Pathology - Research and Practice 215 (2019) 653–659

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Is co-expression of USP22 and HSP90 more effective in predicting prognosis of gastric cancer?

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ARTICLE INFO

Keywords:
Ubiquitin-specific peptidase 22
Heat shock protein 90
Gastric cancer
Metastasis
Prognosis

ABSTRACT

The ubiquitin-specific peptidase 22 (USP22) belongs to the largest subfamily of deubiquitylases and recent studies indicate that overexpression of USP22 may promote gastric cancer progression and predict prognosis. But little is known about the interaction network of USP22 in gastric cancer. In this study, we applied bioinformatics methods and found that USP22 was correlated with the heat shock protein 90 (HSP90) which is now considered to be a biomarker to predict the prognosis of gastric cancer. Then the siRNA transfection and western blotting were used to testify the correlation of USP22 and HSP90 in gastric cancer cells. The immunohistochemistry staining of the microarrays was applied to confirm the correlation of USP22 and HSP90 expression in gastric cancer tissue and further analysis showed that co-expression of USP22 and HSP90 was related to lymph node metastasis and more effective in predicting the prognosis of gastric cancer. In summary, our data demonstrate that correlation exists between USP22 and HSP90 expressions in gastric cancer and co-expression of USP22 and HSP90 may be more effective in predicting prognosis of gastric cancer.

1. Introduction

Gastric cancer is responsible for over 1,000,000 new cancer cases and 783,000 cancer deaths worldwide annually, which imposes a considerable global health burden. Although substantial progress has been made in the diagnosis and treatment of gastric cancer, the prognosis is still unsatisfactory [1,2]. Also, it is necessary to explore effective methods to improve the prognostic prediction. Many proteins have been found to be capable of predicting prognosis of gastric cancer and may be used as putative biomarkers, but there are still no current validated prognostic protein biomarkers in routine use in the clinical setting [3,4]. Some may have the synergetic effect, but whether the combined application of the synergetic proteins can better predict the prognosis of gastric cancer is still unknown.

The ubiquitin-specific peptidase 22 (USP22) belongs to the largest subfamily of deubiquitylases, which remove ubiquitin moieties from different substrates to regulate protein activity and cell homeostasis. Aberrant expression of the USP22 has been proved to be associated with poor cancer prognosis [5]. Moreover, the results of recent researches indicate that overexpression of USP22 may promote gastric cancer progression and predict prognosis [6,7]. The interaction network of USP22 in cancers is still unclear. The expression of USP22 is positively correlated with the phosphorylation of AKT in cancer cells, and the knockdown of USP22 can cause significant G1 arrest through the down-regulation of p-AKT, p-GSK-3β, and cyclinD1 [8,9]. USP22 can also negatively regulates p53 activation by deubiquitinating Sirt1 [10]. As we know, Akt and p53 are two important oncogenic client proteins of the heat shock protein 90 (HSP90) in a variety of cancer cells [11]. Thus, there may exist some correlation between USP22 and HSP90 in cancers.

HSP90 is a key regulator of proteostasis under stress conditions in eukaryotic cells. HSP90 has hundreds of client proteins that include steroid hormone receptors, kinases, E3 ubiquitin ligases, transcription factors and others. Some of them are substantially involved in cancer

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https://doi.org/10.1016/j.prp.2018.12.020
Received 1 October 2018; Received in revised form 1 December 2018; Accepted 24 December 2018

cell growth, such as p53, SRC, hypoxia-inducible factor 1α, etc. HSP90 acts as a central modulator and plays an important part in the survival of cancer cells. HSP90 has been proved to be associated with gastric cancer growth, angiogeneisis, invasion and metastasis. Overexpression of HSP90 is now considered to be correlated with poor prognosis of gastric cancer [12–14]. The expression of HSP90 has the influence on the transforming activity of c-Myc in cancer cells [15], while USP22 is shown to be necessary for the transcriptional activity of c-Myc [5]. Recent research has reported that the inhibition of HSP90 can significantly reduce the expression of BMI1 [16], while our data and other reported results have confirmed that activation of USP22 and BMI1 may be associated with gastric cancer progression and poor prognosis [7,17].

On the basis of these informations, there is some possibility that USP22 and HSP90 may have synergistic effect during gastric cancer progression. Here, we intend to verify the relationship between USP22 and HSP90 expression in gastric cancer as well as the validity of these two proteins co-expression in gastric cancer progression and prognosis prediction.

2. Materials and methods

2.1. Protein–protein interaction network and module construction

Interaction networks between HSP90 and USP22 were identified by applying BioGRID 3.4 (thebiogrid.org/) database and were visualized by Cytoscape v3.5.1.

2.2. Cell culture

The poorly differentiated human gastric cancer cell line BGC-823 was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were grown in RPMI1640 (Sigma,St. Louis, MO, USA) supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO_2.

2.3. siRNA transfection and western blotting

Human HSP90 and USP22 siRNA and siRNA nonspecific control were synthesized by GenePharma Co.,Ltd (Shanghai,China). X-tremeGENE siRNA transfection reagent (Roche) was used to transiently transfect siRNAs into the cells according to the manufacturer's instruction. Briefly described as following: 5×10^5 cells were added to each well of a six-well culture plate and cultured overnight for the cell attachment, then were washed with PBS. The mixture of transfection reagent (10 μl) and siRNA (2 μg) was added to each well and the cells were harvested during 24–48 h time intervals for the subsequent analysis. The designed human HSP90 and USP22 siRNA as well as the control-siRNA sequences were listed below:

HSP90: sense, 5′-CCCAGUUGAUGUCAUUGAUTT-3′
antisense,5′-AUCAAUGACAUCAACUGGGTT-3′
Sense, 5′-GCUUGACAGAUCCCAGUAATT-3′
antisense, 5′-UUACUGGGAUCUGUCAAGCTT-3′
sense, 5′-GCUGGUGCAGAUAUCUCUATT-3′
antisense, 5′-UAGAGAUAUCUGCACCAGCTT-3′
USP22: sense, 5′-GCAUCAUAGACCAGAUCUUTT-3′
antisense, 5′-AAGAUCUGGUCUAUGAUGCTT-3′
sense, 5′-GGAGAAAGAUCACCUCGAATT-3′
antisense, 5′-UUCGAGGUGAUCUUUCUCCTT-3′
sense, 5′-GCAGCUUCAAGGUGGACAATT-3′
antisense, 5′-UUGUCCACCUUGAAGCUGCTT-3′
control: sense, 5′-UUCUCCGAACGUGUCACGUTT-3′
antisense, 5′-ACGUGACACGUUCGGAGAATT-3′

The western blotting analysis was performed as previously described [18]. Transfected BGC-823 gastric cancer cells were harvested. Then proteins were extracted and quantified. Equal amounts of proteins were separated on 12% SDSPAGE gels and transferred to nitrocellulose membranes. The membranes were blocked overnight in 1% fat-free powdered milk in TBST buffer at 4 °C, followed by being incubated with a dilution of primary antibody (anti- HSP90, Abcam, ab13495, 1:500; anti-USP22, Abcam, ab4812, 1:200) at 4 °C overnight. After washing in TBST for three times, the membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody solution for 1 h at room temperature. The protein complex was revealed with enhanced chemiluminescence method. GAPDH was used as a normalization.

2.4. Tissue microarrays

The microarrays (HStm-Ade180Sur-06) containing gastric cancer and paired adjacent tissues were obtained from Shanghai Outdo Biotech Co.,Ltd. The patients' clinicopathological characteristics and survival information including operation time, survival time, age, sex, tumor grade (1.well

differentiated, 2. Moderately differentiated, 3. Poorly differentiated), pT stage, pN stage, M stage, pTNM stage etc were all available.

2.5. Immunohistochemistry staining

Two-step method was used to perform the immunohistochemitry staining procedure. The microarray slides were heated at 60 °C for 1 h, deparaffinized in xylene and rehydrated by graded alcohol. Antigen retrieval was achieved by incubating the slides in EDTA buffer, continued with a blocking treatment by endogenous peroxidase blocking solution for 15 min. The primary antibody of HSP90 (Abcam, ab13495) or USP22(Abcam, ab217968) was added to each slide and incubated overnight at 4 °C. The negative control was incubated without the primary antibody. Then the second antibody (DAKO,EnVision™ + /HRP) was added and incubated for 30 min. Antibody staining was visualized with DAB (DAKO) and hematoxylin counterstain. The staining intensity was scored from 0 to 3 as follows: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The percentage of positively stained cells was scored on a scale of 0 to 4 as follows: 0 (negative), 1 (< 70%), 2 (70%–79%), 3 (80–89%) and 4 (90%–100%). The scores for percentages of positive cells and staining intensities were then multiplied. High expression was defined as the product ≥4 and low expression was defined as the product < 4.

2.6. Statistical analysis

Statistical analyses were conducted using SPSS v21.0 software. The chi-square test was used for HSP90 and USP22 expression. The survival curves were calculated using the Kaplan-Meier method and analysis was performed using the log-rank test. Correlation between HSP90 and/or USP22 expression and clinicopathological characteristics were statistically analyzed using Spearman's rank correlation coefficient. The univariate analysis was performed by using the Cox regression model. $p < 0.05$ was considered statistically significant.

3. Results

3.1. HSP90 and USP22 interaction network

According to BioGRID database, nine co-interactors between HSP90 and USP22 were selected as follows, RP11-57G10.3, ADMIO, c-Myc, TADA2A, MRP1, DITHP, NXF1, P34CDC2, RCAN1. Then the HSP90 and USP22 interaction network was established based on the above co-interactors by Cytoscape (Fig. 1).

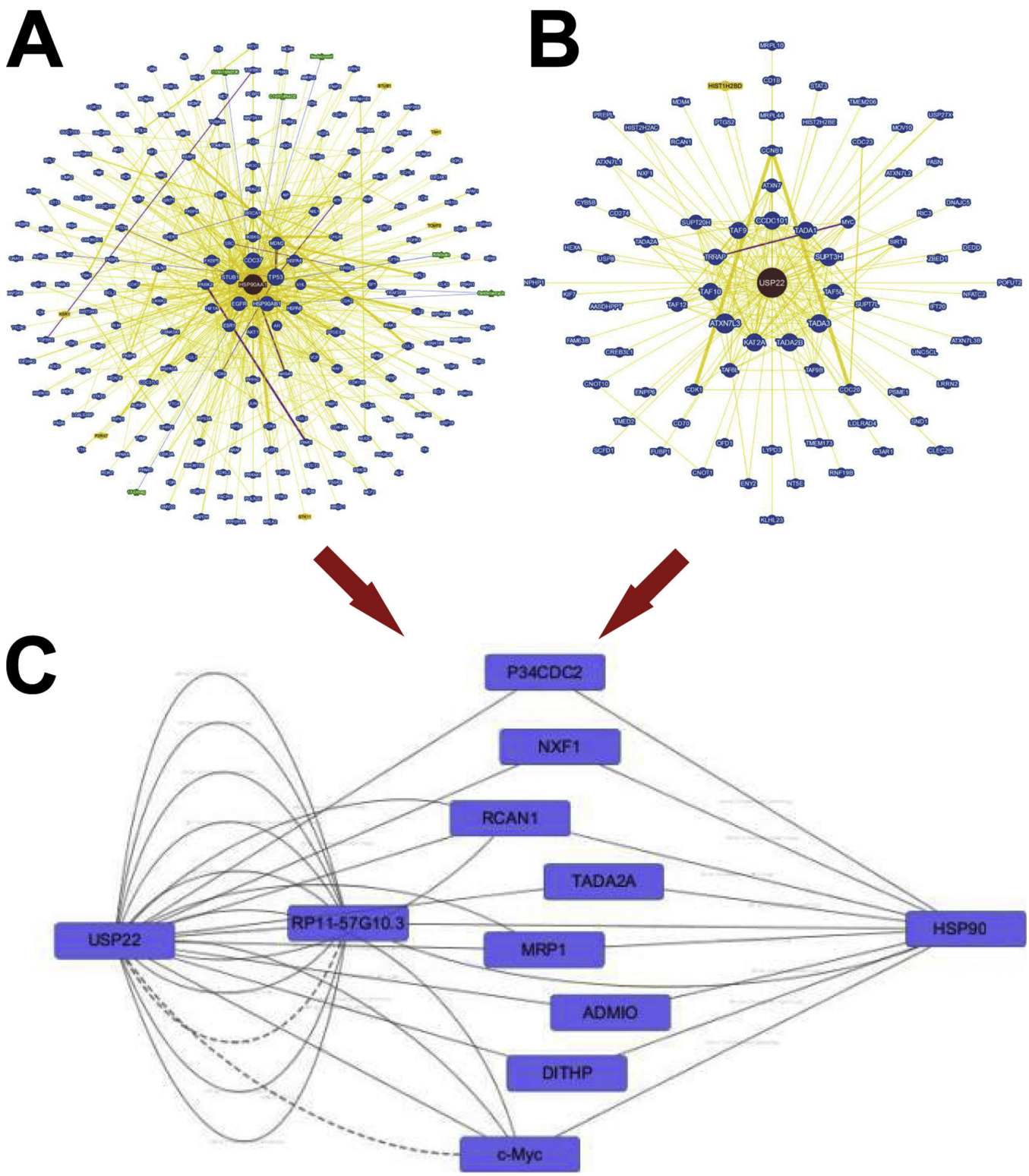

Fig. 1. Interaction networks between HSP90 and USP22. A: Interaction network of HSP90. B: Interaction network of USP22. C: Interaction networks between HSP90 and USP22, nine co-interactors were selected as follows, RP11-57G10.3, ADMIO, c-Myc, TADA2A, MRP1, DITHP, NXF1, P34CDC2, RCAN1.

3.2. siRNA transfection and western blotting

The transfected siRNA could significantly inhibit the protein expression of HSP90 and USP22. There was no significant difference in the expression of HSP90 after downregulating USP22 expression. However, downregulated expression of HSP90 could significantly reduce USP22 expression (Fig. 2). This may, to some degree, prove the existence of the relation between these two putative biomarkers in gastric cancer.

3.3. Correlation of HSP90 and USP22 expression in gastric cancer

According to the immunohistochemistry staining results, HSP90 staining was mainly localized in cytoplasm. USP22 staining was

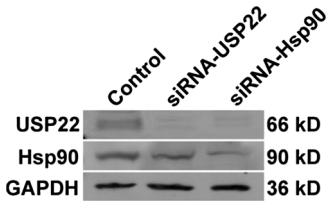

Fig. 2. Results of siRNA transfection and western blotting. The transfected siRNA could significantly inhibit the protein expression of HSP90 and USP22. Downregulated expression of HSP90 could significantly reduce USP22 expression.

observed in both cytoplasm and nuclear, but mainly localized in cytoplasm. Low expressions of HSP90 and USP22 were observed in paracancerous tissue. Both HSP90 and USP22 expressions were significantly increased in gastric cancer than in paracancerous tissue (Fig. 3). There was a statistical correlation between HSP90 and USP22 expressions in gastric cancer ($p < 0.001$, Table 1).

3.4. Relationships between the clinicopathologic features and the expression of HSP90 and USP22

Both the expressions of HSP90 and USP22 showed significant correlation with pN stage and pTNM stage ($p < 0.05$). Neither the expression of HSP90 nor the expression of USP22 was significantly associated with the following features: Age, Sex, Tumor grade, pT stage, M stage (Tables 2 and 3). We also investigated the relationship between the clinicopathologic features and the co-expression of HSP90 and USP22. The results showed that co-expression of HSP90 and USP22 was also significantly associated with pN stage and pTNM stage ($p < 0.05$). And there was no significant difference between the co-expression and the features as Age, Sex, Tumor grade, pT stage, M stage (Table 4).

3.5. Survival analysis

Since the results indicated that the expressions of HSP90 and USP22 as well as the co-expression showed the significant correlation with pN stage and pTNM stage, we further investigated the influence of HSP90 and USP22 on the survival. The survival analysis demonstrated that the patients with high expression of HSP90 or USP22 indicated worse overall survival rate than those with low expression (Fig. 4). Moreover, the patients with co-expression of HSP90 and USP22 had the worst overall survival rate. The univariate analysis by the Cox regression model revealed that co-expression of HSP90 and USP22 was one of the independent prognosis factors in gastric cancer patients (Table 5).

Table 1
Association between the expression of HSP90 and USP22.

variable	n	HSP90 expression		r_s	p value
		High	Low		
USP22					
High	58	44	14	0.395	< 0.001*
low	26	9	17		

* Statistically significant ($p < 0.05$).

Table 2
Correlation between HSP90 expression and clinicopathological characteristics.

	variables	HSP90 expression		total	r_s	p value
		high	low			
Age (year)					−0.016	0.877
	≤60	24	14	38		
	>60	32	20	52		
Sex					0.001	0.992
	female	23	14	37		
	male	33	20	53		
Grade					0.014	0.900
	1	1	0	1		
	2	27	16	43		
	3	27	14	41		
	dull			5		
T stage					0.054	0.613
	T1	2	1	3		
	T2	7	4	11		
	T3	31	22	53		
	T4	16	7	23		
N stage					0.303	0.004*
	N0	10	13	23		
	N1	6	6	12		
	N2	11	7	18		
	N3	29	8	37		
M stage					0.057	0.595
	M0	53	33	86		
	M1	3	1	4		
TNM stage					0.211	0.045*
	I	6	3	9		
	II	11	16	37		
	III	36	14	50		
	IV	3	1	4		

* Statistically significant ($p < 0.05$).

4. Discussion

USP22 and HSP90 are now considered as putative biomarkers

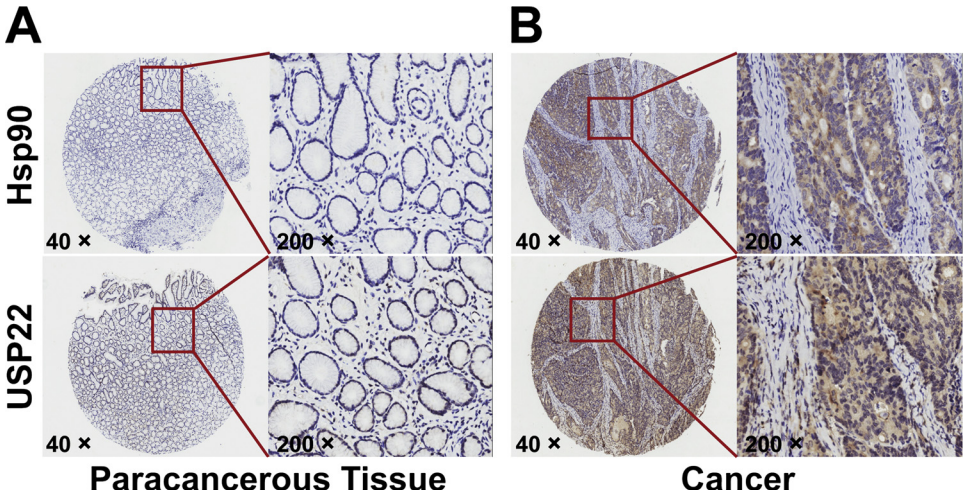

Fig. 3. The microarrays immunohistochemistry staining results. A: Low expressions of HSP90 and USP22 in paracancerous tissue. B: HSP90 and USP22 expressions were significantly increased in gastric cancer tissue. HSP90 staining was mainly localized in cytoplasm. USP22 staining was observed in both cytoplasm and nuclear, but mainly localized in cytoplasm.

Table 3
Correlation between USP22 expression and clinicopathological characteristics.

	variables	USP22 expression		total	r_s	p value
		high	low			
Age(year)					−0.044	0.694
	≤60	25	10	35		
	>60	33	16	49		
	dull			6		
Sex					−0.185	0.092
	female	27	7	34		
	male	31	19	50		
	dull			6		
Grade					0.096	0.396
	1	1	0	1		
	2	27	14	41		
	3	29	9	38		
	dull			10		
T stage					0.040	0.721
	T1	2	1	3		
	T2	6	5	11		
	T3	36	13	49		
	T4	14	7	21		
	dull			6		
N stage					0.454	< 0.001*
	N0	9	13	22		
	N1	8	3	11		
	N2	8	8	16		
	N3	33	2	35		
	dull			6		
M stage					0.150	0.174
	M0	54	26	80		
	M1	4	0	4		
	dull			6		
TNM stage					0.333	0.002*
	I	5	4	9		
	II	12	13	25		
	III	37	9	46		
	IV	4	0	4		
	dull			6		

* Statistically significant ($p < 0.05$).

because their aberrant expressions are usually correlated with the tumorigenesis, progression and metastasis of cancer [5,19]. Here, our results indicate that aberrant expression of USP22 or HSP90 is related to pN stage and pTNM stage as well as the prognosis of gastric cancer patients, which is consistent with the previous reports [6,7,14]. However, our results further demonstrate that there is some correlation between the expression of USP22 and HSP90, and the co-expression of these two biomarkers may be more effective in the prognosis prediction of gastric cancer patients.

According to the bioinformatic analysis, we identified nine co-interactors between HSP90 and USP22. And some of them have been proved to be associated with gastric cancer. RP11-57G10.3 mediates autophagy and has a dual role in gastric cancer. Some researches indicate that RP11-57G10.3 is highly expressed in gastric cancer and significantly correlated with tumor stage, lymph node metastasis, tumor invasion, histologic types and poor prognosis. But the opposite results can be observed in some other researches. The contradictory results may be caused by the tumor stage, cellular context, the presence of other factors or its subcellular localization. And further studies are needed to identify the exact role of RP11-57G10.3 in gastric cancer [20–23]. Overexpression of c-Myc can be observed in nearly 40% of gastric cancer and is the predictor of poor prognosis. It is the regulator of gastric cancer cell proliferation and can help maintain the malignant phenotype [24–26]. MRP1 plays a role in multidrug resistance and high expression of MRP1 is inversely correlated with the chemosensitivity of gastric cancer cells against some anticancer drugs [27]. Downregulation of P34CDC2 can cause gastric cancer cell growth inhibition and P34CDC2 can also be involved in the genesis or progression of gastric cancer [28,29]. Some of the other co-interactors, such as TADA2A, NXF1 and RCAN1, are now believed to be associated with cancer. And future researches may prove the exact roles of these co-interactors in gastric cancer [30–32].

Our results proved the existence of the correlation between HSP90 and USP22 in gastric cancer. Since the abnormal expression of HSP90 and USP22 can be observed in various types of cancer, we consider that the correlation may also exist in other cancers. The co-interactor RP11-57G10.3, c-Myc and MRP1 have been proved to be downregulated by USP22 silencing in some cancer cell lines [33–35]. And the inhibition of HSP90 can influence the expression or function of some co-interactors, such as ADMIO and P34CDC2 [36,37]. These regulatory relationships may be very important to cancer prognosis. c-Myc is found to be overexpressed and it's oncogenic role has been proved in a variety kinds of human cancers [38]. Moreover, recent report demonstrated that MYC expression was significantly elevated in high-burden metastases and metastatic progression was also associated with increased MYC expression [39]. Previous studies have proved the c-Myc and HSP90 interaction, for example, HSP90 may be important in the subcellular trafficking of c-Myc and overexpression of HSP90 increased the transforming activity of c-Myc [15,40]. USP22 is necessary for the transcriptional activity of c-Myc [5]. Kim et al. [34] recently reported that c-Myc physically interacts with USP22 in cancer cells and the c-Myc protein levels in cancer cells can be reduced by the depletion of USP22, and c-Myc levels can also be increased due to the overexpression of USP22. Moreover, USP22 mediates deubiquitination of poly-ubiquitinated c-Myc, promoting c-Myc stability. Deubiquitination of c-Myc via USP22 is positively associated with cancer cell growth, progression, and angiogenesis. And we also find some other clues concerning the prognosis of cancer and the co-interactors,such as RP11-57G10.3 and RCAN1 [41,42]. Co-expression of USP22 and HSP90 may further enhance the stability and function of these cancer-related co-interactors, which may explain our result that the patients with co-expression of these two biomarkers had the worst overall survival rate.

Until now, many proteins have been found to be related to the prognosis prediction of gastric cancer, but there is still no widely recognized one to be used in the clinical setting [3,4]. Since the proteins regulatory networks in cancer are so complicated that it is hard to find the key protein as the predictive biomarker. Here we used bioinformatics methods to predict the possible relationship between two existing putative biomarkers of gastric cancer, then we found the combined application of these two biomarkers might be related to pN stage and more effective in predicting the prognosis. Thus, we consider that the combined application of the existing putative biomarkers may be more effective and economical. There may be a significant amount of effort to confirm how to use two or more biomarkers according to the existing data, however, the bioinformatics database as well as the analysis tools may be very helpful.

Interestingly, the results of siRNA transfection and western blotting showed that downregulated expression of HSP90 could significantly reduce USP22 expression in gastric cancer cells, which indicates that HSP90 might be the upstream regulatory protein of USP22. Some reports have confirmed that the inhibiton of either USP22 or HSP90 has the anticancer effects against gastric cancer cells [7,43]. Though HSP90 has several hundred protein clients [12], to date there is no evidence to support the direct regulation of USP22 by HSP90. And these two proteins could not be proved to bind directly. We consider that the correlation is indirectly through the co-interactors. Whether this regulation depends on some of the existing co-interactors or there are other regulatory pathways needs further investigation to figure out.

In summary, in this study, we verify the possible relationship between USP22 and HSP90 expression in gastric cancer and co-expression of these two proteins is related to lymph node metastasis and may be more effective in predicting prognosis of gastric cancer patients. All these may indicate that the combined application of the existing putative biomarkers is feasible and bioinformatics methods can help find

Table 4
HSP90 and USP22 co-expression and clinicopathological characteristics.

Variables		HSP90 + /USP22 + (n = 44)	HSP90 + /USP22 − (n = 9)	HSP90-/USP22 + (n = 14)	HSP90 − /USP22 − (n = 17)	rs	p
Age(year)						−0.033	0.769
	≤60	19	3	6	7		
	>60	25	6	8	10		
Sex						−0.126	0.254
	female	20	1	7	6		
	male	24	8	7	11		
Grade						0.075	0.507
	1	1	0	0	0		
	2	20	6	7	8		
	3	22	3	7	6		
	dull	1			3		
T stage						0.084	0.450
	T1	1	1	1	0		
	T2	5	2	1	3		
	T3	25	4	11	9		
	T4	13	2	1	5		
N stage						0.457	< 0.001*
	N0	5	5	4	8		
	N1	5	1	3	2		
	N2	8	2	0	6		
	N3	26	1	7	1		
M stage						0.128	0.245
	M0	41	9	13	17		
	M1	3	0	1	0		
TNM stage						0.344	< 0.001*
	I	4	2	1	2		
	II	6	5	6	8		
	III	31	2	6	7		
	IV	3	0	1	0		

* Statistically significant ($p < 0.05$).

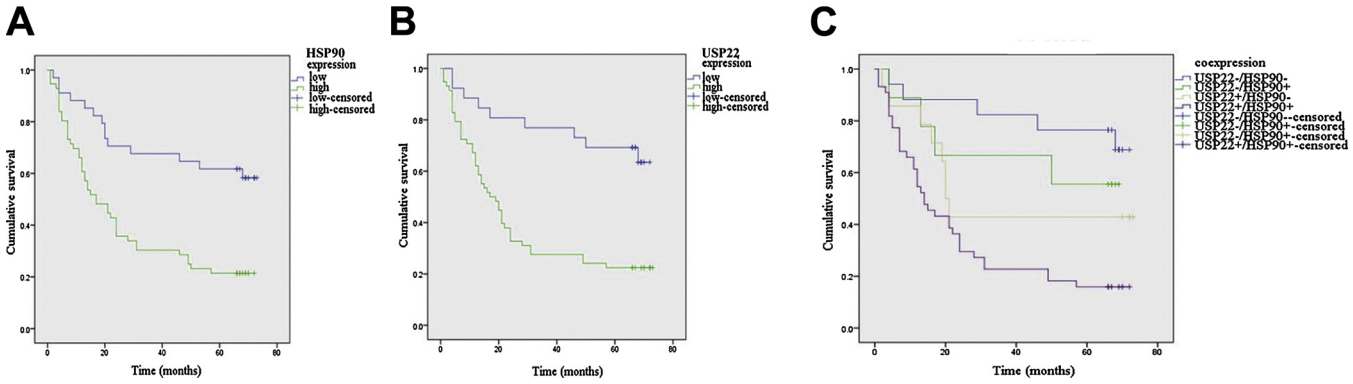

Fig. 4. The survival analysis related to the expressions of HSP90 and USP22. A: The patients with high expression of HSP90 indicated worse overall survival rate than those with low expression. B: The patients with high expression of USP22 indicated worse overall survival rate than those with low expression. C: The patients with co-expression of HSP90 and USP22 had the worst overall survival rate.

Table 5
Univariate analysis of the factors correlated with overall survival.

Variables	Univariate analysis		
	HR	95% CI	p Value
Co-expression	1.751	1.321–2.322	0.000*
Sex	0.689	0.411–1.154	0.157
Grade	1.618	0.964–2.714	0.069
Age	0.622	0.371–1.045	0.073
T stage	1.943	1.310–2.881	0.001*
N stage	2.772	2.038–3.770	0.000*
M stage	3.966	1.385–11.356	0.010*
TNM stage	4.533	2.848–7.216	0.000*

* Statistically significant ($p < 0.05$).

the clues.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

For this type of study formal consent is not required. This article does not contain any studies with human participants or animals performed by any of the authors.

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