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Expression and prognosis of MYOZ2 in gastric cancer

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Abstract. – OBJECTIVE: To investigate the expression of human myozenin 2 (MYOZ2) in cancer tissues and its effect on the prognosis of patients with gastric cancer.

PATIENTS AND METHODS: Gastric cancer tissue and adjacent normal tissue specimens were obtained from a total of 258 patients together with complete clinicopathological data. Those patients were treated in Harbin Medical University Cancer Hospital from March 2007 to March 2012. Quantitative Real Time-Polymerase Chain Reaction (RT-PCR) was used to detect the expression of MYOZ2 messenger RNA (mRNA) in gastric cancer tissues and normal gastric tissues, and correlations between the expression level of MYOZ2 in gastric cancer tissues and the clinicopathological parameters of patients were analyzed. MYOZ2 protein expression levels in different gastric cancer cells were detected by Western blotting; transwell chamber assay was used to detect the effect of MYOZ2 expression on the *in-vitro* migration and invasion abilities of gastric cancer cells; 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was applied to examine the *in-vitro* proliferation and growth abilities of gastric cancer cells.

RESULTS: The expression level of MYOZ2 mRNA in gastric cancer tissues was significantly higher than that in cancer-adjacent tissues in 258 cases ($p<0.05$). Western blotting results showed that the expression level of MYOZ2 protein in gastric cancer tissues was significantly increased compared with that in the corresponding adjacent healthy tissues ($p<0.05$). In-vitro growth, migration and invasion abilities of MYOZ2 positive gastric cancer cells were significantly higher than those of normal tissue cells. Univariate analysis showed that the high expression level of MYOZ2 in gastric cancer tissues was closely related to tumor size, lymph node metastasis, and pathological tumor-node-metastasis (pTNM) staging ($p<0.05$), but not associated with gender, age, differentiation degree, and tumor location ($p>0.05$). The 1-, 3- and 5-year survival rates of patients with low expression level of MYOZ2 (n=130) were higher than those of patients with high expression level of MYOZ2 (n=128). Multivariate analysis revealed that the expression level of MYOZ2 ($p=0.000$), lymph node metastasis ($p=0.002$), and pTNM staging ($p=0.015$) were independent risk factors influencing the prognosis of gastric cancer.

CONCLUSIONS: Our results showed that the expression level of MYOZ2 may play an important role in the occurrence and development of gastric cancer, and can also provide references for the clinical diagnosis and treatment of gastric cancer.

Key Words:
Gastric cancer, MYOZ2, PCR, Western blotting, Prognosis.

Introduction

Gastric cancer is a type of malignant tumor with the highest incidence rate and mortality rate worldwide. Gastric cancer is difficult to be diagnosed during early stages, so most patients are diagnosed at advance stages, leading to poor outcomes of surgery and chemotherapy as well as poor prognosis[1,2]. At present, the pathogenesis of gastric cancer still hasn't been elucidated. Mutations of certain genes, such as p53, may be related to the occurrence and development of gastric cancer[3-5]. In spite of those progresses, the mechanism of the occurrence of gastric cancer is still largely unknown. Human myozenin 2 (MYOZ2), also known as Calsarcin-1, is widely distributed in various types of human cells. MYOZ2 is a member of the calcium-regulated neuropeptide-binding protein family, which is mainly involved in the regulation of various cell functions[6,7]. The function of MYOZ2 in heart and skeletal muscle has been elucidated through studies using transgenic animals8, and those studies have shown that MYOZ2 could negatively regulate expression of calcineurin in pathological hyper-

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trophy remodeling[9,10]. However, the correlation between MYOZ2 expression and gastric cancer is still unclear. TCGA database screening showed that MYOZ2 was differentially expressed in gastric cancer. Therefore, Real Time-Polymerase Chain Reaction (RT-PCR), Western blotting, and other methods were used in this work to detect the expression of MYOZ2 in gastric cancer and cancer-adjacent normal tissues. Also, correlations between MYOZ2 expression and clinicopathological parameters of gastric cancer patients were investigated to explore the effects of MYOZ2 expression on the prognosis of gastric cancer.

Patients and Methods

Patients

A total of 258 gastric cancer patients with complete clinicopathological data who were treated in Harbin Medical University Cancer Hospital from March 2007 to March 2012 were enrolled. Gastric cancer tissue and adjacent normal gastric tissue specimens were collected during surgical operation and immediately stored in liquid nitrogen. Those patients were confirmed with gastric cancer by pathological examinations using tumor tissues. Those patients included 167 males and 91 females, aged from 29 to 82 years, with a mean age of 58.4 years. The study was approved by the Ethics Committee of Harbin Medical University Cancer Hospital, and all participants signed the informed consent. None of those patients received chemotherapy before admission, and clinicopathological data including age, gender, tumor size, tumor location, lymph node metastasis, and pathological tumor-node-metastasis (pTNM) staging were collected. Patients were followed-up regularly through outpatient service, telephone, etc. Follow-up was not stopped until patients' deaths or until March 2017. Patients out of contact during follow-up and patients died in accidents were excluded (Table I).

Experimental Reagents and Materials

TRIzol Separation Reagent (Thermo Fisher Scientific, Waltham, MA, USA); reverse transcription kits, SYBR Green and PCR amplification kits were provided by Shanghai Kang Lang Biological Technology Co., Ltd (Shanghai, China); primers [Sangon Biotechnology (Shanghai) Co., Ltd., Shanghai, China]; siRNA Kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA); bicinchoninic acid (BCA) protein concentration detection kits and bovine serum albumin (BSA) detection kits (Shanghai Yu Bo Biological Technology Co., Ltd., Shanghai, China); Lipofectamine 2000 Kit (Invitrogen, Carlsbad, CA, USA); hu-

Table I. Clinicopathological data of 258 patients with gastric cancer.

General feature		N	%
Gender	Male	167	64.7
	Female	91	35.3
Age (years old)	≥60	143	55.4
	<60	115	44.6
Body mass index	Low	91	35.3
	Normal	89	34.5
	High	78	30.2
Smoking (400 cigarettes/year)	No	92	35.7
	<400	67	30.0
	≥400	99	38.3
Helicobacter pylori	Positive	162	62.8
	Negative	96	37.2
Carcinoembryonic antigen (CEA)	Normal or deceased (≤5.0 ng/ml)	127	49.2
	Increased (>5.0 ng/ml)	131	50.8
Cancer antigen 19-9 (CA 19-9)	Normal or deceased (≤3.7 kU/L)	77	29.8
	Increased (>3.7 kU/L)	181	70.1

man gastric cancer cell lines, MKN28, SGC-7901, and MKN45 were provided by the Cell Bank of Shanghai Academy of Social Sciences (Shanghai, China).

Experimental Methods

Cell culture

Gastric cancer cell lines, MKN28, SGC-7901, MKN45, and normal human gastric tissue cell line GES-1 were subcultured in an incubator with cell culture medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin (37°C, 5% CO_2 and 95% relative humidity). Cultured cells were subcultured every 24-48 h, and cells in the logarithmic growth phase were collected for subsequent experiments.

Extraction of the RNA

Gastric cancer and normal gastric tissues stored in liquid nitrogen were ground into powder, followed by the addition of TRIzol reagent. They were mixed completely, and chloroform was then added, followed by centrifugation to collect supernatant, which was mixed with isopropanol, followed by centrifugation at 4°C. Finally, diethyl pyrocarbonate (DEPC)-treated water was used to dissolve RNA, and optical density at the wavelength of 260 nm (OD260 nm) and OD280 nm were measured using an ultraviolet spectrometer. The ratio of OD260/OD280 between 1.8 and 2.0 represented satisfactory RNA quality. RNA samples were stored at -84°C before use.

Quantitative RT-PCR

Complementary DNA (cDNA) was synthesized by reverse transcription, and PCR reaction system was prepared using SYBR Green Upstream primers of human MYOZ2 genes: 5'-TAAGATGCGACAAAGAAGAT-3'; downstream primers: 5'-TAGGAGGAGTAAATGGTGCT-3', and the amplified fragment length was 155 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was the endogenous control. Upstream primers of GAPDH: 5'-GTCACCAACTGGGACGACA-3'; downstream primers: 5'-AGGCGTACAGGGACAGCA-3'; the length of products was 118 bp. PCR reaction conditions: 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. 2-ΔΔCt method was used to analyze the expression level of MYOZ2 messenger RNA (mRNA) [ΔCt = Ct (MYOZ2) mean value – Ct (endogenous control GAPDH) mean value; ΔΔCt = ΔCt (MYOZ2 in random normal gastric tissues) mean value – ΔCt (the endogenous control GAPDH) mean value. Patients with a relative expression level of MYOZ2 mRNA higher than median value were grouped into high expression group, and the remaining patients were grouped into the low expression group.

Western Blotting

BCA protein detection kit was used to quantify total protein. GAPDH was used as endogenous control. Polyclonal antibodies of MYOZ2 proteins (Abcam, Cambridge, MA, USA) were used at a dilution of 1/500, and GAPDH monoclonal antibodies were used at a dilution of 1/1000. 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12 μg protein from each sample. OD was measured to estimate the relative expression level of MYOZ2.

Cell Transfection

SiRNA was used to interfere with the expression of MYOZ2 in cells. MKN45 cells were collected during logarithmic growth and seeded into 6-well plate with 5.0X104 per well. After incubation for 24 h, cell culture medium containing 5% serum was replaced by serum-free medium. Transfection master mixture was prepared according to the instructions of Lipofectamine 2000, and cells were mixed with transfection master mixture. After incubation for 5-8 h, cells were transferred into culture medium containing 5% serum.

Detection of in-vitro Proliferation of Gastric Cancer Cells by MTT Assay

MKN45 cells and MKN45 cells with MYOZ2 knockdown were collected during logarithmic growth phase to prepare single cell suspension. Cells were inoculated into 96-well cell culture plate, and 20 μL MTT (5 mg/mL) were added into each well 6 h later. After incubation for 4 h at 37°C, dimethylsulfoxide was added, and OD values at wavelength of 570 nm were measured using a microplate reader. The above procedures were repeated at 12 h, 24 h, 36 h, 48 h and 72 h, respectively.

Detection of the Migration and Invasion Abilities of Gastric Cancer Cells by Transwell Chamber Assay

Transwell cell migration and invasion assay (BD Biosciences, Franklin Lakes, NJ, USA) were performed to measure cell migration and invasion ability. Briefly, the upper chamber was filled

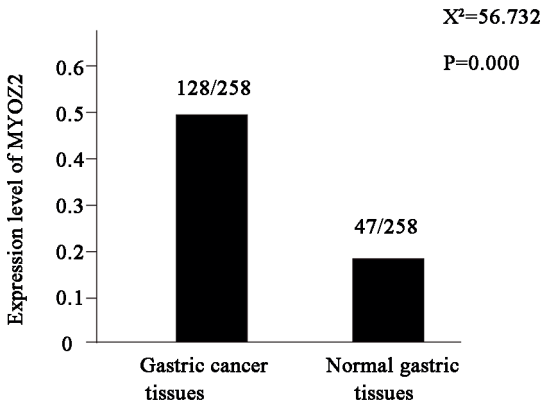

Figure 1. Comparison of the expression level of MYOZ2 between gastric cancer tissues and normal gastric tissues. Expression level of MYOZ2 mRNA in gastric cancer tissues was higher than that in adjacent normal tissues (p<0.01).

with 5x104 cells. Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 20% FCS (Sigma-Aldrich, St. Louis, MO, USA) was used to fill the lower chamber. After incubation for 24 h, membranes were collected and stained with 0.5% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) for 20 min. Stained cells were counted under an optical microscope (Olympus, Tokyo, Japan). Upper chamber was pre-coated with Matrigel (356234, Millipore, Billerica, MA, USA) in invasion assay.

Statistical Analysis

Data were processed by Statistical Product and Service Solutions (SPSS Inc., Armonk, NY, USA) 22.0, and the t-test was used for comparisons of measurement data (expressed as $\bar{x} \pm s$). The χ^2-test was used for comparisons of count data. Univariate analysis was performed by Log-rank test, and variables with statistical significance (p<0.1) were subjected to Cox multivariate analysis. Survival curves were plotted using Kaplan-Meier method. p<0.05 represented differences with statistical significance.

Results

Expression of MYOZ2 mRNA in Gastric Cancer Tissues

The expression of MYOZ2 in gastric cancer tissues and adjacent normal tissues was detected by RT-PCR. Results showed that the expression level of MYOZ2 mRNA in gastric cancer tissues was higher than that in adjacent normal tissues in more patients (128/258). Only a few patients had higher expression level of MYOZ2 mRNA in adjacent normal tissues than that in cancer tissues (47/258). The difference was statistical significant (Figure 1). Those data suggest that MYOZ2 genes are most highly expressed in gastric cancer tissues than that in adjacent normal tissues.

Expression of MYOZ2 Proteins in Gastric Cancer Line

Western blot was used to detect the expression of MYOZ2 protein in different cell lines with GAPDH protein as endogenous control. Results showed that the MYOZ2 protein expression level in MKN45 cells was 0.854 ± 0.58, while in normal human gastric cell line GES-1 was 0.142 ± 0.14, significant difference was found between them (p<0.05) (Figure 2).

Detection of the Proliferation Ability of MKN45 Cells by MTT Assay

MTT method was used to detect the proliferation of MKN45 cells with and without MYOZ2 knockdown. The growth curve showed that after 6 h, proliferation rate of MKN45 cells with MYOZ2 knockdown was significantly lower than that of MKN45 cells without MYOZ2 knockdown (p<0.05) (Figure 3).

MKN45 Cell Invasion Detected by Transwell Invasion Assay

The number of MKN45 cells invaded the membrane was (73.2 ± 5.6) and the number of cells MKN45 with MYOZ2 knockdown invaded the

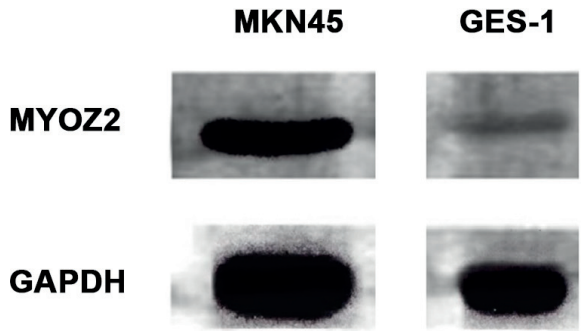

Figure 2. Comparisons of the expression level of MYOZ2 proteins between MKN28, SGC-7910 and MKN45 gastric cancer cells and the GES-1 normal gastric tissue cells. The expression level of MYOZ2 proteins was significantly increased in gastric cancer cells, and the increase was most significant in MKN45.

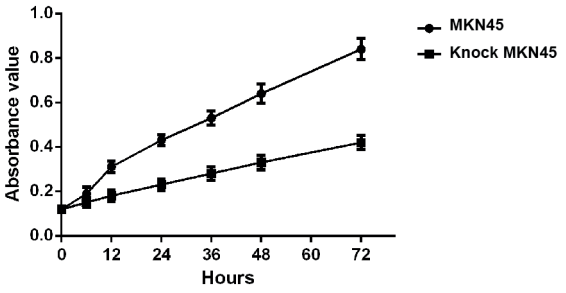

Figure 3. Comparison of the proliferation ability between MKN45 and MKN45 cells with MYOZ2 knockdown. MTT assay was used to detect MKN45 and MKN45 cells with MYOZ2 knockdown. Results showed that the proliferation rate of MKN45 cells was significantly higher than those of MKN45 cells with MYOZ2 knockdown; the differences are statistically significant ($p<0.05$).

membrane was 38.2±5.1. Significant difference was found between them ($p<0.05$) (Figure 4).

Correlations Between Expression of MYOZ2 in Gastric Cancer and Clinicopathological Features

No significant correlations of high expression of MYOZ2 with age, gender, tumor location, differentiation degree, smoking, and Helicobacter pylori infection were found ($p>0.05$). However, high expression of MYOZ2 was significantly correlated with tumor diameter ($\chi^2=5.173$, $p=0.023$), lymph node metastasis ($\chi^2=14.802$, $p=0.001$), pTNM staging ($\chi^2=14.879$, $p=0.002$), carcinoembryonic antigen level ($\chi^2=3.978$, $p=0.046$) and CA19-9 level ($\chi^2=67.55$, $p=0.001$) (Table II).

Multivariate Cox Regression Analysis of Factors Related To The Survival of 258 Patients With Gastric Cancer

Factors with statistical significance in univariate analysis ($p<0.01$) were further subjected to multivariate Cox regression analysis. Results showed that the expression of MYOZ2, lymph node metastasis and pTNM staging were the independent prognostic factors for gastric cancer ($p<0.05$). However, tumor size showed no significant influence on survival (Table III). In 258 patients with gastric cancer in this study, 1 patient died in the perioperative period, and 19 patients were out of touch and died for other causes. Follow-up completion rate was 92.6%, and follow-up lasted for 60 months. The 5-year survival rate in the MYOZ2 high expression group was 41.4%, while that in the MYOZ2 low expression group was 60.8%, and the difference was statistically significant ($\chi^2=11.303$, $p<0.01$) (Figure 5).

Discussion

Genetic studies[11,12] have shown that the development of different types of cancer are affected by the function of certain gene. With the highest incidence and mortality rate among all gastrointestinal cancers in our country, gastric cancer is usually diagnosed at advanced stages. Understanding the pathogenesis of gastric cancer is a key step for the treatment of this disease[13-15]. This study aimed to investigate the correlations between MYOZ2 expression and the clinicopathological features of gastric cancer, and to explore the effects of MYOZ2 on tumor cell proliferation and invasion. It has been confirmed that increased expression level of MYOZ2 is a leading cause of hypertrophic diseases16-18. MYOZ2 is also highly expressed in a variety of tumor cells and cardiomyocytes[16-18], indicating that MYOZ2 could affect the prognosis of cancer. At present, MYOZ2 expression pattern in gastric cancer and its effect on prognosis of this disease is still unclear. In this study, results of RT-PCR and Western blot showed that expression level of MYOZ2 was significantly higher in gastric cancer tissue than that in normal gastric tissue. In vitro experiments showed that MYOZ2 knockdown can inhibit the proliferation and invasion of gastric cancer cells. A chip screening carried out by Jia et al[19] showed

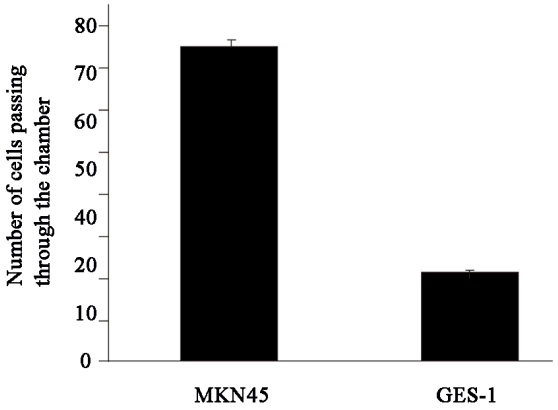

Figure 4. Comparison of the cell invasion ability between MKN45 cells and MKN45 cells with MYOZ2 knockdown. Transwell chamber assay was performed to detect the cell invasion ability. The number of MKN45 cells passed through the chamber was significantly bigger than that of MKN45 cells with MYOZ2 knockdown ($p<0.05$).

Table II. Correlations between expression of CSDAP1 in gastric cancer and clinicopathological features.

Parameter	People (n=258)	%	MYOZ2 High expression (n=128)	Low expression (n=130)	χ^2	*p*
Gender						
Male	167	64.7	83	84		
Female	91	35.3	45	46	0.001	0.969
Age (years old)						
<60	115	44.6	59	56		
≥60	143	55.4	69	74	0.238	0.626
Tumor size						
<5 cm	113	43.8	47	66		
≥5 cm	145	56.2	81	64	5.173	0.023
Tumor location						
Upper part	52	20.1	30	22		
Medium part	61	23.6	33	28		
Lower part	135	52.3	65	70	1.569	0.456
Differentiation degree						
No or low differentiation	142	55.0	75	67		
Medium and high differentiation	116	45.0	53	63	1.297	0.255
Lymph node metastasis						
No	64	24.8	27	37		
Perigastric area	94	36.4	38	56		
Beyond perigastric area	100	38.8	63	37	11.755	0.003
pTNM staging						
Stage I	32	12.4	8	24		
Stage II	73	28.3	31	42		
Stage III	105	40.7	58	47		
Stage IV	48	18.6	31	17	14.879	0.002
Smoking (400 cigarettes/day)						
No	92	35.7	54	48		
<400	67	30.0	30	25		
≥400	99	38.3	44	55	3.113	0.210
Helicobacter pylori						
Positive	162	62.8	84	78		
Negative	96	37.2	45	51	0.597	0.439
Carcinoembryonic antigen (CEA)						
Normal or decreased (≤ 5.0 ng/ml)	127	49.2	55	72		
Increased (>5.0 ng/ml)	131	50.8	73	58	3.978	0.046
CA19-9						
Normal or decreased (3.7 kU/L)	77	29.8	8	69		
Increased (>3.7 kU/L)	181	70.1	120	61	67.55	0.001

that MYOZ2 was highly expressed in gastric cancer cells. Zhang et al2[0] found that increased expression of calcium and phosphorus regulatory proteins in ovarian cancer significantly shortened the cell cycle time and induced cell apoptosis stagnation, suggesting that MYOZ2 can affect proliferation of tumor cells. Our findings and previous studies showed that upregulation of MYOZ2

Table III. Multivariate Cox regression analysis influenced by the survival of 258 patients with gastric cancer.

Factor	Estimated value	Standard error	Wald value	*p*-value	Odds ratio (OD)	95% confidential interval (95% CI)
MYOZ2 expression	-0.598	0.183	11.542	0.000	0.638	0.384-0.798
Tumor size	-0.072	0.202	0.187	0.548	0.987	0.695-1.422
Lymph node metastasis	0.523	0.169	10.872	0.002	1.709	1.139-2.015
TNM staging	0.368	0.121	9.014	0.015	1.308	1.036-1.997

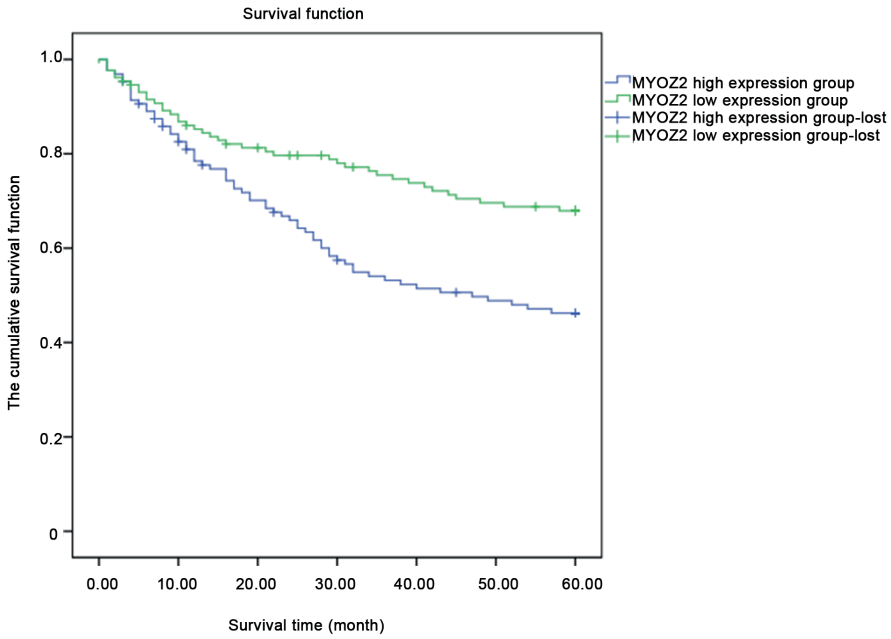

Figure 5. Survival of 258 patients with gastric cancer. The 5-year survival rate of patients with low expression level of MYOZ2 was significantly higher than that of patients with high expression level of MYOZ2 ($p<0.01$).

expression in gastric cancer may promote the development and progression of tumor. However, the mechanism of the role of MYOZ2 in gastric cancer remains to be further studied.

Correlation analysis between MYOZ2 and clinicopathological characteristics showed that MYOZ2 was associated with tumor diameter, lymph node metastasis and pTNM staging, indicating that high expression level of MYOZ2 may indicate poor prognosis. Follow-up study showed that the 5-year survival rate of patients with high MYOZ2 expression level was significantly lower than that of patients with low MYOZ2 expression level, indicating that MYOZ2 may have important prognostic value for gastric cancer.

There are still some shortcomings in this study. The sample size is small and geographical differences were not excluded. Our future study will include more patients in different races and from different regions to further confirm the conclusions.

Conclusions

We found that MYOZ2 can be used as prognostic factor for gastric cancer. MYOZ2 is lowly expressed in normal tissues, but highly expressed in cancer tissues, and MYOZ2 gene may play an important role in the occurrence and development of gastric cancer. Detection of MYOZ2 may provide references for the prognosis of gastric cancer.

Acknowledgements

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Ethics approval and consent to participate

The study was approved by the Ethics Committee of Harbin Medical University Cancer Hospital. Signed written informed consents were obtained from the patients and/or guardians

Conflict of Interest

The Authors declare that they have no conflict of interest.

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