Clinic Correlation and Prognostic Value of P4HB and GRP78 Expression in Gastric Cancer

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Abstract

Background: Prolyl 4-hydroxylase, beta polypeptide (P4HB) and Glucose-regulated protein 78 (GRP78) are represent for poor prognosis of various cancers. However, rare research investigate the correlation of them in cancer. This study aimed to investigate the correlation and prognostic value of P4HB and GRP78 expression along with clinical features in gastric cancer (GC).

Methods: A total of 150 GC tissue samples separately evaluated P4HB and GRP78 protein expression by immunohistochemistry. Association of P4HB and GRP78 expression with clinicopathological features was analyzed. Bioinformatics analyses were performed to find correlation between mRNAs and pathways. Kaplan-Meier analyses were taken to compare survival curves. Univariate and multivariate Cox regression models used to analyze the impact of prognostic factors on overall survival (OS). A prognostic nomogram was constructed based on the multivariate Cox regression model, and compared to TNM stage in discrimination ability and clinical usefulness.

Results: P4HB and GRP78 expression were both associated with tumor invasion and lymph node metastasis. P4HB protein positively correlated with GRP78, as well as the same tendency of mRNAs in both GC tissue and cell databases. Bioinformatics analyses indicated that P4HB and GRP78 may be associated with protein folding and response to ER stress, and the protein processing in ER pathway. High single or co-expression of P4HB and GRP78 represented for a shorter OS. When stratified by postoperative adjuvant chemotherapy, the high co-expression was only related to unfavorable prognosis of with chemotherapy group, especially in advanced stage. Multivariate Cox regression analysis identified co-expression of P4HB and GRP78 as an independent prognostic factor for OS. The nomogram including the co-expression was better than TNM stage in discrimination ability and clinical usefulness through the receiver operating characteristic (ROC) and decision curve analysis (DCA) curves.

Conclusion: P4HB was positively correlated with GRP78 expression, co-expression of them was an independent prognostic factor, could serve as a predictive marker for GC patients with postoperative adjuvant chemotherapy in advanced stage.

Introduction

Gastric cancer (GC) is the fifth common diagnosed cancer and the third most frequently cause of cancer death global wide, relating to over one million new cases and an estimated nearly zero point eight million deaths in 2018, along with the incidence rate is still markedly elevating in Eastern Asia [1]. Since the best treatment period of most GC patients had already missed when they clinically diagnosed, the prognosis of GC remains poor [2]. The grim fact that survival trends were little increased in most countries between 1995–1999 and 2010–2014 disappoints us, despite efforts to improve treatment strategies [3]. Owing to huge biological heterogeneity, even with similar clinical features, the prognoses of GC would perform great differences [4, 5]. Therefore, precisely evaluate the prognosis of GC with the aid of better predictors is urgently needed.

The endoplasmic reticulum (ER) is responsible for calcium dynamic balance, lipid biogenesis and participate in folding of either secretory or membrane bound proteins. Once various stresses such as hypoxia break its homeostasis, the resulting abnormal accumulation of unfolded and misfolded proteins together constitute ER stress [6]. To reconstruction ER homeostasis, a series of signaling pathways named unfolded protein response (UPR) subsequently activates, which makes up of three main branches: 1) PERK (pancreatic ER kinase-like ER kinase, also known as eIF2AK3) – eIF2 α (eukaryotic translation initiation factor 2 α); 2) ATF6 (activating transcription factor-6); 3) IRE1 (inositol-requiring protein 1, also known as ERN1) – XBP1 (X-box binding protein 1) [6–8]. Tumors trend to accompany with hypoxia, acidosis and nutrient deprivation, frequently induce ER stress, together with follow-up activation of UPR play core roles in tumorigenesis, proliferation and survival of cancer cells [6].

Prolyl 4-hydroxylase, beta polypeptide (P4HB, also known as PDIA1) belongs to protein disulfide isomerase (PDI) family, close connects with ER stress and UPR, as an enzymatic molecular chaperone to reconstruct misfolded proteins [9]. P4HB is proved to be upregulated in a variety of cancers, such as lung cancer [10], diffuse glioma [9], colon cancer [11]. As a result, P4HB has been reported by numerous researches to be a potential target for cancer therapy [12–14]. But studies referred to GC were rather limited. Only one recent research showed P4HB positively correlated with hypoxia-associated biomarkers, overexpression of P4HB indicated poor prognosis in GC patients [15].

Glucose-regulated protein 78 (GRP78, also known as HSPA5 or BiP) is also an ER chaperone protein, will dissociate from three specific sensors (PERK, ATF6 and IRE1) in response to ER stress, and subsequently activates UPR [16]. General overexpression of GRP78 in different cancers including GC has been widely accepted, also GRP78 could serve as a good indicator of poor prognosis and contribute a lot to therapy resistance [17, 18].

Xia *et al*/identified that P4HB through downregulating GRP78 promoted hepatocellular cancer tumorigenesis [19]. However, in GC, single overexpression of the two ER chaperone proteins has been reported to represent poor prognosis, meaning P4HB may not negative correlate with GRP78. Due to rare relevant research, we aimed to investigate the correlation between P4HB and GRP78 expression and clinical features, as well as their prognostic implications in GC. Furthermore, we integrated the expression of P4HB and GRP78 with other prognostic variables to predict overall survival (OS) after surgery in GC patients.

Materials And Methods

Patients

Amount to 150 GC patients who underwent gastrectomy from 2007 to 2013 at Affiliated Tumor Hospital of Nantong University (Nantong, China) were enrolled in our retrospective study. All patients confirmed histopathology diagnosis after gastrectomy, and none of them received preoperative chemotherapy or radiotherapy. Patients with incomplete follow-up data and histopathological diagnosed neuroendocrine

or adenosquamous carcinoma were excluded from the cohort. The pathological tumor-node-metastasis (TNM) stage conformed to TNM Staging Manual (7th edition) from the AJCC (American Joint Committee on Cancer). Fluorouracil-based postoperative adjuvant chemotherapy were mainly performed in patients with advanced disease (Ⅲ/Ⅳ TNM stage). The clinicopathological parameters included were shown below: age, gender, Bormann type, tumor size, Lauren's classification, differentiation, histological type, depth of tumor invasion, lymph node metastasis, TNM stage, vessel invasion, perineural invasion and fluorouracil-based postoperative adjuvant chemotherapy (sTable 1). The median follow-up time was 68.0 (range 2.5–92.5) months. Overall survival (OS) was defined as the period between the date of gastrectomy or the death or the last follow-up. If patients ended follow-up in keeping alive, data were censored. The study had achieved the approval from the Ethics Committee of Affiliated Tumor Hospital of Nantong University and the informed consent of all in cohort patients.

Immunohistochemistry (IHC)

The tissue microarrays (TMA) were constructed by duplicate 1.5 mm formalin-fixed paraffin-embedded tissue cores of two different areas. We performed IHC according to the standard protocol for the immunostaining provided by a previous study [20]. P4HB expression used a mouse anti-P4HB monoclonal antibody (1:100, Abcam, Cambridge, UK). While GRP78 IHC used a rabbit anti-GRP78 monoclonal antibody (1:100, Abcam, Cambridge, UK). The mi-quantitative H-score, ranged from 0 to 300, was derived from the staining intensities (0: negative, 1: weak staining, 2: moderate staining, 3: strong staining) and the proportion of stained (0–100%). The H-score was evaluated independently by two experienced pathologists who were blind to clinical information. The median H-score was used as the expression cutoff point (P4HB and GRP78 median H-score both = 200). Under the condition, for both proteins, H-scores less than 200 were considered to low expression, while H-scores on the opposite were considered to low expression, respectively.

Bioinformatics analyses

The Gene Expression Profiling Interactive Analysis (GEPIA, <http://gepia.cancer-pku.cn/index.html>) [21], which is an interactive web server based on The Cancer Genome Atlas (TCGA), was used to find the correlation of mRNAs. The mRNA expression data of totally 1168 cell lines, including 37 GC cell lines, was downloaded from the Broad Institute Cancer Cell Line Encyclopedia (CCLE, <https://portals.broadinstitute.org/ccle>) [22]. Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed by R package “clusterProfiler”. The correlation coefficients of genes visualized in KEGG pathway maps were performed by R package “pathview”.

Statistical Analysis

SPSS version 25.0 (IBM, Armonk, NY) was applied to for the χ^2 test to explore the correlations among biomarker variables (P4HB and GRP78) and clinicopathological variables, the Spearman rank correlation

to evaluate the correlation between H-scores of P4HB and GRP78, the univariate and multivariate analyses by the stepwise Cox proportional hazard regression model to estimate the crude hazard ratios (HRs) and 95% CI of HRs. MedCalc Software version 19.0.4 (MedCalc, Mariakerke, Belgium) was applied to compare two correlation coefficients. The R packages “rms”, “survival”, “foreign”, “survivalROC” and “survminer” were used for plotting the nomogram, the calibration curves to evaluate the calibration ability of the nomogram, the ROC curves to assess the discrimination ability of the nomogram, Kaplan–Meier curves to estimate prognosis and calculate the Harrell’s C-index also to evaluate the discrimination ability of the nomogram. Decision curve analysis (DCA) curves were plotted by R package “stdca”, whose source file was from www.mskcc.org, to analyze the clinical usefulness of the nomogram. Two-sided P value less than 0.05 was considered statistically significant.

Results

1. Association of P4HB and GRP78 protein expression with clinicopathological characteristics in GC

We detected the expression of P4HB and GRP78 separately by immunohistochemistry (IHC) in 150 GC tissues. The representative images of P4HB and GRP78 staining are shown in Fig. 1. A total of 74 patients (49.3%) were P4HB low expression and 76 patients (50.7%) were P4HB high expression, as for GRP78 expression, there were 71 low cases (47.3%) and 79 high cases (52.7%) respectively. Furthermore, the expression of P4HB was correlated with age ($P = 0.021$), Bormann type ($P = 0.029$), depth of tumor invasion ($P = 0.001$), lymph node metastasis ($P = 0.018$), postoperative adjuvant chemotherapy ($P = 0.008$), while GRP78 expression was only correlated with depth of tumor invasion ($P = 0.041$) and lymph node metastasis ($P = 0.043$) (Table 1). And P4HB expression was strongly correlated with GRP78 ($P < 0.001$). Subsequent Spearman rank correlation analysis of P4HB and GRP78 H-score proved there existed a significant positive correlation ($R = 0.587$, $P < 0.001$) (sFigure 1A). By investigating the correlation coefficients of different clinicopathological characteristics, the correlation coefficients were significantly elevated in tumor size ≥ 5 cm ($P = 0.037$), TNM ≥ 4 stage ($P = 0.037$) and positive vessel invasion ($P = 0.049$) subgroups, as shown in sTable 2.

Table 1
Relationship between P4HB/GRP78 protein expression and clinicopathological characteristics in GC patients

Clinicopathological parameters	P4HB expression				GRP78 expression			
	Low	High	χ^2	<i>P</i> value	Low	High	χ^2	<i>P</i> value
	74	76			71	79		
Age(year)			5.369	0.021			2.404	0.121
< 65	48	35			44	39		
≥ 65	26	41			27	40		
Gender			0.408	0.523			0.008	0.931
Male	49	54			49	54		
Female	25	22			22	25		
Bormann type			4.743	0.029			0.018	0.894
Ⅰ/Ⅱ	32	20			25	27		
Ⅲ/Ⅳ	42	56			46	52		
Tumor size(cm)			0.455	0.500			0.318	0.573
< 5	43	40			41	42		
≥ 5	31	36			30	37		
Lauren's classification			5.489	0.064			2.263	0.323
Intestinal	17	14			12	19		
Diffuse	40	31			38	33		
Mixed	17	31			21	27		
Differentiation			0.218	0.641			0.040	0.841
Well/Moderate	31	29			29	31		
Poor	43	47			42	48		
Histological type			2.599	0.107			3.071	0.080
Adenocarcinoma	66	73			63	76		
Mucinous/Signet-ring cell	8	3			8	3		
Depth of tumor invasion			11.786	0.001			4.192	0.041

Clinicopathological parameters	P4HB expression				GRP78 expression			
	Low	High	χ^2	<i>P</i> value	Low	High	χ^2	<i>P</i> value
T1/T2/T3	61	43			55	49		
T4	13	33			16	30		
Lymph node metastasis			5.561	0.018			4.092	0.043
Negative	32	19			30	21		
Positive	42	57			41	58		
TNM stage			3.209	0.073			0.914	0.339
I/II	41	31			37	35		
III/IV	33	45			34	44		
Vessel invasion			0.064	0.800			0.609	0.435
Negative	53	53			48	58		
Positive	21	23			23	21		
Perineural invasion			0.080	0.777			0.028	0.867
Negative	56	59			54	61		
Positive	18	17			17	18		
Postoperative adjuvant chemotherapy			7.055	0.008			0.334	0.563
No	17	33			22	28		
Yes	57	43			59	51		
GRP78 expression			34.562	< 0.001				
Low	53	18						
High	21	58						

2. Bioinformatics analyses of P4HB and GRP78 expression in GC

We explored TCGA-STAD (stomach adenocarcinoma) data by GEPIA and analyzed of extracted GC cell lines data (amount to 37 lines) from CCLE successively. It turned out P4HB mRNA was also positively

correlated with GRP78 in both GC tissue and cell databases ($R = 0.61$, $P < 0.001$, and $R = 0.356$, $P = 0.031$, respectively) (sFigure 1B and 1C), in accordance with the correlation of proteins. Then we performed GO and KEGG analysis of these two genes. The top 5 items of each GO term was shown in sFigure 1D. Only items involve with both two genes were taken into account. Finally, protein folding and response to ER stress were discovered in biological process (BP) term, ER chaperone complex, melanosome, pigment granule, ER-Golgi intermediate compartment and ER lumen were discovered in cellular component (CC) term, together with only cell adhesion molecule binding was discovered in molecular function (MF) term. KEGG pathway analysis showed both two genes were enriched in the pathway “protein processing in ER” (sFigure 1E). As the occurrence and development of GC was most likely related to ER stress and UPR, we made further efforts to search correlation of P4HB or HSPA5 with genes within UPR, originated from the “protein processing in ER” KEGG pathway map (hsa04141), by explore TCGA–STAD cohort in GEPIA (sFigure 2–3). Except for P4HB and HSPA5 both positively correlated with typical genes within UPR from STAD (Table 2), correlation coefficients of most upstream genes were higher than downstream genes, as shown in sFigure 1F and 1G.

Table 2
Correlation between P4HB/HSPA5 with genes in UPR pathways from TCGA–STAD cohort

Pathway	Name in pathway	Typical encoded gene	P4HB		HSPA5	
			R	P value	R	P value
PERK pathway						
	PERK	EIF2AK3	0.48	< 0.001	0.52	< 0.001
	eIF1a	EIF2S1	0.41	< 0.001	0.53	< 0.001
	ATF4	ATF4	0.19	< 0.001	0.22	< 0.001
	GADD34	PPP1R15A	0.22	< 0.001	0.30	< 0.001
	NRF2	NFE2L2	0.27	< 0.001	0.31	< 0.001
ATF6 pathway						
	ATF6	ATF6	0.35	< 0.001	0.52	< 0.001
	S1P	MBTPS1	0.30	< 0.001	0.41	< 0.001
	S2P	MBTPS2	0.29	< 0.001	0.41	< 0.001
	WFS1	WFS1	0.25	< 0.001	0.18	< 0.001
IRE1 pathway						
	IRE1	ERN1	0.47	< 0.001	0.29	< 0.001
	TRAF2	TRAF2	0.41	< 0.001	0.41	< 0.001
	ASK1	MAP3K5	0.20	< 0.001	0.31	< 0.001
	MKK7	MAP2K7	0.22	< 0.001	0.11	0.021
	JNK	MAPK8	0.31	< 0.001	0.42	< 0.001
	XBP	XBP1	0.48	< 0.001	0.38	< 0.001

3. Association of P4HB and GRP78 expression with OS in GC

We applied Kaplan-Meier survival analysis and log-rank test to compare OS according to P4HB and GRP78 expression. The results revealed that high expression of P4HB (Fig. 2A) and GRP78 (Fig. 2B) alone was related to an obvious shorter OS ($P < 0.001$ and $P = 0.012$, respectively). Further stratification

analysis showed that the prognosis was the best when P4HB and GRP78 were both low expressed, the worst prognosis was correlated with high expression of both P4HB and GRP78, while the prognosis was in between when one of P4HB and GRP78 was high expressed (Fig. 2C, $P = 0.002$). When analyzing of co-expression of P4HB and GRP78, OS of both or one high expression was sharp lower than that of both low expression (Fig. 2D, $P = 0.002$).

Since P4HB and GRP78 were both reported to be associated with chemotherapy, we also explored the prognosis of fluorouracil-based postoperative adjuvant chemotherapy together with co-expression of P4HB and GRP78, which contained both variables might more precise. We observed the prognosis of group with postoperative adjuvant chemotherapy was better than the other group (Fig. 2E, $P = 0.011$). High level of P4HB and/or GRP78 exhibited significant worse in the former group (Fig. 2F, $P = 0.007$), but no prognostic significance in the latter (Fig. 2G, $P = 0.231$). Once the group with postoperative adjuvant chemotherapy was further divided according to TNM stage, it exhibited no prognostic significance in TNM $\geq$ stage (Fig. 2H, $P = 0.859$), on the contrary, a significant worse OS in TNM $\geq$ stage (Fig. 2I, $P = 0.004$). This indicates that GC patients in TNM $\geq$ stage may not benefit from postoperative adjuvant chemotherapy if P4HB and/or GRP78 high expressed.

4. Construction of a nomogram based on stepwise Cox regression analysis

Univariate analyses were performed for clinicopathological characteristics to estimate postoperative survival in our study group. According to the Table 3, we observed that OS was significantly correlated with Bormann type ($P = 0.004$), tumor size ($P = 0.028$), differentiation ($P = 0.004$), depth of tumor invasion ($P < 0.001$), lymph node metastasis ($P < 0.001$), TNM stage ($P < 0.001$), postoperative adjuvant chemotherapy ($P = 0.012$), P4HB expression ($P = 0.001$), GRP78 expression ($P = 0.013$), P4HB and GRP78 co-expression ($P = 0.002$). These statistically significant variables were subsequent recruited to forward stepwise COX analysis. The result showed that differentiation ($P = 0.020$, HR = 1.846, 95% CI: 1.099–3.101), TNM stage ($P < 0.001$, HR = 3.124, 95% CI: 1.870–5.217), postoperative adjuvant chemotherapy ($P = 0.005$, HR = 0.507, 95% CI: 0.317–0.810) and P4HB and GRP78 co-expression ($P = 0.002$, HR = 2.304, 95% CI: 1.355–3.915) were independent prognostic factors in GC patients (Table 3). Then four independent prognostic factors were included to construct a nomogram for predicting OS probability of GC patients (Fig. 3A). Harrell's C-index for nomogram was 0.733 (95% CI: 0.680–0.786). And calibration plots for nomogram were close to the 45-degree line (the perfect calibration model) at 3-year and 5-year (Fig. 3B and 3C), indicated the model was well calibrated.

Table 3

Univariate and multivariate analyses of prognostic characteristics for overall survival in patients with gastric cancer

Variables	Univariate analysis			Multivariate analysis		
	HR	(95%CI)	P value	HR	(95%CI)	P value
Age(year)						
≥ 65 vs. <65	1.246	0.791–1.963	0.343			
Gender						
Female vs. Male	1.081	0.665–1.756	0.754			
Bormann type						
Ⅱ/Ⅲ vs. Ⅰ/Ⅳ	2.142	1.271–3.609	0.004			
Tumor size(cm)						
≥ 5 vs. <5	1.662	1.056–2.616	0.028			
Lauren's classification			0.214			
Intestinal	1.000	Reference				
Diffuse	1.818	0.932–3.545	0.079			
Mixed	1.613	0.790–3.293	0.190			
Differentiation						
Poor vs. Well/Moderate	2.111	1.274–3.497	0.004	1.846	1.099–3.101	0.020
Histological type						
Mucinous/Signet-ring cell vs. Adenocarcinoma	0.592	0.216–1.622	0.308			
Depth of tumor invasion						
T4 vs. T1/T2/T3	2.536	1.601–4.019	< 0.001			
Lymph node metastasis						

Variables	Univariate analysis			Multivariate analysis		
	HR	(95%CI)	P value	HR	(95%CI)	P value
Positive vs. Negative	3.824	2.060–7.101	< 0.001			
TNM stage						
Ⅱ/Ⅲ vs. Ⅳ/Ⅴ	3.200	1.942–5.275	< 0.001	3.124	1.870–5.217	< 0.001
Vessel invasion						
Positive vs. Negative	1.385	0.861–2.229	0.180			
Perineural invasion						
Positive vs. Negative	1.279	0.766–2.135	0.346			
Postoperative adjuvant chemotherapy						
Yes vs. No	0.556	0.351–0.879	0.012	0.507	0.317–0.810	0.005
P4HB expression						
High vs. Low	2.266	1.414–3.630	0.001			
GRP78 expression						
High vs. Low	1.799	1.130–2.862	0.013			
P4HB and GRP78 co-expression						
Both/one high vs. Both low	2.260	1.338–3.817	0.002	2.304	1.355–3.915	0.002

5. Evaluate the nomogram and its clinical value with TNM stage

To evaluate the discrimination ability and clinical usefulness of the nomogram, we compared it with widely clinical used TNM stage. From Fig. 4A and 4B, we found areas under ROC (receiver operating characteristic) curve (AUROCs) of the 3-year and 5-year OS probability for nomogram model were 0.790 and 0.760, respectively, much better than that for TNM stage (0.684 and 0.671, respectively). Since DCA has superiorities over AUROC for estimating alternative prognostic strategies, we performed the 3-year

and 5-year DCA curves individually for the nomogram and TNM stage. As shown in Fig. 4C and 4D, compared to TNM stage, even at a very small threshold probability, the nomogram offered a high net benefit in 3-year and 5-year, respectively.

Discussion

ER is not only high in calcium-dependent molecular chaperones such as GRP78 for helping stabilize protein-folding intermediates, also has an oxidative environment for the formation of disulphide bonds with the aid of PDI and for the proper folding of proteins [23, 24]. Under non-stressed conditions, GRP78 combines with three UPR sensors (PERK, ATF6 and IRE1) keeping them inactive. Once ER stress occurs, GRP78 preferentially binds to the unfolded proteins, dissociates from the sensors, resulting in activation of the UPR transducers [6, 25]. The outcome of it involves in protein folding and transporting, as well as increasing unfolded protein clearance through pathways such as ER-associated degradation (ERAD) and autophagy. Upon unresolved stress, cells enter apoptosis [8].

PDI family is a part of the thioredoxin (TRX) superfamily, mainly act as catalysts for the formatting and rearranging S-S bonds, also can function as chaperones involved in ERAD [26], playing an important role in the maintenance and regulation of proteostasis. P4HB, also known as PDIA1 (usually referred as PDI), as the archetype PDI protein, the chaperone activity of who is independent of catalytic activity, while the former is vital for many client proteins [27]. There is sufficient evidence supporting that PDIA1 high expressed in a wide variety of cancers tissues [9–11, 24] and cancer cells [28]. And the upregulated PDIA1 is closely correlated with cancer metastasis and invasiveness [27].

GRP78 is encoded by HSPA5, as the most abundant protein of heat shock protein-70 (Hsp70) family, mainly exists in ER lumen, corrects the folding and assembly, as well as prevents the transport of misfolded proteins or protein subunits [29, 30]. GRP78 works as a molecular chaperone in cells, its high expression is crucial for proliferation, invasion, metastasis of many cancers, proves to be correlated with tumor resistance, recurrence, and a low OS [17, 18, 31–33]. With the help of GRP78, tumor cells can organize the stimulation of processes including macroautophagy, combat reactive oxygen species (ROS), and activate pro-survival signaling pathways [29]. Thus, GRP78 will be a useful prognostic biomarker and a promising target for cancer.

P4HB and GRP78 are both ER chaperone proteins involved in UPR, and represent for poor prognosis of various cancers from the above mentioned. However, rare research pays attention to the correlation of them, especially in cancer. An early study shows that BiP and PDI perform synergistically in vitro in the oxidative folding of antibodies, BiP bind to the unfolded polypeptide chains to keep them in a conformation where cysteine residues are accessible for PDI [34]. Different from P4HB negatively correlated with GRP78 in hepatocellular cancer in vitro [19], we confirmed that P4HB and GRP78 showed a positive correlation in protein (from our cohort) and mRNA (from the databases) levels, with disease progress, the two proteins might more closely related since the correlation coefficients were significantly elevated in tumor size ≥ 5 cm, TNM ≥ 4 stage and positive vessel invasion subgroups. Together with

single or combined high expression of P4HB and GRP78 represented for poor prognosis in GC. The specific mechanisms need further exploration.

A lot of studies have shown in a wide range of primary tumors, there exists a sustained and high-level activation of all three branches within UPR [35]. From bioinformatics analyses, we also found P4HB and GRP78 might participate in UPR, since they positively correlated with all typical genes within UPR, as well as correlation coefficients of most upstream genes were higher than downstream genes. UPR may act as a key driver in resistance to chemotherapy [36, 37]. A new encouraging research finds that activation of the PERK branch with UPR is required for colon cancer cells surviving from treatment of 5-fluorouracil, the usage of PERK inhibitor synergizes with 5-FU could suppress the growth of colon cancer cells in vivo [38]. It may explain in fluorouracil-based postoperative adjuvant chemotherapy subgroup, GC patients with a high co-expression of P4HB and GRP78 had a shorter OS of our cohort. When stratified by TNM stage, only patients in TNM $\geq$ stage had a significant shorter OS, one reason might be that in early stage GC, the positive effects from chemotherapy could cancel out the negative effects from UPR, while in advanced GC, the negative effects probably exceeded.

Our nomogram integrated co-expression of P4HB and GRP78 with other prognostic variables, was a simple visualized tool for predicting OS after surgery of patients with OS. Comparing to TNM stage, the discrimination ability and clinical usefulness of the nomogram was much better through AUROCs and DCA curves of 3-year and 5-year, may be great valuable for clinic.

There still remain several limitations in our study. Firstly, this is a retrospective study of small sample group of GC patients, exists the statistical limitations. Secondly, further researches are required to confirm the correlation and regulation mechanisms of P4HB and GRP78 expression of GC in vitro and in vivo. Finally, it needs an independent external cohort to verify our research findings.

In conclusion, we revealed that P4HB expression is positively correlated with GRP78 expression, the relationship may closer in advanced GC; P4HB and GRP78 may involve in UPR of GC from bioinformatics analyses; high co-expression of P4HB and GRP78 predicts a shorter OS with fluorouracil-based postoperative adjuvant chemotherapy in the advanced stage; co-expression of P4HB and GRP78 can independently predict unfavorable OS for GC patients; the prognostic nomogram based on stepwise Cox regression model is much better than TNM stage in the discrimination ability and clinical usefulness.

Declarations

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Authors' contributions

Zhang CF and Zhao WJ performed the majority of experiments and wrote the manuscript; Yang L and Chen XD designed and supervised the study; Zhang XS and Wu TY assisted in conducting the IHC assays; Zhang XL and Xu WW collected and analyzed the data; Song L helped to correct the manuscript.

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Availability of data and materials

All data and material are available from the corresponding author.

Ethics approval and consent to participate

This research was reviewed and approved by the Ethics Committee of Affiliated Tumor Hospital of Nantong University

Consent for publication

The publication of this manuscript has been approved by all authors.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Representative immunohistochemical staining images of P4HB and GRP78 in gastric cancer (GC) tissues. A: Low expression of P4HB in the cytoplasm of a GC case. B: High expression of P4HB in the cytoplasm of a GC case. C: Low expression of GRP78 in the cytoplasm of a GC case. D: High expression of GRP78 in the cytoplasm of a GC case. Magnifications in all figures were 400 ×.

Figure 2

Kaplan-Meier analyses of OS in GC patients. A: Kaplan-Meier analysis of OS according to single P4HB expression. B: Kaplan-Meier analysis of OS according to single GRP78 expression. C and D: Kaplan-Meier analysis of OS according to combined P4HB and GRP78 expression. E: Kaplan-Meier analysis of OS according to postoperative adjuvant chemotherapy. F: Kaplan-Meier analysis of OS in patients without postoperative adjuvant chemotherapy according to co-expression of P4HB and GRP78. G: Kaplan-Meier analysis of OS in patients with postoperative adjuvant chemotherapy according to co-expression of P4HB and GRP78. H: OS curves of stage I/II patients with postoperative adjuvant chemotherapy stratified by co-expression of P4HB and GRP78. I: OS curves of stage III/IV patients with postoperative adjuvant chemotherapy stratified by co-expression of P4HB and GRP78.

Figure 3

Nomogram and calibration plots for OS prediction in GC patients. A: Nomogram for predicting 3-year and 5-year OS probabilities; B: Calibration plot for predicting 3-year OS; C: Calibration plot for predicting 5-year OS.

Figure 4

ROC and DCA curves of nomogram and TNM stage for OS prediction in GC patients. A: ROC curves for predicting 3-year OS; B: ROC curves for predicting 5-year OS; C: DCA curves for predicting 3-year OS; D: DCA curves for predicting 5-year OS.

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