

Research Article

Serum CYR61 as a potential biomarker for the diagnosis of esophagogastric junction tumor

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Background: Esophagogastric junction tumor (EGJ) is a rare but fatal disease with a rapid rising incidence worldwide in the late 20 years, and it lacks a convenient and safe method for diagnosis. The present study aimed to evaluate the potential of serum CYR61 as a biomarker for the diagnosis of EGJ tumor.

Methods: Enzyme-linked immunosorbent assay (ELISA) was used to estimate CYR61 levels in sera of 152 EGJ tumor patients and 137 normal controls. Receiver operating characteristics (ROC) was carried out to evaluate the diagnostic accuracy. The Mann–Whitney's *U* test was used to compare the difference of serum levels of CYR61 between groups. And chi-square tests were employed to estimate the correlation of the positive rate of serum CYR61 between/among subgroups.

Results: Serum CYR61 levels were statistically lower in EGJ tumor and early-stage EGJ tumor patients than those in normal controls ($P < 0.0001$). The sensitivity, specificity and the area under the curve (AUC) of this biomarker in EGJ tumor were 88.2%, 43.8% and 0.691, respectively, and those for early stage of EGJ tumor were 80.0%, 66.4% and 0.722, respectively. Analyses showed that there was no correlation between the clinical data and the levels of CYR61 ($P > 0.05$).

Conclusion: The present study showed that CYR61 might be a potential biomarker to assist the diagnosis of EGJ tumor.

Introduction

Esophagogastric junction (EGJ) tumor is a rare but fatal disease with a rapid rising incidence worldwide in the late 20 years [1]. Studies have shown that incidence rate of EGJ tumor in China is higher than that in western countries [2–4]. Adenocarcinoma is the most common histology type, accounting for more than 90% of all EGJ tumors [5,6]. Due to the lack of epidemiological available data and public supervision, the diagnosis of EGJ tumor has always been complex. So far, the primary strategy in clinic of early detection for EGJ tumor is endoscopy that is invasive, unacceptable to some patients and proved to have side effects [7]. In western countries, patients with EGJ tumors are always diagnosed as advanced cancer with poor prognosis because of the nonspecific symptoms at early stage [1]. What's more, despite a variety of treatment options, such as radical surgery, chemotherapy and radiotherapy, patients with EGJ tumor still appear extremely low survival rate [8–10]. Five-year overall survival (OS) rates with surgery alone are gloomy at approximately 25% [11]. Thus, a reliable and sensitive early detection method that has clinical value for effective treatment and improving the prognosis of patients is urgently needed for EGJ tumor patients.

CCN1/CYR61 is a protein from CCN family, which contains five parts: an N-terminal secretory signal peptide and four functional domains: an insulin-like growth factor-binding protein domain (IGFBP),

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a Von Willebrand factor domain (VWC), a thrombospondin type-1 repeat module (TSP-1) and a CT [12]. It can be induced rapidly by growth factors. As an angiogenic inducer that can promote tumor growth and vascularization, it plays an important part in promoting cell survival, proliferation, differentiation, angiogenesis and inducing apoptosis and senescence by binding directly to the integrins and heparin sulfate proteoglycans or activating multiple signaling transduction pathways [13–15]. This suggests that CYR61 might be useful as a biomarker or therapeutic target in certain diseases. Some studies have indicated that high expression of CYR61 was related to colorectal cancer [16], prostate cancer [17,18], ovarian cancer [19], glioma [20], osteosarcoma [21], gastric cancer [22] and breast cancer [23,24]. Meanwhile, it was proved that the expression of CYR61 reduced in high-grade chondrosarcomas [25], advanced gastric cancers [25], endometrial cancer [26] and lung cancer [27]. What's more, multiple studies showed that CYR61 could be a metastatic biomarker for prediction of poor prognosis of EGJ tumors [28] and a potential diagnosis biomarker for colorectal cancer [29] and endometrial cancer [15]. However, the application of serum CYR61 as a clinical biomarker in the diagnosis of EGJ tumor patients has rarely been reported. The aim of our study is to examine the use of serum CYR61 as a potential biomarker for the diagnosis of EGJ tumor.

Materials and methods

Study sample

In the present study, we set the sample size required for the EGJ tumor group and the normal control group to be equal. In order to estimate the sample size, we calculate it according to the following formula [30,31].

$$\text{Sample size}(n)\text{based on Sensitivity} = \frac{[Z_{1-\alpha/3}^2 \times S_N \times (1 - S_N)]}{(L^2 \times P)}$$

$$\text{Sample size}(n)\text{based on Specificity} = \frac{[Z_{1-\alpha/2}^2 \times S_P \times (1 - S_P)]}{[L^2 \times (1 - P)]}$$

$Z_{1-\alpha/2}$ is the value of Z when the cumulative probability in the normal distribution is equal to $\alpha/2$, When α is 0.05, $Z_{1-\alpha/2}$ is 1.96, and when α is 0.01, $Z_{1-\alpha/2}$ is 2.58. L is the width of the 95% interval of sensitivity or specificity that we allow, which is artificially designated by the researcher, and is generally set at 0.03–0.1. Here, we set the allowable error (L) as 0.1 and α as 0.05. In the preliminary experiment, we concluded that the sensitivity (S_N) of *cyr61* for EGJ diagnosis is 0.4, the specificity (S_P) is 0.9, and the disease prevalence (P) is 0.6.

When we use the sensitivity to estimate the sample size, according to the formula:

$$N1 = \frac{[1.96^2 \times 0.4 \times (1 - 0.4)]}{(0.1^2 \times 0.6)} \approx 154$$

When we use the specificity to estimate the sample size, according to the formula:

$$N2 = \frac{[1.96^2 \times 0.9 \times (1 - 0.9)]}{[0.1^2 \times (1 - 0.6)]} \approx 86$$

Because $N1 > N2$, according to the principle of which general election, and taking $N1$ as a reference, it reminds us that we need to include at least 154 research subjects. For some other reasons, the actual sample size we collected was 152 cases in the EGJ tumor group and 137 cases in the normal control group. Among the 152 serum samples from EGJ tumor patients, 81 were diagnosed at the First Affiliated Hospital of Shantou University Medical College and 71 were diagnosed at Cancer Hospital of Shantou University Medical College from October 2017 to December 2019. And the 137 serum samples from normal controls were selected from the First Affiliated Hospital of Shantou University Medical College. All the normal samples were healthy subjects without cancer signs. After being coagulated at room temperature for 30 min and centrifuged at 1250g for 5 min, all the serum samples were stored at -80°C until use.

The diagnosis of EGJ tumor was confirmed by histopathology and the tumor stage was referred to the eighth edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual [32]. In the present study, EGJ tumor with TNM stage 0 + I + IIA was defined as early-stage EGJ tumor.

Enzyme-Linked Immunosorbent Assay (ELISA) for CYR61

The Serum concentrations of CYR61 were tested by ELISA Kit based on manufacturer's directions. Reagents, samples and standards were prepared as instructed. The CYR61 standard concentrations for standard curve were 0, 78, 156,

Table 1 Participant information and clinicopathological characteristics

Group	EGJ tumor patients (n=152)	Normal controls (n=137)
Age, years		
Mean ± SD	64.34 ± 9.578	48.32 ± 12.529
Range	22-93	24-81
Gender		
Male	125	65
Female	27	72
Smoke		
Yes	25	
No	72	
Unknown	55	
TNM Stage		
0	2	
I	13	
II	6	
III	29	
IV	44	
Unknown	58	
Histological stage		
High (Grade 1)	5	
Middle (Grade 2)	19	
Low (Grade 3)	27	
Unknown	101	
Depth of tumor invasion (T staging)		
Tis	2	
T1	12	
T2	9	
T3	22	
T4	45	
Unknown	62	
Regional lymph nodes (N staging)		
N0	27	
N1	19	
N2	16	
N3	27	
Unknown	63	
	Metasstasis	
M0	83	
M1	9	
Unknown	60	

Abbreviation: EGJ tumor, esophagogastric junction tumor.

312, 625, 1250, 2500 and 5000 pg/ml, respectively. It was proved in our preliminary experiments that the most appropriate dilution ratio was 1:1. After adding 100 µl standards and serum samples (a 2-fold dilution) per well, the 96-well plate were incubated for 2 h at 37°C. Then the liquid was removed and 100 µl of biotin-antibody (1×) was added to each well and incubated for 1 h at 37°C followed by washing the plate for three times using microplate washer with water buffer. Before accomplishing the same washing procedure for another five times, 100 µl of HRP-avidin was added to each well and incubated for 1 h at 37°C. After adding 90 µl TMB substrate to each well, the plate was incubated for 20 min at 37°C protected from light. Color formation was stopped by 50 µl Stop Solution, and the optical density (OD) value was read at wavelength of 450 and 590 nm on a plate microplate reader within 5 min. Corresponding concentrations were converted from OD values using standard curve method (Table 1). Each serum sample was tested twice and the average value was taken for analysis.

Table 2 Comparison between three groups

	N	Mean ± SD	P value	95%CI
EGJ tumor	152	258.515 ± 191.736	*(<0.0001)	227.788–289.243
Early-stage EGJ tumor (0+I+IIA)	15	225.146 ± 114.316	*(<0.0001)	161.840–288.453
Normal controls	137	429.115±273.432		382.917–475.312

*compared with normal controls; EGJ tumor, esophagogastric junction tumor.

Statistical analysis

With Microsoft Excel, SPSS (version 20.0), GraphPad Prism 8.0 and Sigma Plot 10.0 software, data analyses were performed statistically. The Mann–Whitney’s *U* test was used to compare the difference of serum levels of CYR61 between EGJ tumor group and normal group, early-stage EGJ tumor group and normal group. And chi-square tests were used to estimate the correlation between different clinical data and the positive rate, and the correlation between different groups. Plotting ROC curves and calculating the area under ROC curves (AUC) [33] with 95% confidence interval were used to analyze the accuracy of diagnostic value. The optimum cut-off values were obtained from the Youden’s indexes of the ROC curves and the maximum indexes were calculated by the sum of sensitivity and specificity minus 1. And sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV), false positive rate (FPR), false negative rate (FNR), positive likelihood ratio (PLR) and negative likelihood ratio (NLR) were calculated using the optimum cut-off values to further evaluate the diagnostic value. $P < 0.05$ (two-sided) was considered as statistically significant in all the analyses.

Results

The levels of serum CYR61 in EGJ tumor patients and normal controls

In our study, 289 serum samples were tested, including EGJ tumor group ($n=152$) and normal control group ($n=137$), with the mean age of 64 years old and 48 years old respectively. (Table 1) The mean concentration of serum CYR61 in EGJ tumor group ($n=152$), early-stage EGJ tumor group ($n=15$) and normal group ($n=137$) was 258.515 ± 191.736 ng/ml, 225.146 ± 114.316 ng/ml, and 429.115 ± 273.432 ng/ml, respectively (Table 2). There was a difference between the distribution of EGJ tumor group and normal control group. The EGJ tumor group accounted for more histogram volume at low concentration while normal group accounted for more at high concentration (Figure 1). More intuitive distribution and dispersion could be seen in combined scatter plot and box plot (Figure 2). Confirmed by statistics, the level of serum CYR61 in EGJ tumor group was lower than that in normal controls ($P < 0.0001$), and there was also a significant difference between early-stage EGJ tumor group and normal control group ($P < 0.0001$). (Table 2)

The diagnostic value of serum CYR61 in EGJ tumor and early-stage EGJ tumor

ROC curve was established to evaluate the diagnostic value of CYR61 in EGJ tumor. According to the ROC curve of EGJ tumor group and normal group (Figure 3), AUC was 0.691, and the optimized cutoff value of 445.708 ng/ml was selected with specificity of 43.8% and sensitivity of 88.2%. For early-stage EGJ tumor group with AUC of 0.722 and the cutoff value of 281.947 ng/ml, specificity and the sensitivity were 66.4% and 80.0%, respectively. And the positive rates of EGJ tumor and early-stage EGJ tumor were much higher than that of the normal controls (Table 2). In order to better explain the clinical value, more relevant indicators were calculated and the results were displayed with 95% confidence interval, including false positive rate, false negative rate, positive predictive value, negative predictive value, positive likelihood ratio and negative likelihood ratio (Table 3).

Correlation between serum concentration of CYR61 and clinical data in patients with EGJ tumor

The association between serum CYR61 level of patients with EGJ tumor and clinical data variables was shown in Table 4. There was no statistically significant correlation between positive rate of serum CYR61 and clinical data, including age, gender, smoking status, depth of tumor invasion, lymph node status, metastasis, histological grade and early-stage or advanced-stage of EGJ tumor (all $P > 0.05$).

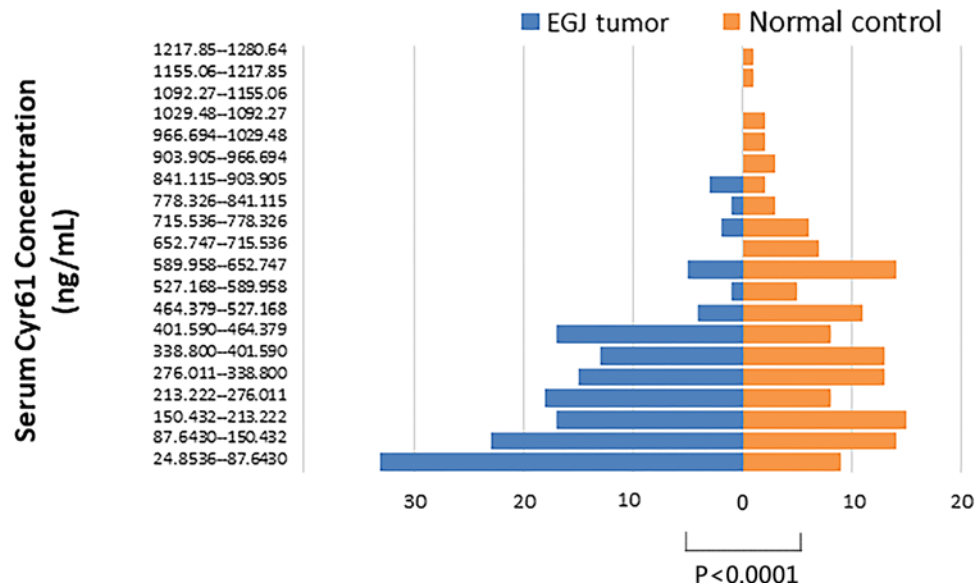


Figure 1. Bar chart of concentration of CYR61 from EGJ tumor serum and normal serum

The diagram of EGJ tumor ($n=152$) is in blue; the one of normal control ($n=137$) is in orange. The lowest concentration was 24.85 ng/ml in EGJ tumor and the highest one was 1280.64 ng/ml in normal control. The concentration was divided into 20 sections equally. EGJ tumor stands for more histogram volume on lower concentration while normal control accounts for more on higher concentration.

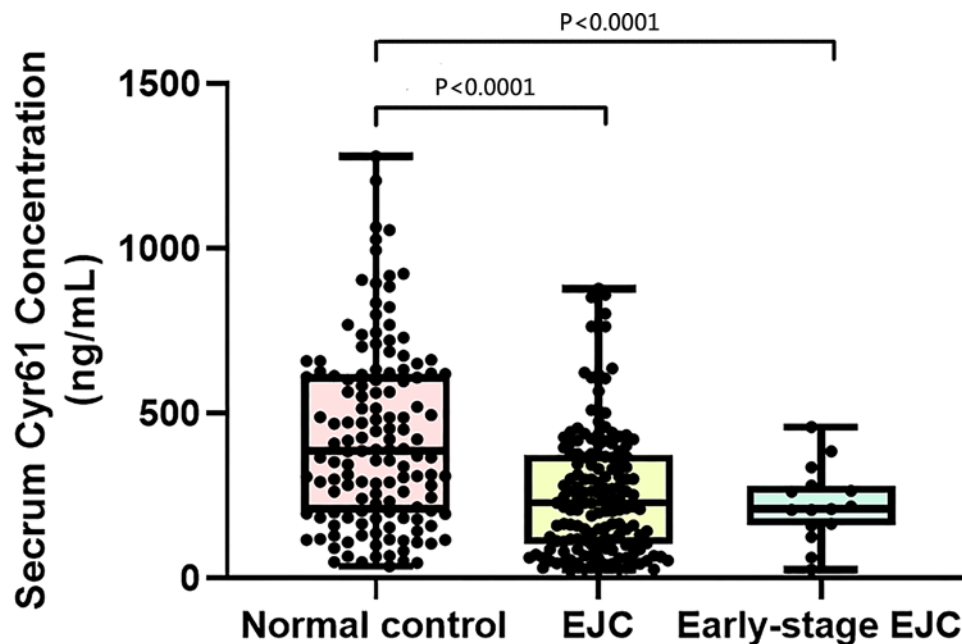


Figure 2. Scatter plots and box plots of concentration of CYR61 from EGJ tumor serum, early-stage EGJ tumor serum and normal serum

Every sample of the concentration of serum CYR61 in three groups were shown in scatter plots and box plots ($P<0.0001$). The central line is median. It showed the degree of dispersion. The lines up and down are the extremum; EGJ tumor, esophagogastric junction tumor. CYR61 is a protein from CCNs family.

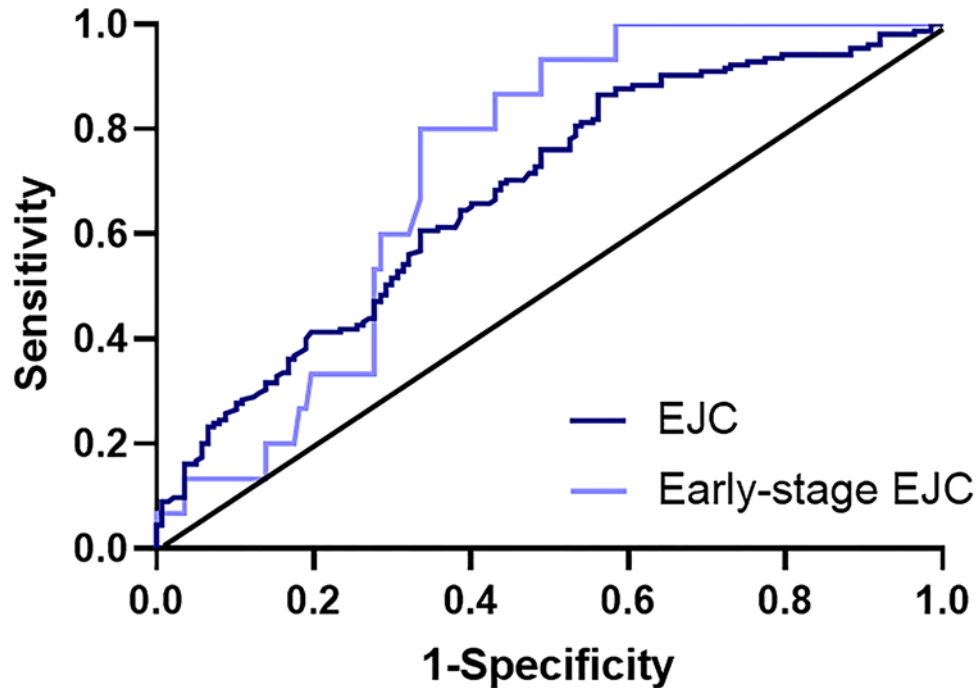


Figure 3. ROC curve analysis in the diagnosis of EGJ tumor and early-stage EGJ tumor

Two groups versus normal controls group are in different colors. The area under the red line is 0.5 for reference; ROC curve, receiver operating characteristic curve; EGJ tumor, esophagogastric junction tumor.

Table 3 Evaluation of the detection value of CYR61 in the diagnosis of EGJ tumor

	AUC	SEN	SPE	FPR	FNR	PPV	NPV	PLR	NLR
EGJ tumor vs. NC	0.691	88.2% (81.7–92.6%)	43.8% (35.4–52.5%)	56.2% (47.5–64.6%)	11.8% (7.4–18.3%)	63.5% (56.6–69.9)	76.9% (65.8–85.5%)	1.57 (1.34–1.84)	0.27 (0.17–0.42)
Early-stage EGJ tumor vs. NC	0.722	80.0% (51.4–94.7%)	66.4% (57.8–74.1%)	33.6% (25.9–42.2%)	20.0% (5.3–48.6%)	20.7% (11.6–33.7%)	96.8% (90.3–99.2%)	2.38 (1.68–3.36)	0.30 (0.11–0.83)

95% CI were given in brackets for each group. AUC, area under the ROC curve; EGJ tumor, esophagogastric junction tumor; FNR, false negative rate; FPR, false positive rate; NC, normal controls; NLR, negative likelihood ratio; NPV, negative predictive value; PLR, positive likelihood ratio; PPV, positive predictive value; SEN, sensitivity; SPE, specificity.

Discussion

At present, the diagnostic examination choice for EGJ tumor is upper esophagogastrroduodenoscopy [1,5], an invasive method with serious side effects, which is not suitable for the screening and detection of asymptomatic population. With the development of clinical medicine, the detection of serum tumor biomarkers, as a painless, convenient, and most importantly, non-invasive detection method, has been widely developed in clinical diagnosis. Tumor biomarkers are a kind of substances reflecting the existence of tumors. When these substances reach a certain level *in vivo*, they can predict the existence of some tumors, which makes it possible to diagnose EGJ tumor early [34]. In this regard, our study found that CYR61 might be a potential biomarker for the diagnosis of EGJ tumor.

CYR61 plays an important role in tumor angiogenesis, tumor cell proliferation, apoptosis and tumor metastasis, which closely participates in the occurrence and development of tumors [13–15]. An increasing number of studies have proved CYR61 to be a metastatic biomarker for prediction of prognosis in osteosarcoma [21], gastric cancer [22], colorectal cancer [16], laryngeal tumor [35], ovarian carcinoma [36] and prostate cancer [37]. As for EGJ tumors, a study has suggested that CYR61 might serve as a metastatic predictor of poor prognosis and provide a potential molecular target for anti-metastatic therapy of EGJ tumor [28]. In addition, many other studies have shown that CYR61 could also act as a diagnosis predictor in patients with colorectal cancer [29] and endometrial cancer [15]. However, CYR61 as a potential biomarker for diagnosis of EGJ tumor has not yet been reported. In the present study,

Table 4 Correlation between CYR61 and clinical data in EGJ patients

	N	Positive	%	95%CI	P
Age					0.823
≥60	115	101	87.8%	80.1–92.9	
<60	37	33	89.2%	73.6–96.4	
Gender					0.598
Male	125	111	88.8%	81.6–93.5	
Female	27	23	85.2%	65.4–95.1	
Smoke					0.780
Yes	25	21	84.0%	63.1–94.7	
No	72	64	88.9%	78.7–94.7	
Unknown	55	49	89.1%	77.1–95.5	
T					0.819
Tis+T1+T2	23	21	91.3%	70.5–98.5	
T3+T4	67	58	86.6%	75.5–93.3	
Unknown	62	55	88.7%	77.5–95.0	
N					0.360
N0	27	22	81.5%	61.3–93.0	
N1+N2+N3	62	57	91.9%	81.5–97.0	
Unknown	63	55	87.3%	76.0–94.0	
M					0.119
M0	83	74	89.2%	79.9–94.6	
M1	9	6	66.7%	30.9–91.0	
Unknown	60	54	90.0%	78.8–95.9	
Grade					0.818
G1	5	5	100.0%	46.3–100	
G2	19	17	89.5%	65.5–98.2	
G3	27	23	85.2%	65.4–95.1	
Unknown	101	89	88.1%	79.8–93.4	
TNM stage					0.658
Early	15	14	93.3%	66.0–99.7	
Advanced	79	68	86.1%	76.0–92.5	
Unknown	58	52	89.7%	78.2–95.7	

Abbreviations: EGJ tumor, esophagogastric junction tumor.

ROC results showed that AUC was 0.691, specificity was 43.8%, and sensitivity was 88.2%, suggesting the diagnostic value of serum CYR61 for EGJ tumor. Similar results could also be demonstrated in early EGJ tumor. Taking other diagnostic evaluation indices into consideration contributes to better understanding of the diagnostic value of serum CYR61 in EGJ tumor, including false positive rate (FPR) of 56.2% (95%CI: 47.5–64.6%), false negative rate (FNR) of 11.8% (95%CI: 7.4–18.3%), positive predictive value (PPV) of 63.5% (95%CI: 56.6–69.9%), negative predictive value (NPV) of 76.9% (95%CI: 65.8–85.5%), positive likelihood ratio (PLR) of 1.57 (95%CI: 1.34–1.84) and negative likelihood ratio (NLR) of 0.27 (95%CI: 0.17–0.42). Meanwhile, in the present study, the serum CYR61 concentration in EGJ tumor was shown significantly lower than that in healthy control group ($P < 0.001$), which was inconsistent with the results of high expression in the study of colon cancer, esophageal cancer and many other cancers. Therefore, we infer that the expression patterns of CYR61 differ in different types of tumors, as well as different histopathological types may lead to the difference, thereby CYR61 has certain significance for the differential diagnosis of tumors and a broad application prospect as a diagnostic biomarker of tumors.

However, there are still some limitations in the present study. It remains open to be discussed and improved. Relatively low specificity may limit the clinical application of CYR61 in the screening of asymptomatic early EGJ tumor patients, so a single detection of serum CYR61 is unable to meet the clinical demands. As reported, compared with single biomarker, combined detection of multiple serum proteins could help improve the sensitivity or specificity of gastrointestinal cancer screening [38], which provides us with a new research direction: CYR61 could be combined with other tumor markers or even other tests to diagnose EGJ tumor. Because the age and sex of normal control group were mismatching with that of EGJ tumor cases, further study could be carried out according to the corresponding age and sex. However, the P value of the variance test between the age and the concentration of serum CYR61 was

0.153 and the one between the sex and the concentration of serum CRY61 was 0.249, which showed that CYR61 has no significant relationship with the age and the sex. So, the age and the sex bias between the two groups could be reduced. In addition, due to the low clinical incidence of EGJ tumor and difficulty of diagnosing EGJ tumor as early cancer, the sample size of our study is small. Besides, incomplete clinical data and single center study are also likely bias. Our conclusion only suggested the possibility of CYR61 being a potential biomarker in the early detection of EGJ tumor. We hope further in-depth studies with large sample size, complete clinical information and well-matched age and sex controls in multiple institutions could be conducted, which could help better evaluate the diagnostic value of CYR61 as a biomarker.

Conclusion

In summary, our study evaluated the relationship between serum CYR61 and EGJ tumor, and proved that serum CYR61 could be a potential biomarker in the early detection of EGJ tumor.

Data Availability

The data were collected and saved in hospital's medical history management center. Due to the legitimate protection of patients' privacy, our information is not available on public or any private websites, but is available from the corresponding author on reasonable request.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Author Contribution

J.J.X. and L.Y.C. initiated the project and designed the study. J.Y.Z., Y.X.Z. and Y.T.O. conceived, designed and performed the experiments, and analyzed the data. J.Y.Z., Y.X.Z. and Y.T.O. wrote the manuscript. All authors have read and approved the final manuscript.

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Ethics Approval and Consent to Participate

The present work was approved by the Ethics Committee of First Affiliated Hospital of Shantou University Medical College, the Ethics Committee of the Cancer Hospital of Shantou University Medical College. The research has been carried out in accordance with the World Medical Association Declaration of Helsinki. Informed consents were acquired from all the participants.

Abbreviations

AJCC, American Joint Committee on Cancer; AUC, area under the ROC curve; CI, confidence interval; CYR61, cysteine-rich angiogenic inducer 61; EGJ tumor, esophagogastric junction tumor; ELISA, enzyme-linked immunosorbent assay; FNR, false negative rate; FPR, false positive rate; NLR, negative likelihood ratio; NPV, negative predictive value; OD, optical density; PLR, positive likelihood ratio; PPV, positive predictive value; ROC curve, receiver operating characteristic curve.

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