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RESEARCH

Stem Cell Associated Genes Working with One MiRNA Cluster Have Different Clinic Pathologic Values in Gastric Cancer

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Abstract Cancer stem cells are nowadays considered to be the origin of cancer. Also, stem cell associated genes are emerging as predictors of cancer malignancy. We investigated the association of several stemness genes (c-Myc, PTEN, p57 and p21) with clinic pathological parameters and survival in stomach cancer by performing immunohistochemistry on paraffin sections of gastric cancer patients who underwent surgical staging with following-up statistics. We discovered that expression of c-Myc was significantly related to distant metastasis, the combined expression of PTEN and p21 correlated positively to overall survival, while p57 was less useful in overall survival prediction in gastric cancer. Additionally, there is a positive correlation between expressions of p57 and p21. In conclusion, our present study indicated that expression of stemness genes (c-Myc, PTEN, p57 and p21) performed different predictive potential in the evaluation of clinical malignancy levels in gastric cancer.

Keywords Clinic pathologic · PTEN · P21 · P57 · C-Myc · Stemness · Prognosis

Introduction

Nowadays, the most popular theory of cancer associated origin is cancer stem cells (CSCs) or cancer initiating cells (C-IT). CSCs are defined as a small population of cells with stem cell-like features which can escape from the control of their environment and give rise to cancerous growth [1]. These cells are long-lived, capable of self-renewal and giving rise to more differentiated cancer cells. The most well-known property of CSC populations is their ability to self-renew, which is also the hallmark of CSCs [2]. Recently, stem cell self-renewal associated genes (also known as "stemness genes") have emerged as newly diagnostic and prognostic markers in many types of cancers [3], some of which are also prevalently used in the evaluation of clinical statistics [4].

Gastric cancer is one of the leading causes of cancer-related deaths in developing countries. Chemotherapy plays a significant and crucial role in clinical anti- gastric cancer treatment to achieve prolonged prognosis. However, a low median survival time is observed in patients who undergone chemotherapy with intractable gastric cancer. To evaluate the potential significance of stemness genes in predicting the malignancy of gastric cancer, we performed immunohistochemistry using four stemness genes including p21, PTEN, p57 and c-Myc in gastric cancer tissues. And a combined evaluation of survival prediction and a clinic pathological association were observed in the present study.

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Table 1 Results of univariate analysis between clinicaopathological factors and overall survival

Clinical feature	overall survival	
	P value	HR (95%CI)
Tumor stage	0.009	0.403(0.204–0.793)
Lymph node metastasis	0.004	0.397(0.212–0.742)
Distant metastasis	0.002	0.266(0.113–0.626)
Tumor infiltration	0.006	0.380(0.192–0.753)
Tumor differentiation	0.113	0.593(0.311–1.132)
Tumor size	0.050	0.531(0.282–1.000)
Gender	0.231	0.657(0.331–1.305)
Age	0.415	1.285(0.704–2.344)
P21 expression	0.029	2.006(1.075–3.743)
PTEN expression	0.045	1.883(1.014–3.499)
P57 expression	0.326	0.736(0.399–1.357)
C-Myc expression	0.891	0.954(0.487–1.868)

Materials and Methods

Patients

During the period November 2003 to December 2004, 44 patients who received primary surgical treatment for gastric cancer with tissues and following-up data available for review were included in this study. Patients with recurrent tumors or neuroendocrine tumors were excluded. Age, gender, tumor size, tumor infiltration, tumor stage, lymph node metastasis and distant metastasis were evaluated by reviewing medical charts and pathological records. Following-up information were obtained from the patients' medical records. The following-up time were measured from the date of surgery until disease-caused death. All patients were informed consent to use excess pathological specimens for research purposes. The protocols used in this study were approved by the hospital's Protection of Human Subjects Committee. The use of human tissues was approved by the institutional review board of the Fourth Military Medical University and was conformed to the Helsinki Declaration, and to local legislation.

Histological Examination and Clinic Pathological Parameters

Surgical specimens were fixed in 10% formalin and embedded in paraffin. The haematoxylin and eosin (HE) stained sections were reviewed classified by Pathologists who were unaware of the clinical statistics according to the World Health Organization (WHO) guidelines. The prognostic effects of the following clinic pathological parameters were examined: age (<60 years versus >=60 years); gender; tumor size (<5 cm versus>=5 cm); tumor differentiation (well, moderate, moderate poor versus poor); tumor infiltration to placenta (positive versus negative); lymph node metastasis (positive versus negative); and distant metastasis (positive versus negative).

Immunohistochemistry

Gastric cancer tissues and the adjacent normal tissues taken from 44 patients were used for paraffin embedding, and serial 4 μm sections were used for immunohistochemistry staining. Deparaffinized and rehydrated sections were washed in fresh water for 10 min. Heat-induced antigen retrieval was performed for 20 min at 95°C with 10 mM citrate sodium buffer (PH 6.0). After the sections were cooled at room temperature for 40 min, they were blocked in 3% hydrogen peroxide for 20 min and then in normal goat serum confining liquid for 40 min. After this, the sections were allowed to react over night at 4°C with primary antibodies for p21, p57, PTEN and c-Myc (Santa Cruz). After rewarming for 40 min, the slides were reacted with second antibodies (Zhongshan Goldenbridge Biotechnology CO. LTD) for another 40 min at room temperature. Then the products were developed with 3,3'-diaminobenzidine and counterstained with haematoxylin.

Immunohistochemical Scoring

Scoring was completed by a specialist pathologist and a scientist who were blinded to the clinical and pathologic information. In case of discrepancy, a consensus was reached by conferencing. The proportions of positive cells (0 to +3) and the staining intensities (0 to +3) were evaluated separately at a magnification of × 200. The former was calculated by counting the number of stained tumor cells among the total number of tumor cells, for example, when 25% of total cells stained, the proportion score was +1, and 50% stained equals to score +2, 75%

Table 2 Results of multivariate analysis for overall survival

Clinical feature	overall survival	
	P value	HR (95%CI)
Tumor size	0.054	2.079 (0.987–4.377)
Tumor stage	0.654	0.771 (0.248–2.400)
Lymph node metastasis	0.335	1.484 (0.666–3.307)
Distant metastasis	0.212	2.018 (0.670–6.075)
P21 expression	0.283	0.670 (0.323–1.391)
PTEN expression	0.181	0.601(0.285–1.266)
Tumor differentiation	0.508	1.259(0.636–2.494)
Tumor infiltration	0.161	2.336 (0.714–7.647)

Table 3 Association between stem cell associated genes and clinicopathologic parameters

Parameters	patient number	C-Myc positivity			Pten positivity			P57 positivity			P21 positivity		
		+	−	P value	+	−	P value	+	−	P value	+	−	P value
Tumor stage													
I+II	13(29.55%)	2(4.55%)	11(25%)		9(20.45%)	4(9.09%)		7(15.91%)	6(13.64%)		10(22.73%)	3(6.82%)	
III+IV	31(70.45%)	10(22.73%)	21(47.73%)	0.438	11(25%)	20(45.45%)	0.040	14(31.82%)	17(38.64%)	0.599	9(20.45%)	22(50%)	0.003
Lymph node metastasis													
Negative	17(38.64%)	4(9.09%)	13(29.55%)		9(20.45%)	8(18.18%)		11(25%)	6(13.64%)		11(25%)	6(13.64%)	
Positive	27(61.36%)	8(18.18%)	19(43.18%)	0.924	11(25%)	16(36.36%)	0.429	10(22.73%)	17(38.64%)	0.074	8(18.18%)	19(43.18%)	0.022
Distant metastasis													
Negative	37(84.09)	7(15.91%)	30(68.18%)		18(40.91%)	19(43.18%)		18(40.91%)	19(43.18%)		18(40.91%)	19(43.18%)	
Positive	7(15.91%)	5(11.36%)	2(4.55)	0.016	2(4.55%)	5(11.36%)	0.572	3(6.82%)	4(9.09%)	1.000	1(2.27%)	6(13.64%)	0.205
Tumor differentiation													
Well	13(29.55%)	2(4.55%)	11(25%)		7(15.91%)	6(13.64%)		6(13.64%)	7(15.91%)		5(11.36%)	8(18.18%)	
Moderate	10(22.73%)	4(9.09%)	6(13.64%)		5(11.36%)	5(11.36%)		6(13.64%)	4(9.09%)		5(11.36%)	5(11.36%)	
Moderate-poor	7(15.91%)	2(4.55%)	5(11.36%)		2(4.55%)	5(11.36%)		4(9.09%)	3(6.82%)		3(6.82%)	4(9.09%)	
Poor	14(31.82%)	4(9.09%)	10(22.73%)	0.853	6(13.64%)	8(18.18%)	0.349	5(11.36%)	9(20.45%)	0.537	6(13.64%)	8(18.18%)	0.967
Tumor infiltration													
Negative	13(29.55%)	2(4.55%)	11(25%)		7(15.91%)	6(13.64%)		7(15.91%)	6(13.64%)		10(22.73%)	3(6.82%)	
Positive	31(70.45%)	10(22.73%)	21(47.73%)	0.438	13(29.55%)	18(40.91%)	0.469	14(31.82%)	17(38.64%)	0.599	9(20.45%)	22(50%)	0.003
Tumor size													
<5 cm	24(54.55%)	7(15.91%)	17(38.64%)		15(34.09%)	9(20.45%)		8(18.18%)	16(36.36%)		12(27.27%)	12(27.27%)	
>=5 cm	20(45.45%)	5(11.36%)	15(34.09%)	0.757	5(11.36%)	15(34.09%)	0.013	13(29.55%)	7(15.91%)	0.036	7(15.91%)	13(29.55%)	0.317
Gender													
Male	32(72.73%)	8(18.18%)	24(54.55%)		14(31.82%)	18(40.91%)		16(36.36%)	16(36.36%)		12(27.27%)	20(45.45%)	
Female	12(27.27%)	4(9.09%)	8(18.18%)	0.863	6(13.64%)	6(13.64%)	0.711	5(11.36%)	7(15.91%)	0.622	7(15.91%)	5(11.36%)	0.214
Age													
<60	20(45.45%)	7(15.91%)	13(29.55%)		11(25%)	9(20.45%)		8(18.18%)	12(27.27%)		6(13.64%)	14(31.82%)	
>60	24(54.55)	5(11.36%)	19(43.18%)	0.293	9(20.45%)	15(34.09%)	0.246	13(29.55%)	11(25%)	0.349	13(29.55%)	11(25%)	0.107

equals to score +3, 0% equals to score 0. The intensity scoring was evaluated by the color of stained tumor cell nucleus, specifically, score 0 means achromatic, +1 means amber, +2 means yellow, and +3 means brown. Combined scoring +1~+2 were defined "negative", and+3~+6 were considered "positive".

Statistical Analysis

All statistical analysis was conducted using SPSS software (Chicago, Illinois, USA). Chi-square test was used to assess the associations of the expression with various clinical statistics. Cumulative survival were assessed with Kaplan–Meier curves and compared using the log-rank test. The independent predictors for survival were calculated using the Cox regression model. Covariates incorporated into the multivariate analysis were variables reaching $p<0.2$ in univariate analysis. And the correlations among various genes were analyzed with Spearman's correlation analysis. Two-tailed p-values <0.05 were considered statistically significant.

Results

Patient Characteristics and Clinic Pathological Prognostic Factors

The present study included 12 women and 32 men (ratio 1:2.7). Patients' ages ranged from 28 to 82 years (mean 60±14 years; median 61 years). At the time of surgery, 27 patients (61.36%) had lymph node metastasis and 7 (15.91%) had distant metastasis. According to the stage, stage III and IV tumors were the most predominant (31 cases 70.45%). The median survival time was 30 months (range 8 to 67 months). All of the patients died of their diseases during the follow up period. The parameters significantly associated with tumor-related death by univariate analysis were tumor stage (P=0.009), lymph node metastasis (P=0.004), distant metastasis (P=0.002), and tumor infiltration (P=0.006, Table 1). However, multivariate analysis did not support any relationships of these parameters above with overall survival except for tumor size (P=0.057) which had the tendency to be associated with prognosis (Table 2).

Expression of Stemness Genes in Gastric Cancer and Their Association with Clinic Pathological Parameters

Relationships between p21, PTEN, p57, c-Myc expression and clinic pathological factors were indicated in Table 3. Expressions of c-Myc in cancer tissues were significantly related to distant metastasis of gastric cancer (P=0.016), however, statistics do not support c-Myc's correlation with other clinical information such as overall survival, ages, gender, tumor size, tumor infiltration, tumor stage and lymph node metastasis (P>0.05). PTEN expression is significantly correlated to tumor size (P=0.013) and tumor stage (P=0.040), no relationships with other clinical data were examined (P>0.05). There is no statistical significance between p57 and clinical statistics (P>0.05) except for tumor size (P=0.036). And the p21 expression is significantly related to the tumor infiltration (P=0.003), tumor stage (P=0.003) and lymph node metastasis (P=0.022), no correlation with other clinical statistics was detected (P>0.05). In addition, there is a positive correlation between the genes p57 and p21 (P=0.014, Fig. 1).

Fig. 1 Representative photomicrograph of p21 and p57 immunostaining in gastric cancer, photographs showed a positive correlation of p21 and p57 in slides from the same patients, magnification×400. **a c e**, positive expression of p21. **b d f**, positive expression of p57 in gastric cancer. A and B slides are from the same patient. C and D slides are the serial section from one gastric cancer tissue, E and F are also from one patient. G,Scatter plot showing strong correlation between the expression of p57 and p21(P=0.014)

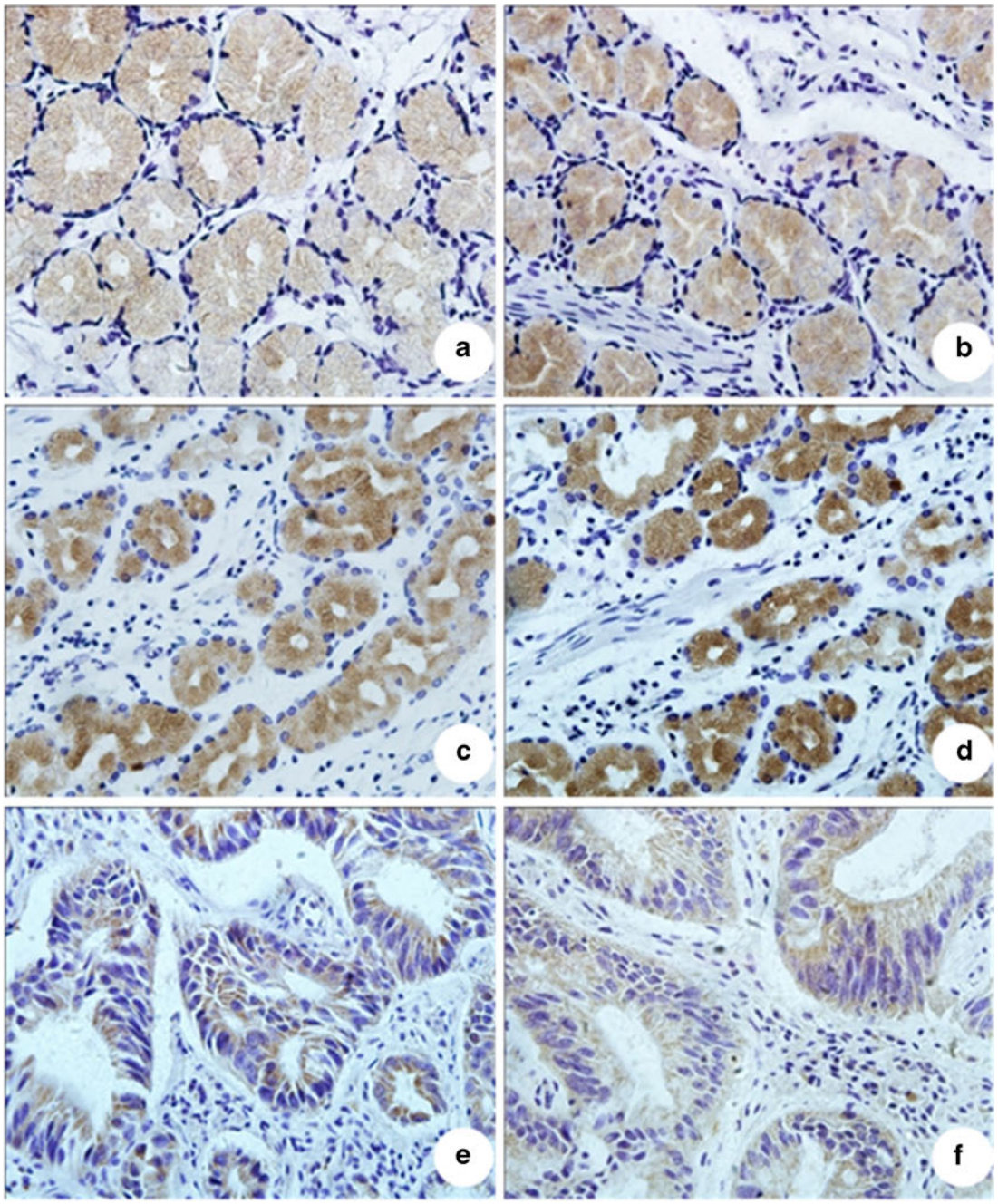
Relationships Between Expressions of Stemness Genes and Overall Survival

The Kaplan-Meier survival curves were illustrated in Fig. 2. The results of log rank tests indicated that both of PTEN and p21 overexpression were independently associated with an increase in overall survival (P=0.045, P=0.029 respectively), however, the COX regression has not identified a single independent predictor. While the positivity of PTEN high combined with p21 high expression was more significant in predicting prognosis in gastric cancer (P=0.002).

Discussion

The ability to predict tumor aggressiveness is essential for the treatment of gastric cancer. However, the biological behavior of gastric cancer has not been elucidated because of the indefinite origin of them. Recently, a generally acknowledged theory of cancer origin – cancer stem cells has emerged. CSCs are defined as a small sub-population of cancer cells with the capability of both self-renewal and pluripotency [5]. Therefore, reliable CSC associated markers (stemness genes) are needed to predict prognosis and optimize treatment plans. In the present study, we examined whether stemness genes would predict tumor aggressiveness and prognosis using a series of clinical histological specimens and statistics.

The stemness genes involve transcription factor c-Myc, cell cycle regulators p21 and p57, and tumor suppressor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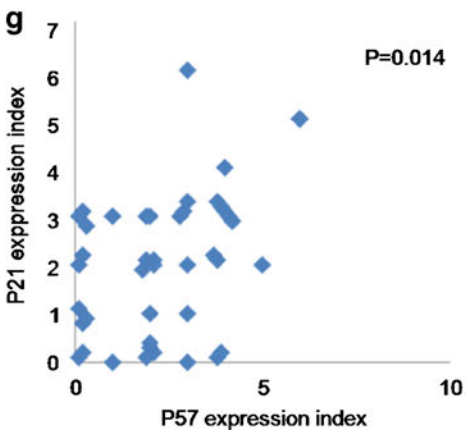
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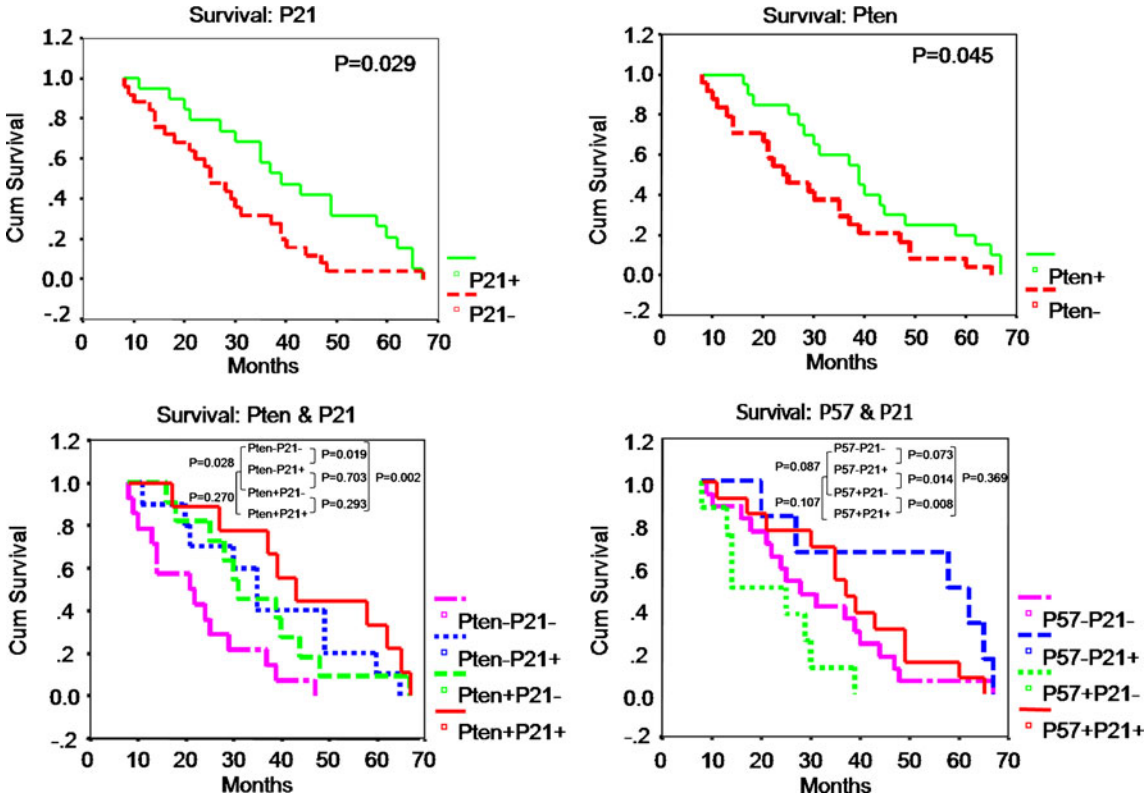

Fig. 2 Kaplan–Meier survival curves showing overall survival of p21 (**a**), PTEN (**b**), combination of PTEN and p21 (**c**), combination of p57 and p21 (**d**)

PTEN. Among them, p21 inhibitors were previously reported to play key roles in spermatogonial stem cells self-renewal and differentiation [6], and were also reported to be repressed by Bmi-1, which could enhance the self-renewal ability of forebrain neural stem cells [7]. Upregulation of p57, another member of the cyclin-dependent kinase inhibitors (CDKIs), leaded to cell cycle arrest, precocious differentiation and depletion of the progenitor pool in pancreatic progenitors [8]. PTEN deletion in adult neural stem cells leaded to enhanced neural stem cell self-renewal [9]. Also, PTEN could control stem/progenitor cell self-renewal and differentiation in glioma [10]. We chose these three genes because they were reported to be the definite targets of a cluster of microRNAs–miR-17-92 cluster [11, 12]. We also chose the up-stream molecule of miR-17-92 cluster [13], the known proto-oncogene c-Myc, which was previously reported to maintain the self-renewal and pluripotency of embryonic stem cells [14] and glioma cancer stem cells [15]. Accordingly, it can be concluded that these four stemness genes are probably involved in one molecular pathway which is connected by miR-17-92 cluster. In the present study, these stemness genes showed different potential in the prediction of aggressiveness in stomach cancer.

The present study indicates that p21 is a useful indicator in predicting the aggressiveness of gastric cancer. P21 is a known cell cycle regulator which plays a role in G1/S checkpoint [16] and is not expressed in the self-renewing embryonic stem cells [17]. Overexpression of p21 can abrogate proliferation of spermatogonial stem cells [6]. Also, p21 is reported to be a direct target of genes or microRNAs which were reported to maintain the self-renewal ability of stem cells [12]. In the present study, negative expression of p21 reflected malignancy in gastric cancer. With p21 overexpression, clinical parameters showed a lower depth of tumor infiltration, a lower tumor stage and less lymph node metastasis. Our results agreed with previously studies in which positive p21 immunostaining was associated with less depth of gastric wall infiltration and the absence of lymphatic [18]. Similar results also indicated in other reports that p21 expression positively correlated with early pTNM stages and good prognosis, whereas it inversely correlated with the lymph node metastasis in stomach cancer [19].

In the present study, our work indicated that although PTEN could not act as a single independent predictor by the results of COX regression, its expression correlated positively with the malignancy of gastric cancer. Similar results were previously reported in endometrial cancer [20], colorectal cancer [21], breast cancer [22], bronchioloalveolar carcinoma and lung adenocarcinoma [23]. Also, PTEN expression in stomach cancer in this study was inversely

correlated with tumor size and tumor stage which agreed with previous studies in colorectal carcinoma [21]. The present study suggested that the results of PTEN and p21 in the correlation of clinical statistics were similar especially in overall survival and tumor infiltration. The reason for this may be that PTEN and p21 share the same molecular pathway axis. However, the expression level of p21 (P= 0.029) predicted clinical outcome of stomach cancer more precisely than that of PTEN (P=0.045) which may be because of the down-stream character of p21 down-regulated by loss of PTEN in the axis [24]. Additionally, our study demonstrated that the combination of PTEN and p21 (P=0.002) predicted prognosis more definitely than any of them alone as indicated by the COX regression.

Although p21 and p57 are both involved in the family of cyclin-dependent kinase inhibitors (CDKI), in our study, loss of p57 did not show as predictive for malignancy as p21. Expression of p57 only showed correlation with the clinical parameters of tumor size. Previous studies showed results regarding p57 expression and clinical outcome differently in different cancers [25]. Our result indicated that no correlation with p57 and overall survival exists in gastric cancer. However, the combination of p21 high p57 low showed significant in prognositic prediction as a result of the positive correlation between p21 and p57 in the present study in stomach cancer.

As the potential up-stream molecule of other three ones, c-Myc showed little predictive value for overall survival. However, in our study, c-Myc overexpression was positively correlated with distant metastasis, which indicated a metastasis-promoting function of this oncogene in stomach cancer. C-Myc is a well known oncogenic molecule in many types of tumors. As the transcriptional suppressor of c-myc, MBP-1 was recently reported to be a suppressor of metastasis in stomach cancer [26], which indirectly demonstrated a predictive value of c-Myc in metastasis of gastric cancer.

In conclusion, the present study indicated that although potentially existing in the same molecular axis, expression of stemness genes including p21, PTEN, p57 and c-Myc have different potential in predicting cancer malignancy in stomach. Furthermore, as known stemness genes, p21 combined with PTEN showed significant to be prognostic factors, while p57 was less useful in overall survival prediction in gastric cancer. And c-Myc, a well-known oncogene, indicated a metastasis predictive potential in gastric cancer. Our work is limited by the small sample size and the retrospective nature of study design, in which the results should be interpreted with caution. However, this study indicated that it may be significant to research more on the stemness genes to further elucidate the definite function of them on cancer stem cells and the relationships with gastric cancer.

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Authors' Contributions Qiong Wu, Yongquan Shi, and Daiming Fan designed the study and carried out the immunohistochemistry studies. Zhiping Yang performed the statistical analysis and participated in the discussion. Sijun Hu, Tao Su and Yanxin An prepared and provided the tumor biological samples and participated in the immunohistochemistry studies. Yongzhan Nie and Xin Wang participated in the discussion. All authors read, discussed and approved the final manuscript.

Competing Interests There are no competing interests in this study.

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