Neoplasma 2022; 69(4): 965–975

doi:10.4149/neo_2022_220303N237

NPM1 is a diagnostic and prognostic biomarker associated with the clinicopathological characteristics of gastric cancer

Chang-An GUO[1,#], Xiao-Lu SU[2,#], Wen-Jie WANG[3], Tian-Hong XIA[4], Xiao-Meng CAO[5], Shao-Bin YUAN[5], Wen-An WANG[5], An ZHANG[5], Hong-Bin LIU[1,*]

[1]Second Clinical Medical College, Lanzhou University, Lanzhou, Gansu, China; [2]Department of Pathology, Lanzhou University Second Hospital, Lanzhou, Gansu, China; [3]Department of General Surgery, Lanzhou University Second Hospital, Lanzhou, Gansu, China; [4]Clinical Medical College, Ningxia Medical University, Yinchuan, The Ningxia Hui Autonomous Region, China; [5]Clinical Medical College, Gansu University of Chinese Medicine, Lanzhou, Gansu, China

**Correspondence: liuhongbin999@163.com*
[#]Contributed equally to this work.

Received March 3, 2022 / Accepted June 8, 2022

NPM1 plays an important role in the occurrence and development of leukemia and various solid tumors. This study aimed to investigate the expression of NPM1 in gastric cancer (GC) and adjacent normal tissues, study the relationship between NPM1 expression and clinicopathological characteristics in GC patients, and explore the impact of NPM1 expression on the diagnosis and prognosis of GC. We used tissue microarray immunohistochemical analysis to examine the expression level of NPM1 in GC and adjacent tissues and analyzed the relationship between NPM1 expression, clinicopathological factors, and GC prognosis. Prognostic values of NPM1 mRNA were also investigated using an online database. qRT-PCR was used to detect the expression of NPM1 mRNA in cancer and adjacent tissues. According to microarray immunohistochemical analysis and qRT-PCR results, NPM1 had a high expression in all adjacent normal tissues. Microarray immunohistochemical analyses demonstrated that the NPM1 was lowly expressed in 75.5% of GC tissues but highly expressed in 24.5% of GC tissues. qRT-PCR results showed NPM1 mRNA low expression in most GC tissues. NPM1 high expression group was associated with a better overall survival rate and disease-free survival rate than the NPM1 low expression group ($p<0.01$). This result is consistent with that of the online database. The receiver operating characteristics curve showed that NPM1 was valuable in the diagnosis of GC. The assessment of NPM1 expression in GC samples may represent a useful tool for GC diagnosis and prognosis assessment.

Key words: gastric cancer, nucleophosmin 1, tissue microarray, diagnosis, prognosis

Gastric cancer (GC), the most common gastrointestinal tumor in the world, is one of the main causes of cancer-related deaths [1, 2]. Since it usually has either non-specific or no symptoms in its early stages, this pathology is usually diagnosed at an advanced stage, resulting in a low five-year survival rate [3–6]. The incidence of GC varies from one region to another, and it is more likely to occur in East Asia where the associated mortality rate is high [7]. Personalized treatment for GC based on markers is considered one of the methods of improving the five-year overall survival (OS) rate of patients with GC. Finding new molecular biomarkers for GC would significantly improve the diagnostic accuracy and treatment efficacy [8, 9].

Nucleophosmin 1 (NPM1, also known as B23) is a nucleolar shuttle phosphoprotein containing 294 amino acids [10]. It can quickly shuttle between the cytoplasm and the nucleus. This function determines that NPM1 can participate in many cellular biological processes and perform different functions [11]. For example, it can be used as a molecular chaperone to regulate the function of histones [12]. It can also regulate the assembly of ribosomes [13, 14], DNA repair [15, 16] and cell apoptosis [17, 18], and so on. Over the years, numerous studies have shown that NPM1 can cause acute myeloid leukemia in the case of a mutation or continuous high expression, and it plays a vital role in the pathogenesis of acute myeloid leukemia (AML) [19–21]. Mutations in the NPM1 gene play a crucial role in the occurrence and development of AML [22–24]. NPM1 can also participate in the pathogenesis of a variety of solid tumors at the same time [10, 25, 26].

So far, there are few studies on the relationship between NPM1 and GC, and controversy still exists between the existing research results. One study reported that the expression level of NPM1 was significantly reduced in GC samples compared to the matched non-tumor tissue samples, and

the low expression of NPM1 is obviously associated with the distant metastasis of GC [27]. Another study reported that NPM1 expression was significantly higher in GC tissues than in adjacent noncancerous tissues and the expression rates of NPM1 were significantly higher in patients with distant metastases and more advanced tumor stages [28]. In view of the important role of NPM1 in neoplastic diseases and the recent contradictory results of research studies on GC, it is necessary to conduct further research on its role and mechanism in GC. In this study, tissue microarray and quantitative real time polymerase chain reaction (qRT-PCR) were used to analyze NPM1 protein expression between GC tissues and matched noncancerous gastric samples. We also assessed the possible association between NPM1 and different clinicopathological features. Furthermore, we analyzed and evaluated the impact of the NPM1 expression level on the prognosis of patients with GC. Finally, a receiver operating characteristic (ROC) curve was generated to investigate the biomarker potential of NPM1 in GC diagnosis.

Patients and methods

Patients. This study was approved by the research ethics committee at the Lanzhou University Second Hospital (NO:2021A-561). One hundred and six patients with primary GC who underwent surgical resection from the Department of General Surgery of Lanzhou University Second Hospital between January 2015 and October 2016 were included in this study. The recruited criteria were as follows: 1) a histological diagnosis of gastric adenocarcinoma, 2) the patients' age ≥18 years, 3) patients undergoing surgical resection for primary GC, 4) the availability of complete pathological, treatment, surgical, and follow-up data, and 5) the patients gave their written informed consent. The exclusion criteria were as follows: 1) patients that died before discharge, 2) receipt of preoperative chemoradiation or neoadjuvant chemotherapy, and 3) patients with multiple cancers within five years. Of the 106 patients in this cohort, 79 had received standard adjuvant postoperative chemotherapy (5-fluorouracil or oxaliplatin-based regimen) within the first month after surgery, and 27 patients had not received treatment due to financial reasons. None of them accepted either neoadjuvant chemotherapy or perioperative chemoradiation. Tumors were histologically staged according to the 7th edition of the TNM classification by the American Joint Committee on Cancer (AJCC). All study participants gave their written informed consent to participate in this study.

Follow-up was conducted through normal outpatient visits and telephone calls. Follow-up was carried out two weeks after discharge and once every three months in the first and second years and every six months over the following three years. The OS time is the time-lapse from surgery to either the end of follow-up or death. Disease-free survival (DFS) was the time-lapse from the date of surgery to the date of recurrence or death.

Tissue microarray construction. Tissue microarray was built from 106 patients with excised specimens of primary gastric tumors. The hematoxylin and eosin-stained pathological sections of the included cases were read, and the H&E stained pathological sections and the representative positions on the corresponding wax blocks were marked. A puncher was used to punch holes on the sample wax block to obtain tissue columns that were loaded in the chip wax block according to the sequence of arrangement. Each sample had 3 multiple points.

Immunohistochemical staining. We used immunohistochemical staining analysis according to the procedure previously described by the manufacturer's instructions [29]. For antigen retrieval, the TMA slides are dewaxed, rehydrated, and boiled in a pressure pot with sodium citrate buffer (pH 6.0). The TMA slides were blocked with an inhibitor (3% hydrogen peroxide) for 30 min at 37 °C after the antigen retrieval. We used the NPM1 antibody for immunohistochemical staining (NPM1, 1:200, Abcam, Cambridge, USA) for 25 min at room temperature and overnight at 4 °C. Then we rewarmed it for 15 min, washed it with TBS three times, added 50 μl of the secondary antibody, and incubated it for 25 min at room temperature. Then, we rinsed it three times with TBS, after which DAB developed a color. This was followed by hematoxylin counterstaining, rinsing with water, and dehydration, after which we mounted the film. We observed the expression of the NPM1 protein under an optical microscope and took photos of the mounted specimen.

Evaluation of immunostaining. Under the microscope, the complete tissue structure could be observed and the brown-yellow particles with obvious distribution in the background cells were judged as positive. The immunoreactive score (IRS) method [30] was used for semi-quantitative scoring according to the degree of staining: 0 = no, 1 = light yellow, 2 = brown, and 3 = dark brown. Then, the percentage of positively stained tumor cells in each field was calculated, and the score was deduced from the percentage range: the score for 0% of positive cells no was 0, that for <10% was 1, that for 10–50% was 2, that for 51–80% was 3, and that for >80% was 4. When the two scores (staining degree score and percentage score) were multiplied, 0 was considered negative (0+), 1 to 4 were considered weakly positive (1+), 5 to 8 were considered moderately positive (2+), and 9 to 12 were considered strongly positive (3+). If there were multiple visual fields with different scores in the same specimen, the average of the maximum and minimum values was taken as the immunohistochemical score. NPM1 expression was considered high when the score was ≥5 and low when the score was <5. All arrays were reviewed by two unsuspecting pathologists. All inconsistent cases were reviewed and discussed until a consensus was reached.

Cancer-related public database searches. Prognostic values of NPM1 mRNA were investigated using an online database Kaplan-Meier plotter (http://kmplot.com/analysis/).

Quantitative real-time polymerase chain reaction. We selected 41 pairs of paired GC tissue and adjacent normal tissue samples to detect the expression level of NPM1 mRNA. The reactions were operated on Mastercycler ep realplex (Eppendorf, Germany). The reaction conditions were as follows: 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 1 min for a total of 40 cycles. Each experiment was repeated three times. The relative mRNA expression of the NPM1 gene was normalized to GAPDH. The primer sequences were as follows: the NPM1 forward primer, 5'-TAGACGTGGCG-CAAACCAGG-3' and reverse primer, 5'-CGGACGGCTCT-GAGCATATA-3'; GAPDH forward primer, 5'-GTTCAACT-GCATAGCGTCGTC-3'; and reverse primer, 5'-AGATC-GTTCGACCATTCGATAC-3'.

Statistical analysis. The relationship between the clinicopathological characteristics and expression level of NPM1 was assessed using Pearson's chi-squared test (χ^2 test) or Fisher's exact probability test. The Kaplan-Meier method was used to construct OS and DFS curves. The significance of the OS and DFS between the NPM1 high and low expression groups was tested using the log-rank test. The Cox proportional hazards regression model was used for univariate and multivariate survival analyses. The ROC curve was used to evaluate the diagnostic value of NPM1 in GC. The threshold for statistical significance was set at $p<0.05$. All statistical analyses were performed using SPSS 23.0 statistical software package (SPSS, Chicago, IL, USA).

Result

The expression of NPM1 in GC and patient's clinicopathological characteristics. NPM1 has a high expression in all adjacent normal tissues. Compared with the corresponding adjacent tissues, NPM1 is lowly expressed in 75.5% (80 persons) of GC tissues but highly expressed in 24.5% (26 persons) of GC tissues. The representative expression levels (0+, 1+, 2+, 3+) of NPM1 in different GC tissues and corresponding adjacent tissues are shown in Figure 1. The cohort consisted of 83 (78.3%) males and 23 (21.7%) females, with a median patient age of 64 (range 31–88) years. Sixty-seven percent of tumors were located in the antrum of the stomach, 22.6% were located in the body of the stomach, and about 10.4% were located at the junction between the cardia and the esophagus. The intestinal type and diffuse type in Lauren classification accounted for 56.6% and 43.4%, respectively. Noticeably, 42 cases (39.6%) were in stages Ib–II, and 64 cases (60.4%) were in stages III–IV (according to the 7th edition of the TNM classification by the AJCC). Seventy-nine patients had received standard adjuvant postoperative chemotherapy (5-fluorouracil or oxaliplatin-based regimen) within the first month after surgery, and 27 patients had not due to financial constraints. The clinicopathologic data and demographic features of the 106 patients with GC are summarized in Table 1.

Table 1. Clinicopathological characteristics of GC patients.

Variables	n=106
Age, median (range)	64 (31-88)
Gender	
Male	83 (78.3%)
Female	23 (21.7%)
Tumor location	
Antrum	71 (67.0%)
Body	24 (22.6%)
Cardia	11 (10.4%)
Tumor size	
≥5 cm	48 (45.3%)
<5 cm	58 (54.7%)
pT stage	
T1–T3	40 (37.7%)
T4	66 (62.3%)
Lymph-node metastasis	
Positive	86 (81.1%)
Negative	20 (18.8%)
Lymphatic invasion	
Positive	78 (73.6%)
Negative	28 (26.4%)
pN stage	
N0	20 (18.8%)
N1	19 (17.9%)
N2	26 (24.5%)
N3	41 (38.7%)
Lauren's classification	
Intestinal type	60 (56.6%)
Diffuse type	46 (43.4%)
AJCC stage	
Ib–II	42 (39.6%)
III–IV	64 (60.4%)
Venous invasion	
Positive	73 (68.9%)
Negative	33 (31.1%)
CEA	
Positive	58 (55.0%)
Negative	48 (45.0%)
CA199	
Positive	44 (41.5%)
Negative	62 (58.5%)
CA724	
Positive	52 (49.1%)
Negative	54 (50.9%)
AFP	
Positive	11 (10.4%)
Negative	95 (89.6%)
CA125	
Positive	22 (20.8%)
Negative	84 (79.2%)
NPM1 expression	
Low	80(75.5%)
High	26(24.5%)
Adjuvant chemotherapy	
Yes	79 (74.5%)
No	27 (25.5%)

Abbreviations: NPM1-nucleophosmin 1; pT stage-pathological assessment of primary tumor; pN stage-pathological assessment of regional lymph nodes; CEA-carcinoembryonic antigen; CA199-carbohydrate antigen 199; CA724-carbohydrate antigen 724; AFP-α-fetoprotein. CA125-carbohydrate antigen 125

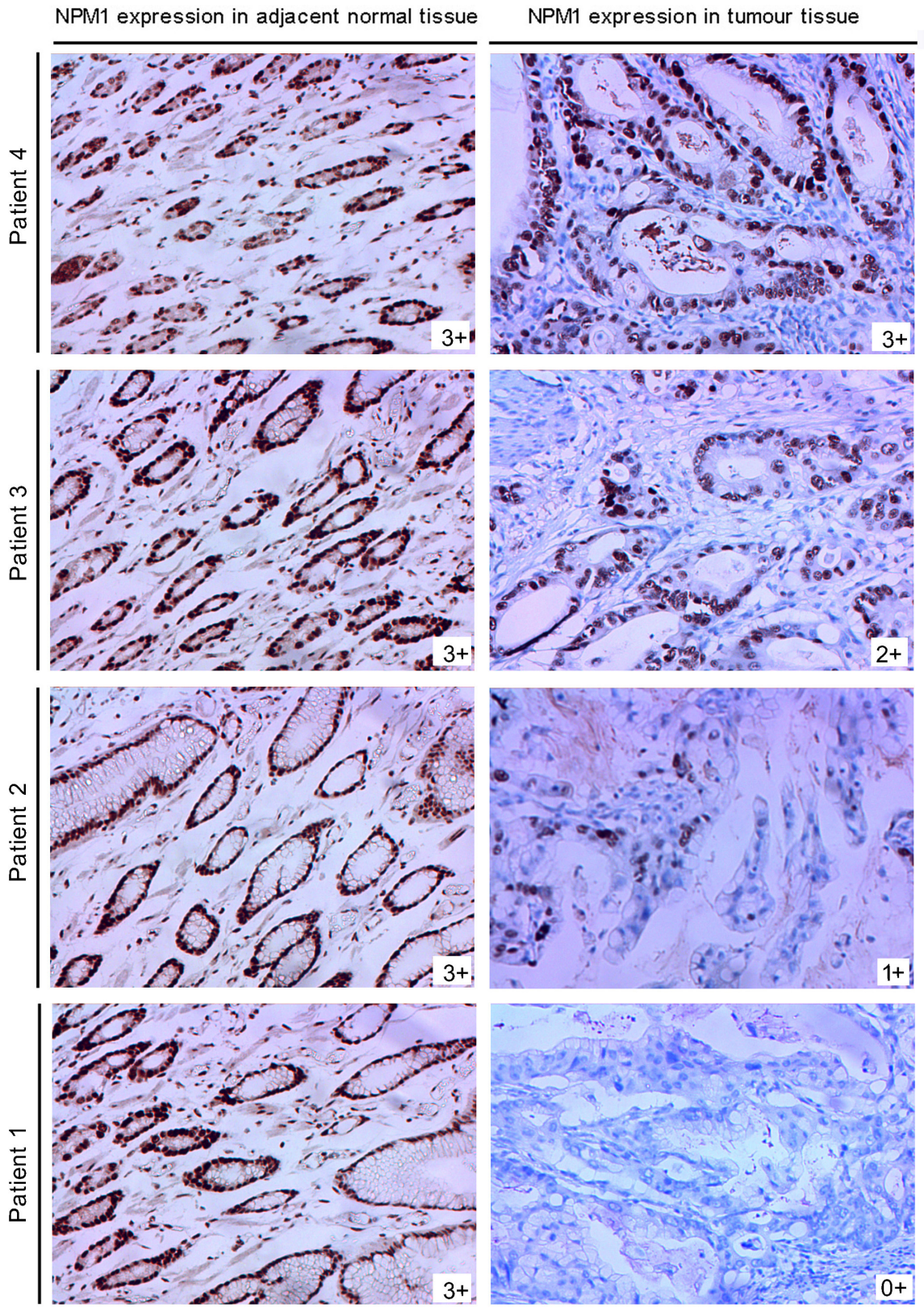

Figure 1. Representative patients and scores of the immunohistochemical staining of NPM1. Expression of NPM1 was observed in the nuclei of cells (200× magnification). Abbreviations: NPM1-nucleophosmin1

Expression of NPM1 mRNA in cancer tissues and adjacent normal tissues. NPM1 mRNA levels were highly expressed in all matched normal tissues, but lowly expressed in most GC tissues. NPM1 mRNA was significantly highly expressed in all matched normal tissues than in tumor tissues ($p<0.001$, Figure 2).

Relationship between clinicopathological factors and NPM1 expression. Fifteen clinicopathological factors of high and low expression of NPM1 in GC tissues were compared separately (Table 2). There were significant differences in the venous invasion and AJCC stage ($p<0.05$).

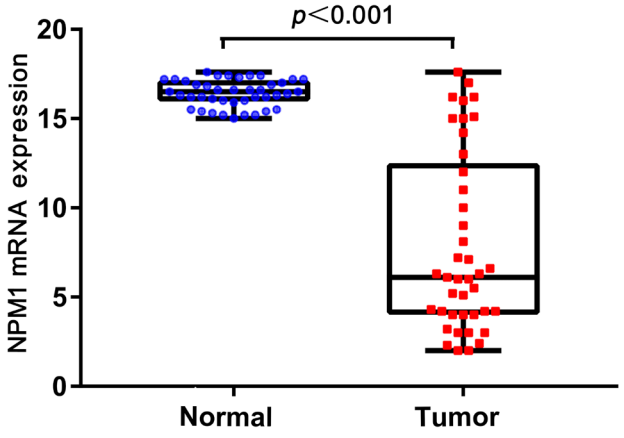

Figure 2. NPM1 mRNA levels in tumor and normal tissue were detected by qRT-PCR. Abbreviations: NPM1-nucleophosmin1; qRT-PCR-quantitative real-time polymerase chain reaction

Relationship between NPM1 expression level and prognosis of GC. Kaplan-Meier curves for OS and DFS rates in the NPM1 high and the NPM1 low expression groups of GC are shown in Figure 3 (data from GC patients of our hospital). The OS rates in patients with high and low NPM1 expression were 49.3% and 33.1%, respectively (Figure 3A). Compared with the low NPM1 expression group, the high NPM1 expression group showed significantly better OS (by log-rank test $p<0.05$). The DFS rates in patients with high and low NPM1 expression were 34.3% and 19.3%, respectively (Figure 3B). Compared with the low NPM1 expression group, the high NPM1 expression group showed significantly better DFS (by log-rank test $p<0.01$).

The prognostic value of NPM1 mRNA expression in GC was evaluated using the online Kaplan-Meier Plotter tool (https://kmplot.com/analysis/). In the entire cohorts of GC patients, higher levels of NPM1 mRNA were correlated with better OS times (HR=0.74, 95% CI, 0.62–0.88, $p<0.001$, Figure 4).

Univariate and multivariate Cox regression analyses of OS and DFS. In the univariate and multivariate Cox regression analyses of OS, we analyzed: age, gender, location of the tumor, tumor size, pT stage (pathological assessment of primary tumor), pN stage (pathological assessment of regional lymph nodes), AJCC stage, lymphatic invasion, Lauren's classification, venous invasion, carcinoembryonic antigen (CEA), carbohydrate antigen 199 (CA199), carbohydrate antigen 724 (CA724), α-fetoprotein (AFP), carbohydrate antigen 125 (CA125), and NPM1 expression. In the univariate analyses, age, tumor size, pT stage, AJCC stage, venous invasion, and NPM1 expression were selected as

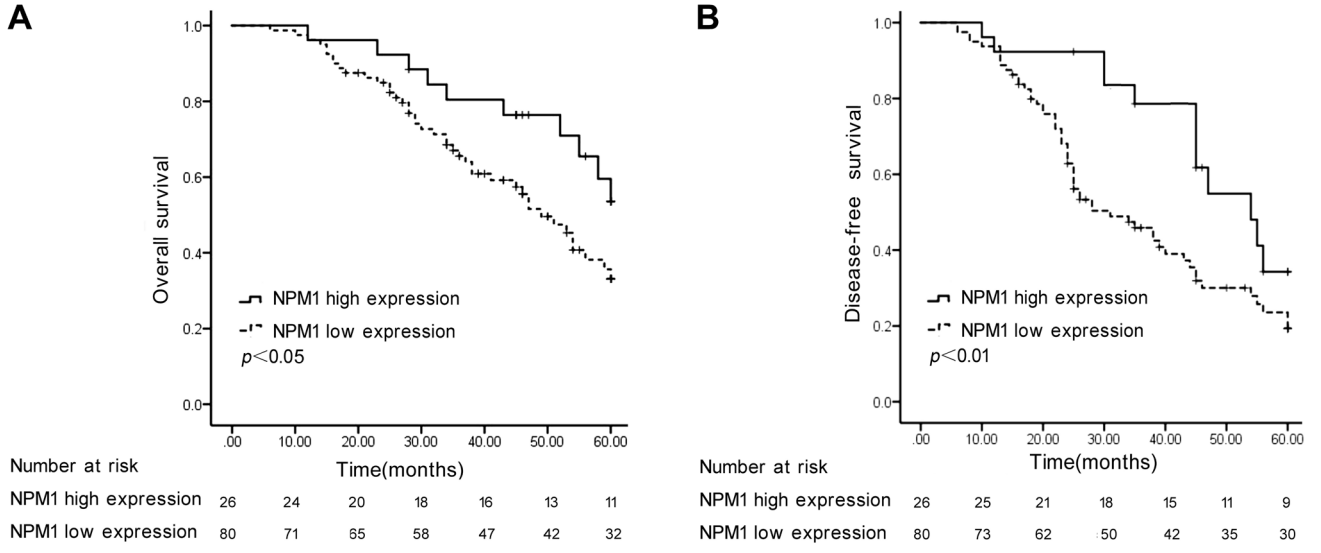

Figure 3. Kaplan-Meier curves for OS and DFS rates in the NPM1 low and the NPM1 high expression groups (data from clinical GC patients of our hospital). The OS rates in patients with high and low NPM1 expression were 49.3% and 33.1%, respectively (A). Compared with the low NPM1 expression group, the high NPM1 expression group showed significantly better OS (by log-rank test $p<0.05$). The DFS rates in patients with high and low NPM1 expression were 34.3% and 19.3%, respectively (B). Compared with the low NPM1 expression group, the high NPM1 expression group showed significantly better DFS (by log-rank test $p<0.01$). Abbreviations: OS-overall survival; DFS-disease-free survival; NPM1-nucleophosmin 1; GC-gastric cancer

factors significantly associated with the OS. Meanwhile, in the multivariate analyses, age, tumor size, pT stage, AJCC stage, venous invasion, and NPM1 expression were independent predictors of OS in patients who underwent curative gastrectomy (Table 3).

In the univariate and multivariate Cox regression analyses of DFS, we analyzed: age, gender, location of the tumor, tumor size, pT stage, pN stage, AJCC stage, lymphatic invasion, Lauren's classification, venous invasion, CEA, CA199, CA724, AFP, CA125, and NPM1 expression. In the univariate analyses, age, pT stage, pN stage, AJCC stage, venous invasion, and NPM1 expression were selected as significant factors of DFS. Meanwhile, in the multivariate analyses, age, pT stage, pN stage, AJCC stage, venous invasion, and NPM1 expression were independent predictors of DFS in curative gastrectomy patients (Table 4).

Table 2. Comparison of clinicopathological parameters between NPM1 low expression and high expression gastric cancer patients.

	NPM1 expression		
	Low (n=80)	High (n=26)	p-value
Age (years), median (range)	66 (32-88)	65 (31-87)	0.102
Gender			
Male	63 (78.8%)	20 (76.9%)	0.158
Female	17 (21.2%)	6 (23.1%)	
Tumor location			
Antrum	56 (70.0%)	15 (57.7%)	0.154
Body	16 (20.0%)	8 (30.8%)	
Cardia	8 (10.0%)	3 (11.5%)	
Tumor size			
≥5 cm	36 (45.0%)	12 (46.2%)	0.149
<5 cm	44 (55.0%)	14 (53.8%)	
Lauren's classification			
Intestinal type	46 (57.5%)	14 (53.8%)	0.264
Diffuse type	34 (42.5%)	12 (46.2%)	
pT stage			
T1–T3	29 (36.3%)	11 (42.3%)	0.056
T4	51 (63.7%)	15 (57.7%)	
pN stage			
N0	15 (18.8%)	5 (19.2%)	0.325
N1	14 (17.5%)	5 (19.2%)	
N2	20 (25.0%)	6 (23.1%)	
N3	31 (38.8%)	10 (38.5%)	
AJCC stage			
Ib–II	26 (32.5%)	16 (61.5%)	**0.010**
III–IV	54 (67.5%)	10 (38.5%)	
Venous invasion			
Positive	64 (80.0%)	9 (34.6%)	**0.023**
Negative	16 (20.0%)	17 (65.4%)	
Lymphatic invasion			
Positive	60 (75.0%)	18 (69.2%)	0.259
Negative	20 (25.0%)	8 (30.8%)	
CEA			
Positive	42 (52.5%)	16 (61.5%)	0.092
Negative	38 (47.5%)	10 (38.5%)	
CA199			
Positive	33 (41.3%)	11 (42.3%)	0.328
Negative	47 (58.7%)	15 (57.7%)	
CA724			
Positive	40 (50.0%)	12 (46.2%)	0.219
Negative	40 (50.0%)	14 (53.8%)	
AFP			
Positive	8 (10.0%)	3 (11.5%)	0.146
Negative	72 (90.0%)	23 (88.5%)	
CA125			
Positive	16 (20.0%)	6 (23.1%)	0.287
Negative	64 (80.0%)	20 (76.9%)	

Abbreviations: NPM1-nucleophosmin 1; pT stage-pathological assessment of primary tumor; pN stage-pathological assessment of regional lymph nodes; CEA-carcinoembryonic antigen; CA199-carbohydrate antigen 199; CA724-carbohydrate antigen 724; AFP-α-fetoprotein; CA125-carbohydrate antigen 125

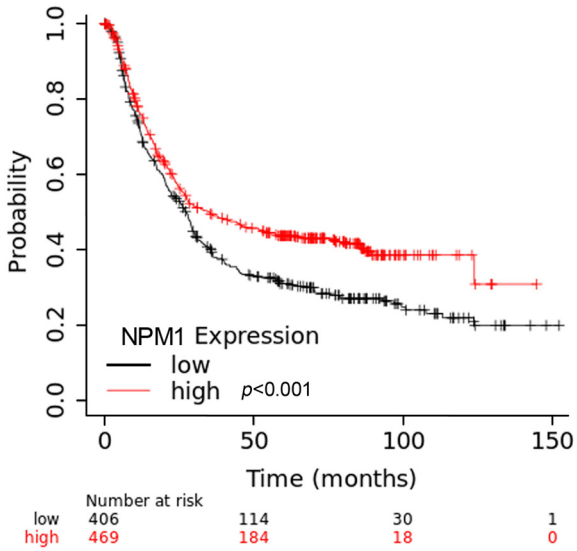

Figure 4. The prognostic value of NPM1 mRNA expression in GC (data from an online database). In the entire cohorts of GC patients, higher levels of NPM1 mRNA were correlated with better OS (HR=0.74, 95% CI, 0.62–0.88, p<0.001). Abbreviations: NPM1-nucleophosmin 1; GC-gastric cancer; OS-overall survival

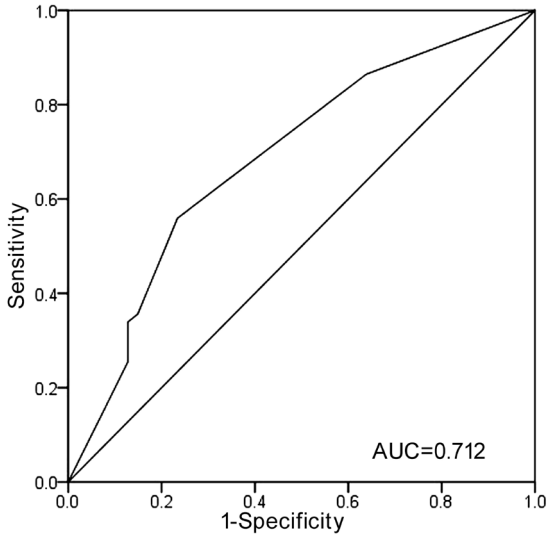

Figure 5. ROC curve was generated to investigate the biomarker potential of NPM1 in GC diagnosis. Abbreviations: NPM1-nucleophosmin 1; ROC curve-receiver operating characteristic curve; GC-gastric cancer

Table 3. Univariate and multivariate Cox regression analyses of OS predictors in patients with GC (n=106).

Variable	N	Univariate Cox regression			Multivariate Cox regression		
		HR	95% CI	p-value	HR	95% CI	p-value
Age (years)				0.015			0.025
<70	33						
≥70	73	2.369	1.733–3.046		2.254	1.856–2.965	
Gender				0.254			
Male	83						
Female	23	0.761	0.463–1.214				
Location of tumor				0.124			
Antrum	71						
Non-antrum	35	1.062	0.743–1.325				
Tumor size				0.002			0.003
≥5 cm	48						
<5 cm	58	2.354	1.698–3.021		1.938	1.249–2.367	
pT stage				0.023			0.005
T1–T3	40						
T4	66	1.785	1.245–2.021		1.865	1.256–2.214	
pN stage				0.156			
N0	20						
N1–N3	86	1.564	1.021–1.986				
AJCC stage				0.029			0.032
Ib–II	42						
III–IV	64	2.325	1.654–3.021		2.158	1.433–2.986	
Lymphatic invasion				0.452			
Positive	78						
Negative	28	1.445	1.015–1.965				
Lauren's classification				0.091			
Intestinal type	60						
Diffuse type	46	1.251	0.856–1.834				
Venous invasion				0.048			0.012
Positive	73						
Negative	33	1.785	1.124–2.215		1.935	1.003–2.465	
CEA				0.069			
Positive	58						
Negative	48	1.255	0.645–1.855				
CA199				0.122			
Positive	44						
Negative	62	0.966	0.542–1.455				
CA724				0.135			
Positive	52						
Negative	54	1.548	0.965–2.122				
AFP				0.253			
Positive	11						
Negative	95	1.223	0.554–1.869				
CA125				0.114			
Positive	22						
Negative	84	0.965	0.258–1.562				
NPM1 expression				0.015			0.032
Low	80						
High	26	1.965	1.354–2.432		1.875	1.324–2.433	

Abbreviations: NPM1-nucleophosmin 1; pT stage-pathological assessment of primary tumor; pN stage-pathological assessment of regional lymph nodes; CEA-carcinoembryonic antigen; CA199-carbohydrate antigen 199; CA724-carbohydrate antigen 724; AFP-α-fetoprotein; CA125-carbohydrate antigen 125; N-Number of patients; HR-Hazard ratio; CI-Confidence interval

Diagnostic accuracy of NPM1 for patients with GC. The ROC curve for the diagnostic accuracy of NPM1, which was used for identifying patients with GC, was presented in Figure 5. NPM1 scores yielded an area under the curve (AUC) of 0.712 (95% CI: 0.616–0.818), with a sensitivity of 77.3%, specificity of 78.2%, and a cutoff value of 2.0 points (Figure 5).

Discussion

For a long time, numerous studies have shown that NPM1 can cause acute myeloid leukemia in the case of a mutation or continuous high expression, and it plays a vital role in the occurrence, development, and prognosis of acute myeloid leukemia [19–21]. NPM1 can also partici-

Table 4. Univariate and multivariate Cox regression analyses of DFS predictors in patients with GC (n=106).

Variable	N	Univariate Cox regression			Multivariate Cox regression		
		HR	95% CI	p-value	HR	95% CI	p-value
Age (years)				**0.023**			**0.036**
<70	33						
≥70	73	2.448	2.021–3.147		2.543	2.035–3.011	
Gender				0.325			
Male	83						
Female	23	0.865	0.395–1.441				
Location of tumor				0.212			
Antrum	71						
Non-antrum	35	1.124	0.632–1.624				
Tumor size				0.062			0.089
≥5 cm	48						
<5 cm	58	1.533	0.744–2.125		1.993	1.147–2.452	
pT stage				**0.012**			**0.011**
T1–T3	40						
T4	66	1.663	1.114–2.142		1.965	1.145–2.359	
pN stage				**0.014**			**0.026**
N0	20						
N1–N3	86	1.978	1.154–2.534		1.895	1.126–2.433	
AJCC stage				**0.035**			**0.022**
Ib–II	42						
III–IV	64	2.547	1.553–3.154		2.248	1.536–3.024	
Lymphatic invasion				0.325			
Positive	78						
Negative	28	1.665	1.125–2.012				
Lauren's classification				0.069			
Intestinal type	60						
Diffuse type	46	1.123	0.733–1.654				
Venous invasion				**0.032**			**0.021**
Positive	73						
Negative	33	1.865	1.235–2.354		1.754	1.102–2.214	
CEA				0.126			
Positive	58						
Negative	48	1.356	0.725–1.965				
CA199				0.232			
Positive	44						
Negative	62	1.133	0.423–1.654				
CA724				0.253			
Positive	52						
Negative	54	1.446	1.325–1.968				
AFP				0.154			
Positive	11						
Negative	95	1.354	0.785–1.954				
CA125				0.091			
Positive	22						
Negative	84	1.032	0.368–1.625				
NPM1 expression				**0.023**			**0.011**
Low	80						
High	26	2.368	1.663–2.996		1.889	1.228–2.521	

Abbreviations: NPM1-nucleophosmin 1; pT stage-pathological assessment of primary tumor; pN stage-pathological assessment of regional lymph nodes; CEA-carcino-embryonic antigen; CA199-carbohydrate antigen199; CA724-carbohydrate antigen 724; AFP-α-fetoprotein; CA125-carbohydrate antigen 125; N-Number of patients; HR-Hazard ratio; CI-Confidence interval

pate in the occurrence and development of a variety of solid tumors at the same time [10, 25, 26]. In oral squamous cell carcinoma, higher NPM1 expression is significantly associated with larger tumor sizes, lymph node metastasis, and advanced clinical stage [31]. Multivariate analysis results show that higher NPM1 expression is associated with worse prognoses [31]. Another study shows that the expression level of NPM1 in lung adenocarcinoma samples was higher than that in adjacent normal paracancerous tissues. NPM1 has high specificity and sensitivity values in the diagnosis and prognosis assessment of lung adenocarcinoma [32]. A recent study reported that NPM1 expression is significantly increased in colorectal cancer and is associated with a poorer five-year OS rate [33]. The above studies are examples of

the high expression of NPM1 in different tumors. Its high expression promotes the biological behavior of the tumor and predicts a poor prognosis. However, it is interesting that there are different reports showing that NPM1 is lower-expressed in some tumors. Its low expression promotes the biological behavior of tumors and predicts a poor prognosis. Luo et al. [34] reported that NPM1 has a low expression in bladder cancer cells, which is also associated with the poor prognosis of bladder cancer. The knockdown of NPM1 expression in bladder cancer cell lines can significantly improve tumor cell migration and invasion capabilities. The silencing of NPM1 will accelerate the tumorigenicity of drug-resistant bladder cancer cells. Karhemo et al. [35] reported that NPM1 expression is low in breast cancer, and the decrease in the NPM1 protein level in breast cancer is associated with a poor prognosis. Histologically, the luminal epithelial cells of normal breasts show high levels of NPM1 expression. The overexpression of NPM1 in breast cancer cells MDA-MB-231 stopped their growth in soft agar. NPM1 has a tumor suppressor effect in breast cancer. It can be seen that NPM1 plays different roles in different types of tumors. It acts as a tumor-promoting factor in some tumors but as a tumor suppressor in others.

In this study, we used the tissue microarray method and qRT-PCR to detect the expression level of NPM1 in GC tissues and the adjacent normal tissues in patients with GC. The tissue microarray result of this study shows that NPM1 has a high expression in all adjacent normal tissues. Among all GC tissue samples, NPM1 showed low expression in 75.5% of GC samples but the high expression in 24.5% of GC tissues. Tissue microarray results showed that the expression of NPM1 was observed in the nucleus compared to the cytoplasm. The localization of NPM1 in cells determines that NPM1 can participate in many cell biological processes and perform different functions [11]. The qRT-PCR result showed NPM1 mRNA low expression in most GC tissues. The results of qRT-PCR are consistent with the results of tissue microarray. It can be seen that NPM1 has a low expression in most GC tissues, and its expression level is significantly lower than that in normal adjacent tissues. The result of low expression of NPM1 in most GC tissues is consistent with the previous study [27].

In addition to the controversial findings between NPM1 and GC in previous studies [27, 28], to the best of our knowledge, there has never been a study on the role of NPM1 in the diagnosis and prognosis of GC. Our study is the first to elucidate the role of NPM1 in the diagnosis and prognosis of GC.

Survival analysis results showed that the low expression of NPM1 in GC was significantly associated with worse OS and DFS, whereas the high expression of NPM1 in GC was significantly associated with better OS and DFS (data from GC patients of our hospital). The prognostic value of NPM1 mRNA expression in GC was also evaluated using the online Kaplan-Meier plotter tool. In the entire cohorts of GC patients, higher levels of NPM1 mRNA were correlated with better OS ($p<0.001$). The results of the online database are completely consistent with our experimental results. Thus, the expression level of NPM1 in GC tissue has a direct impact on OS and DFS in patients with GC. NPM1 may serve as a tumor suppressor and a prognostic biomarker in GC.

In the univariate Cox regression analysis, age, tumor size, pT stage, AJCC stage, venous invasion, and NPM1 expression were identified as factors significantly associated with OS. Meanwhile, in the multivariate Cox regression analysis, age, tumor size, pT stage, AJCC stage, venous invasion, and NPM1 expression were identified as independent predictors of OS in patients who underwent curative gastrectomy. In the univariate Cox regression analysis, age, pT stage, pN stage, AJCC stage, venous invasion, and NPM1 expression were identified as factors significantly associated with DFS. Meanwhile, in the multivariate Cox regression analysis, age, pT stage, pN stage, AJCC stage, venous invasion, and NPM1 expression were independent predictors of DFS in patients who underwent curative gastrectomy.

Finally, a ROC curve was generated from the value of NPM1 expression in GC tissues to investigate the biomarker potential of NPM1 in GC diagnosis. NPM1 scores yielded an AUC of 0.712. The ROC curve shows that NPM1 is valuable in the diagnosis of GC. The assessment of NPM1 expression in GC samples may represent a useful tool for GC diagnosis.

However, our study has some limitations. Firstly, although the number of patients with GC in this study is relatively large (n=106), this is a single-center, retrospective study. To obtain a more reliable analysis of the clinical significance of NPM1 in GC, a multicenter study that includes a larger number of patients is also needed. Secondly, we used immunostaining to examine the expression of NPM1 in the central and peripheral parts of each GC tissue; however, considering the heterogeneity, the expression level of NPM1 at the sampling site may not be representative of the entire tumor area.

In conclusion, NPM1 has a high expression in all adjacent normal tissues. Tissue microarray results in this study show that NPM1 is lowly expressed in 75.5% of GC tissues but highly expressed in 24.5% of GC tissues. Survival analysis results show that low expression of NPM1 in GC was significantly associated with worse OS and DFS. The assessment of NPM1 expression in GC samples may represent a useful tool for GC diagnosis and prognostic assessment.

Acknowledgments: This research was supported by the Gansu Provincial Natural Science Foundation Project (21JR11RA106) and the Gansu Provincial Youth Science and Technology Fund Project (21JR7RA424).

References

[1] SIEGEL RL, MILLER KD, FUCHS HE, JEMAL A. Cancer statistics, 2021. CA Cancer J Clin 2021; 71: 7–33. https://doi.org/10.3322/caac.21654

[2] SUNG H, FERLAY J, SIEGEL RL, LAVERSANNE M, SOERJOMATARAM I et al. Global cancer statistics 2020: Globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021; 71: 209–249. https://doi.org/10.3322/caac.21660

[3] VENERITO M, VASAPOLLI R, ROKKAS T, MALFERTHEINER P. Gastric cancer: Epidemiology, prevention, and therapy. Helicobacter 2018; 23: e12518. https://doi.org/10.1111/hel.12518

[4] XIE JW, LU J, XU BB, ZHENG CH, LI P et al. Prognostic value of tumor regression grading in patients treated with neoadjuvant chemotherapy plus surgery for gastric cancer. Front Oncol 2021; 11: 587856. https://doi.org/10.3389/fonc.2021.587856

[5] BAE JS, CHANG W, KIM SH, CHOI Y, KONG SH et al. Development of a predictive model for extragastric recurrence after curative resection for early gastric cancer. Gastric Cancer 2022; 25: 255–264. https://doi.org/10.1007/s10120-021-01217-1

[6] ELORANTA S, SMEDBY KE, DICKMAN PW, ANDERSSON TM. Cancer survival statistics for patients and healthcare professionals – a tutorial of real-world data analysis. J Intern Med 2021; 289: 12–28. https://doi.org/10.1111/joim.13139

[7] HONG S, WON YJ, LEE JJ, JUNG KW, KONG HJ et al. Cancer statistics in korea: Incidence, mortality, survival, and prevalence in 2018. Cancer Res Treat 2021; 53: 301–315. https://doi.org/10.4143/crt.2021.291

[8] PANDEY I, MISRA V, PANDEY AT, RAMTEKE PW, AGRAWAL R. Artificial intelligence technologies empowering identification of novel diagnostic molecular markers in gastric cancer. Indian J Pathol Microbiol 2021; 64: S63–S68. https://doi.org/10.4103/IJPM.IJPM_950_20

[9] MOLINARI C, TEDALDI G, REBUZZI F, MORGAGNI P, CAPELLI L et al. Early gastric cancer: Identification of molecular markers able to distinguish submucosa-penetrating lesions with different prognosis. Gastric Cancer 2021; 24: 392–401. https://doi.org/10.1007/s10120-020-01135-8

[10] GRISENDI S, MECUCCI C, FALINI B, PANDOLFI PP. Nucleophosmin and cancer. Nat Rev Cancer 2006; 6: 493–505. https://doi.org/10.1038/nrc1885

[11] BOX JK, PAQUET N, ADAMS MN, BOUCHER D, BOLDERSON E et al. Nucleophosmin: From structure and function to disease development. BMC Mol Biol 2016; 17: 19. https://doi.org/10.1186/s12867-016-0073-9

[12] OKUWAKI M, MATSUMOTO K, TSUJIMOTO M, NAGATA K. Function of nucleophosmin/b23, a nucleolar acidic protein, as a histone chaperone. FEBS Lett 2001; 506: 272–276. https://doi.org/10.1016/s0014-5793(01)02939-8

[13] MAGGI LB, JR., KUCHENRUETHER M, DADEY DY, SCHWOPE RM, GRISENDI S et al. Nucleophosmin serves as a rate-limiting nuclear export chaperone for the mammalian ribosome. Mol Cell Biol 2008; 28: 7050–7065. https://doi.org/10.1128/MCB.01548-07

[14] POZZO F, BITTOLO T, VENDRAMINI E, BOMBEN R, BULIAN P et al. Notch1-mutated chronic lymphocytic leukemia cells are characterized by a myc-related overexpression of nucleophosmin 1 and ribosome-associated components. Leukemia 2017; 31: 2407–2415. https://doi.org/10.1038/leu.2017.90

[15] LOPEZ DJ, RODRIGUEZ JA, BANUELOS S. Nucleophosmin, a multifunctional nucleolar organizer with a role in DNA repair. Biochim Biophys Acta Proteins Proteom 2020; 1868: 140532. https://doi.org/10.1016/j.bbapap.2020.140532

[16] LOPEZ DJ, DE BLAS A, HURTADO M, GARCIA-ALIJA M, MENTXAKA J et al. Nucleophosmin interaction with ape1: Insights into DNA repair regulation. DNA Repair (Amst) 2020; 88: 102809. https://doi.org/10.1016/j.dnarep.2020.102809

[17] LI S, ZHANG X, ZHOU Z, HUANG Z, LIU L. Downregulation of nucleophosmin expression inhibited proliferation and induced apoptosis in salivary gland adenoid cystic carcinoma. J Oral Pathol Med 2017; 46: 175–181. https://doi.org/10.1111/jop.12482

[18] JEON YJ, CHO JH, LEE SY, CHOI YH, PARK H et al. Esculetin induces apoptosis through egfr/pi3k/akt signaling pathway and nucleophosmin relocalization. J Cell Biochem 2016; 117: 1210–1221. https://doi.org/10.1002/jcb.25404

[19] FALINI B, SCIABOLACCI S, FALINI L, BRUNETTI L, MARTELLI MP. Diagnostic and therapeutic pitfalls in npm1-mutated aml: Notes from the field. Leukemia 2021; 35: 3113–3126. https://doi.org/10.1038/s41375-021-01222-4

[20] FALINI B, BRUNETTI L, MARTELLI MP. How i diagnose and treat npm1-mutated aml. Blood 2021; 137: 589–599. https://doi.org/10.1182/blood.2020008211

[21] ZARKA J, SHORT NJ, KANAGAL-SHAMANNA R, ISSA GC. Nucleophosmin 1 mutations in acute myeloid leukemia. Genes (Basel) 2020; 11: 649. https://doi.org/10.3390/genes11060649

[22] LA MANNA S, SCOGNAMIGLIO PL, ROVIELLO V, BORBONE F, FLORIO D et al. The acute myeloid leukemia-associated nucleophosmin 1 gene mutations dictate amyloidogenicity of the c-terminal domain. FEBS J 2019; 286: 2311–2328. https://doi.org/10.1111/febs.14815

[23] LA MANNA S, ROVIELLO V, SCOGNAMIGLIO PL, DIAFERIA C, GIANNINI C et al. Amyloid fibers deriving from the aromatic core of c-terminal domain of nucleophosmin 1. Int J Biol Macromol 2019; 122: 517–525. https://doi.org/10.1016/j.ijbiomac.2018.10.210

[24] DI NATALE C, LA MANNA S, MALFITANO AM, DI SOMMA S, FLORIO D et al. Structural insights into amyloid structures of the c-terminal region of nucleophosmin 1 in type a mutation of acute myeloid leukemia. Biochim Biophys Acta Proteins Proteom 2019; 1867: 637–644. https://doi.org/10.1016/j.bbapap.2019.01.010

[25] LIM MJ, WANG XW. Nucleophosmin and human cancer. Cancer Detect Prev 2006; 30: 481–490. https://doi.org/10.1016/j.cdp.2006.10.008

[26] KARIMI DERMANI F, GHOLAMZADEH KHOEI S, AFSHAR S, AMINI R. The potential role of nucleophosmin (npm1) in the development of cancer. J Cell Physiol 2021; 236: 7832–7852. https://doi.org/10.1002/jcp.30406

[27] LEAL MF, MAZZOTTI TK, CALCAGNO DQ, CIRILO PD, MARTINEZ MC et al. Deregulated expression of nucleophosmin 1 in gastric cancer and its clinicopathological implications. BMC Gastroenterol 2014; 14: 9. https://doi.org/10.1186/1471-230X-14-9

[28] ZHOU F, CHEN E, YOU D, SONG Y, SUN Z et al. Both high expression of nucleophosmin/b23 and crm1 predicts poorer prognosis in human gastric cancer. APMIS 2016; 124: 1046–1053. https://doi.org/10.1111/apm.12604.

[29] GU J, ZHANG S, HE X, CHEN S, WANG Y. High expression of pig11 correlates with poor prognosis in gastric cancer. Exp Ther Med 2021; 21: 249. https://doi.org/10.3892/etm.2021.9680

[30] SEGAMI K, AOYAMA T, HIROSHIMA Y, KOMORI K, HASHIMOTO I et al. Clinical significance of tap1 and dll4 expression in patients with locally advanced gastric cancer. In Vivo 2021; 35: 2771–2777. https://doi.org/10.21873/invivo.12562

[31] PENG HH, KO HH, CHI NC, WANG YP, LEE HC et al. Upregulated npm1 is an independent biomarker to predict progression and prognosis of oral squamous cell carcinomas in taiwan. Head Neck 2020; 42: 5–13. https://doi.org/10.1002/hed.25971

[32] ZHOU LM, YUAN LL, GAO Y, LIU XS, DAI Q et al. Nucleophosmin 1 overexpression correlates with (18)f-fdg pet/ct metabolic parameters and improves diagnostic accuracy in patients with lung adenocarcinoma. Eur J Nucl Med Mol Imaging 2021; 48: 904–912. https://doi.org/10.1007/s00259-020-05005-4

[33] YU ACY, CHERN YJ, ZHANG P, PASILIAO CC, RAHMAN M et al. Inhibition of nucleophosmin 1 suppresses colorectal cancer tumor growth of patient -derived xenografts via activation of p53 and inhibition of akt. Cancer Biol Ther 2021; 22: 112–123. https://doi.org/10.1080/15384047.2020.1839278

[34] LUO C, LEI T, ZHAO M, MENG Q, ZHANG M. The role of npm1 in the invasion and migration of drug resistant bladder cancer. Urol J 2021; 18: 452–459. https://doi.org/10.22037/uj.v18i.6087

[35] KARHEMO PR, RIVINOJA A, LUNDIN J, HYVONEN M, CHERNENKO A et al. An extensive tumor array analysis supports tumor suppressive role for nucleophosmin in breast cancer. Am J Pathol 2011; 179: 1004–1014. https://doi.org/10.1016/j.ajpath.2011.04.009