

The loss of PTEN expression and microsatellite stability (MSS) were predictors of unfavorable prognosis in gastric cancer (GC)

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The predictors for the prognosis of gastric cancer (GC) are not clear. We intended to explore the role of PTEN and microsatellite (MS) in GC, showing their potential as prognostic markers. Primary gastric cancer tissues, adjacent normal tissues, and clinicopathological parameters of 187 patients were collected. The expression level of PTEN and MS status were classified by immunohistochemistry (IHC). The relationship among indicators was compared by the chi-square test. The survival curves were delineated by Kaplan-Meier and tested by Log-rank methods. The impact of multiple factors on prognosis was determined by COX proportional hazards model. Significantly, PTEN expression was associated with lymphatic invasion ($p=0.011$) and Lauren grade ($p=0.015$). MS status was associated with differentiation ($p=0.006$) and Lauren grades ($p=0.035$). TNM stage was associated with MS status under positive PTEN expression ($p=0.014$). Patients with microsatellite stability (MSS) had worse median overall survival (OS) than that with microsatellite instability (MSI) ($p=0.013$). Patients with negative PTEN expression had a worse median OS than that with positive PTEN expression ($p<0.001$). The tumor subtype of PTEN negative-MSS ($p<0.001$) and PTEN negative-MSI ($p=0.042$) were strong predictors of poor survival. Negative PTEN expression and MSS might lead to unfavorable prognosis due to their association with clinicopathological parameters of GC. PTEN expression and MS status could be predictors for the prognosis of GC.

Key words: PTEN, microsatellite, prognosis, predictor, clinicopathological parameters

Gastric cancer (GC) is one of the most common tumors in the world and the main cause of this disease is *Helicobacter pylori* infection [1]. At present, the treatment based on surgery is often used in clinical practice, however, the prognosis is still discouraging [1]. According to previous studies, the prognosis of gastric cancer is affected by many factors, among which clinicopathological factors are important indicators for predicting the prognosis of GC patients, including tissue subtypes, lymph node metastasis, TNM staging, and so forth [2]. The understanding of the predictors of prognosis for GC would greatly benefit the intervention of GC.

PTEN is a homolog of tyrosine phosphatase, located in the 10q23 region of human chromosomes and plays a role as a tumor suppressor in many kinds of tumors [3]. Previous researches have reported that PTEN can affect the apoptosis, proliferation, migration, and invasion of tumor cells, as well as inhibit tumor growth by affecting the activation of angiogenic signaling pathways [4]. Recently, Xu et al. revealed that PTEN lipid phosphatase inactivation could induce gastric tumorigenesis, which was linked to the Hippo and phosphatidylinositol 3 kinase/protein kinase B (AKT) PI3K/AKT

pathways [5]. Therefore, further studies on the role of PTEN in GC are still valuable.

Microsatellite (MS) is a simple tandem DNA sequence including 1 to 6 nucleotides with a high degree of polymorphism [6, 7]. MS is distributed more widely in the non-coding regions of the eukaryotic genome than in the coding regions [6, 7]. The functions of MSs are complex and various [6, 7]. It is accepted that it plays an important role in the formation and recombination of chromosome structure, which can affect the replication and expression of target genes [6, 7]. Microsatellite instability (MSI) is a phenomenon when the sequence of MS is inserted or lost bases due to DNA mismatch, which is related to the defects of the mismatch repair (MMR) system [8]. Currently, it is found that MMR-related proteins are composed of at least seven proteins (MLH1, MLH3, MSH2, MSH3, MSH6, PMS1, and PMS2), which specifically bind with each other to form functional heterodimers that can recognize the mismatched base pairs and small nucleotides (1 to 4 base pairs) during the DNA replication [8, 9]. Besides, MS without a mismatch is defined as microsatellite stability (MSS) [8]. Nowadays, the assessment of MSI is not

considered to be a marker in the routine evaluation of GC patients due to the existence of a controversial perspective about its prognostic role in GC [10]. Thus, the confirmation of the role of MSI in GC becomes an imperative requirement.

In view of the circumstance described above, we intended to explore the association of PTEN or MS with clinicopathological parameters or prognosis, aiming to exhibit the potential of PTEN and MS as prognostic markers of GC in the treatment of GC.

Patients and methods

The collection of tissue samples and clinicopathological parameters. The protocol of the present study was approved by the ethics board of the People's Hospital of Quzhou. Patients signed informed consent before the study. Primary gastric cancer tissues, adjacent normal tissues, and clinicopathological characteristics of 187 patients were collected from September 2nd, 2012 to September 2nd, 2013 in the People's Hospital of Quzhou. Patients ranged in age from 26 to 89 years, with an average age of 57.41 ± 14.38 . The clinicopathological characteristics of 187 patients are presented in Tables 1, 2, or 3. All patients were confirmed as gastric adenocarcinoma by postoperative pathology, had no family history of tumors and did not receive radiotherapy or chemotherapy before surgery. All patients underwent D2 radical gastrectomy and achieved R0 resection. The degree of TNM staging and tissue differentiation in gastric cancer was determined according to the criteria established by The Union for International Cancer Control (UICC). Primary gastric cancer tissues and adjacent normal tissues collected from the patients were embedded in optimal cutting temperature compound (OCT, Tissue-Tek O.C.T. Compound, #4583, SAKURA FINETEK, Torrance, USA) and stored in a -80°C liquid nitrogen immediately. The follow-up was ended at September 2nd, 2018, and there were no cases lost to follow-up. The patient was followed up once a week in the first month after discharge, once every half month in the second month, once every month after 3 months, every 3 months after half a year, and every 6 months after 2 year later. A total of follow-up of 3 to 60 months was made.

Immunohistochemistry (IHC). The tissue was sectioned on a cryostat machine and soaked in distilled water for 5 min. To each section, 50 μl of 3% hydrogen peroxide solution was added and incubated for 10 min at room temperature (RT) to block the endogenous peroxidase activity. Sections were washed with PBS (P5368-10PAK, Sigma, Burlington, USA) 3 times for 5 min. Sections were incubated in 50 μl of Anti-PTEN antibody (ab32199, Abcam, Cambridge, USA, 1:1000), Anti-MLH1 antibody (ab92312, Abcam, Cambridge, USA, 1:1000), Anti-MSH2 antibody (ab227941, Abcam, Cambridge, USA, 1:1000), Anti-MSH6 antibody (ab92471, Abcam, Cambridge, USA, 1:1000), or Anti-PMS2 antibody (ab110638, Abcam, Cambridge, USA, 1:1000) in a humidified box overnight at 4°C in a refrigerator. Sections

were washed with PBS 3 times for 3–5 min, and rabbit specific HRP/DAB (ABC) Detection IHC kit (ab64261, Abcam, Cambridge, USA) was used to detect the primary antibody. After washing with PBS 3 times for 5 min, sections were observed under a light microscope. When the staining was clear, sections were washed with tap water for 5 min to stop the reaction. Hematoxylin was used for counterstaining for 1 min, and sections were washed with PBS 3 times for 5 min and added 0.5% ammonia water for returning blue for 30 sec. The sections were then subjected to gradient alcohol (75% alcohol for 2 min, 80% alcohol for 2 min, 95% absolute alcohol for 2 min) for dehydration and xylene solution for transparency for 2 min, and then sealed by neutral balsam. Sections were observed under the microscope using the magnification of $\times 100$ and $\times 200$ and photographed. The presence of brownish-yellow granules in tumor tissues was considered positive staining.

Evaluation of PTEN immunohistochemistry and MS status. According to the proportion of positive staining, the tissue with positive PTEN staining was scored as: 1) 0, no staining; 1, more than one-third of cells were stained; 2, one-third to two-thirds of cells were stained; 3, more than two-thirds of cells were stained. 2) According to the intensity of positive staining, the tissue with positive PTEN staining was scored as: 0, no staining; 1, light yellow; 2, brownish-yellow; 3, dark brown. The final classifications were determined as a combination of the two scores above: 0, negative; 2-3, weak positive (+); 4, moderate positive (++); 5–6, strong positive (+++).

According to the definition of MSI, tissue samples that exhibited positive staining for all four proteins were considered to be MSS, while samples that were clear-cut negative for at least one of the four proteins were classified as MSI [11]. Thus, the status of MS of patients was classified.

Statistical analysis. Statistical data were analyzed using SPSS13.0 and GraphPad 7.0 software. The relationship among PTEN expression level, MS, and clinicopathological parameters of gastric cancer patients were compared by the chi-square test. The survival curve was delineated by the Kaplan-Meier method and the Log-rank method was used for the significant test. The COX proportional hazards model was used to determine the impact of multiple factors on prognosis. $\alpha=0.05$ was set as a significance level, and $p<0.05$ was considered to be statistically significant.

Results

The classification of PTEN expression and association with clinicopathological parameters. In order to classify the expression level of PTEN, we measured the expression of PTEN by IHC. Representative images of positive PTEN staining in cancer or adjacent normal tissues are shown in our results (Figure 1). The expression of PTEN in cancer tissues was lower than that in adjacent tissues (Figure 1). In order to figure out the association of PTEN with clini-

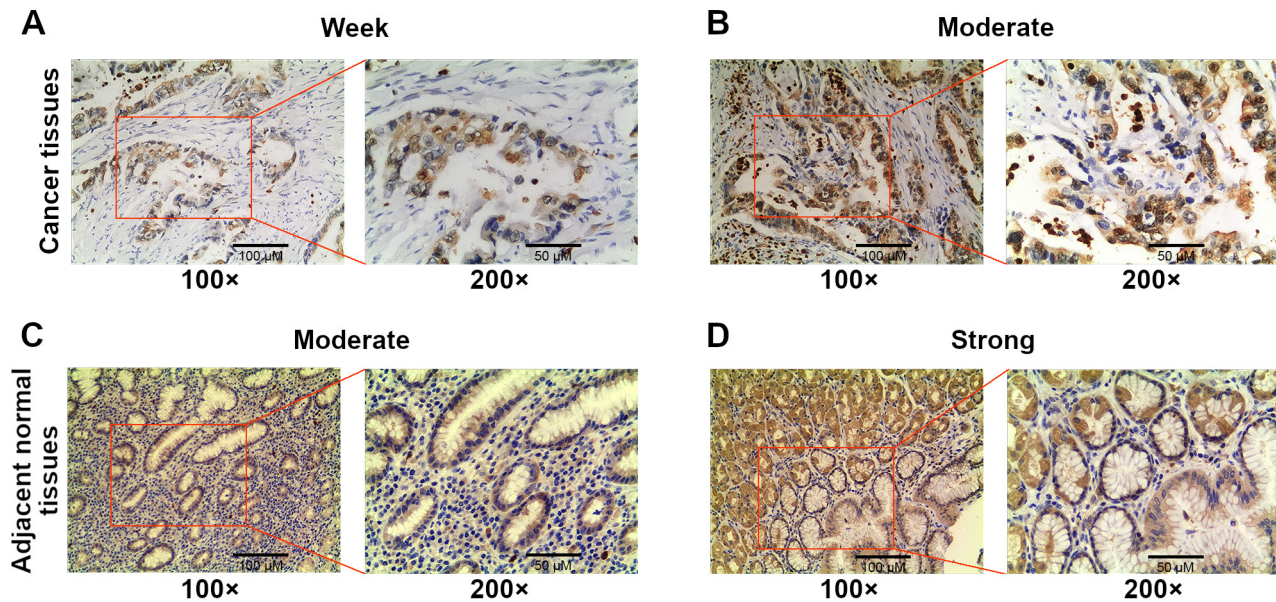


Figure 1. Representative photographs of different expression levels of PTEN by IHC in cancer or adjacent normal tissues. A) The weak expression level of PTEN in cancer tissues. B) The moderate expression level of PTEN in cancer tissues. C) The moderate expression level of PTEN in adjacent normal tissues. D) The strong expression level of PTEN in adjacent normal tissues. Magnification of $\times 100$ and $\times 200$ is presented.

copathological parameters, the characteristics of patients we chose were collected. All associations with clinicopathological parameters were given (Table 1). The negative PTEN expression was found in 48.66% of total patients. PTEN expression was only significantly associated with lymphatic invasion ($p=0.011$) and Lauren grade ($p=0.015$). There was no significance between PTEN expression and age ($p=0.250$), gender ($p=0.951$), tumor size ($p=0.223$), differentiation grade ($p=0.165$), TNM stage ($p=0.084$), and Ki-67 expression ($p=0.130$).

The classification of MSI or MSS and association with clinicopathological parameters. To identify the MSI status of patients, we measured the expression of four major mismatch repair proteins (MMRPs), namely, MLH1, PMS2, MSH2, and MSH6, by IHC. Representative images of the positive staining in cancer tissues are shown (Figure 2). To investigate the association of the status of MS with clinicopathological parameters, all characteristics of patients and their associations with MS were given (Table 2). MSI constituted 35.29% in total patients while MSS was 64.71%. The status of MS was significantly associated with differentiation grade ($p=0.006$) and Lauren grade ($p=0.035$). However, there was no significance between the status of MS and age ($p=0.772$), gender ($p=0.476$), tumor size ($p=0.470$), TNM stage ($p=0.181$), lymphatic invasion ($p=0.084$), Ki-67 expression ($p=0.657$).

The association among PTEN expression, status of MS, and clinicopathological parameters. To further explore the relationship among PTEN expression, status of MS, and clinicopathological parameters, the associations among them

Table 1. The classification of PTEN expression and association with clinicopathological parameters.

Clinicopathological parameters	n	PTEN expression		p-value
		Negative (n=91)	Positive (n=96)	
Age				0.250
<average	82	36	46	
\geq average	105	55	50	
Gender				0.951
Female	60	29	31	
Male	127	62	65	
Tumor size (cm)				0.223
<5	101	45	56	
≥ 5	86	46	40	
Differentiation grade				0.165
High-Moderate	69	29	40	
Low	118	62	56	
TNM stage				0.084
I+II	84	35	49	
III+IV	103	56	47	
Lymphatic invasion				0.011
No	75	28	47	
Yes	112	63	49	
Lauren grade				0.015
Intestinal	115	47	68	
Diffuse	23	12	11	
Mixed	49	32	17	
Ki-67 expression				0.130
Negative	72	30	42	
Positive	115	61	54	

p-values < 0.05 are in bold

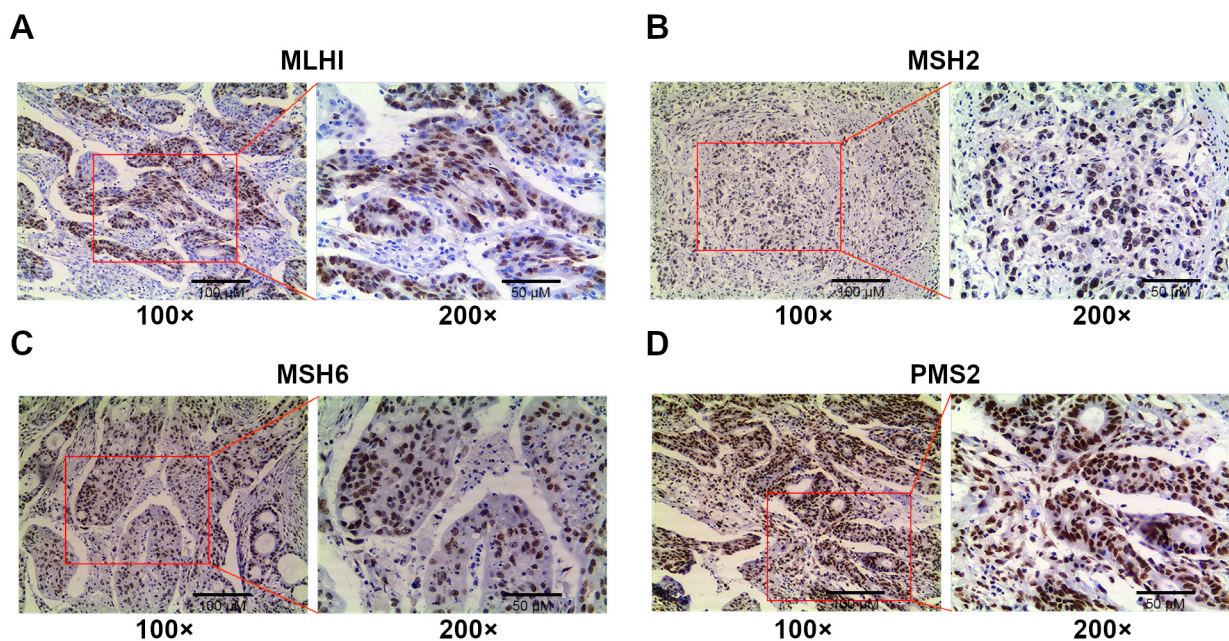
Table 2. The classification of MSI or MSS and association with clinicopathological parameters.

Clinicopathological parameters	n	MS		p-value
		MSI (n=66)	MSS (n=121)	
Age				0.772
<average	82	28	54	
≥average	105	38	67	
Gender				0.476
Female	60	19	41	
Male	127	47	80	
Tumor size (cm)				0.470
<5	101	38	63	
≥5	86	28	58	
Differentiation grade				0.006
High-Moderate	69	33	36	
Low	118	33	85	
TNM stage				0.181
I+II	84	34	50	
III+IV	103	32	71	
Lymphatic invasion				0.084
No	75	32	43	
Yes	112	34	78	
Lauren grade				0.035
Intestinal	115	49	66	
Diffuse	23	5	18	
Mixed	49	12	37	
Ki-67 expression				0.657
Negative	72	24	48	
Positive	115	42	73	

p-values <0.05 are in bold

were analyzed and given (Table 3). TNM stage was significantly associated with the status of MS when PTEN expression was positive ($p=0.014$). Age ($p=0.876$ or 0.522), gender ($p=0.869$ or 0.268), tumor size ($p=0.759$ or 0.756), differentiation grade ($p=0.077$ or 0.078), lymphatic invasion ($p=0.804$ or 0.104) Lauren grade ($p=0.113$ or 0.375), Ki-67 expression ($p=0.413$ or 0.367) were neither associated with the status of MS under negative PTEN expression nor positive expression.

Survival analysis. In order to investigate the effect of the status of MS and PTEN expression on overall survival of patients with GC, we drew the overall survival curve of patients with MSI or MSS as well as the patients with positive PTEN expression or not (Figures 3A–3B). As shown in the results, patients with MSS had a worse median overall survival than the patients with MSI (Figure 3A, $p=0.013$). Moreover, patients with negative PTEN expression had a worse median overall survival than the patients with positive PTEN expression (Figure 3B, $p<0.001$). To further find out the risk factor affecting the survival rate of patients, patients were divided into four groups as four different tumor subtypes, namely, PTEN positive-MSI, PTEN positive-MSS, PTEN negative-MSI, and PTEN negative-MSS groups, according to the expression of PTEN and the status of MS. The patients with PTEN negative-MSI and PTEN negative-MSS had a worse median overall survival than the patients with PTEN positive-MSI and PTEN positive-MSS (Figure 3C). Multi-variate analysis was conducted by COX proportional hazards model (Table 4). The tumor subtype of PTEN negative-MSS (HR 3.011, 95% CI: 1.691–5.363, $p<0.001$) and PTEN negative-MSI (HR 2.197, 95% CI: 1.028–4.696, $p=0.042$) were strong predictors of poor survival.

**Figure 2. Representative photographs of the expression of MLH1, MSH2, MSH6, and PMS2 in cancer tissues. A) The expression of MLH1. B) The expression of MSH2. C) The expression of MSH6. D) The expression of PMS2. Magnification of $\times 100$ and $\times 200$ is presented.**

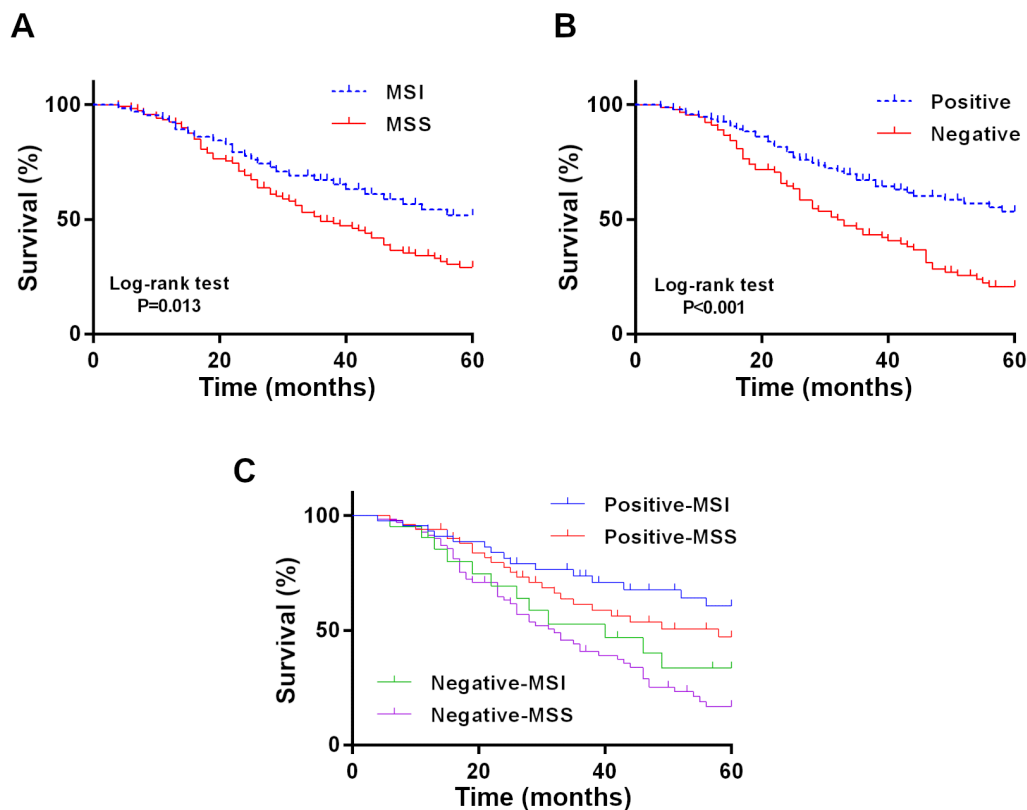


Figure 3. Survival analysis of PTEN and MS. A) The overall survival curves of the status of MS. B) The overall survival curves of PTEN. C) The overall survival curves of the status of MS with or without the expression of PTEN. $p < 0.05$ was considered statistically significant

Discussion

In this study, we measured and classified the expression of PTEN and the status of MS in tissues from patients with GC, and further explored the association of PTEN or MS with clinicopathological parameters or prognosis. Our study provided significant evidence that PTEN and MS might be prognostic markers of GC in the future.

Lauren's histological classification divides GC into intestinal type with the presence of gland formations and a diffuse type with a pattern of poorly cohesive cells, which are related to prognosis, epidemiology, and pathogenic perspective [12]. We found that the PTEN expression was linked to lymphatic invasion and Lauren grade. Zhang et al. revealed that microRNA-21 was significantly associated with lymph node metastasis via regulating PTEN in GC [13]. Li et al. reported that the positive rate of PTEN was related to lymph node metastasis in GC [14]. Furthermore, Zheng et al. demonstrated that intestinal-type gastric cancer always accompanied the strong expression of PTEN [15]. Zheng et al. proved that PTEN expression was linked to Lauren's classification negatively [16]. It indicated that PTEN expression might be closely related to the lymphatic invasion and Lauren grade.

The associations of the status of MS with differentiation grade and Lauren grade were also been seen in the present study. Jahng et al. revealed that the cases with two or more microsatellite instability (MSI) mutations (MSI-H) in the early stage of gastric cancer were associated with histologic differentiation and Lauren grade [17]. Moreover, Arai et al. showed that a much higher amount of cases with MSI were observed with poorly differentiated gastric adenocarcinoma than other subtypes [18]. Polom et al. reported that the status of MS was associated with Lauren histotype [19]. Thus, it suggested that the status of MS was linked to the differentiation and Lauren grade of GC.

Further, we found that there was a significant association of the status of MS with differentiation grade or TNM stage when PTEN expression was negative or positive, respectively. It was proved that the differentiation of gastric cancer cells to intestinal cells could be modulated by PTEN [20]. Investigations also revealed that there was a correlation between reduced nuclear PTEN expression and increasing TNM classification in GC patients [21]. In addition, Choi et al. reported that there was a close relationship between the microsatellite genotype and the TNM stage [22]. Therefore, we speculated that there was a strong linkage between PTEN, the status of MS, and the differentiation grade or TNM stage.

Table 3. The association among PTEN expression, status of MS and clinicopathological parameters.

Clinicopathological parameters	n	PTEN Negative (91)		p-value	PTEN Positive (96)		p-value 0.001
		MSI (n=21)	MSS (n=70)		MSI (n=45)	MSS (n=51)	
Age				0.876			0.522
< average	82	8	28		20	26	
≥ average	105	13	42		25	25	
Gender				0.869			0.268
Female	60	7	22		12	19	
Male	127	14	48		33	32	
Tumor size (cm)				0.759			0.756
< 5	101	11	34		27	29	
≥ 5	86	10	36		18	22	
Differentiation grade				0.077			0.078
High-Moderate	69	10	19		23	17	
Low	118	11	51		22	34	
TNM stage				0.116			0.014
I+II	84	5	30		29	20	
III+IV	103	16	40		16	31	
Lymphatic invasion				0.804			0.104
No	75	6	22		26	21	
Yes	112	15	48		19	30	
Lauren grade				0.113			0.375
Intestinal	115	15	32		34	34	
Diffuse	23	2	10		3	8	
Mixed	49	4	28		8	9	
Ki-67 expression				0.413			0.367
Negative	72	9	21		15	27	
Positive	115	12	49		30	24	

p-values <0.05 are in bold

Table 4. The effect of the status of MS and PTEN expression on overall survival of patients with GC.

Tumor subtype	n	Overall survival HR (95% CI)	p-value
PTEN positive-MSI	45	1	
PTEN positive-MSS	51	1.486 (0.775–2.849)	0.233
PTEN negative-MSI	21	2.197 (1.028–4.696)	0.042
PTEN negative-MSS	70	3.011 (1.691–5.363)	<0.001

p-values <0.05 are in bold

However, the underlying mechanism was still needed to be further investigated.

The patients with MSS exhibited worse prognosis than the ones with MSI as observed by the study of the follow-up data. Zhao et al. found that low-frequency microsatellite instability correlates with a poor prognosis in gastric cancer [23], which supported our findings. One possible reason to explain the outcome was that the status of MS was associated with the differentiation and Lauren grades, which might further affect the prognosis of GC patients. Several investigations had revealed the fact that there were correlations between differentiation and prognosis of GC as well as between Lauren

grade and prognosis of GC, which could be the evidence to prove the possibilities. For instance, Chen et al. reported that Lauren's classification was an independent prognostic factor in GC patient undergoing gastrectomy [24], and Adachi et al. found that the overall survival rate of 5 years for patients with well-differentiated GC was higher than that for patients with poorly differentiated GC [25].

The more unfavorable prognosis of patients with negative PTEN expression than the ones with positive PTEN expression was found in our study as well. Chen et al. reported that aberrant expression of PTEN was observed in GC that correlated with poor overall survival [26]. Similarly, we hypothesized that the prognosis of the cases with negative PTEN expression was affected due to the presence of lymphatic invasion and Lauren's classification, which were associated with the expression of PTEN. It could be proved by the fact that lymph node metastasis is a critical marker of prognosis of gastric cancer patients [27]. Moreover, Bu et al. reported that lymphatic vascular invasion is an independent correlated factor for the prognosis of resectable T2 gastric cancer patients [28]. In addition, researches revealed that the patients with intestinal-type gastric carcinomas had a more favorable prognosis [15], while the patients with diffuse-

subtype tumors had the worst prognosis among all cases in the study of GC [29].

Our study revealed that the cases carrying MSI or MSS with positive PTEN expression had a lower overall survival HR value than the cases with negative PTEN expression, which showed the significance in COX proportional hazards model. It could be explained that the prognosis was influenced by the differentiation grade of GC which was affected by the status of MS under the condition of the loss of PTEN expression. The outcomes also implied the critical role of PTEN expression in the development of GC, affecting the prognosis of patients. Salvesen et al. found that the PTEN methylation was associated significantly with MS unstable phenotype [30]. This research might be circumstantial evidence to explain the outcome in the present study to some extent, however, further investigations are needed to fully understand the relationship between PTEN, MS, and prognosis of GC patients.

In conclusion, negative PTEN expression and MSS might lead to unfavorable prognosis due to their association with clinicopathological parameters of GC patients. The expression of PTEN and the status of MS could be seen as predictors of prognosis for GC patients.

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