Exploring the clinicopathological significance of YKL-39 in gastric adenocarcinoma based on tumor angiogenesis

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Abstract

Background

Gastric cancer is a malignant tumor with a high incidence and mortality rate. Angiogenesis is necessary for tumor infiltration and metastasis, and also affects patient prognosis. YKL-39 has monocyte chemotactic activity and pro-angiogenic activity in some of the tumors. In this study, we will investigate the relationship between YKL-39 and tumor-associated macrophages and microangiogenesis in gastric cancer and explore the value of YKL-39 as a prognostic biomarker for gastric cancer.

Methods

A total of 119 patients with gastric cancer who had undergone gastrectomy at the 940th Hospital of the Joint Security Force between 2013 and 2019 were included in this study, and the protein expression of YKL-39, CD68, and CD34 was detected by immunohistochemistry, and intracellular expression of YKL-39 and CD68 was detected by immunofluorescence.

Results

Our results showed that YKL-39 was expressed in both nucleus and cytoplasm of gastric cancer tissues and tumor mesenchyme, and the expression of YKL-39 was positively correlated with CD68 and CD34, as well as the high expression of YKL-39 was associated with poor prognosis of gastric cancer patients.

Conclusion

In gastric cancer, YKL-39 expression positively correlated with the degree of tumor-associated macrophage infiltration and angiogenesis, and it could be a potential prognostic marker and therapeutic target for gastric cancer.

1. Introduction

Gastric cancer (GC) is one of the most common gastrointestinal malignancies, ranking fifth in incidence and third in lethality globally[1]. With advances in technology such as endoscopy, more and more patients with GC are being diagnosed and treated in a timely manner, but the overall 5-year survival rate is still less than 40%[2]. Most GC patients are already in the middle or late stages when they are diagnosed, with a median overall survival (OS) less than 12 months[3]. Angiogenesis is one of the most fundamental factors that promote the growth and metastasis of CG cells by providing nutrients and oxygen[4]. When the tumor has advanced metastasis or cannot be excised, the current treatment is based on palliative chemotherapy, when anti-angiogenic therapy can be used as an effective adjuvant treatment[5]. The commonly used anti-angiogenic drugs include bevacizumab, which targets vascular endothelial growth factor-A (VEGF-A), ramucirumab, which targets VEGFR2, and ziv-aflibercept, which targets VEGF-A isoforms, placental growth factor (PLGF), and VEGF-B etc. [6]. However, tumors can develop resistance to anti-angiogenic drugs through a variety of mechanisms, including upregulation of alternative pro-angiogenic signaling pathways, resistance of tumor stromal cells to anti-angiogenic drugs, adaptation of tumor cells to hypoxic environments and alternative mechanisms of tumor vascularization[7]. Therefore, it is of great significance to find new anti-angiogenic targets.

Tumor-associated macrophages (TAM) are the most common tumor-infiltrating immune cells in the tumor microenvironment (TME), accounting for more than 50% of immune cells in the TME and promoting tumorigenesis through various mechanisms such as stimulating angiogenesis, increasing tumor cell invasion and migration, and inhibiting anti-tumor immunity[8, 9]. Macrophages are stimulated by different chemokines released by tumors and stromal cells to differentiate into two phenotypes with dramatic differences: M1 macrophages with antitumor effects and M2 macrophages with pro-tumor effects[8]. M2 macrophages, which occupy the majority of TAM, can produce a variety of pro-angiogenic factors such as VEGF-A and tumor necrosis factor α (TNFα) in hypoxic areas to maintain tumor growth[10]. TAM infiltration in multiple tumors is positively correlated with angiogenesis[11, 12]. And it has been shown that the emergence of anti-VEGF therapy resistance is associated with the aggregation of TAMs in TME[13, 14].

YKL-39, also known as CHI3L2, belongs to the family of chitinase-like proteins (CLPs) that function as both cytokines and growth factors[15]. The CLPs in the human body include YKL-40, YKL-39 and SI-CLP. YKL-39 was originally found in human synoviocytes and chondrocytes and plays a role in regulating autoimmunity and participating in tissue remodeling[16]. It has been shown that YKL-39 expression is elevated in degenerative pathologies and diseases characterized by tissue remodeling, such as osteoarthritis, multiple sclerosis, Alzheimer's disease and amyotrophic lateral sclerosis[17–19]. Recent studies have reported that YKL-39 has monocyte chemotactic and pro-angiogenic activity and is expressed in M2 macrophages from breast, glioma and kidney cancers, affecting tumor angiogenesis, and overexpressed YKL-39 is also associated with poor prognosis[20–22]. However, there are no reports on the relationship between YKL-39 expression and GC biological behavior and prognosis of GC patients.

In this study, we used immunohistochemistry (IHC) and immunofluorescence (IF) to detect the expression of YKL-39, CD68 and CD34 in GC tissues to verify the relationship between YKL-39 expression and macrophage infiltration and its effect on angiogenesis. Our findings support that YKL-39 is expressed in both M2 macrophages and tumor cells with stimulated angiogenesis that correlates with poor prognosis in GC. In summary, our results confirm that YKL-39 has the potential to become a target for anti-TAMs aggregation and anti-tumor angiogenesis in GC.

2. Material And Methods

2.1 Patients and tissue samples

This study included 119 patients diagnosed with GC through histopathologic evaluation on gastroscopic biopsy or surgical tissue specimens. 101 cases were male and 18 cases were female, ranging in age from 29–81 years, with a median age of 59.03 years. All patients underwent gastrectomy between 2014 to 2018 at the Gansu Wuwei Tumor Hospital, Gansu, China. The patients who had preoperative treatment, such as radiotherapy, chemotherapy, or other medical interventions and those diagnosed with autoimmune diseases were excluded from the study. Formalin-fixed, paraffin-embedded (FFPE) GC tissue samples were obtained from these patients after surgery. Demographic and Clinicopathologic characteristics, including age, sex, TNM stage, histologic grade and lymph node metastases were assessed. The patients were followed up for 5 years by inpatient, outpatient and telephone contact. In this study, we used OS to evaluate prognosis. OS was defined as the period from the date of surgery to the date of death or last visit. The median follow-up time was 44.18 months (range 3-114 months). Informed consent was obtained from all patients.

2.2 Tissue microarray (TMA) construction

FFPE specimens were stained with H&E and examined by our experienced pathologists. At least three representative regions were selected and marked on H&E slices each tumor. Then the corresponding part on the wax block was selected as the material selection site according to the mark on the H&E slice. TMAs containing the 119 FFPE specimens were constructed by using Manual Tissue Arrayer (TM1, boyikang, Bejing, China). A cylindrical spotting tissue with a diameter of 2.0 mm was dug using a TMA instrument and placed into the acceptor wax block from top to bottom in a right-to-left order.

2.3 Immunohistochemistry

Firstly, All TMA slides with a thickness of 4 μm were deparaffinized using xylene and rehydrated using graded ethanol. Then, the slides were immersed and boiled in Ethylene Diamine Tetraacetic Acid (pH 9.0) for 30 min in a pressure cooker for antigen retrieval. The endogenous peroxidase was inhibited by 3% H_2O_2 for 15 min. For YKL-39 staining, recombinant monoclonal rabbit anti-human YKL-39 (1:150; ThermoFisher, USA) was applied. For TAM evaluation, monoclonal mouse anti-human CD68 (ready-to-use; maixin, China) was applied. For Tumor micro vessel density (MVD) evaluation, monoclonal mouse anti-human CD34 (ready-to-use, maixin, China) was applied. Specimens were incubated with all antibodies overnight at 4℃. After washing with PBS, the slides were incubated with secondary antibody at 37°C for 10 minutes. The following steps were performed: color development, counterstaining, differentiation, dehydration, and transparency. Finally, the slides fixed with neutral resin.

IHC slices were examined at a low magnification (100x), the most representative 5 high-magnification (400x) fields were selected for staining assessment. A semi-quantitative IHC scoring criterion was used to determine the YKL-39 protein expression levels in tumor. The percent positivity of staining cells ranges from 0 to 4: 0, none; 1, 1%-25%; 2, 26%-50%; 3, 51%-75%; 4, 76%-100%. The intensity of staining was graded from 0 to 3 (0, no staining; 1, weak; 2, moderate and 3, strong). Then, we obtained the final IHC score by multiplying the proportion score by the intensity score of staining. A median score of 6 (> 6 or ≤ 6) was selected as the cutoff point to distinguish patients with YKL-39 positive or YKL-39 negative expression results. CD68 expression was determined by the number of positive cells expressed. MVD was determined by counting the number of vessels stained by CD34.

2.4 Immunofluorescent staining and confocal microscopy

Formalin fixed, paraffin embedded sections were deparaffinized by xylene and rehydrated. The slices are placed in a pressure cooker with sodium citrate and boiled, then cooled naturally to room temperature to complete the antigen repair. 0.3% Triton + 10 mg/mL bovine serum albumin + PBS was used to prepare the blocking solution and blocked for 1h. Configure the primary antibody according to the instructions. The primary antibodies used include YKL-39 (1:100, Bioss, China) and CD68 (1:50, BOSTER, China). Drop the diluted antibody onto the tissue and incubate overnight in a 4°C refrigerator. The secondary antibodies used were goat anti mouse - Alexa Fluor 594 (1:200) and goat anti rabbit - Alexa Fluor 488 (1:200) according to the instructions. The secondary antibodies were dropped onto the tissues and incubated for 2h at room temperature and protected from light. After cleaning the slides with PBS dilution, add an amount of anti-fluorescence quenching sealing liquid (including DAPI), cover the coverslip over the tissue, and store the slides at 4°C in the refrigerator protected from light.

Confocal laser scanning microscopy was performed with a Leica TCS SP8 laser scanning spectral confocal microscope, equipped with a 63 £ 1.32 objective. Excitation was with laser emitting at 405nm, 488nm and 559nm. All two-or three-color images were acquired using a sequential scan mode.

2.5 Statistical analysis

SPSS 25.0 was used for statistical analysis. Correlation analysis using Spearman's rank correlation coefficient. Survival analyses were conducted using the Kaplan-Meier method and differences in survival were examined using the log-rank test. Multivariate survival analyses were conducted using the Cox proportional hazards regression model. Statistical significance was defined as a value of $p < 0.05$.

3. Results

3.1 Relationship between YKL-39, CD68 and CD34 expression and clinicopathological characteristics of gastric cancer patients

Among the 119 IHC staining results, 69(57.98%) cases showed elevated expression of YKL-39, 68(57.14%) cases showed elevated expression of CD68, and 59(49.58%) cases showed elevated expression of CD34.

We analyzed the correlation between YKL-39, CD68, and CD34 expression and patients' gender, age, depth of tumor infiltration, lymph node metastasis, distant metastasis, TNM stage, and degree of differentiation. YKL-39 protein expression was associated with the depth of tumor infiltration ($p = 0.018$), lymph node

metastasis (p = 0.029) and TNM stage (p = 0.002). Tumor infiltration was deeper, lymph node metastasis was more frequent, and TNM stage was higher, and YKL-39 expression was higher. CD68 protein expression was associated with lymph node metastasis (p = 0.048) and TNM stage (p = 0.03), with more lymph node metastasis and higher TNM stage associated with higher CD68 expression. There was no significant correlation between CD34 expression and clinicopathological characteristics (Table 1).

Table 1
Association of YKL-39, CD68, CD34 protein expression with clinical and pathological parameters (n = 119).

Clinical factors	total	Expression of YKL-39		$\chi2$	p value	Expression of CD68		$\chi2$	p value	Expression of CD34		$\chi2$
		Low (n = 50)	High (n = 69)			Low	High			Low	High	
Gender				0.555	0.456			1.31	0.252			2.239
Male	101(84.87)	41(34.45)	60(50.42)			46(38.66)	55(46.22)			48(40.34)	53(44.54)	
Female	18(15.13)	9(7.56)	9(7.56)			5(4.20)	13(10.92)			12(10.08)	6(5.04)	
Age(years)				0.125	0.724			1.754	0.185			1.011
>60	57(47.90)	23(19.33)	34(28.57)			28(23.53)	29(24.37)			26(21.85)	31(26.05)	
≤60	62(52.10)	27(22.69)	35(29.41)			23(19.33)	39(32.77)			34(28.57)	28(23.53)	
Depth of tumor invasion				**5.602**	**0.018**			0.474	0.491			1.416
T1-T3	61(51.26)	32(26.89)	29(24.37)			28(23.53)	33(27.73)			34(28.57)	27(22.69)	
T4	58(48.74)	18(15.13)	40(33.61)			23(19.33)	35(29.41)			26(21.85)	32(26.89)	
Lymph node metastasis				**9.005**	**0.029**			**7.88**	**0.048**			5.312
N0	30(25.21)	19(15.97)	11(9.24)			18(15.13)	12(10.08)			19(15.97)	11(9.24)	
N1	25(21.01)	11(9.24)	14(11.76)			13(10.92)	12(10.08)			15(12.61)	10(8.40)	
N2	34(28.57)	12(10.08)	22(18.49)			11(9.24)	23(19.33)			14(11.76)	20(16.81)	
N3	30(25.21)	8(6.72)	22(18.49)			9(7.56)	21(17.65)			12(10.08)	18(15.13)	
Distant metastases				2.24	0.134			0.706	0.401			0.708
M0	79(66.39)	37(31.09)	42(35.29)			36(30.25)	43(36.13)			42(35.29)	37(31.09)	
M1	40(33.61)	27(22.69)	13(10.92)			15(12.61)	25(21.01)			18(15.13)	22(18.49)	
TNM stage				**14.243**	**0.002**			**8.888**	**0.03**			6.48
I	12(10.08)	11(9.24)	1(0.84)			10(8.40)	2(1.68)			10(8.40)	2(1.68)	
II	22(18.49)	9(7.56)	13(10.92)			9(7.56)	13(10.92)			12(10.08)	10(8.40)	
III	44(36.97)	17(14.29)	27(22.69)			16(13.45)	28(23.53)			20(16.81)	24(20.17)	
IV	41(34.45)	13(10.92)	28(23.53)			16(13.45)	25(21.01)			18(15.13)	23(19.33)	
Degree of differentiation				0.496	0.481			2.707	0.1			0.218
Well differentiation	55(46.22)	25(21.01)	30(25.21)			28(23.53)	27(22.69)			29(24.37)	26(21.85)	
Poor differentiation	64(53.78)	25(21.01)	39(32.77)			23(19.33)	41(34.45)			31(26.05)	33(27.73)	

3.2 Correlation between YKL-39 Expression and TAMs Infiltration and angiogenesis in GC tissue.

IHC staining showed that YKL-39 was expressed in both the nucleus and cytoplasm of GC tumor cells and stroma cells (Fig. 1A). IHC staining for CD68 showed cytoplasmic staining, diffusely distributed in the tumor stroma cells, and was used to label TAMs (Fig. 1A). MVD is an important index of tumor angiogenic activity and intensity. To evaluate tumor angiogenesis, MVD was evaluated by IHC staining with CD34. To determine the relationship between YKL-39 protein expression and TAMs infiltration and MVD, we performed a correlation analysis with Spearman's correlation coefficient (Table 2). The results showed that positive reactions for both CD68 and CD34 were increased in the samples with higher YKL-39 expression. YKL-39 expression significantly correlates with CD68 and CD34 expression in GC (p < 0.001).

In order to determine the sites of expression of YKL-39 and CD68 in GC tissues, IF and confocal microscopy analysis was performed on samples from 10 patients with GC. The results showed that YKL-39 was expressed in both tumor cells and tumor mesenchyme, and co-expressed with macrophages in the tumor mesenchyme. (Fig. 1B**).**

Table 2
Association of YKL-39, CD68, CD34 protein expression.

		total	YKL-39 expression			
			low	high	r	*p* value
CD68	low	51	38	13	0.666	< 0.001
	high	68	12	56		
					0.662	< 0.001
CD34	low	60	42	18		
	high	59	8	51		

3.3 Survival analysis and the prognostic value of YKL-39, CD68 and CD34 expression in patients with GC

Among the 75 patients with follow-up, 28 patients had died at the last follow-up period. To explore the prognostic effects of YKL-39, CD68 and CD34 protein expression in GC, we plotted Kaplan-Meier survival curves and performed log-rank test (Fig. 2A-C). The results showed that patients with high YKL-39 expression had a significantly worse prognosis than those with low YKL-39 expression ($p < 0.001$, Hazard Ratio = 1.4). Patients expressing high CD68 and CD34 have a worse prognosis than those with low expression ($p < 0.001$).

As shown in Fig. 2D, when combining CD68 and YKL-39 to analyze prognosis, patients in the high CD68 low YKL-39 group had a significantly better prognosis than those in the high CD68 high YKL-39 group. The same situation also occurred when combining CD34 and YKL-39 to analyze the prognosis of patients (Fig. 2E) ($p < 0.001$).

To further discern the independent prognostic role of YKL-39 in GC, we performed Cox regression analysis. The results of the univariate independent prognostic analysis showed a statistically significant relationship between age, N-stage, YKL-39 expression, MVD and survival outcome ($p < 0.05$) (Fig. 2F). Multivariate COX regression results showed that only YKL-39 expression was an independent prognostic factor for GC ($p < 0.05$) (Fig. 2G). Our results demonstrate that YKL-39 may serve as an independent prognostic factor for GC.

4. Discussion

Presently, despite advances in diagnostic and therapeutic techniques, GC remains a class of malignant tumors that threatens human health, with a median survival of less than 12 months for patients with advanced GC. Therefore, there is an urgent need to explore new biomarkers to predict the prognosis of GC patients as well as to guide individualized treatment. In this study, we detected the expression of YKL-39, CD68, and CD34 in GC cells and mesenchyme by immunohistochemistry. The correlation analysis confirmed that the expression of YKL-39 correlated with TAMs and angiogenesis. The Kaplan-Meier survival curve and log-rank test demonstrated that YKL-39 expression correlated with prognosis of GC patients, and Multivariate Cox regression analysis proved that YKL-39 expression in tumors was an independent predictor of GC progression. These results demonstrate that YKL-39 is a valuable prognostic biomarker for GC.

Both chitinases and CLPs contain Glyco-18 structural domains, and in humans, chitinases include acidic mammalian chitinase (AMCase) and chitotriosidase (CHIT1), which cleave chitin polymers into oligosaccharides and release monosaccharides from the ends of chitin polymers[23]. CLPs do not possess glycosyl hydrolase activity due to the substitution of the catalytic residue glutamate in the active site DxxDxDxE motif terminus by leucine, isoleucine or tryptophan, but due to the Glyco-18 structural domain, they still have the sugar-binding properties[24–26]. The human CLPs include YKL-40, YKL-39 and SI-CLP, of which YKL-40 (CHI3L1) is the most, well-studied which is upregulated in a variety of inflammatory and neurodegenerative diseases and cancers[16]. YKL-40 mediates activation of MAPK and PI3K pathways through phosphorylation of ERK1/2 and AKT. It has been demonstrated that the expression of YKL-40 is elevated in a variety of cancers, including breast cancer, colon cancer, lung cancer, prostate cancer, bladder cancer, and GC, and promotes tumor progression by promoting tumor cell proliferation, rapid invasion and migration, and angiogenesis[27]. YKL-39 and YKL-40 show some similarity in size and amino acid sequence, which suggests that YKL-39 may have a similar biological function to YKL-40[15]. It has been demonstrated that YKL-39 can directly activate vascular endothelial cells in vitro and exert strong pro-angiogenic effects[26]. Moreover, in a variety of cancers, YKL-39 expression correlates with patient prognosis and chemotherapy resistance[20, 21].

As the most vital immune cells in the tumor microenvironment, the infiltration status of TAMs significantly affects the patient's response to treatment and prognosis[28, 29]. TAMs can stimulate solid tumor development and invasion by secreting a variety of pro-angiogenic factors, such as VEGF-α, PLGF and transforming growth factor-β[30]. It has been demonstrated that the level of TAMs is positively correlated with the degree of MVD in a variety of tumors[11, 31]. Previous studies have confirmed that anti-YKL-40 treatment showed better anti-angiogenic effects in animal tumor models both in vivo and in vitro[32]. In recent studies, YKL-39 has been identified in breast cancer as having monocyte chemotactic activity and pro-angiogenic activity. YKL-39 does not work directly on tumor cells but can influence tumor progression by altering the state of the tumor microenvironment, and elevated YKL-39 levels are associated with

increased risk of metastasis and post-chemotherapy drug resistance[21]. In gliomas, YKL-39 has likewise been shown to be associated with tumor immune infiltration and affect patient prognosis[20]. YKL-39 can stimulate monocytes to migrate to tumor sites and further differentiate into TAMs[26]. There were no studies to validate the relevant role of YKL-39 in gastric cancer, and for the first time, our study confirmed that YKL-39 expression in gastric cancer correlates with TAMs and angiogenesis through immunohistochemical staining. The co-expression relationship between TAMs and YKL-39 in tumors was also confirmed by immunofluorescence staining. Also, our experiments have limitations, the specific function of YKL-39 in gastric cancer needs to be further confirmed by cellular experiments.

5. Conclusion

In summary, our results support that YKL-39 expression in gastric cancer correlates with TAMs infiltration and tumor angiogenesis and is significantly associated with prognosis of gastric cancer patients. It indicates the potential of YKL-39 as a prognostic biomarker and therapeutic target for gastric cancer.

List Of Abbreviations

Gastric cancer (GC), Overall survival (OS), Vascular endothelial growth factor-A (VEGF-A), Placental growth factor (PLGF), Tumor-associated macrophages (TAM), Tumor microenvironment (TME), Tumor necrosis factor α (TNFα), Chitinase-like proteins (CLPs), Immunohistochemistry (IHC), Immunofluorescence (IF), Formalin-fixed, paraffin-embedded (FFPE), Tissue microarray (TMA), Micro vessel density (MVD)

Declarations

Ethical approval retrospective studies

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee (Medical Ethics Committee of Gansu Wuwei Tumor Hospital) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

L.X., PF.W. and XW.Z. wrote the main manuscript text and L.X and W.C. prepared all figures and tables. All authors reviewed the manuscript.

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Figures

Figure 1

YKL-39, CD68 and CD34 IHC staining result and IF staining results. (A) IHC staining images of high and low expression of YKL-39, CD68 and CD34 protein in gastric adenocarcinoma. (200×, horizontal lines represent 50 μm). (B) IF staining showed co-expression of YKL-39 (green fluorescence) and CD68 (red fluorescence) in gastric adenocarcinoma tissue (20×, horizontal lines represent 500 μm).

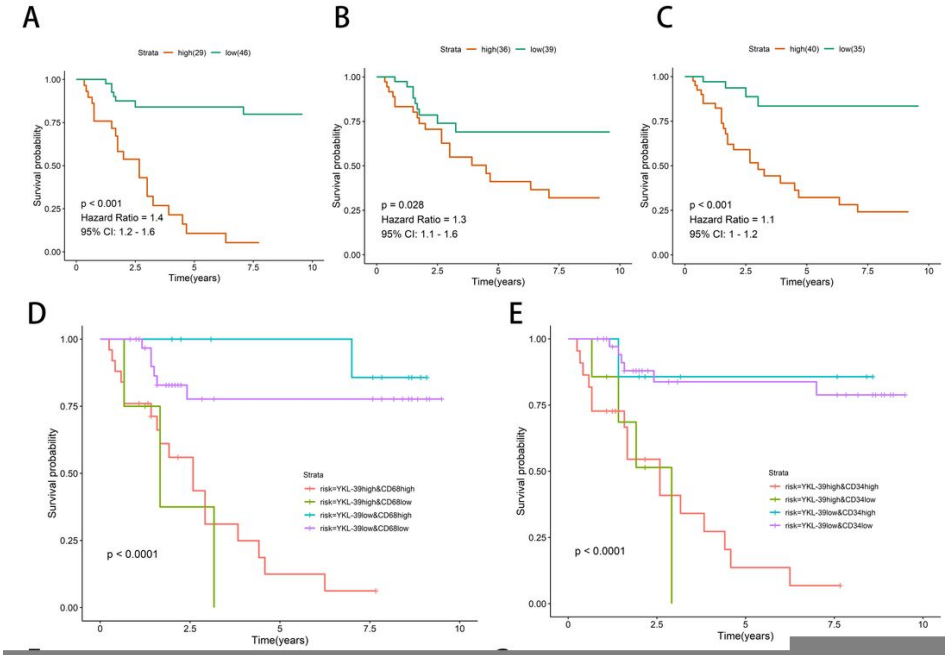

Figure 2

Survival analysis and the prognostic value of YKL-39, CD68 and CD34 expression in patients with GC. Kaplan–Meier survival curves of **(A)**YKL-39, **(B)**CD68, **(C)**CD34 expression in GC patients. **(D)**Kaplan-Meier survival curves of overall survival among four patient groups stratified by YKL-39 and CD68 expression. **(E)**Kaplan-Meier survival curves of overall survival among four patient groups stratified by YKL-39 and CD34 expression. **(F)** Results of univariate COX regression analysis of YKL-39, CD68, CD34 expression and clinicopathological information. **(G)** Results of multivariate COX regression analysis of YKL-39, CD68, CD34 expression and clinicopathological information.