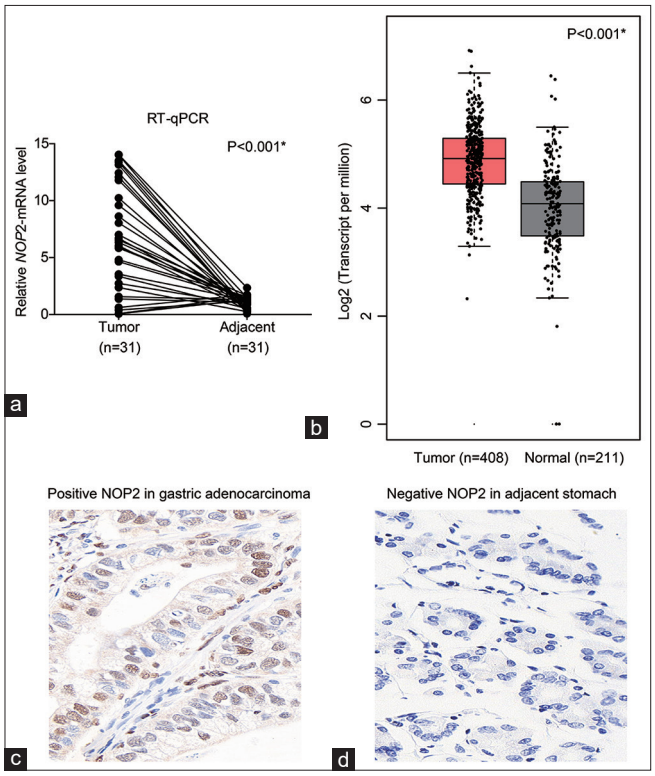

Figure 1: mRNA and protein expression of NOP2 in gastric cancer (a) RT-qPCR data showed that gastric cancer tissues exhibited higher NOP2 expression than that in paired adjacent tissues. Data were acquired from 31 paired tissues and compared by paired Student's *t* test. (b) The mRNA level of NOP2 was retrieved from the TCGA database and presented as transcripts per million, which showed a higher NOP2-mRNA level in gastric cancer tissues than that in normal stomach tissues. Data were analyzed by GEPIA web server and compared by unpaired Student's *t* test. (c) Represents high NOP2 expression in gastric adenocarcinoma tissues. (d) Represents negative NOP2 expression in normal stomach tissues

Prognostic significance of NOP2 in gastric adenocarcinoma

Next, we conducted survival analysis using the Kaplan–Meier method to investigate the clinical significance of NOP2 in gastric adenocarcinoma. As shown in Table 2, the average survival time of patients in the low-NOP2 group was 52.2 ± 3.8 months and decreased to 29.2 ± 3.1 months in patients in the high-NOP2 group ($P < 0.001$). Consistently, the 5-year overall survival rate was significantly higher in the low-NOP2 group (53.4%) than that in the high-NOP2 group [17.1%, Figure 2a]. Besides the protein expression of NOP2, we also analyzed the prognostic role of its mRNA level using in silico method according to the TCGA datasets. As a result, patients with lower NOP2 mRNA levels showed significantly better overall survival and progression-free survival than those with higher NOP2 mRNA levels [Figure 2b, 2c; both $P < 0.001$]. Taken together, we concluded that higher expression of

Table 1: Correlations between NOP2 expression with patients' characteristics

Characteristics	Cases	NOP2 protein expression		*P*
	(*n*=148)	Low (*n*=74)	High (*n*=74)	
Age				
<65 yrs	65	37	28	0.136
≥65 yrs	83	37	46	
Sex				
Female	34	17	17	1.000
Male	114	57	57	
Localization				
Cardia/fundus	68	39	29	0.099
Body/antrum/pylorus	80	35	45	
Tumor diameter				
<2.0 cm	23	18	5	0.006*
2.0–5.0 cm	84	41	43	
>5.0 cm	41	15	26	
Differentiation				
Well	10	6	4	0.250
Moderate	54	31	23	
Poor	84	37	47	
T stage				
T1	32	24	8	0.011*
T2	23	12	11	
T3	75	31	44	
T4	18	7	11	
N stage				
N0	57	39	18	0.001*
N1	38	19	19	
N2	34	11	23	
N3	19	5	14	
Gastrectomy				
Total/subtotal	33	15	18	0.554
Partial	115	59	56	
Chemotherapy				
Absent	55	31	24	0.234
Accepted	93	43	50	

* indicates $P<0.05$ by Chi-square test or Fisher exact test

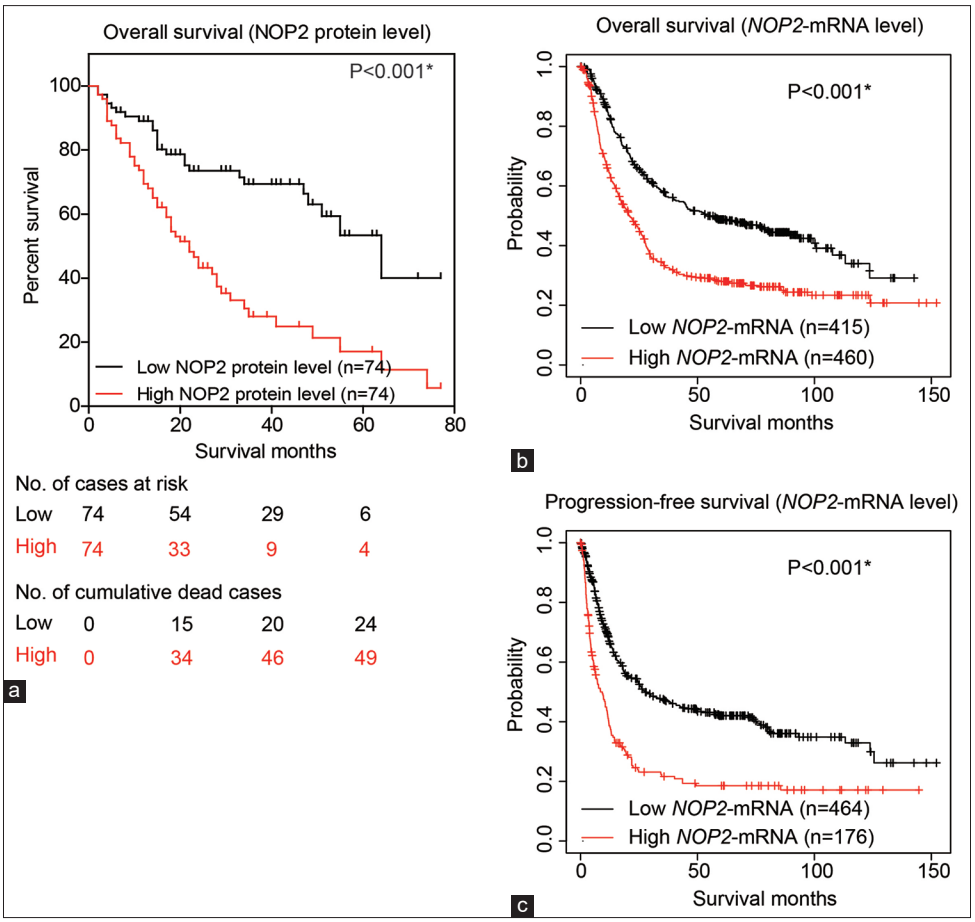

Figure 2: Prognostic prediction role of NOP2 in gastric cancer. (a) According to our retrospective cohort, gastric cancer patients with higher NOP2 protein expression in tumor tissues exhibited poorer overall survival than those with lower NOP2 protein expression. (b) According to the TCGA dataset, gastric cancer patients with higher NOP2 mRNA levels in tumor tissues exhibited poorer overall survival than those with lower NOP2 mRNA levels. (c) According to the TCGA cohort, gastric cancer patients with higher NOP2 mRNA levels in tumor tissues exhibited poorer progression-free survival than those with lower NOP2 mRNA levels. Data were compared using log-rank test. *$P < 0.05$ was considered statistically significant

NOP2 can help predict a poorer prognosis of gastric adenocarcinoma.

In addition, we analyzed the prognostic significance of other retrieved variables [Table 2, Figure 3]. Patients with larger tumor size ($P = 0.015$), poorer differentiation grade ($P = 0.001$), advanced T stage ($P < 0.001$), or advanced N stage ($P < 0.001$) exhibited shorter overall survival time. All significant factors ($P < 0.05$) based on univariate analysis (including tumor diameter, differentiation, T stage, N stage, and NOP2 expression level) were subjected to a Cox hazard regression model for multivariate analysis. According to the multivariate test, advanced T stage showed an independent effect on unfavorable prognosis [Table 3, $P < 0.05$]. Of note, higher NOP2 expression also independently contributed to a poorer overall survival (Hazard ratio = 2.221, 95% confidence interval: 1.310–3.765, $P = 0.003$).

NOP2 promotes gastric cancer progression both *in vitro* and in vivo

These clinical findings allowed us to further explore the tumor-related effects of NOP2 in gastric adenocarcinoma. After validating the knockdown efficiencies of shRNAs targeting NOP2 by immunoblotting [Figure 4a], cells were subjected to CCK-8 assay and colony formation assay to test their proliferation capacities. NOP2-knockdown significantly attenuated the proliferation processes of MKN28 and MKN45 gastric adenocarcinoma cell lines [Figure 4b, 4c].

Moreover, we generated the xenograft mice model by subcutaneously injecting cells into nude mice. By monitoring the *in vivo* tumor growth, we found that silencing NOP2 resulted in a slower growth rate of xenografts [Figure 4d]. Therefore, we concluded that NOP2 can promote gastric cancer progression and help predict patients' prognosis after surgical resection.

Table 2: Kaplan–Meier analyses of overall survival (OS)

Characteristics	Cases (*n*=148)	OS months (Mean±SEM)	5-year OS (%)	*P*
Age				
<65 yrs	65	45.2±3.8	45.9%	0.085
≥65 yrs	83	36.7±3.3	28.6%	
Sex				
Female	34	34.3±4.8	19.1%	0.112
Male	114	43.0±3.1	41.6%	
Localization				
Cardia/fundus	68	38.8±4.0	34.2%	0.601
Body/antrum/pylorus	80	41.9±3.5	36.5%	
Tumor diameter				
<2.0 cm	23	58.4±5.9	65.4%	0.015*
2.0-5.0 cm	84	39.8±3.6	32.7%	
>5.0 cm	41	32.1±4.3	24.9%	
Differentiation				
Well	10	67.3±8.9	88.9%	0.001*
Moderate	54	48.8±4.4	46.6%	
Poor	84	31.6±2.7	21.5%	
T stage				
T1	32	60.2±4.7	69.5%	<0.001*
T2	23	47.5±6.3	32.3%	
T3	75	32.4±2.9	24.2%	
T4	18	17.4±3.1	0%	
N stage				
N0	57	54.9±4.1	64.1%	<0.001*
N1	38	37.9±4.1	26.5%	
N2	34	27.6±4.5	10.7%	
N3	19	23.6±5.0	20.3%	
Gastrectomy				
Total/subtotal	33	33.4±4.5	14.3%	0.209
Partial	115	42.8±3.0	40.9%	
Chemotherapy				
Absent	55	41.7±4.3	41.5%	0.858
Accepted	93	40.7±3.4	30.3%	
NOP2 expression				
Low	74	52.2±3.8	53.4%	<0.001*
High	74	29.2±3.1	17.1%	

* indicates $P<0.05$ by log-rank test

Table 3: Multivariate analysis

Variables	Hazard ratio	95% CI	*P*
Tumor diameter			
<2.0 cm	Reference		
2.0-5.0 cm	0.854	0.332-2.196	0.744
>5.0 cm	0.858	0.308-2.393	0.770
Differentiation			
Well	Reference		
Moderate	2.060	0.444-9.544	0.356
Poor	2.709	0.583-12.593	0.204
T stage			
T1	Reference		
T2	1.493	0.497-4.486	0.475
T3	2.724	1.069-6.939	0.036*
T4	4.004	1.339-11.976	0.013*
N stage			
N0	Reference		
N1	1.150	0.567-2.334	0.698
N2	1.429	0.697-2.930	0.330
N3	1.438	0.629-3.286	0.389
NOP2 expression			
Low	Reference		
High	2.221	1.310-3.765	0.003*

* indicates $P<0.05$ by Cox regression test

DISCUSSIONS

The prognosis of gastric cancer patients is largely dependent on tumor stages, although great improvement has been achieved on adjuvant therapies. However, even patients in the same stage may exhibit completely different clinical outcomes because gastric cancer is a highly heterogeneous disease. Therefore, identifying more prognostic predictive biomarkers is essential for personalized follow-up instruction and treatment. Here, we initially tested the expression profile and clinical relevance of NOP2 in gastric cancer. In our cohort and TCGA cohort, higher NOP2 was observed in gastric cancer tissues compared to adjacent nontumorous stomach tissues, indicating its participation in tumorigenesis. Interestingly, higher NOP2 was more frequent in tumors with a large size and deeper invasion depth, thus suggesting its role in tumor growth. Indeed, cellular and mice data demonstrated that silencing NOP2 can remarkably inhibit the proliferation capacity of gastric cancer cells. Considering its role in ribosomal assembly, NOP2 may functions by regulating cell cycle, which needs further experimental validation. Interestingly, Yang *et al*.[26] reported a reduced methylation level in NOP2-knockdown HeLa cells, indicating its role as a critical mRNA m^5C methyltransferase which may thus participate in tumorigenesis. Indeed, a recent study by Mei *et al*.[27] demonstrated that NOP2 can promote gastric cancer cell proliferation by repressing Cyclin-dependent kinase inhibitor 1B (CDKN1B, $p27^{Kip1}$) in an m^5C-dependent manner, which is consistent with our major findings.

Considering its tumor-promoting role in various malignancies, targeting NOP2 may serve as a novel direction for drug development. One example is that the ribozyme against p120 mRNA can suppress glioma cell growth.[4] In contrast, expression and function of NOP2 can be modulated by multiple upstream regulators. For instance, oncofetal long noncoding RNA PVT1 was reported to promote proliferation and stem cell-like property of hepatocellular carcinoma cells by stabilizing NOP2.[28] Similarly, long noncoding RNA LINC00963 induces NOP2 expression by sponging tumor suppressor miR-542-3p to promote metastasis in prostate cancer.[29] In addition to long noncoding RNAs, telomerase can also activate transcription of cyclin D1 gene through an interaction with NOP2,[30] thereby promoting cell proliferation. All these upstream regulators and their crosstalk with NOP2 deserve further investigation in gastric cancers.

Besides its functional mechanisms, we focused more on NOP's clinical significance in this study. Accordingly, higher

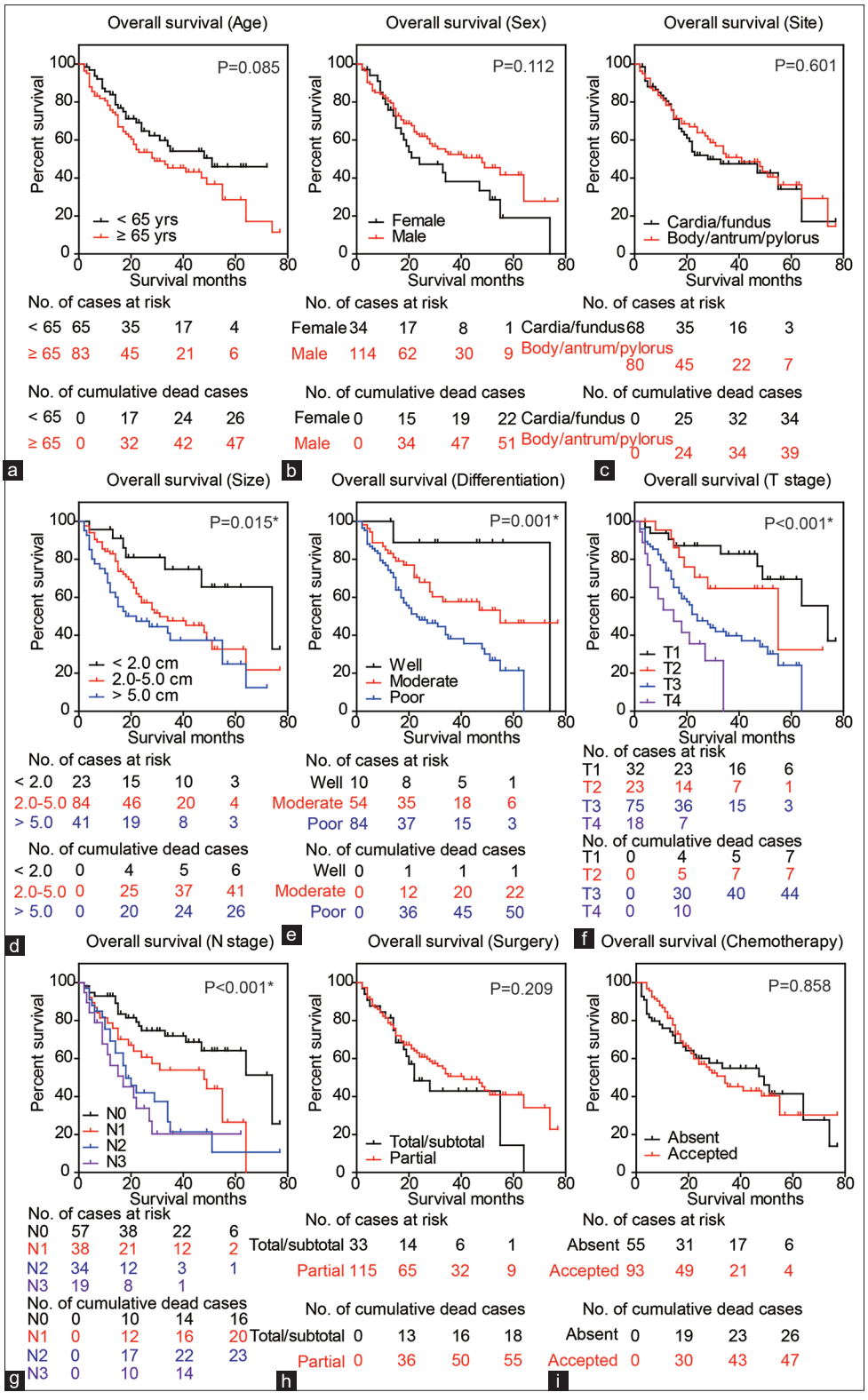

Figure 3: Overall survival analyses of our retrospective gastric adenocarcinoma cohort. The prognostic significance of each enrolled variable was evaluated, including patients age (a), sex (b), tumor site (c), tumor size (d), tumor differentiation (e), T stage (f), N stage (g), gastrectomy (h), and postoperative chemotherapy (i). Data were compared using log-rank test. *$P < 0.05$ was considered statistically significant

expression of NOP2 in gastric cancer tissues was significantly correlated with unfavorable prognosis on either mRNA level or protein level. Moreover, multivariate analysis confirmed the independent contribution of NOP2 on poorer survival of gastric cancer patients. Therefore, NOP2 may serve as a novel biomarker to help predict the prognosis of gastric cancer.

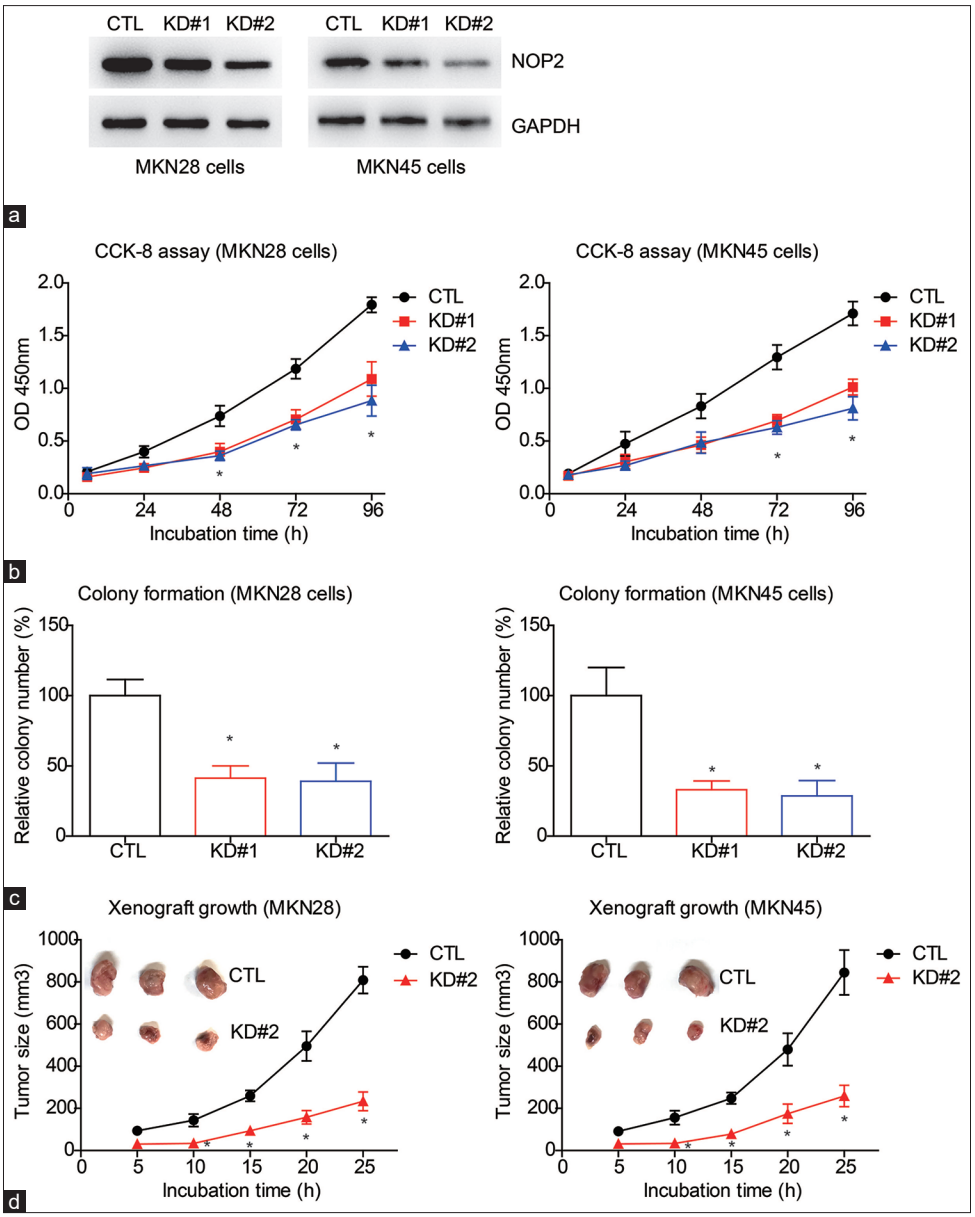

Figure 4: Silencing NOP2 inhibits gastric cancer progression both *in vitro* and *in vivo*. (a) Immunoblotting was conducted to test the protein expression level of NOP2 after shRNA transduction. Both NOP2-shRNA#1 and NOP2-shRNA#2 resulted in decreased expression of NOP2 compared to the control-shRNA (CTL). (b) CCK-8 experiments were performed to evaluate the proliferation capacities of gastric cancer cells. (c) Colony formation assays revealed a significant effect of NOP2-shRNA on inhibiting gastric cancer cell proliferation. (d) Subcutaneous injection of different gastric cancer cells led to distinct growth curves of xenografts, indicating that silencing NOP2 attenuated tumor growth *in vivo*. Data were obtained from three independent repeats and compared using Student's *t* test

CONCLUSIONS

Our study established the tumor-promoting role of NOP2 in gastric adenocarcinoma progression and highlighted its clinical significance as an independent prognostic predictor.

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Conflicts of interest

There are no conflicts of interest.

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