Plasma lncRNA-GACAT2 is a valuable marker for the screening of gastric cancer

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Abstract. Long non-coding RNAs (lncRNAs) are crucial in contributing to gastric tumorigenesis and development. However, the diagnostic value of the majority of lncRNAs in gastric cancer (GC) are not clear. The present study investigated the diagnostic value of gastric cancer associated transcript 2 (GACAT2), a lncRNA that is aberrantly expressed in GC tissues. A total of 343 plasma samples from 80 healthy individuals, 29 patients with gastric dysplasia (GD) and 117 paired preoperative and postoperative patients with GC were collected. Plasma GACAT2 levels were subsequently measured by reverse transcription-quantitative polymerase chain reaction. Finally, the associations between plasma GACAT2 levels and various clinicopathological features of patients with GC were assessed. The results demonstrated that plasma GACAT2 levels in preoperative patients with GC were significantly higher than those in the postoperative group (P=0.031). Compared with healthy individuals, plasma GACAT2 levels were significantly increased in patients with GD (P<0.001) and preoperative patients with GC (P=0.040). Moreover, the individual relative changes of plasma GACAT2 expression following surgery were significantly associated with lymphatic metastasis (P=0.034), distal metastasis (P=0.035) and perineural invasion (P=0.039). Therefore, the results of the current study suggest that plasma-based GACAT2 may be developed as a tumor marker to screen and predict the prognosis of GC patients.

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Introduction

Gastric cancer (GC) is one of the most prevalent malignant tumors (1). A number of cancer prevention studies have demonstrated that the most effective way to reduce GC-associated mortality is through early diagnosis and appropriate treatment (2-4). However, the diagnosis rate of early GC remains extremely low (<10%) (5). Patients with GC are usually diagnosed following identification of tumors on B-mode ultrasound, computerized tomography, or magnetic resonance imaging scans. However, early GC tumors are often not large enough to be detected by conventional imaging techniques. Furthermore, the sensitivity and specificity of traditional tumor markers are not satisfactory (6). Thus, investigations into new, more efficient tumor markers are important to improve early diagnosis of this disease.

Long non-coding RNAs (lncRNAs) are a class of regulatory non-coding RNAs (7). Previous studies have investigated the roles of lncRNAs in tumorigenesis and cancer development. For example, Zang *et al* (8) reported that lncRNA-PEG10 expression was upregulated in esophageal cancer tissues, and that it regulated the proliferation and invasion of cancer cells. Furthermore, Shao *et al* (9) demonstrated that lncRNA-AA174084 expression was downregulated in gastric cancer tissues. A previous study determined that gastric cancer associated transcript 2 (GACAT2) was not only significantly downregulated in gastric cancer tissue, but was also aberrantly expressed in gastric precancerous lesions and gastric cell lines (10).

Blood is one of the most commonly used samples in cancer screening, therefore in the present study, plasma GACAT2 expression was compared among groups of healthy individuals, patients with GD, and patients with preoperative or postoperative GC. The aim of the current study was to investigate whether plasma GACAT2 levels may be used as a novel tumor marker to screen and predict the prognosis of patients with GC.

Materials and methods

Plasma and clinical data collection. A total of 343 plasma samples were collected from 80 healthy individuals,

29 patients with GD, 117 preoperative and 117 postoperative patients with GC at the Department of Gastroenterology, Affiliated Hospital of Ningbo University School of Medicine (Ningbo, China) between March 2013 and July 2014. Post-operative plasma samples were collected 14 days following surgery. No patients underwent chemotherapy or radiotherapy ahead of sample collection. Tumors were classified using the tumor-node-metastasis staging system (11). Histological grade was evaluated by the National Comprehensive Cancer Network clinical practice guidelines (V.1.2011) (12). Written informed consent was provided by all patients, and all aspects of the study were approved by the Human Research Ethics Committee of Ningbo University (IRB No. 20120303).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from plasma using the TRIzol® LS reagent (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol as previously reported (13). Complementary DNA was subsequently generated using the GoScript™ Reverse Transcription system (Promega Corporation, Madison, WI, USA), following the manufacturer's protocol, as previously reported (14). In order to detect plasma GACAT2 levels, RT-qPCR was performed with GoTaq qPCR Master mix (Promega Corporation) on a Mx3005P qPCR system (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA) following the manufacturer's protocol. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression, which maintains a stable level in human plasma (9), was used as a control for plasma GACAT2 detection. Primers for GAPDH and GACAT2 were synthesized by Sangon Biotech, Co., Ltd. (Shanghai, China). The primer sequences were as follows: GACAT2 forward, 5'-TGGATGCTTACAAAGGAC TGG-3' and reverse, 5'-CTGCAATTACGGAAAGAGCTG-3'; GAPDH forward, 5'-ACCCACTCCTCCACCTTTGAC-3' and reverse, 5'-TGTTGCTGTAGCCAAATTCGTT-3'. The conditions of thermal cycling were as follows: 95˚C at 5 min for a hot-start, followed by 45 cycles at 94˚C for 15 sec, 55˚C for 30 sec and 72˚C for 30 sec. The level of GACAT2 was calculated using the $2^{-\Delta\Delta Cq}$ method (9). A lower quantification cycle (Cq) indicates a higher level of plasma GACAT2. The ΔΔCq method ($\Delta Cq_{post\text{-}op}$-$\Delta Cq_{pre\text{-}op}$) was used to evaluate individual relative changes of plasma GACAT2 following surgery, and all results were expressed as the mean ± standard deviation of three independent experiments.

Detection of serum carcinoembryonic antigen (CEA) and serum carbohydrate antigen (CA)19-9. To measure levels of CA19-9 and CEA, an Elecsys 2010 machine (Roche Diagnostics, Basel, Switzerland) was used. The cut-off values of CA19-9 and CEA were set at 35 U/ml and 5 ng/ml, respectively (15).

Statistical analysis. Data were analyzed using SPSS software v19.0 (IBM SPSS, Armonk, NY, USA). The difference in plasma GACAT2 levels among healthy controls, patients with GD, and preoperative and postoperative patients with GC were analyzed by one-way analysis of variance (ANOVA). Associations between plasma GACAT2 levels and clinicopathological factors were analyzed by ANOVA and Student's t-test. Graphs were plotted using SigmaPlot v12.1.0 (Systat Software Inc., San

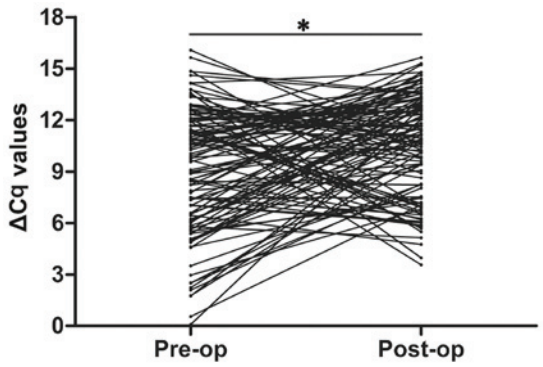

Figure 1. Reverse transcription-quantitative polymerase chain reaction was used to detect GACAT2 levels in pre-op and post-op patients with gastric cancer. Plasma GACAT2 expression decreased following surgery (*P<0.05). A higher ΔC_q indicates a higher level of plasma GACAT2. GACAT2, gastric cancer associated transcript 2; Cq, quantification cycle; pre-op, preoperative; post-op, postoperative.

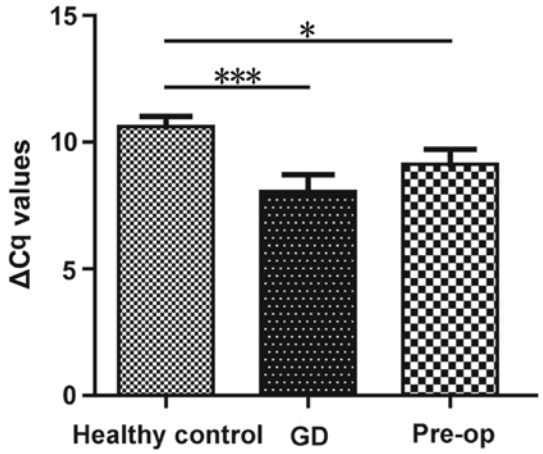

Figure 2. Plasma GACAT2 levels were increased in GD and pre-op patients with gastric cancer as compared with healthy controls (*P<0.05 and ***P<0.001). A lower ΔC_q indicates a higher level of plasma GACAT2. Healthy volunteers, n=80; GD, n=29; pre-op patients, n=117; GACAT2, gastric cancer associated transcript 2; GD, gastric dysplasia; pre-op, preoperative; Cq, quantification cycle.

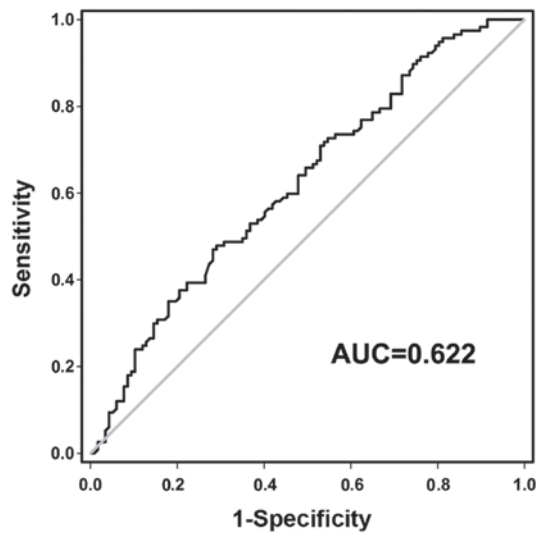

Figure 3. ROC curve for differentiating gastric cancer plasma from healthy controls. ROC, receiver operating characteristic; AUC, area under the curve.

Table I. Association of plasma GACAT2 level changes (ΔΔCq) between postoperative and preoperative patients with gastric cancer and clinicopathological factors.

Characteristics	N	Mean ± SD	P-value
Age, years			0.889
≥60	81	1.544±4.253	
<60	36	1.340±3.996	
Gender			0.449
Male	81	1.143±4.443	
Female	36	2.243±3.344	
Tumor location			0.969
Sinuses ventriculi	57	1.547±4.149	
Cardia	21	1.027±4.205	
Corpora ventriculi	21	1.206±3.103	
Others	18	2.123±5.797	
Diameter, cm			0.252
≥5	60	0.735±4.393	
<5	57	2.266±3.777	
Differentiation			0.057
Well	6	5.070±1.725	
Moderate	57	2.608±4.290	
Poor	54	-0.108±3.571	
TNM stage			0.326
Early	24	2.775±3.990	
Advanced	93	1.147±4.155	
Borrmann type			0.224
I and II	33	2.386±3.091	
III and IV	60	0.466±4.567	
Pathological diagnosis			0.342
Signet ring cell cancer	18	-0.117±5.671	
Adenocarcinoma	99	1.752±3.831	
Invasion			0.223
T_1 and T_2	36	2.701±3.917	
T_3 and T_4	81	0.939±4.168	
Lymphatic metastasis			0.034[a]
N_0	45	3.232±3.805	
N_{1-3}	72	0.387±4.005	
Distal metastasis			0.035[a]
M_0	96	2.070±3.720	
M_1	21	-1.210±5.093	
Venous invasion			0.246
Negative	60	2.236±3.916	
Positive	57	0.687±4.292	
PNI			0.039[a]
Negative	63	2.731±3.326	
Positive	54	0.022±4.563	
Blood CEA			0.767
Positive	51	1.722±5.144	
Negative	66	1.295±3.246	
Blood CA19-9			0.793
Positive	39	1.232±5.011	
Negative	78	1.606±3.708	

[a]P<0.05. $\Delta\Delta Cq=\Delta Cq_{post\text{-}op}-\Delta Cq_{pre\text{-}op}$. TNM, tumor-node-metastasis; PNI, perineural invasion; CEA, carcinoembryonic antigen; CA, carbohydrate antigen; SD, standard deviation; GACAT2, gastric cancer associated transcript 2; Cq, quantification cycle.

Jose, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Plasma GACAT2 levels are significantly decreased following surgery. GACAT2 levels in preoperative and postoperative patients with GC were detected by RT-qPCR. As presented in Fig. 1, the level of plasma GACAT2 in the postoperative gastric cancer patients was significantly lower than that in the preoperative group (P=0.031), indicating that GACAT2 expression significantly decreased following surgery.

Plasma GACAT2 levels are significantly increased in patients with GD and GC. Plasma GACAT2 levels were measured at two stages of gastric carcinogenesis to gain further information regarding their effect on gastric mucosal damage. The data indicated that plasma GACAT2 levels in patients with GD (P<0.001) and patients with preoperative GC (P=0.040) were significantly higher than those in the healthy controls (Fig. 2). However, there was no significant difference in GACAT2 expression between the two patient groups.

To investigate whether preoperative plasma GACAT2 may be used as a tumor biomarker for GC screening, a receiver operating characteristic (ROC) curve was constructed to evaluate its clinical value. The area under the curve (AUC) was 0.622 (95% confidence interval, 0.551-0.694; P<0.05; Fig. 3), and the optimal cut-off value was 6.625, with which the sensitivity and specificity were 87.2 and 28.2%, respectively (Fig. 3). This implies that plasma GACAT2 levels may be used as a reliable biomarker in the screening of GC.

Identification of RT-qPCR products of plasma GACAT2. Blood is the primary material used in the diagnosis of diseases; therefore, to confirm the results and prove the existence of GACAT2 in plasma, the RT-qPCR products of plasma GACAT2 were sequenced. Fig. 4 demonstrates that the sequence of plasma GACAT2 was completely consistent with that from the genome database (http://www.ncbi.nlm.nih.gov/nuccore/NR_120598.1).

Association between plasma GACAT2 levels and clinicopathological factors of patients with GC. Based on the aforementioned results, associations between plasma GACAT2 levels and the clinicopathological features of patients with GC were investigated. The individual relative changes of plasma

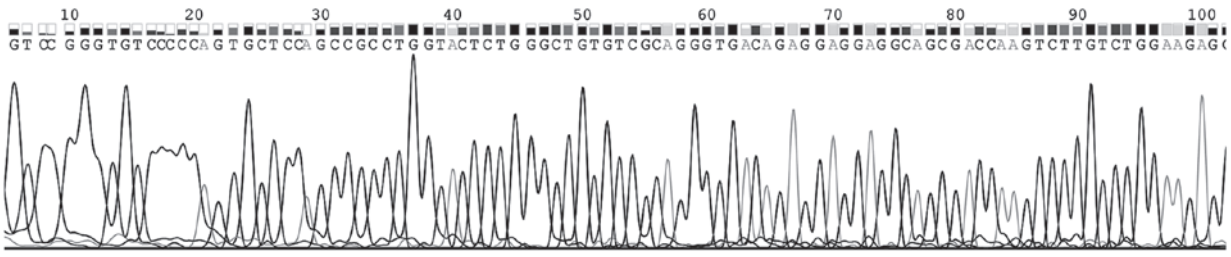

Figure 4. Sequencing result of the product of plasma GACAT2 by RT-qPCR, demonstrating that the sequence was the same as the sequence of GACAT2 in the genomic database. GACAT2, gastric cancer associated transcript 2; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

GACAT2 following surgery were significantly associated with lymphatic metastasis (P=0.034), distal metastasis (P=0.035) and perineural invasion (P=0.039) (Table I), indicating a correlation between GACAT2 and invasive GC.

Discussion

Previous studies have identified the differential expression of lncRNAs in various diseases and suggested their possible associations with tumorigenesis (16). Advanced cancer genomic techniques have demonstrated that lncRNAs are important in determining GC occurrence and development (17).

Blood plasma is the most commonly used sample in clinical diagnosis (18). A number of changes in tumor cells may be detected by measuring the expression of different proteins in plasma. RT-qPCR may provide a method to detect changes in the expression of DNA or RNA associated with tumorigenesis. The present study investigated whether plasma-based lncRNAs could be used as biomarkers for the screening of GC and prediction of prognosis in patients with the disease. Plasma GACAT2 levels between preoperative and postoperative patients with GC were compared. The results demonstrated that plasma GACAT2 levels in postoperative patients with GC were significantly lower than those in the preoperative group (Fig. 1). To obtain further information regarding the change in GACAT2 expression during gastric tumorigenesis, plasma GACAT2 levels in two stages of gastric carcinogenesis were measured. Compared with healthy controls, plasma GACAT2 levels were not only increased in the gastric cancer group as a whole (P=0.040), they were also significantly higher in patients with GD (P<0.001; Fig. 2). The AUC was 0.622 (Fig. 3). When the optimal cut-off value was 6.625, the sensitivity and specificity were 87.2 and 28.2%, respectively. Taken together, the results of the current study indicated that plasma GACAT2 has the potential value for the early screening of GC.

A previous study demonstrated that GACAT2 expression was significantly downregulated in gastric tissue and gastric cancer cell lines (10). The reason plasma GACAT2 expression increased in patients with GC and GD in the current study remains unknown. A recent study compared lncRNA-LINC00152 levels between plasma and exosomes extracted from the blood and observed that no difference between the two (19). One hypothesis suggests that lncRNAs that are stable in human plasma are protected by exosomes (19). It has been demonstrated that exosomes serve an important role in the protection and secretion of non-coding RNAs, including miRNAs and lncRNAs (20,21), and cells affected by stimuli, such as oxidative stress, may increase the secretion of exosomes (22,23). In addition, GC cells are able to release related RNAs into the extracellular environment via exosomes during tumorgenesis (24). Therefore, GACAT2 may be released in a similar manner, leading to elevated GACAT2 levels in the blood plasma of patients with malignant GC.

Lymphatic metastasis, distal metastasis and perineural invasion are important factors affecting GC prognosis (25,26). The present study demonstrated that individual relative changes of plasma GACAT2 following surgery were significantly associated with lymphatic metastasis (P=0.034), distal metastasis (P=0.035) and perineural invasion (P=0.039). Higher GACAT2 levels in plasma therefore are correlated with a worse pathological situation; therefore plasma-based GACAT2 may be used as a tumor marker to predict the prognosis of patients with GC.

In conclusion, our study demonstrated that plasma GACAT2 levels were significantly increased in patients with GD and preoperative patients with GC. In addition, the individual relative changes of plasma GACAT2 expression following surgery were significantly associated with lymphatic metastasis, distal metastasis and perineural invasion. Our findings suggested that plasma-based GACAT2 as a tumor marker has a potential diagnostic value for the screening of GC and prediction of prognosis.

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