ORIGINAL ARTICLE

Expression and prognostic analysis of AEG-1, NKD-1 and β-catenin in gastric cancer tissues

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ABSTRACT

Objective: To investigate the expressions of AEG-1, NKD-1 and β-catenin in gastric cancer (GC), and analyze their relationship with clinicopathological features and prognosis and the correlation among the three proteins.
Methods: Fifty GC tissues and 50 adjacent normal tissues of patients who underwent radical gastrectomy in the Third Affiliated Hospital of Inner Mongolia Medical University during December 2015 to June 2017 were collected. The expressions of AEG-1, NKD-1 and β-catenin were detected by immunohistochemical SP method and qRT-PCR. The relationship between positive expression and clinical features and survival time of patients was analyzed, and the correlation between the three was analyzed.
Results: The positive expression rates of AEG-1 and β-catenin in GC were 74% (37/50), 60% (30/50), respectively, which was significantly higher than that of paracancer tissue (18% [9/50] and 26% [13/50]). The positive expression rate of NKD1 in GC tissues was 20% (10/50), which was significantly lower than that in adjacent tissues [88% (44/50)]; and the differences were statistically significant ($p <$. 05). AEG-1 mRNA expression level (1.0148$\pm$0.0019) and β-catenin mRNA expression level (1.0000$\pm$0.0005) in GC tissues were significantly higher than that of AEG-1 mRNA expression level (1.0002$\pm$0.0036) and β-catenin expression level in adjacent tissues mRMA expression level (0.9984$\pm$0.0012), and the differences were statistically significant ($p <$ .05). The expression level of NKD1 mRNA in GC tissues (1.0008$\pm$0.0073) was significantly lower than that in adjacent tissues (1.0291$\pm$0.0041), and the differences were statistically significant ($p <$ .05). The positive expression of AEG-1, NKD1 and β-catenin in GC tissues was not related to age, sex or tumor size ($p >$ .05), but was related to differentiation degree, TNM stage and lymph node metastasis ($p <$ .05). In GC, there was a positive correlation between AEG-1 and β-catenin protein expression (r = .678, $p <$ .001), and a negative correlation between AEG-1 and NKD1 protein expression (r = -.323, p = .012). β-catenin expression was negatively correlated with NKD1 expression (r = -.347, p = .007). The survival time of patients with positive expression of AEG-1 and β-catenin was significantly shortened compared with those with negative expression ($p <$ .05), while the survival time of patients with positive expression of NKD1 was longer than that of patients with negative expression of NKD1 ($p <$ .05).
Conclusions: The abnormal expression of AEG-1, NKD-1 and β-catenin is closely related with the occurrence, development, invasion, metastasis and prognosis of GC, which provide a therapeutic target and may become markers for prognosis evaluation.

Key Words: Gastric cancer, AEG-1, NKD-1, β-catenin

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1. INTRODUCTION

The gastric cancer (GC) is one of common malignant tumors in the world. The number of onset and death rank the second in our country, and it is one of the most aggressive malignant tumors.[1] Due to the slow growth of GC, no obvious symptoms and signs in the early stage and unclear pathogenesis of GC, the early detection rate is low, the long-term prognosis is poor, and the mortality is high. Therefore, the research on the regulatory mechanism of GC has gained time for the early diagnosis and treatment of GC. In recent years, it has been found that Wnt signaling is involved in stem cell development, homeostasis and many other aspects, and its abnormal regulation may lead to the occurrence of GC.[2] Therefore, in this study, the expression of core factor β-catenin, passive antagonist NKD1 and AEG-1 in GC tissues, clinicopathological features, long-term prognosis and the correlation among the three factors were discussed, and the possible role of AEG-1, NKD1 and β-catenin proteins in the occurrence and development of GC was further discussed. It provides theoretical basis for early diagnosis, treatment and prognosis.

2. DATA AND METHODS

2.1 Source of cases

Fifty cases of GC tissues surgically removed in the Third Affiliated Hospital of Inner Mongolia Medical University from December 2015 to June 2017 were selected. The patients were 30-70 years old, with an average age of 50 years old, including 28 males and 22 females. Their medical records were complete, and none of them received antitumor therapy before surgery. The pathological diagnosis was GC. There were 20 cases of low differentiation, 23 cases of medium differentiation and 7 cases of high differentiation. According to the 7^{th} edition of the International Union Against Cancer (2009) TNM staging of GC:[3] 10 cases of stage I, 19 cases of stage II, 21 cases of stage III; There were 15 cases with lymph node metastasis and 35 cases without lymph node metastasis. In addition, 50 patients with adjacent normal tissues (more than 5 cm away from the tumor) were collected as control group.

2.2 Reagents and instruments

Rabbit anti-human AEG-1, NKD1 polyclonal antibody, mouse anti-human β-catenin monoclonal antibody were purchased from Beijing Boosen Biotechnology Co., LTD., SP two-step assay kit was purchased from Fuzhou Maixin Reagent Co., LTD. RNA extraction kit, reverse transcription kit, real-time fluorescence quantitative kit, PCR primers, RIzol RNA separation reagent, etc., were purchased from Invitrogen, USA. Real-time fluorescence quantitative PCR instrument was purchased from ABI, USA, and optical microscope was purchased from OLYMPUS, Japan.

2.3 Methods

2.3.1 *Immunohistochemical staining*

The expression of AEG-1, NKD-1 and β-catenin was detected by immunohistochemical SP staining. For specific steps, refer to the instructions. The main steps are described as follows: conventional dewaxing, hydration, antigenic repair with citrate buffer solution at high temperature and high pressure, blocking endogenous peroxidase with 3% hydrogen peroxide, adding primary antibody overnight at 4°C, conventional DAB color development, hematoxylin restaining, neutral gum sealing. The positive signals of AEG 1 and NKD1 staining were located in the cytoplasm, while the positive signals of β-catenin staining were located in the cytoplasm and/or nucleus, which were yellow or brownish yellow granules. PBS buffer was used as the negative control instead of primary antibody and known GC positive tablets were used as the positive control. According to the semi-quantitative integral method interpretation results: staining intensity: unstained 0 point; Light yellow 1point; Brownish yellow 2 points; Brown 3 points; Score according to the proportion of positive cells: the proportion of positive cells $\leq$5% : 0 point, 6%-25%: 1 point; 26%-50%: 2 points; 51%-75%: 3 points; > 75% is 4 points; Multiply the two scores: 0-1 is negative, 2-12 is positive. Section scoring was performed independently by two pathologists using a double-blind method and unified standards.

2.3.2 *Detection of AEG-1, NKD-1 and β-catenin mRNA expression by real-time quantitative PCR*

Total RNA of AEG-1, NKD1 and β-catenin were extracted from fresh GC tissues and corresponding adjacent tissues according to the operation guide of total RNA extraction kit. Reverse transcription (RNA prep-pure TissueKit, Quantc DNA and Quantone step qRT-PCR purchased from Invitrogen, USA) was used for post-cDNA amplification and analysis. Using β-actin as internal reference, the upstream and downstream products of NKD1 gene were: 5'-GGGAAACTTCACTCCAAGCC-3', 5'-CTGTCTCCCGATCCACTCCT-3' (120 bp), upstream and downstream products of AEG-1 gene: 5'-ACTGCACAGGACACAGAAGAA-3', 5'-GGCTCGGTAGAAGTAGCAGG-3' (153 bp), upstream and downstream products of β-catenin gene: 5'-GCTCTTGTGCGTACTGTCCT-3', 5'-TGGGCCATCTCTGCTTCTTG 3' (110 bp); upstream and downstream products of β-actin: 5'- GCACTCTTCCAGCCTTCCTT-3', 5'-AATGCCAGGGTACATGGTGG-3' (150 bp). PCR reaction

conditions: 95°C, 10 min, 95°C, 15 s, 60°C, 1 min, a total of 40 cycles. Expression levels of AEG-1, NKD-1 and β-catenin mRNA were determined by $2^{-\Delta\Delta ct}$ assay.

2.4 Follow-up

The follow-up period was August 2, 2022. The follow-up methods included outpatient review, telephone and WeChat group follow-up for survival. Among them, 3 cases were lost to follow-up.

2.5 Statistical processing

Statistical software SPSS19.0 was used to analyze the experimental data. Measurement data with $\bar{x}\pm$ s said that the comparison between the measurement data set t, χ^2 test analysis data. Spearman correlation analysis was used to analyze the correlation of AEG-1, NKD1 and β-catenin, and Kaplan-Meier survival curve was drawn. Log-rank χ^2 test was used for comparison, and $p < .05$ was considered statistically significant.

3. RESULTS

3.1 Expression levels of AEG-1, NKD1 and β-catenin in GC and para cancer tissues

The mRNA relative expressions of AEG-1 and β-catenin in GC tissues (mRNA 1.0148±0.0019, mRNA 1.0000±0.0005) were higher than those in para cancer tissues (mRNA 1.0002±0.0036, mRNA 0.9984±0.0012). The comparison between groups was statistically significant ($p < .001$, $p = .017$). The relative expression of NKD1 mRNA in para cancer tissues (1.0291±0.0041) was higher than that in GC tissues (mRNA 1.0008±0.0073), and the difference between groups was statistically significant ($p = .001$) (see Table 1). The positive expression rates of AEG-1 and β-catenin in GC tissues (74%, 60%) were higher than those in para cancer tissues (18%, 26%), with $p < .001$ between the two groups, and the difference was statistically significant. The positive expression rate of NKD1 in para cancer tissues (20%) was higher than that in GC tissues (88%), and the difference was statistically significant ($p = .001$), as shown in Table 1 and Figures 1-6.

Table 1. Expression of AEG-1, NKD1 and β-catenin in GC and para cancer tissues

Group	Positive expression rate of AEG-1 (%)	Positive expression rate of NKD1 (%)	Positive expression rate of β-catenin (%)	Relative expression level of AEG-1 protein ($\bar{x} \pm s$)	Relative expression of NKD1 protein ($\bar{x} \pm s$)	Relative expression level of β-catenin protein ($\bar{x} \pm s$)
Gastric cancer tissue	37 (74.0%)	10 (20.0%)	30 (60.0%）	1.0148±0.0019	1.0008±0.0073	1.0000±0.0005
Paracancer tissue	9 (18.0%)	44 (88.0%)	3 (26.0%)	1.0002±0.0036	1.0291±0.0041	0.9984±0.0012
χ^2/t	31.56	11.79	46.54	4.207	3.962	1.408
p	< .001	.001	< .001	< .001	.001	.017

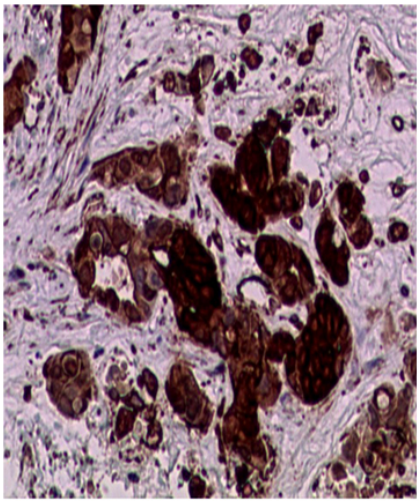

Figure 1. Immunohistochemical results of AEG-1 in gastric cancer (×400)

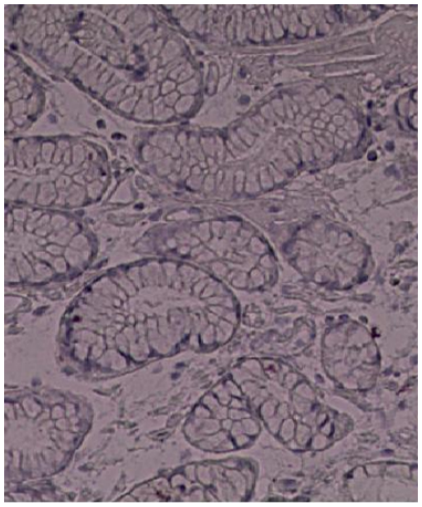

Figure 2. Immunohistochemical results of NKD1 in gastric cancer tissues (×400)

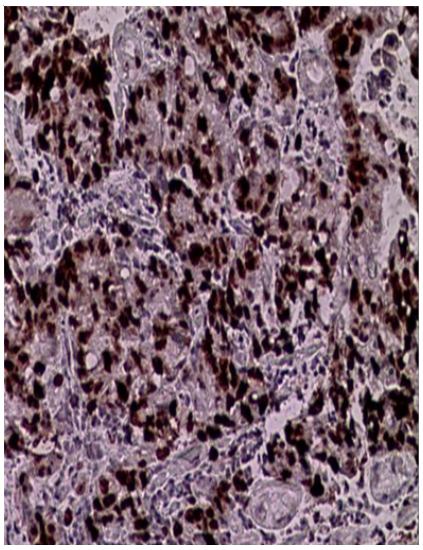

Figure 3. Immunohistochemical results of β-catenin in gastric cancer tissues ($\times$400)

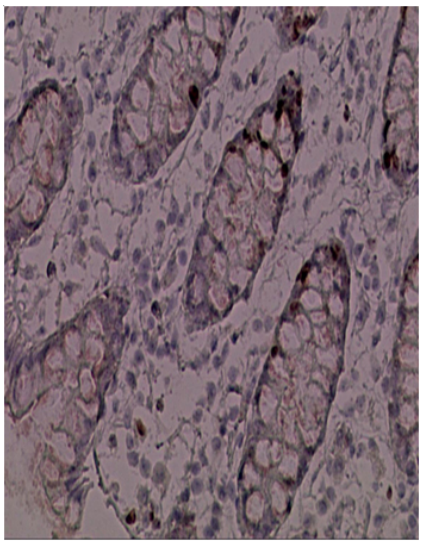

Figure 4. Immunohistochemical results of AEG-1 in para cancer tissues ($\times$400)

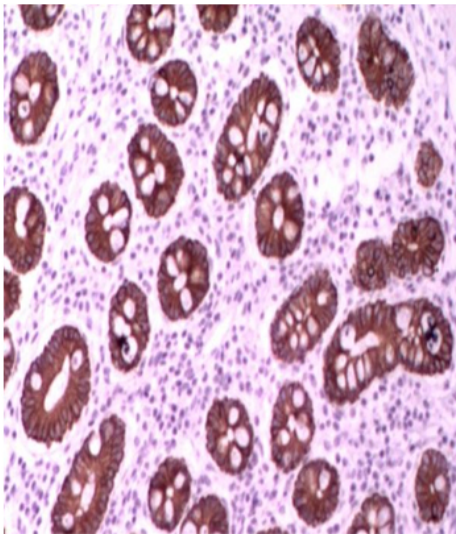

Figure 5. Immunohistochemical results of NKD1 in para cancer tissues ($\times$400)

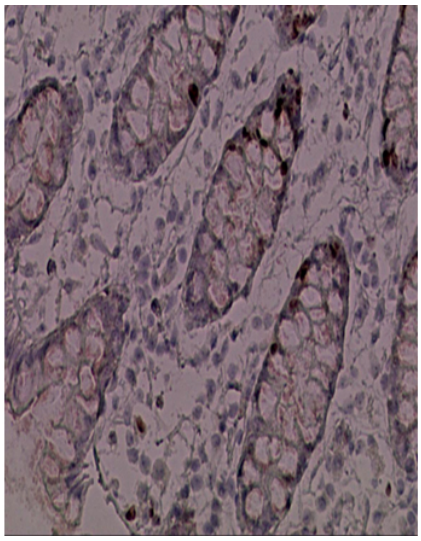

Figure 6. Immunohistochemical results of β-catenin in para cancer tissues ($\times$400)

3.2 Relationship between expression of AEG-1, NKD1 and β-catenin and clinicopathologic features of GC

The positive expressions of AEG-1, NKD1 and β-catenin in GC tissues were not correlated with age, sex or tumor size ($p > .05$, as shown in Table 2). The positive expression of AEG-1, NKD1 and β-catenin in GC was related to the degree of tumor differentiation, TNM staging and lymph node metastasis ($p < .05$, see Table 2).

3.3 Correlation of AEG-1, NKD1 and β-catenin expression in GC

Spearma correlation analysis showed that there was a positive correlation between AEG 1 and β-catenin expression in GC ($r = .678$, $p < .001$) (see Figure 7), and a negative correlation between AEG 1 and NKD1 expression ($r = -.323$, $p = .012$) (see Figure 8). β-catenin expression was negatively correlated with NKD1 expression ($r = -.347$, $p = .007$) (see Figure 9).

3.4 Relationship between expression of AEG-1, NKD1 and β-catenin and prognosis of GC patients

The median follow-up time of the 50 patients was 35 (95%CI: 32.29-43.34) months, and 3 of them were lost to follow-up, with a follow-up rate of 94%. The median survival time of 37 patients with AEG-1 positive expression was 35 months (95%CI: 24.62-45.38), and the 5-year survival

rate was 21.15%. The median survival time of 13 patients with negative expression of AEG-1 was 58 months (95%CI: 47.26-68.74), and the 5-year survival rate was 48.078%. The difference in survival curve was statistically significant (p = .045), as shown in Figure 10. The median survival time of 10 patients with positive expression of NKD1 was 55 months (95%CI: 40.13-69.88), and the 5-year survival rate was 54%. The median survival time of 40 patients with negative expression of NKD1 was 35 months (95%CI: 24.59-45.40), and the 5-year survival rate was 23.19%. The difference in survival curve was statistically significant (p = .046), as shown in Figure 11. The median survival time of 30 patients with β-catenin positive expression was 32 months (95%CI: 14.82-49.18), and the 5-year survival rate was 16.53%. The median survival time of 20 patients with β-catenin negative expression was 55 months (95%CI: 17.44-92.56), and the 5-year survival rate was 48%. The difference of survival curve was statistically significant (p = .047) (see Figure 12).

Table 2. Relationship between expression of AEG-1, NKD1 and β-catenin and clinicopathologic features of GC

Clinicopathologic parameter	n	AEG-1		χ^2	*p*	NKD1		χ^2	*p*	β-catenin		χ^2	*p*
		+	-			+	-			+	-		
Age (years)													
<50	30	19	11	0.06	.812	13	17	0.06	.815	17	13	0.35	.556
≥50	20	12	8			8	12			13	17		
Gender													
Male	28	18	12	0.08	.773	10	18	0.4	.525	15	13	0.01	.945
Female	22	15	7			6	16			12	10		
Cancer size													
< 2 cm	27	15	12	0.48	.487	12	15	0.14	.704	15	12	0.06	.811
≥ 2 cm	23	15	8			9	14			12	11		
Degree of differentiation													
Low differentiation	30	12	18	5.92	.015	19	11	11.38	.001	8	22	5.56	.018
Medium-high differentiation	20	15	5			3	17			12	8		
TNM staging													
Stage I+II	29	10	19	15.68	.001	18	11	11.42	.001	9	20	7.96	.005
Stage III	21	19	2			3	18			15	6		
Lymph node metastasis													
Yes	15	13	2	7.23	.007	2	13	8.18	.004	14	1	13.35	.001
No	35	16	19			20	15			13	22		

4. DISCUSSION

At present, there is no common understanding of the carcinogenic mechanism of GC, and no specific characteristics of gene expression of GC have been found. Therefore, it is an urgent problem for us to understand the molecular mechanism of the progression of GC, and to find and determine a specific target gene that can make effective early diagnosis, early prognostic markers and new therapeutic targets. Dysregulation of Wnt/β-catenin signaling is a common feature of many human cancers. In this study, related protein factors in GC tissues may participate in the occurrence and development of GC by regulating Wnt pathway from the level of protein and gene.

AEG-1 is crucial in tumor transformation, escape from apoptosis, invasion, metastasis and angiogenesis, and is regarded as a multifunctional factor that can regulate a variety of signal transduction pathways.[4] In this study, the expression of AEG-1 protein and mRNA in GC tissues and corresponding paracancer tissues was detected at protein and gene levels. The results showed that the positive expression rate and mRNA relative expression level of AEG-1 in GC tissues were significantly higher than those in paracancer tissues. Consistent results were obtained at the protein and gene levels. This is consistent with the research results of Huang Yong.[5] Further study on the relationship between the abnormal expression of AEG-1 and different pathological features of GC

tissue showed that AEG-1 was highly expressed in the poorly differentiated GC tissue group, lymph node metastasis group, and TNM stage III group. Huang Yong et al.[4] found that the positive expression of AEG-1 in GC tissues was related to the degree of differentiation, TNM staging, and lymph node metastasis. Wu[6] found that AEG-1 promoted the epithelial-mesenchymal transformation (EMT), migration and invasion of GC cells, and the increased expression of AEG-1 was related to metastasis in GC tissues. Therefore, we speculate that AEG-1 plays the role of an oncogene and may promote the occurrence and development of tumors by regulating the Wnt/β-catenin signaling pathway. In addition, it can promote the invasion and metastasis of tumor and contribute to the prognosis evaluation of GC.

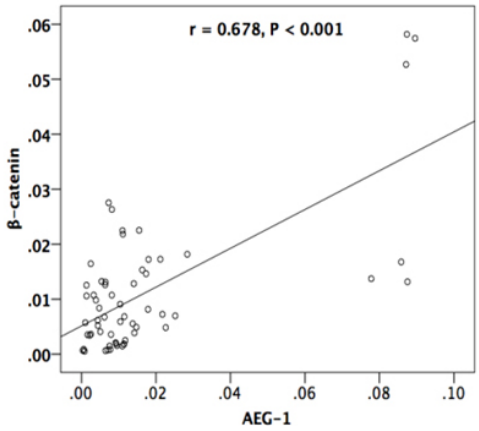

Figure 7. Correlation between expression of AEG-1 and β-catenin in gastric cancer tissues

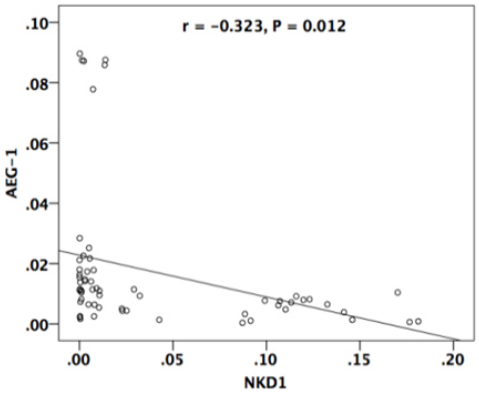

Figure 8. Correlation between expression of AEG-1 and NKD1 in gastric cancer tissues

NKD1 is a negative regulator of the classic Wnt/β-catenin pathway, and exerts a tumor inhibitory effect by negatively regulating the expression of the core factor β-catenin in the Wnt pathway.[7] NKD1 - mediated tumor inhibition has received much attention in recent years. Studies have confirmed that NKD1 is low expressed in tumor tissues of breast cancer, colorectal cancer and small cell lung cancer,[8–10] which is closely related to the occurrence of tumors. In this study, it was found that the positive expression rate of NKD1 and the relative expression level of mRNA in the GC group were significantly lower than those in the paracancer cancer group, which was consistent with the above research reports. In this study, the positive expression of NKD1 in GC tissues was closely related to the degree of differentiation, TNM staging and lymph node metastasis. Studies have shown that[11] miR-532 promotes the migration and invasion of GC cells by inhibiting NKD1 and activating Wnt/β-catenin pathway, similar to the results of this study. It is suggested that NKD1 gene may act as a tumor suppressor gene to inhibit the occurrence of GC, which provides a potential target for the treatment of GC.

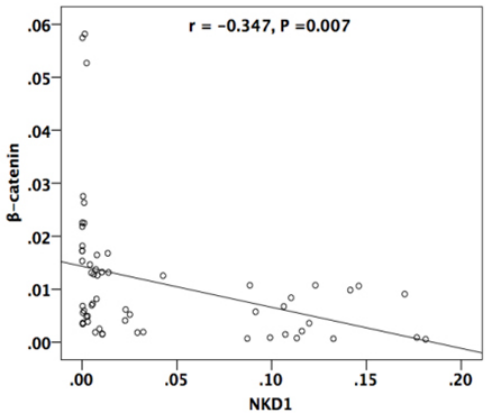

Figure 9. Correlation between β-catenin and NKD1 expression in gastric cancer

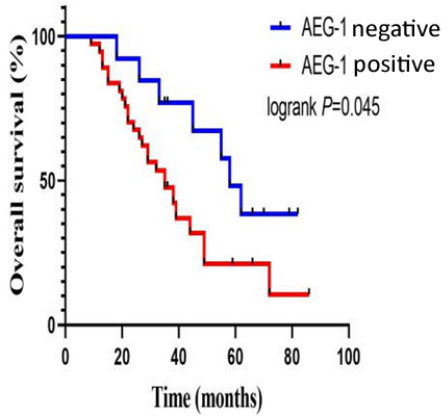

Figure 10. Survival curve of patients with negative and positive expression of AEG-1

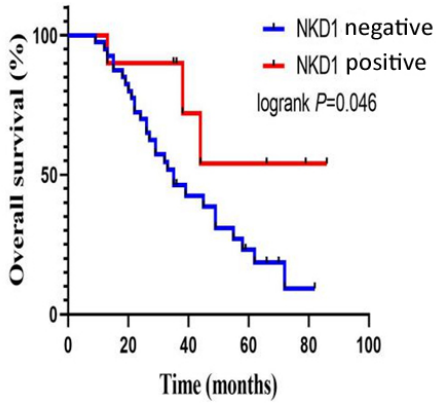

Figure 11. Survival curves of patients with negative and positive expression of NKD1

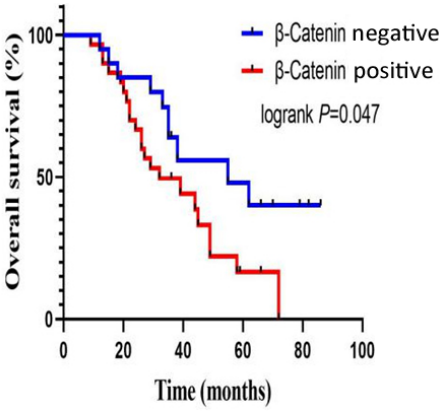

Figure 12. Survival curves of patients with negative and positive expression of β-catenin

β-catenin is the core factor of the classical Wnt/β-catenin signaling pathway, which acts as a hub to connect upstream and downstream genes, and acts as a transcriptional activator to transmit Wnt signals. At the same time, β-catenin is also involved in epithelial-mesenchymal transformation[12] (EMT) to promote tumor invasion and metastasis, and the abnormal β-catenin can be regarded as a sign of promoting cancer. Li[13] reported that compared with normal gastric mucosa, the positive expressions of miR-214 and β-catenin in GC tissues were significantly increased. In this study, β-catenin positive expression in GC tissues was higher than that in paracancer tissues, which was consistent with the above literature reports. Moreover, β-catenin was highly expressed in GC tissues in the TNM stage III group, the poorly differentiated group, and the group with lymph node metastasis, which was basically consistent with the findings of Qi Hongxia.[14] Therefore, it can be speculated that the abnormal expression of β-catenin protein significantly promotes the proliferation, diffusion and metastasis of GC cells. Correlation studies showed that the expressions of AEG-1, NKD1 and β-catenin were significantly correlated in GC tissues. Studies have reported that in CRC, the high expression of AEG 1 is positively correlated with β-catenin expression,[15] while the expression of NKD1 and β-catenin is negatively correlated,[8] which is consistent with the results of this study. Further follow-up showed that the 5-year survival rate and median survival time of AEG I and β-catenin positive expression group were significantly lower than those of AEG I and β-catenin negative expression group, while the 5-year survival rate and median survival time of NKD1 positive expression group were higher than those of NKD1 negative expression group. These results suggest that the high expression of AEG-1, β-catenin and low expression of NKD1 are significantly correlated with the prognosis of patients with GC, and may become a new marker to evaluate the prognostic value of patients with GC.

In conclusion, AEG-1 plays a carcinogenic role in the generation and development of GC by affecting the key factor β-catenin in the Wnt pathway. AEG-1 overexpression may be a useful prognostic factor in patients with GC, and targeted inhibition of AEG-1 may provide a novel therapeutic strategy for GC. NKD1 exerts anti-cancer effect by negatively regulating β-catenin, a key factor of Wnt pathway. The combined detection of the three may have far-reaching significance for understanding the biological behavior of GC, and intervening its interaction pathway may become a new way to block the progression of the disease and treat GC. At the same time, it may also be a marker for prognostic evaluation of GC. Due to the small sample size of this study, more comprehensive studies are needed to confirm the relationship between the three and the specific carcinogenic mechanism of GC.

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CONFLICTS OF INTEREST DISCLOSURE

The authors declare that they have no known competing financial interests or personal relationships that could have

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Obtained.

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DATA SHARING STATEMENT
No additional data are available.

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