Int J Clin Exp Pathol 2017;10(3):3151-3156
www.ijcep.com /ISSN:1936-2625/IJCEP0046280

Original Article

Identification of tissue-specific circRNA hsa_circ_0000705 as an indicator for human gastric cancer

Yongfu Shao[1], Yunben Yang[2], Rongdan Lu[1,2], Binxiu Xiao[2], Guoliang Ye[1], Junming Guo[2]

[1]*Department of Gastroenterology, The Affiliated Hospital of Ningbo University School of Medicine, Ningbo, China;* [2]*Department of Biochemistry and Molecular Biology and Zhejiang Key Laboratory of Pathophysiology, Ningbo University School of Medicine, Ningbo, China*

Received December 10, 2016; Accepted December 27, 2016; Epub March 1, 2017; Published March 15, 2017

Abstract: Circular RNAs (circRNAs) are a special class of endogenous noncoding RNAs. Recent studies indicated that circRNAs play an important role in human carcinogenesis. However, clinical significances of most circRNAs in gastric cancer are still unknown. In this study, we chose hsa_circ_0000705 as a targeted circRNA to investigate its clinical significances in gastric carcinogenesis. Using real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR), hsa_circ_0000705 expression levels in 311 tissue samples from various stages of gastric carcinogenesis are explored; and the potential relationship between hsa_circ_0000705 levels and clinical clinicopathological factors were investigated. Then, a receiver operating characteristic (ROC) curve was constructed for evaluating the diagnostic value of hsa_circ_0000705. Hsa_circ_0000705 expression was significantly down regulated in 79.2% (76/96) gastric cancer and 84% (21/25) dysplasia tissues, but no difference in gastric ulcer and erosive gastritis tissues. Combined with clinical pathological factors, hsa_circ_0000705 expression level was strongly associated with tumor location, tumor stage, Borrmann type, pathologic diagnosis, and tissue CA19-9 expression. The area under the curve was up to 0.719. The optimal cutoff value was 9.125, with which the sensitivity and specificity were 64.58% and 69.79%, respectively. Taken together, all these results imply that hsa_circ_0000705 plays a crucial role in gastric carcinogenesis; and could be used as indicator of gastric cancer.

Keywords: Gastric cancer, circular RNA, hsa_circ_0000705, molecular diagnosis

Introduction

Gastric cancer is the fifth most common malignancy and third leading cause of cancer death in the world [1]. As clinical symptoms of gastric cancer are dormant and difficult to be found at the early stage, most patients are diagnosed at advanced stages and often lost the opportunity for surgery [2]. The 5-year overall survival rate of gastric cancer patients is still less than 30% although clinical treatments are constantly improved [3]. This is mainly due to the absence of effective methods for early diagnosis. Thus, raising the discovery rate of early gastric cancer and precancerous lesions is the key to improve clinical patients' prognosis.

Circular RNAs (circRNAs), a special class of endogenous RNAs, are characterized by jointing 3' and 5' ends together via exon or intron circularization [4, 5]. Compared with traditional linear RNA, circRNAs have many unique features in structure and function. They are enormously abundant, evolutionally conserved and more stable in cytoplasm [6, 7]. Majority of circRNAs are resistant to exonucleases and often exhibit tissue and developmental stage specific expression [8]. These features confer numerous functions to circRNAs, such as acting as microRNA sponges, regulating gene transcription and protein production [7, 9]. Moreover, accumulating evidence indicate that circRNAs can associate with cancer-related miRNAs to regulate cancer-related pathways and serve as diagnostic or predictive biomarkers of some cancers [7].

Hsa_circ_0000705 (Alias, hsa_circ_000212, http://circrna.org/), with 559 nt in spliced sequence length, is one of circular RNAs aber-

rantly expressed in gastric cancer tissues, identified by our microarray. Its gene is located at human chr16:58593707-58594266; and its associated-gene symbol is *CNOT1* (CCR4-NOT transcription complex subunit 1). In this study, we investigated the clinical significance of hsa_circ_0000705 expression in gastric cancer. Firstly, hsa_circ_0000705 expression levels in gastric cancer tissues and its marched adjacent normal tissues were detected by real-time reverse transcription-polymerase chain reaction (RT-PCR). Then, its expression in various stages of gastric tumorigenesis was explored; and the potential relationship between hsa_circ_0000705 expression levels and clinicopathological factors were further investigated. Finally, a receiver operating characteristic (ROC) curve was constructed for evaluating the diagnostic value of hsa_circ_0000705. Our results indicated that hsa_circ_0000705 is a potential biomolecular involved in the process of gastric carcinogenesis and development.

Materials and methods

Specimens

A total of 311 specimens were collected from the Center for Gastroenterology of the Affiliated Hospital of Ningbo University School of Medicine, China, from November 2014 to February 2016. The 96 gastric cancer tissues and their matched adjacent non-tumorous tissues 5 cm from the edge of cancer were obtained from surgical operation. The 35 healthy gastric mucosa, 16 gastric ulcer tissues, 18 erosive gastritis tissues, and 25 paired gastric dysplasia tissues were obtained from biopsy specimens. All tissue specimens were preserved in RNA fixer (Bioteke, Beijing, China) at -80°C until RNA isolation. No patient received clinical treatment before surgery or endoscopy examination.

Diagnosis of every tissue specimen in this study was histopathologically confirmed. Tumors were classified according to the tumor-node-metastasis (TNM) staging system (7^{th} ed). Histological grade was assessed following the National Comprehensive Cancer Network (NCCN) Clinical Practice Guideline of Oncology (V.1.2012). This study was approved by The Human Research Ethics Committee of Ningbo University School of Medicine. Informed consent was obtained from all subjects.

Total RNA extraction and reverse transcription reaction

TRIzol reagent (Ambion, Carlsbad, CA, USA) was used to extract total RNA from all the gastric tissue specimens, following the manufacturer's instructions. A260/A280 ratio and 1.5% agarose gel electrophoresis were used to evaluate the quality of total RNA. cDNA was synthesized by random primers and GoScript Reverse Transcription (RT) System (Promega, Madison, WI, USA). The conditions of RNA reverse transcription were as follows: incubation at 42°C for 1 h, 70°C for 10 min and 4°C for forever. No template reaction was used as the control at the same time.

Quantitative detection of circRNA

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to detect targeted circRNA expression by GoTaq qPCR Master Mix (Promega) on an Mx3005P real-time PCR System (Stratagene, LaJolla, CA, USA). Divergent primers of targeted circRNA were designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd. The sequences of primers were as follows: 5'-ACCCACTCCTCCACCTTTGAC-3' (sense) and 5'-TGTTGCTGTAGCCAAATTCGTT-3' (antisense) for GAPDH; 5'-TAACTGGGGCTCTTCAGCTC-3' and 5'-TGGTGGTTGTCTGGCCTTAT-3' for hsa_circ_0000705. Conditions of thermal cycling were as follows: 95°C at 5 min for a hot-start, then 45 cycles at 94°C for 15 s, 56°C for 30 s, and 72°C for 30 s. ΔC_t method was used to calculate targeted circRNA expression level, and larger ΔC_t value indicate lower level [10]. All results were expressed as the Means ± SD of three independent experiments.

Statistical analysis

All statistical data were analyzed using Statistical Product and Service Solutions (SPSS) 20.0 software (SPSS, Chicago, IL, USA). To evaluate diagnostic value, SigmaPlot12.3 software (Systat Software, San Jose, CA, USA) were used to construct receiver operating characteristic (ROC) curve of targeted circRNA. Student's t-test, one way analysis of variance (ANOVA) test and rank-sum test were flexible used as appropriate. $P<0.05$ was considered statistically significant.

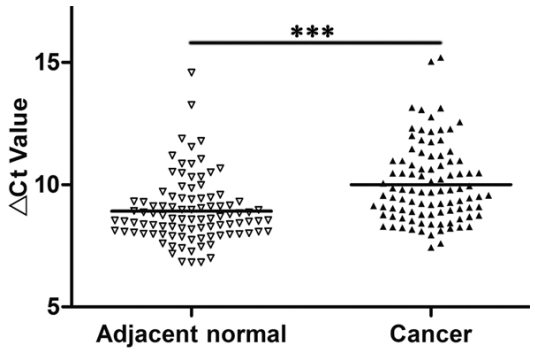

Figure 1. Hsa_circ_0000705 expression in gastric cancer tissues and adjacent normal tissues. Compared with the adjacent normal tissues, hsa_circ_0000705 was significantly down regulated in 79.2% (76/96) gastric cancer tissues (n=96, ***P<0.001). Data are expressed as the Means ± SD of three independent experiments.

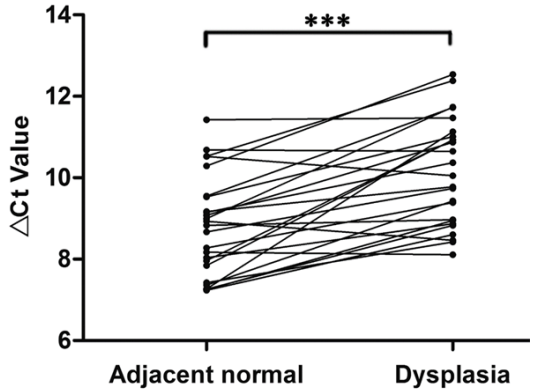

Figure 2. Hsa_circ_0000705 expression in dysplasia tissues. Hsa_circ_0000705was significantly decreased in 84% (21/25) dysplasia compared with healthy control group (n=25, ***P<0.001). Data are expressed as the Means of three independent experiments.

Results

Expression of hsa_circ_0000705 in gastric cancer tissues

To verify whether hsa_circ_0000705 was aberrantly expressed during the course of gastric carcinogenesis, we firstly used qRT-PCR method to detect its expression levels in 96 gastric cancer tissues and their matched adjacent non-tumorous tissues 5 cm from the edge of cancer. The results showed that, compared with the adjacent normal tissues, hsa_circ_0000705 was significantly down regulated in 79.2% (76/96) gastric cancer tissues (P<0.001, **Figure 1**). Similar to the situations in gastric cancer tissues, hsa_circ_0000705 expression had also been changed in gastric mucosal dysplasia. It was significantly decreased in 84% (21/25) dysplasia compared with healthy control group (P<0.001, **Figure 2**).

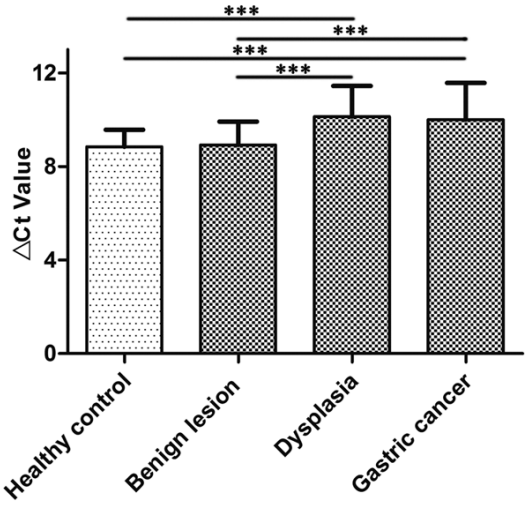

Figure 3. Hsa_circ_0000705 expression levels in the tissues from various stages of gastric carcinogenesis. Hsa_circ_0000705 expression was only significantly decreased in gastric cancer tissues (n=96) and gastric dysplasia (n=25) compared with healthy controls (n=35) and benign lesions (n=34). Data are expressed as the means ± SD of three independent experiments (***P<0.001).

Then, we further explore hsa_circ_0000705 expression in the tissues from various stages of gastric carcinogenesis to obtain more information about its expression and gastric mucosal damage. As indicated in **Figure 3**, hsa_circ_0000705 only significantly decreased in dysplasia and gastric cancer tissues. There was no difference in gastric ulcer tissues and erosive gastritis tissues.

Relationship between hsa_circ_0000705 levels and clinicopathological factors

Based on the above findings, analyses were performed to assess the relationship between tissue hsa_circ_0000705 and clinical features. As shown in **Table 1**, the expression level of hsa_circ_0000705 was related to tumor location (P=0.031), tumor stage (P=0.024), Borrmann type (P=0.005), pathologic diagnosis (P=0.045), and tissue CA19-9 expression (P=0.010). However, it was not associated with

Table 1. Relationship of Hsa_circ_0000705 expression levels (ΔC_t) in cancer tissues with clinicopathological factors of gastric cancer patients

Characteristics	No. of case (%)	Mean ± SD	*P* value
Age (y)			
≥60	64 (66.7)	9.963±1.527	0.746
<60	32 (33.3)	10.074±1.702	
Gender			
Male	65 (67.7)	10.110±1.671	0.326
Female	31 (32.3)	9.769±1.364	
Tumor location			
Sinuses ventriculi	45 (46.9)	10.249±1.574	0.031
Cardia	13 (13.5)	10.350±1.728	
Corpora ventriculi	26 (27.1)	9.223±1.235	
Others	12 (12.5)	10.370±1.714	
Diameter (cm)			
≥5	49 (51.0)	10.175±1.658	0.270
<5	47 (49.0)	9.818±1.489	
Differentiation			
Well	13 (13.5)	9.819±2.003	0.770
Moderate	47 (49.0)	10.116±1.246	
Poor	36 (37.5)	9.914±1.822	
Stage			
Early	23 (24.0)	9.454±1.151	0.024
Advanced	73 (76.0)	10.172±1.662	
Borrmann type			
I & II	9 (9.4)	11.591±1.277	0.005
III & IV	64 (90.6)	9.972±1.619	
Pathologic diagnosis			
Signet ring cell cancer	13 (13.5)	9.187±1.298	0.045
Adenocarcinoma	83 (86.5)	10.127±1.589	
Invasion			
T_1 & T_2	33 (34.4)	9.854±1.439	0.516
T_3 & T_4	63 (65.6)	10.076±1.654	
Lymphatic metastasis			
N_0	36 (60.0)	9.881±1.404	0.571
N_{1-3}	60 (40.0)	10.071±1.684	
Distal metastasis			
M_0	83 (86.5)	9.991±1.458	0.917
M_1	13 (13.5)	10.060±2.286	
Venous invasion			
Absent	49 (51.0)	10.071±1.577	0.655
Present	47 (49.0)	9.926±1.595	
Perineural invasion			
Absent	42 (43.8)	9.883±1.270	0.507
Present	54 (56.2)	10.091±1.790	
CEA (Tissue)			
Positive	88 (91.7)	9.954±1.595	0.345
Negative	8 (8.3)	10.508±1.388	
CA19-9 (Tissue)			
Positive	57 (59.4)	9.642±1.367	0.010
Negative	39 (40.6)	10.523±1.735	

other clinicopathologic factors such as tumor invasion, lymphatic metastasis and cell differentiation.

ROC curve of hsa_circ_0000705

To estimate whether hsa_circ_0000705 could be used as an indicator for gastric cancer, an ROC curve was built for differentiating gastric cancer group from other groups. As shown in **Figure 4**, the area under the curve was up to 0.719 (95% CI, 0.648-0.791; P<0.001) (**Figure 4**). The optimal cutoff value was 9.125, sensitivity was 64.58%; and specificity was 69.79%.

Discussion

CircRNAs are a special class of endogenous noncoding RNAs. Recent studies have demonstrated that circRNAs participate in the carcinogenesis and the malignant behavior of cancers [11]. Some of them act as oncogenic or tumor suppressor roles in human tumorigenesis [11-13]. Several associations between cancer dysregulated circRNAs and clinical significance have been found at the same time [14-16]. Qin et al. found that hsa_circ_0001649 was significantly down regulated in hepatocellular carcinoma; and its expression was correlated with tumor size and the occurrence of tumor embolus [14]. Wang et al. showed that the dysregulated hsa_circ_001988 in colorectal cancer was significantly correlated with differentiation and perineural invasion [15]. In previous studies, we have reported that the downregulated hsa_circ_002059 in gastric cancer were correlated with distal metastasis, TNM stage, gender and patients' age [16]. More importantly, circulating hsa_circ_002059 is highly stable in human plasma; and may be a potential biomarker for the diagnosis of gastric cancer [16]. All these mounting findings strongly suggest an important role of altered circRNAs in cancer pathophysiology.

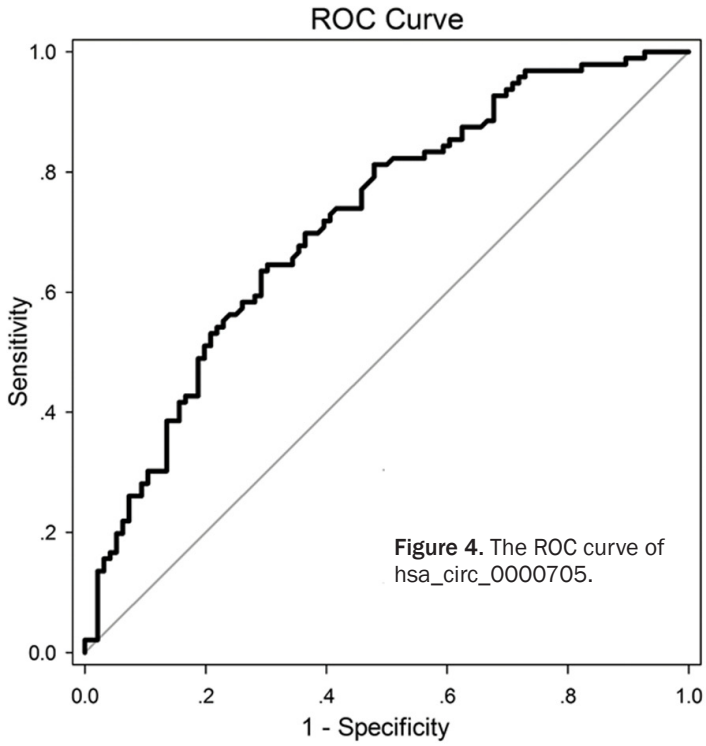

Figure 4. The ROC curve of hsa_circ_0000705.

Hsa_circ_0000705 is one of circRNAs aberrantly expressed in gastric carcinogenesis. In the current study, we found that hsa_circ_0000705 expression was significantly down regulated in 79.2% (76/96) gastric cancer and 84% (21/25) dysplasia tissues, but no difference in gastric ulcer and erosive gastritis tissues (**Figures 2** and **3**). Combined with clinical pathological factors, hsa_circ_0000705 expression level was strongly associated with tumor location, tumor stage, Borrmann type, pathologic diagnosis, and tissue CA19-9 expression (**Table 1**). Moreover, the ROC curve showed that the area under the curve was up to 0.719 (**Figure 4**). The optimal cutoff value was 9.125, with which the sensitivity and specificity were 64.58% and 69.79, respectively. Taken together, all these results imply that hsa_circ_0000705 plays a crucial role in gastric carcinogenesis, and could be used as indictor of gastric cancer.

Tumor stage and histological pathologic diagnosis are independent prognostic factors for all gastric cancer patients [17]. Borrmann type is associated with patients' lymph node metastasis and is a valuable predictor for survival in advanced gastric cancer patients [18]. Tumor location is the only significant prognostic factor for early gastric cancer patients [19]. Aoyama et al. report that the overall survival rate of 5-years was 81.8% when tumor was located in the upper third of the stomach and was 95.5% when located in the middle or lower third of the stomach [19]. In our study, we found hsa_circ_0000705 expression level was strongly associated with tumor location, tumor stage, Borrmann type, and pathologic diagnosis. This indicates that hsa_circ_0000705 may be used as indicator for clinical prognosis prediction.

In conclusion, our study showed that hsa_circ_0000705 is one of circRNAs significantly down regulated in gastric cancer and dysplasia lesion. The down regulation of hsa_circ_0000705 expression was strongly associated with tumor location, tumor stage, Borrmann type, pathologic diagnosis, and tissue CA19-9 expression. Our finding suggested that hsa_circ_0000705 plays a crucial role in gastric carcinogenesis, and could be used as indictor of gastric cancer.

Acknowledgements

This study was supported by grants from the Social Development Research Project of Ningbo (No. 2016C51015), the Medical Research Project of the Affiliated Hospital of Ningbo University School of Medicine (No. XYY16022), the Foundation of Zhejiang Key Laboratory of Pathophysiology (No. 201601), Applied Research Project on Nonprofit Technology of Zhejiang Province (No. 2016C33177), and the K. C. Wong Magna Fund in Ningbo University.

Disclosure of conflict of interest

None.

Address correspondence to: Junming Guo, Department of Biochemistry and Molecular Biology and Zhejiang Key Laboratory of Pathophysiology, Ningbo University School of Medicine, Ningbo 315211, China. Tel: +86-574-87600756; Fax: +86-574-87608638; E-mail: guojunming@nbu.edu.cn; Dr. Guoliang Ye, Department of Gastroenterology, The

Affiliated Hospital of Ningbo University School of Medicine, Ningbo 315020, China. Tel: +86-574-87035171; Fax: +86-574-87380487; E-mail: ndfyy-gl@126.com

References

[1] Li T, Mo X, Fu L, Xiao B and Guo J. Molecular mechanisms of long noncoding RNAs on gastric cancer. Oncotarget 2016; 7: 8601-8612.

[2] Majeed W, Iftikhar A, Khaliq T, Aslam B, Muzaffar H, Atta K, Mahmood A and Waris S. Gastric carcinoma: recent trends in diagnostic biomarkers and molecular targeted therapies. Asian Pac J Cancer Prev 2016; 17: 3053-3060.

[3] Ohtsu A. Chemotherapy for metastatic gastric cancer: past, present, and future. J Gastroenterol 2008; 43: 256-264.

[4] Wang F, Nazarali AJ and Ji S. Circular RNAs as potential biomarkers for cancer diagnosis and therapy. Am J Cancer Res 2016; 6: 1167-1176.

[5] Petkovic S and Muller S. RNA circularization strategies in vivo and in vitro. Nucleic Acids Res 2015; 43: 2454-2465.

[6] Ebbesen KK, Kjems J and Hansen TB. Circular RNAs: Identification, biogenesis and function. Biochim Biophys Acta 2016; 1859: 163-168.

[7] Li J, Yang J, Zhou P, Le Y, Zhou C, Wang S, Xu D, Lin HK and Gong Z. Circular RNAs in cancer: novel insights into origins, properties, functions and implications. Am J Cancer Res 2015; 5: 472-480.

[8] Qu S, Yang X, Li X, Wang J, Gao Y, Shang R, Sun W, Dou K and Li H. Circular RNA: a new star of noncoding RNAs. Cancer Lett 2015; 365: 141-148.

[9] Salzman J. Circular RNA expression: its potential regulation and function. Trends Genet 2016; 32: 309-316.

[10] Shao Y, Ye M, Jiang X, Sun W, Ding X, Liu Z, Ye G, Zhang X, Xiao B and Guo J. Gastric juice long noncoding RNA used as a tumor marker for screening gastric cancer. Cancer 2014; 120: 3320-3328.

[11] Zhao ZJ and Shen J. Circular RNA participates in the carcinogenesis and the malignant behavior of cancer. RNA Biol 2015; 1-8.

[12] Xie H, Ren X, Xin S, Lan X, Lu G, Lin Y, Yang S, Zeng Z, Liao W, Ding YQ and Liang L. Emerging roles of circRNA_001569 targeting miR-145 in the proliferation and invasion of colorectal cancer. Oncotarget 2016; 7: 26680-26691.

[13] Guarnerio J, Bezzi M, Jeong JC, Paffenholz SV, Berry K, Naldini MM, Lo-Coco F, Tay Y, Beck AH and Pandolfi PP. Oncogenic role of fusion-circRNAs derived from cancer-associated chromosomal translocations. Cell 2016; 166: 1055-1056.

[14] Qin M, Liu G, Huo X, Tao X, Sun X, Ge Z, Yang J, Fan J, Liu L and Qin W. Hsa_circ_0001649: a circular RNA and potential novel biomarker for hepatocellular carcinoma. Cancer Biomark 2016; 16: 161-169.

[15] Wang X, Zhang Y, Huang L, Zhang J, Pan F, Li B, Yan Y, Jia B, Liu H, Li S and Zheng W. Decreased expression of hsa_circ_001988 in colorectal cancer and its clinical significances. Int J Clin Exp Pathol 2015; 8: 16020-16025.

[16] Li P, Chen S, Chen H, Mo X, Li T, Shao Y, Xiao B and Guo J. Using circular RNA as a novel type of biomarker in the screening of gastric cancer. Clin Chim Acta 2015; 444: 132-136.

[17] Wang W, Li YF, Sun XW, Chen YB, Li W, Xu DZ, Guan XX, Huang CY, Zhan YQ and Zhou ZW. Prognosis of 980 patients with gastric cancer after surgical resection. Chin J Cancer 2010; 29: 923-930.

[18] Li C, Oh SJ, Kim S, Hyung WJ, Yan M, Zhu ZG and Noh SH. Macroscopic Borrmann type as a simple prognostic indicator in patients with advanced gastric cancer. Oncology 2009; 77: 197-204.

[19] Aoyama T, Yoshikawa T, Fujikawa H, Hayashi T, Ogata T, Cho H, Yamada T, Hasegawa S, Tsuchida K, Yukawa N, Oshima T, Oba MS, Morita S, Rino Y and Masuda M. Prognostic factors in stage IB gastric cancer. World J Gastroenterol 2014; 20: 6580-6585.