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Original Article

P1-HNF4α expression negatively correlates with HER2 levels and is associated with good prognosis in gastric adenocarcinoma

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Abstract: HNF4α, a member of the steroid/thyroid nuclear receptor super family, is a transcriptional factor expressed in various tissues and cells. In this study, we aimed to investigate the clinical significance of P1-HNF4α protein expression in gastric adenocarcinoma. We examined P1-HNF4α and HER2 protein levels in the tissue of 245 gastric adenocarcinoma samples by immunohistochemistry, and analyzed the association between P1-HNF4α levels, and clinicopathologic factors or prognosis. In gastric adenocarcinoma, positive staining of P1-HNF4α was shown in 150 (61.2%) of 245 cases, while positive expression of HER2 was shown in 36 (14.7%) of 245 cases as detected by immunohistochemistry. The expression of P1-HNF4α was negatively correlated with that of HER2. Furthermore, P1-HNF4α and HER2 highly expressed in intestinal-type adenocarcinoma according to the Lauren classification, tubular adenocarcinoma according to WHO classification, and well-to-moderately differentiated tumors. In gastric adenocarcinoma, P1-HNF4α was significantly associated with histological type, Lauren grade, degree of tumor differentiation, vascular invasion, lymph node metastases, and pTNM stage. Moreover, survival analysis showed that P1-HNF4α expression was an independent prognostic factor of good survival in gastric adenocarcinoma ($P<0.05$). Our results indicate that a negative correlation exists between P1-HNF4α and HER2 expression levels and P1-HNF4α is significantly correlated with tumor progression and a good prognosis in gastric adenocarcinoma.

Keywords: Gastric adenocarcinoma, HNF4α, HER2, clinical significance

Introduction

Gastric cancer is a highly heterogeneous disease and the 5th most common cancer, making it the 3rd leading cause of cancer death worldwide [1, 2]. Gastric adenocarcinoma is the most common histologic type of gastric cancer, and imposes a considerable global health burden [3]. Previous studies have yielded a large amount of new information for potential exploitation. However, measures against gastric adenocarcinoma have lagged compared with other tumors, so more research is necessary to overcome the obstacles to prolong survival of patients with advanced gastric adenocarcinoma.

Hepatocyte nuclear factor 4 alpha (HNF4α), a highly conserved member of the steroid/thyroid nuclear receptor superfamily, plays a key role in the establishment and maintenance of hepatocyte differentiation [4]. HNF4α is also related to fat metabolism and maturity onset diabetes of the young 1 (MODY-1) [5, 6]. Several splice variants of HNF4α are generated by alternative promoter (proximal promoter P1 and distal promoter P2) usage and distinct 3' splicing events [7]. The promoter-driven HNF4α isoforms exhibit tissue-specific expression patterns in the gastrointestinal tract, and normal tissues and tumor tissues show alternative expression patterns of P1 and P2 promoter-driven HNF4α. In the small intestine and colon, both P1-driven HNF4α (P1-HNF4α) and P2-driven HNF4α (P2-HNF4α) are expressed. P2-HNF4α is expressed in the normal stomach, while P1-HNF4α is expressed in intestinal gastric cancer but rarely expressed in diffuse gastric cancer [8, 9]. P1-HNF4α was shown to be involved in intestinal gastric adenocarcinoma,

but little is known about the effect of P1-HNF4α on prognosis.

Human epidermal growth factor receptor 2 (HER2), also known as erbB2, is a membrane-associated tyrosine kinase. Phosphorylation of HER2 can lead to activation of signal transduction systems resulting in the cellular events associated with cancer progression [10]. HER2 is usually underexpressed in normal tissues, but in some tumors, such as gastric cancer, it is abnormally overexpressed and activated [11]. High expression level of HER2 has been significantly correlated with increased tumor growth, invasion, metastasis, resistance to chemotherapy, and poor prognosis [12]. In clinical practice we found HER2 gene amplification and HER2 protein was overexpressed in partial gastric adenocarcinoma. Both P1-HNF4α and HER2 are associated with intestinal gastric adenocarcinoma. Nevertheless, the relationship between P1-HNF4α and HER2 remains elusive. To understand whether P1-HNF4α expression is related to the prognosis of patients and HER2 expression is mediated by P1-HNF4α, we carried out a series of studies of human gastric adenocarcinoma.

In this study, we describe that P1-HNF4α and HER2 expression levels are negatively correlated in gastric adenocarcinoma tissues. Furthermore, the clinicopathologic significance of P1-HNF4α expression of gastric adenocarcinoma is further evaluated using archival tissue specimens and statistical analysis. We found that P1-HNF4α is an independent prognostic factor, also a potential novel biomarker and index for tumor invasion, lymph node metastasis and pTNM stage. Our data will facilitate an understanding of gastric adenocarcinoma and mining biomarkers for the diagnosis and treatment of this disease.

Materials and methods

Ethics statement

The present study was approved by the Guizhou Provincical People's Hospital Review Board (Guiyang, China), and all enrolled patients providing tumor tissue as well as normal gastric tissue samples signed an informed consent to participate in the study prior to surgical removal of the gastric carcinoma to allow for this research to be undertaken.

Patients enrolled and follow-up

A total of 245 patients who had surgery for gastric adenocarcinoma between January 2013 and December 2016 at the Guizhou Provincial People's Hospital were selected for this study. No therapy was administered to any of the patients before definitive surgery. All of patients enrolled in this study had complete and detailed clinicopathological data, including gender, age, tumor site, Lauren's classification, WHO classification, degree of differentiation of tumors, nerve infiltration, vascular invasion, extension of tumor invasion, lymph node metastasis, distant metastasis and pTNM stage. Specimens derived from patients were collected and archived under protocols approved by the Institutional Review Boards of the Guizhou Provincial People's Hospital. The diagnosis of gastric adenocarcinoma was confirmed by at least two pathologists and specifically the challenging cases were reviewed by trained gastrointestinal pathologist to confirm their status.

Follow-up data were obtained from the database of Guizhou Provincial People's Hospital. Follow-up time was calculated as the time of the initial surgery for the primary tumor until mortality or May 31, 2017. Overall survival (OS) time was defined as the interval from the date of initial treatment to clinically proven recurrence or metastasis and death.

IHC staining and FISH analysis for gastric adenocarcinoma

For P1-HNF4α and HER2 staining, serial sections from the same paraffin blocks were immunostained with anti-P1-HNF4α (dilution 1:500, R&D Systems, Minneapolis, MN, USA) and mouse anti–HER2 (dilution 1:100, ZSGB-BIO, Beijing, China) monoclonal antibody. Immunostaining was performed using the Leica Bond-Max automated stainer and and Leica Refine detection kit (Leica Biosystems, Bannockburn, IL, USA) as our previously described [13]. Any definitive nuclear or membrane staining of P1-HNF4α or HER2 respectively in the tumor cell was considered positive reaction.

All of the selected tumor specimens scoring "equivocal (2+)" were subjected to HER2 FISH analysis. Amplification of the HER2 gene was determined by dual-color FISH procedure using the PathVysion HER2 DNA probe Kit (PathV-

Table 1. P1-HNF4α expression in relation to the status of HER2 expression

	P1-HNF4α				γ	P
	0	1+	2+	3+		
0	8	1	21	12	-0.197	0.045
1+	4	1	15	8		
2+	7	4	7	4		
3+	4	2	4	2		

ysion, Abbott Molecular, Des Plaines, Illinois, USA) according to the manufacturer's protocol.

Evaluation of immunohistochemical staining and FISH analysis

The diagnosis of IHC staining of P1-HNF4α and HER2 was confirmed by two senior pathologists. The extent of P1-HNF4α expression in the area of gastric adenocarcinoma was graded semi-quantitatively as follows: 0+, <1% positive staining; 1+, 1%-10% positive staining; 2+, 10%-50% positive staining; 3+, more than 50% positive staining of the total gastric adenocarcinoma area. Expression of HER2 was assessed using semi-quantitative scoring method as below: 0 (no staining or weak membranous staining in fewer than 10% of tumor cells), 1+ (weak staining), 2+ (moderate staining), 3+ (strong membranous staining in more than 10% of tumor cells). Tumor specimens scoring 0 and 1+ were considered negative and that scored 3+ were considered positive. Tumor specimens scoring 2+ were considered "equivocal", which were subjected to HER2 FISH analysis by FISH.

The HER2 gene amplification was assessed as described using previously described standard criteria [14]. Briefly, the ratio of orange HER2 signals to green CEP17 signals was calculated and amplification defined as HER2/CEP17 >2.00 or the average HER2 signal ≥6. Cases scoring IHC 2+ but FISH negative were classified as HER2 negative and those FISH positive were regarded as HER2 positive.

Statistical analysis

The X^2 test and Spearman rank correlation analysis were used to analyze the relationship between P1-HNF4α and HER2 expression and clinicopathological factors. The survival time was calculated by the Kaplan-Meier method and the differences between the survival curves were examined by the Log-Rank test. Analyses were performed using Software SPSS17.0. P<0.05 was considered significant.

Results

General clinical information and pathological characteristics of enrolled patients

As shown in **Table 2**, the general clinical information and pathological characteristics of enrolled patients are as follows. The 245 enrolled patients included 71 women and 174 men, with a median age of 59 years and an average age of 58.7 (range 30-85). The location of the tumor was: 12 cases at the esophagogastric junction, 33 cases at the gastric fundus and body and the other 200 cases at the gastric antrum and pylorus. Lauren classification: 106 cases of intestinal type, 107 cases of diffuse type and 32 cases of mixed type. WHO classification of tumors (2010 version): 1 case of papillary adenocarcinoma, 93 cases of tubular adenocarcinoma, 12 cases of mucous adenocarcinoma, 107 cases of low-adhesion adenocarcinoma and 32 cases of mixed adenocarcinoma. Tumor differentiation: 12 cases of highly differentiated adenocarcinoma, 67 cases of moderately differentiated adenocarcinoma, and 166 cases of low differentiated adenocarcinoma. Tumor invasion: nerve invasion was seen in 115 cases and vascular invasion in 147 cases. pTNM staging (WHO 2010 Edition): stage I 38 cases, stage II 67 cases, stage III 126 cases, and stage IV 14 cases.

Correlation between the expressions of P1-HNF4α and HER2 in gastric adenocarcinoma

IHC stained sections of 245 gastric adenocarcinoma tissues were graded for their staining intensity for P1-HNF4α and HER2 protein respectively. Tumor cells of gastric adenocarcinoma showed typical membrane staining of HER2 and nuclear staining of P1-HNF4α respectively (**Figure 1**). Positive staining of P1-HNF4α was shown in 150 (61.2%) of 245 specimens, HER2 was 36 (14.7%). Amplification of HER2 gene of equivocal cases was determined by dual-color FISH assays. HER2 amplification was ascertained in 19 of 38 equivocal cases (data not shown). We analyzed the association between the expression of P1-HNF4α and HER2 in gastric adenocarcinoma. Our analysis showed positive staining for both P1-HNF4α and HER2 in 22 of 245 specimens and negative staining for both

Table 2. Correlation of P1-HNF4α and HER2 expression with clinicopathologic parameters in gastric cancer

Variables	Cases	HER2		*P* value	HNF4αP1		*P* value
		+ (%)	-		+ (%)	-	
Gender							
Male	174	23 (13.2)	151	0.307	107 (61.5)	67	0.892
Female	71	13 (18.3)	58		43 (60.6)	28	
Age (years)							
≤59	125	15 (12.0)	110	0.224	76 (60.8)	49	0.889
>59	120	21 (17.5)	99		74 (61.7)	46	
Location							
Esophagogastric junction	12	3 (25.0)	9	0.457	7 (58.3)	5	0.671
Gastric fundus and body	33	6 (18.2)	27		18 (54.5)	15	
Gastric antrum and pylorus	200	27 (13.5)	173		125 (62.5)	75	
Lauren typing							
Intestinal type	106	28 (26.4)	78	0.000	75 (70.7)	31	0.027
Mixed type	32	2 (6.3)	30		18 (56.3)	14	
Diffuse type	107	7 (6.5)	100		57 (53.3)	50	
WHO classification※							
Tubular adenocarcinoma	93	24 (25.8)	69	0.001	67 (72.0)	26	0.05
Mucous adenocarcinoma	12	2 (16.7)	10		7 (58.3)	5	
Mixed adenocarcinoma	32	3 (9.3)	101		18 (56.3)	50	
Low-adhesion adenocarcinoma	107	6 (5.6)	29		57 (53.3)	14	
Tumor differentiation							
Highly-moderately differentiated	79	19 (24.1)	60	0.004	59 (74.7)	20	0.003
Low differentiated	166	17 (10.2)	149		91 (54.8)	75	
Nerve invasion							
Yes	115	15 (13.0)	99	0.526	66 (57.4)	49	0.247
No	130	21 (16.2)	110		84 (64.6)	46	
Vascular invasion							
Yes	147	27 (18.4)	120	0.047	78 (53.1)	69	0.001
No	98	9 (9.2)	89		72 (73.5)	26	
Extension of tumor invasion							
PT1	25	4 (16.0)	21	0.539	21 (84.0)	4	0.006
PT2	26	5 (19.2)	21		14 (53.8)	12	
PT3	104	11 (10.6)	93		70 (67.3)	34	
PT4	90	15 (16.7)	75		45 (50.0)	45	
LN metastasis							
PN0	86	8 (9.3)	78	0.127	63 (73.3)	23	0.006
PN1	31	3 (9.7)	28		22 (70.9)	9	
PN2	53	12 (22.6)	41		28 (52.8)	25	
PN3	75	13 (17.3)	62		37 (49.3)	38	
Distant metastasis							
M0	230	33 (14.3)	197	0.549	144 (62.6)	86	0.082
M1	15	3 (20.0)	12		6 (40.0)	9	
PTNM stage							
I	38	6 (15.8)	32	0.146	27 (71.1)	11	0.004
II	67	5 (7.5)	62		50 (74.6)	17	
III	126	24 (19.1)	102		68 (53.9)	58	
IV	14	1 (7.1)	13		5 (35.7)	9	

※: 1 case of papillary adenocarcinoma was not included.

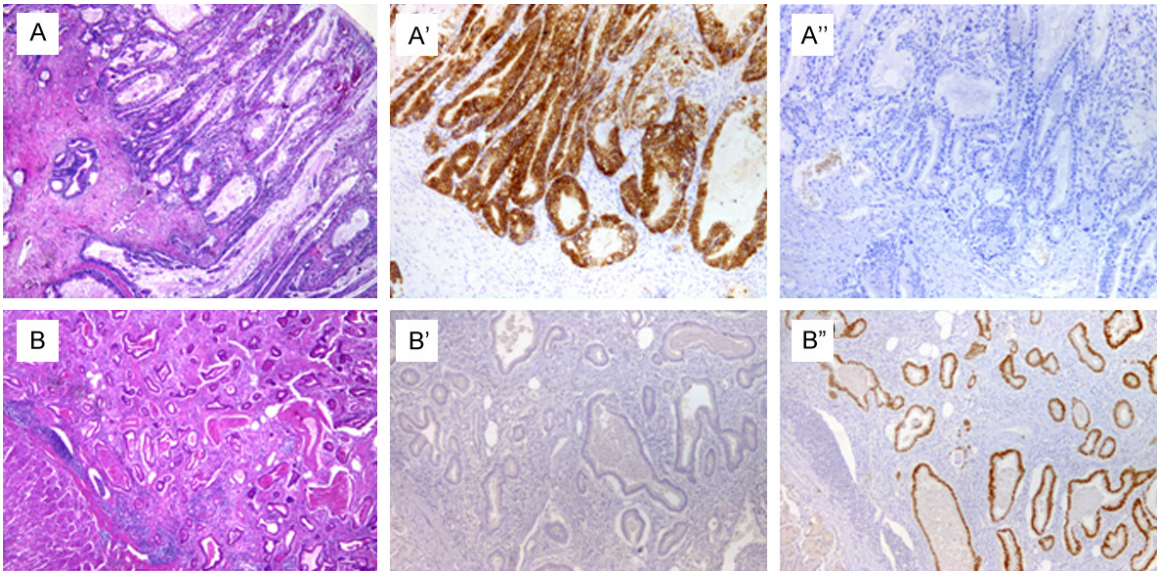

Figure 1. P1-HNF4α and Her2 expression in gastric adenocarcinoma. Moderately (A) and well (B) differentiated intestinal-type adenocarcinoma was stained for HER2 and P1-HNF4α. A strong positive expression of Her2 (A') and negative expression of P1-HNF4α (A") were frequently observed in moderately differentiated intestinal-type adenocarcinoma, while negative expression of Her2 (B') and strong positive P1-HNF4α (B") were found in well differentiated intestinal-type adenocarcinoma. (A and B ×40, A', A", B' and B" ×100).

proteins in 81 of 245 specimens. Spearman's correlation analysis indicated no correlation between the expressions of P1-HNF4α and HER2 in these two groups of specimens (γ= 0.07, P=0.91). Further analysis showed that there are 128 P1-HNF4α positive specimens in HER2 negative group (209 cases), and there are 14 HER2 positive cases in P1-HNF4α negative group (95 cases). As is shown by our data, the expression of P1-HNF4α has a significant negative correlation with that of HER2 (P<0.05) in the immunohistochemical tissue analysis in this group (**Table 1**).

Association between P1-HNF4α and clinicopathological factors

In this study, 245 gastric adenocarcinoma patients with sufficient tumor materials and detailed clinicopathologic data were available. Statistical parameters measured for these patients are described in **Table 2**. We found that expression of P1-HNF4α and HER2 in intestinal type adenocarcinoma specimens was higher than that of the diffuse type and mixed type according to Lauren classification; and expression of P1-HNF4α and HER2 in tubular adenocarcinoma specimens was higher than that of other types according to WHO classification. In addition, expression of both proteins in moderate-well differentiated adenocarcinoma was higher than that of poorly differentiated adenocacinoma. The increased expression of P1-HNF4α and HER2 was both significantly associated with histological type, tumor differentiation, and vascular invasion. Moreover, P1-HNF4α was significantly correlated with the extent of tumor invasion, regional lymph node metastasis, and pTNM staging (**Table 2**). However, no significant correlation was observed between P1-HNF4α expression and other parameters including gender, age, location, perineural invasion, and distant metastasis.

Expression of P1-HNF4α as an independent prognostic marker in gastric adenocarcinoma

There were 139 cases (58.3%) in 245 patients (66 cases of intestinal type, 77 cases of mixed and diffuse type) were interviewed in this study. The median duration of the follow-up was 14 months (range 5-53 months). Of the 139 patients analyzed, statistically significant differences in OS were seen, with a good outcome for patients with positive staining of P1-HNF4α (γ=7.770, P=0.005) (**Figure 2A**), but a poor prognosis for patients with HER2 positive cases (γ=5.984, P=0.014, **Figure 2B**). To further characterize relationships between P1-HNF4α/

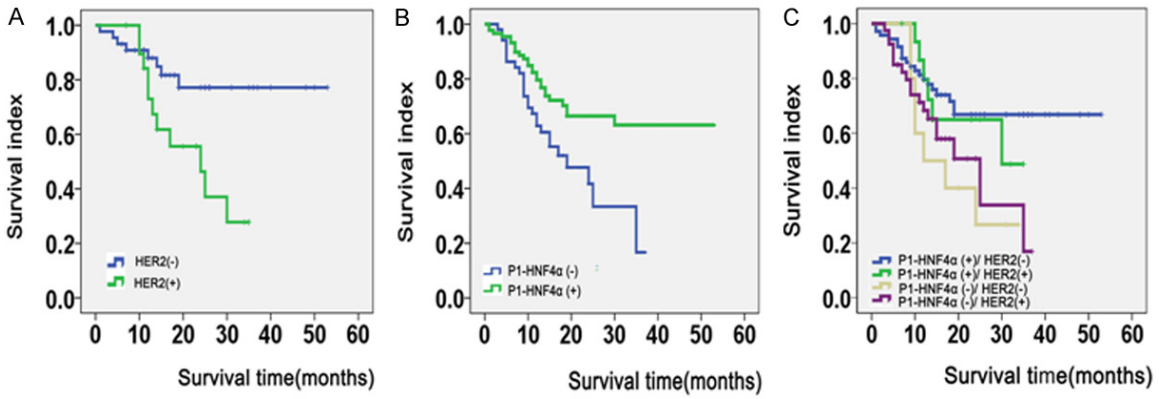

Figure 2. Kaplan-Meier survival analysis of follow-up data. A. Survival rate of HER2 positive and negative expression cases, B. Survival rate of P1-HNF4α positive and negative expression cases, C. Effects of HER2 and P1-HNF4α expression on survival of intestinal-type adenocarcinoma patients.

HER2 expression and prognosis, patients were grouped according to the two protein expression levels: P1-HNF4α (+)/HER2 (-), P1-HNF4α (+)/HER2 (+), P1-HNF4α (-)/HER2 (-) and P1-HNF4α (-)/HER2 (+). The results showed that group with P1HNF4α (+)/HER2 (-) had the best prognosis and the group with P1-HNF4α (-)/HER2 (+) had worst prognosis (γ=11.289, P=0.010) (**Figure 2C**).

Discussion

Gastric adenocarcinoma is one of the most common malignant tumors of the digestive tract. The treatment of this cancer has improved during the last few decades with regard to the advance of surgical skills and tumor targeted strategies. However, the general outcome for patients of gastric adenocarcinoma is still not ideal. The assessment of prognostic factors is of clinical importance, especially for a disease with poor outcome such as gastric adenocarcinoma. The current study assessed the expression characteristics of P1-HNF4α in gastric adenocarcinoma specimens, and confirmed that P1-HNF4α was an independent good prognostic factor in gastric adenocarcinoma. In addition, the present study showed, for the first time, a negative relationship between the expressions of P1-HNF4α and HER2 in gastric adenocarcinoma.

HER2 status is associated with the prognosis of gastric cancer and evidence of the role of HER2 over-expression in patients with gastric cancer is growing, but whether it is an independent prognostic factor remains controversial. The HER2 positive rate of gastric adenocarcinoma is different according to tumor heterogeneity and regional difference [15-17]. The positive rate of HER2 is 14.7% in this study, which is consistent with a previous report [18]. Grabsch and his colleagues analyzed the expression of HER2 in 924 cases of primary gastric cancer in two independent institutions in Germany and the UK, and results indicated that HER2 expression is not related to prognosis in gastric cancer [19]. Meta-analysis showed that HER2 expression is related to Lauren classification, tumor differentiation, TNM stage and lymph node metastasis [20]. He *et al* suggested that HER2 is an independent prognostic factor of intestinal type gastric cancer and HER2 negative intestinal type adenocarcinoma has the best prognosis, while the HER2 positive diffuse adenocarcinoma has the worst prognosis [21]. Our results agree with this viewpoint because HER2 expression in the present study was associated with poor prognosis in gastric adenocarcinoma (**Figure 2B**).

HNF4α plays an important role in the development of tumors and down-regulation of HNF4α is related to the development of hepatocellular carcinoma and colorectal cancer [22-24]. There are limited studies focused on HNF4α in gastric diseases. Tanaka *et al* found that P1-HNF4α was not expressed in normal gastric mucosa, but was expressed in intestinal metaplasia of the gastric mucosa, and the positive rate of P1-HNF4α was 57.1% (8/14) in gastric carcinoma, which was mainly expressed in intestinal type adenocarcinoma, but not detected in the diffuse type [8]. In our previous study, we also found that P1-HNF4α is not expressed in nor-

mal gastric mucosa, but expressed in intestinal metaplasia and a high level of expression of P1-HNF4α was associated with the severity of chronic gastritis [13]. Kojima *et al* studied 35 cases of gastric adenocarcinoma, and found that the positive rate of P1-HNF4α was 42.9% in gastric-type differentiated adenocarcinoma, 31.4% in mixed adenocarcinoma, and 25.7% in intestinal adenocarcinoma respectively [25]. Uozaki and his colleagues found that the positive rate of P1-HNF4α in gastric adenocarcinoma was 24.9% (63/253), and there were associations between P1-HNF4α expression and clinicopathological factors such as age, tumor size, lymphavascular invasion, and lymph node metastasis, but no correlation with Lauren's classification [26]. Our results showed that the positive rate of P1-HNF4α in gastric adenocarcinoma was 61.2% (150/245), which was similar to that of Tanaka [8], but not consistent with Kojima [25] and Uozaki [26]. The difference in the number of cases and the criteria for interpretation may lead to this discrepancy. In addition, our follow-up data showed that the prognosis of cases with positive P1-HNF4α expression was better than those with negative expression. The relationship between P1-HNF4α expression and the prognosis of intestinal type adenocarcinoma, mixed type, and diffuse adenocarcinoma was further analyzed. Result of log rank test showed no significant difference ($P>0.05$), but the survival curve for P1-HNF4α-positive patients tended to be better than the negative ones.

In summary, our results showed that P1-HNF4α and HER2 expression levels are negatively correlated in gastric adenocarcinoma. The clinicopathologic significance of P1-HNF4α expression of gastric adenocarcinoma was further evaluated. We found that P1-HNF4α is an independent prognostic factor, also a potential novel biomarker and index for tumor invasion, lymph node metastasis and pTNM stage. Our data will facilitate understanding of gastric adenocarcinoma and mining new biomarkers such as P1-HNF4α for the diagnosis and treatment of this disease.

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Disclosure of conflict of interest

None.

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