Identification of PGC-related ncRNAs and their relationship with the clinicopathologic features of gastric cancer

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Primary research

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Abstract

Background Pepsinogen C (PGC) is considered to be the final product of mature differentiated gastric mucosa and the expression level of PGC in gastric mucosa decreased obviously in the courses of gastric cancer (GC) development. The mechanism of the down-regulation of PGC is still unclear and needs to be excavated. This study aims to identify the PGC-related ncRNAs, which may have potential to act as PGC post-transcriptional regulator, and further explore the relationship between these ncRNAs and clinicopathological parameters of GC.

Methods Bioinformatics software was used to predict the target binding miRNAs for PGC and target binding circRNAs for candidate miRNAs. Dual-luciferase reporter assay was performed to validate the targeted complete complementary relationship. QRT-PCR was applied to detect the expression levels of PGC and PGC-related ncRNAs in GC tissues. Kaplan-Meier and Cox regression were used for the analysis of the relationship between these ncRNAs and prognostic significance of GC

Results Hsa-let-7c was predicted to binding to PGC gene, hsa_circ_0001483 and hsa_circ_0001324 were targeted binding to hsa-let-7c, which was verified by dual-luciferase reporter assay. The hsa_circ_0001483 / hsa_circ_0001324 -hsa-let-7c-PGC axis was confirmed in GC tissues by qRT-PCR. The expression of hsa_circ_0001483 was correlated with peritumoral inflammatory cell infiltration level and lymphatic metastasis.

Conclusions Hsa_circ_0001483, hsa_circ_0001324 and let-7c were newly identified and validated as PGC-related ncRNAs and were associated with the clinicopathological features of GC in some ways. The hsa_circ_0001483 / hsa_circ_0001324-hsa-let-7c-PGC axis was existed in GC, which may explain the downregulation of PGC in GC tissues.

1. Background

Pepsinogen C (PGC), one of the mature forms of pepsinogen (PG), belongs to the aspartic protease family and is activated under acid conditions [1–3]. In normal physiological conditions, PGC is mainly expressed in the whole stomach and is considered to be the final product of mature differentiated gastric mucosa [4]. Multiple studies have demonstrated that PGC could play important roles in maintaining the normal morphology and physiological function of gastric epithelial cells [5, 6]. According to our previous studies, PGC expression level in the pathological conditions changed significantly, which means that PGC expression in gastric mucosa is decreased considerably during the transformation from superficial gastritis (SG) to atrophic gastritis (AG) and even absent in gastric cancer (GC) [15849808]. And low PGC combined with high expression of the malignant phenotype markers such as MG7-Ag and MMP9 could be important molecular events in malignant transformation of gastric mucosa [6]. In addition, evidence from ProteinChip Arrays and SELD1-TOF MS has demonstrated that PGC is obviously reduced in GC compared to normal gastric mucosa, which was confirmed by 2D electrophoresis and immunohistochemistry [7]. The genome wide association studies have been conducted for searching the GC susceptibility loci and PGC was found to be a key gene in the epithelial differentiation process of stomach [8]. These findings suggested that PGC expression is negatively related to GC occurrence and development [7]. However, the mechanism of the down-regulation of PGC is still unclear. The illumination of PGC gene expression regulatory mechanism can help understand the occurrence of GC in-depth and find early diagnosis indicators and new treatment molecular targets for GC.

Noncoding RNAs (ncRNAs) play important roles in the post-transcriptional regulation of gene expression [9, 10]. MiRNAs are the most well- known ncRNAs involved in the process which can target regulate gene mRNA translation by binding to the 3’-untranslated region (3’-UTR) to lead mRNA transcript degradation or suppression [11, 12]. CircRNAs are another kind of ncRNAs which exist widely in the eukaryotic cells and have tissues expression difference [13, 14]. Many circRNAs contain miRNAs binding sites, which can competitive binding with miRNA response element (MRE), thus sequestering miRNAs from their target genes and further increasing the expression level of the target genes [13, 15]. Due to the role of miRNAs sponge, circRNAs have shown the close relationship with miRNAs which has attracted a lot of attention to explore the relationships among circRNAs -miRNAs-mRNAs network.

As an effective molecular marker from normal to precancerous lesions GC, studies on the regulation of PGC expression by upstream ncRNAs can provide new sights on understanding the occurrence and development of GC. However, there is currently no data about ncRNAs network that regulates PGC gene expression. In this study, we predicted and verified the ncRNAs which were involved in PGC regulation. We next explored the relationships between these PGC-related ncRNAs and clinicopathologic characteristics of GC. This study will suggest clues for elucidating the regulatory mechanisms of PGC as well as provide evidence for finding ncRNAs markers of GC diagnosis and prognosis.

2. Material And Methods

2.1 Target miRNAs and circRNAs for PGC by bioinformatics analysis

In this study, the bioinformatics analysis consists of two steps to find the target miRNAs and circRNAs for PGC. Firstly, we predicted the possible target miRNAs for PGC by using eight kinds of software (including Mirtarbase, http://mirtarbase.mbc.nctu.edu.tw/php/index.php; Diana-microt, http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microtv4/index; MicroRNA.ORG, http://www.microrna.org/microrna/getMirnaForm.do; MiRDB, http://www.mirdb.org/; RNA22-HAS, https://cm.jefferson.edu/rna22/; Targetminer, https://www.isical.ac.in/~bioinfo_miu/targetminer20.htm; Targetscan-vert, http://www.targetscan.org/vert_71/ and Pictar-vert, https://pictar.mdc-

berlin.de/), and as our inclusion criteria when three or more software suggested that the miRNA might be a target ncRNAs of the PGC gene [16–23]. In the part of possible target circRNAs prediction for miRNAs, we used miRanda prediction algorithm (http://www.microrna.org/microrna/home.do, Targetscan (http://www.targetscan.org/) and Starbase (http://starbase.sysu.edu.cn/) to select the possible targets circRNAs for candidate miRNAs [22, 24]. Targetscan predicted miRNA target based on seed region, miRanda mainly based on the binding free energy of circRNAs and miRNAs, the lower the free energy, the stronger the binding ability of both.

2.2 The binding interactions among circRNAs, miRNAs and PGC by Dual-luciferase reporter assay

Prior to the dual-luciferase reporter assays, transfection experiment was conducted to narrow down the range of PGC targets miRNAs. First, the luciferase report gene system was used to construct the luciferase reporter plasmid (pmitGLO-PGC) in the PGC 3′-UTR region, and the mimics of candidate miRNAs from bioinformatics analysis were also constructed (Genoarray Technology, Soochow, China). Both were then co-transfected into the tool cell Hela, and the intensity of the reporter gene was quantitatively measured within 48 hours. And the positive miRNAs in the above experiments were synthesized into overexpressed plasmids and co-transfected with the PGC 3′-UTR region report plasmid into another tool cell Ad293 for verification. MiRNAs which were positive in both cell lines can be the candidate miRNAs for dual-luciferase validation experiments.

Dual-luciferase reporter assay was used to verify the targeted binding relationships between circRNAs and miRNAs, miRNAs and PGC. The wild-type or mutant-type fragments in the PGC 3′-UTR associated with each candidate target miRNAs were designed, synthesized and inserted into GV306 vector (Genechem Co.,Ltd, Shanghai, China). Then, the GV306 vectors and the overexpressed plasmids of candidate miRNAs were co-transfected into Ad293 cell to determine the miRNA target of PGC gene. After 48 hours, the firefly and renilla luciferase activities were measured, and the ratio of firefly luciferase intensity to renilla luciferase intensity was calculated (E2910, Promega). SV40-firefly_luciferase-MCS vector carrying either wild or mutated hsa_circ_0001483, hsa_circ_0001324, hsa_circ_0001051 and hsa_circ_0001614 were co-transfected with hsa-let-7c overexpression plasmid and plasmid NC into Ad293 cell to identify circRNAs with targeted binding ability to hsa-let-7c (Genechem Co.,Ltd, Shanghai, China). After 48 hours, we detected the luciferase activities of these reporters according to the dual-luciferase reporter assay system (E2910, Promega). The dual-luciferase reporter experiment was repeated three times in this round, and three times for each hole.

2.3 Detection of circRNAs, miRNAs and PGC expression in vivo and in vitro experiments

2.3.1 Collection of clinical specimens and accessory information

A total of 66 pairs of tissue samples and accessory information were collected from the first hospital of China Medical University. Overall survival (OS) was followed up for 2 years. GC and their matched distant normal tissues were pathologically diagnosed with two pathologists. The informed consent of these patients has been obtained prior to specimen collection. This study was approved by the ethics committee of the first hospital of China Medical University. In accordance with the Declaration of Helsinki and its subsequent amendments, the written informed consent was obtained from all the participants.

2.3.2 Cell culture

The GC cell line AGS were purchased from ATCC and cultured in F12 medium with 10% fetal bovine serum (HyClone, USA), then incubated at 37°C and 5% CO2.

2.3.3 RNA isolation and qRT-PCR

Total RNA was extracted by using RNAiso Plus reagent according to the manufacturer's protocol (TaKaRa, Japan). The cDNA for circRNA and mRNA was synthesized by PrimeScript RT Master Mix and following the system: 37°C for 15 min and 85°C for 5 sec (Perfect Real Time, cat#RR036A, TaKaRa, Japan). Relative expression level of circRNAs and mRNA were detected by using TB Green Premic EX Taq II (TliRNaseH Plus, cat#RR820A, TaKaRa, Japan). For miRNAs, cDNA was synthesized through miRcute Plus miRNA First-strand cDNA Kit (cact#KR211, TIANGEN) and the relative expression level was measures by using miRcute Plus miRNA qPCR Retection Kit (cat#FP411-02, TIANGEN). β-actin and miR-16 were used as the endogenous reference control. All of the qRT-PCR curves were with single peak. The $2^{-\Delta Ct}$ method was used to compute the relative expression level of cDNA. Primers for circRNAs were tabulated in Supplementary Table 1.

2.4 Statistics analysis

Statistical analysis was mainly performed by SPSSv18.0 (IBM, SPSS, and Chicago, IL, USA) and GraphPad Prism V5.0 software (GraphPad software, USA). And p-value 0.05 was considered statistically significant. For normally distributed data, the Student's t-test was used to analyze the data differences between two groups, while the rank sum test was used for skewed distribution data. Kaplan-Meier was used to analyze the relationship between ncRNAs expression and OS, and Cox regression was performed to identify the prognostic factors of OS.

3. Results

3.1 Identification of miRNAs targeted to PGC gene

In this study, a total of eight bioinformatics prediction software were used to find the target miRNAs for PGC gene. Through screening, 39 possible target miRNAs were found (Supplementary Table 2). We then co-transfected the mimics of these candidate miRNAs with pmirGLO-PGC plasmid into tool cell and nine miRNAs were screened out when the luciferase activity of PGC was downregulated to 90% or less as the selection threshold (Supplementary Table 3). The overexpressed plasmids of these nine miRNAs (hsa-miR-662, hsa-miR-365, hsa-let-7f, hsa-miR-98, hsa-miR-525-5p, hsa-miR-520a-5p, hsa-let-7i, hsa-miR-126-5p and hsa-let-7c) were synthesized and co-transfected with pmirGLO-PGC. And five miRNAs (hsa-miR-365, hsa-miR-520a, hsa-let-7f, hsa-let-7c and hsa-miR-98) were selected out, and the fluorescence activity of PGC was down-regulated to below 85% (Supplementary Table 4). The dual-luciferase reporter assay results showed that hsa-miR-520a, hsa-let-7c and hsa-miR-98 could impair the luciferase activity of the PGC-UTR wild-type reporter, but not of the mutant-type (P = 0.002, P = 0.050 and P = 0.020, respectively, Fig. 1).

In the next phase, AGS cells were transfected individually with PGC overexpression plasmid and ov-NC plasmid. Compared with the control group, only hsa-let-7c was significantly down-regulated in the PGC overexpressed group (p = 0.05, Fig. 2).

3.2 Identification of circRNAs targeted to PGC–related miRNAs

Based on three bioinformatics software (Targetscan, miRanda and Starbase), we finally obtained 12 candidate circRNAs with targeted binding sites to hsa-let-7c for qRT-PCR validation. The expression of four circRNAs (hsa_circ_0012126, hsa_circ_0000365, hsa_circ_0000149 and hsa_circ_0002557) was too low in the tissues to be detected by qRT-PCR, while the other seven circRNAs (hsa_circ_0001483, hsa_circ_0001610, hsa_circ_0001614, hsa_circ_0001685, hsa_circ_0000504, hsa_circ_0001324 and hsa_circ_0001051, Table 1) showed significant differences in expression between 30 pairs GC tissues and normal tissues. We then choose four circRNAs (hsa_circ_0001614, hsa_circ_0001483, hsa_circ_0001324 and hsa_circ_0001051) with the lowest P-value for subsequent dual-luciferase reporter assay for validation of the targeted binding relationship with hsa-let-7c. Based on the predicted binding sites from RNAhybrid, the wild-type or mutant-type of the four circRNAs fragments were constructed, inserted into the downstream of the reporter gene, and co-transfected with hsa-let-7c overexpression plasmid into 293T cell. The luciferase activity was significantly reduced in hsa_circ_0001483 and hsa_circ_0001324 wild-type reporter gene group, respectively (p = 0.002, p□0.001, Fig. 3).

Table 1
Expression of 8 circRNAs in 30 pairs of gastric cancer and non-cancer tissues

Variable	CON		GC		
	N	ΔCt $P_{50}(P_{25}, P_{75})$	N	ΔCt $P_{50}(P_{25}, P_{75})$	P
hsa_circ_0001483	30	8.98(8.18, 10.58)	30	12.11(10.97, 13.31)	0.001
hsa_circ_0001610	30	5.45(4.80, 6.06)	30	6.22(5.44, 7.39)	0.035
hsa_circ_0001614	30	5.73(5.14, 8.97)	30	7.93(6.61, 8.97)	< 0.001
hsa_circ_0001685	30	7.66(7.08, 8.57)	30	9.54(8.57, 10.71)	0.002
hsa_circ_0000504	30	7.43(6.49, 8.12)	30	8.58(7.65, 10.19)	0.012
hsa_circ_0001324	30	4.78(3.76, 5.86)	30	7.37(5.97, 8.52)	< 0.001
hsa_circ_0001051	30	6.16(5.13, 6.53)	30	8.01(6.49, 8.83)	0.001
hsa_circ_0001355	30	3.63(2.64, 4.28)	30	4.51(3.11, 5.73)	0.250

Note: This table showed the expression differences of the 8 circRNAs between gastric cancer and normal tissues. The higher ΔCt value indicates the lower expression. CON, control; GC, gastric cancer.

3.3 PGC-related ncRNAs expression level and clinicopathological features in GC

QRT-PCR results from 30 pairs GC and normal tissues suggested that hsa-let-7c was up-regulated in GC tissues (p = 0.003 Fig. 4). The expression levels for hsa_circ_0001483, hsa_circ_0001324 and PGC in 66 paired GC tissues and distant normal tissues were also detected in this study by qRT-PCR. As shown in Fig. 5, the results suggested both of these two circRNAs and PGC were down-regulated in GC (p□0.001). In addition, we found a positive correlation between hsa_circ_0001324 and PGC in GC and normal tissues (p□0.001, r = 0.480 and p□0.001, r = 0.456, respectively, Table 2). In normal tissues, hsa_circ_0001483 was positively correlated with PGC expression (p = 0.009, r = 0.317, Table 2). While, as far as the current results, the hsa-let-7c expression level had no correlation with PGC and hsa_circ_000483 and hsa_circ_0001324.

Table 2
Correlation among hsa-let-7c, hsa_circ_0001324, hsa_circ_0001483 and PGC

	CON		GC	
	r	P	r	P
hsa-let-7c and hsa_circ_0001483	0.155	0.415	-0.253	0.177
hsa-let-7c and hsa_circ_0001324	0.124	0.514	0.000	0.999
hsa-let-7c and PGC	0.057	0.766	-0.027	0.888
hsa_circ_0001483 and PGC	0.317	0.009	0.202	0.104
hsa_circ_0001324 and PGC	0.456	< 0.001	0.480	< 0.001

Note: The correlation analysis was performed by Spearman's correlation coefficient for the skewed distribution data. CON, control; GC, gastric cancer; PGC, pepsinogen C; r, Spearman's correlation coefficient.

In view of the great diagnostic and prognostic roles of ncRNAs in GC, we also explored the clinical value of these PGC-related ncRNAs. Results suggested that hsa_circ_0001483 expression level was negatively corrected with lymphatic metastasis situation (p = 0.044, r=-0.249, Supplementary Table 5). In addition, hsa_circ_0001483 was highly expressed in the group with more server pericarcinoma inflammatory cell infiltration condition and group with lymphatic metastasis (p = 0.02, p = 0.044, Table 3). In addition, hsa_circ_0001324 was negatively correlated with patients age (p = 0.004, r=-0.350, Supplementary Table 5) and the expression level of hsa_circ_0001324 in the ≥ 60 years old group was lower than that in the 60 years old group (p = 0.005, Table 3). However, the expression level of hsa-let-7c was not significantly correlated with these clinicopathological features in this study (Table 3). We further detected whether the dysregulation of these PGC-related ncRNAs could predict the prognosis of GC patients while the results suggested there were no significant association between these ncRNAs and GC overall survival in this study (Supplementary Table 6 and Supplementary Table 7).

Table 3
Relationship between the expression level of hsa-let-7c, hsa_circ_0001324 and hsa_circ_0001483 in gastric cancer patients and clinicopathological factors

Variability	Patient Number	hsa_circ_0001324(ΔCt) p50(p25, p75)	P($2^{-\Delta Ct}$)	Patient Number	hsa_circ_0001483(ΔCt) p50(p25, p75)	P($2^{-\Delta Ct}$)	Patient Number	hsa-let-7c($2^{-\Delta Ct}$) p50(p25, p75)	P($2^{-\Delta Ct}$)
Gender			0.910			0.189			0.901
Male	42	12.36(11.41, 13.77)		42	18.20(17.40, 19.68)		16	3.76(0.98, 10.39)	
Female	24	12.41(11.15, 13.37)		24	19.11(17.78, 20.61)		14	2.50(1.21, 28.84)	
Age			0.005			0.586			0.917
≥ 60	38	12.65(11.81, 14.10)		38	18.93(17.51, 20.37)		13	2.35(1.11, 14.06)	
< 60	28	11.40(10.77, 12.82)		28	18.30(17.44, 20.00)		17	2.97(1.29, 16.53)	
Smoking			0.748			0.271			0.464
Ever Smoker	16	12.30(11.67, 14.19)		16	19.65(17.59, 20.81)		4	1.52(0.46, 36.32)	
Never Smoker	50	12.43(11.26, 13.26)		50	18.27(17.44, 19.70)		26	2.97(1.21, 12.27)	
Drinking			0.758			0.433			0.454
Ever Drinker	9	11.72(11.68, 14.28)		9	19.67(17.07, 20.93)		2	NA	
Never Drinker	57	12.44(11.26, 13.39)		57	18.27(17.50, 19.92)		28	2.97(1.16, 15.84)	
Family History			0.866			0.449			0.707
Yes	15	12.52(11.22, 14.05)		15	18.59(18.24, 20.47)		8	2.33(1.46, 4.62)	
No	51	12.22(11.28, 13.34)		51	18.15(17.44, 20.07)		22	3.31(0.96, 26.33)	
Location			0.767			0.418			0.891
Body	20	12.28(11.29, 13.41)		20	18.09(16.94, 19.66)		13	2.03(1.28, 7.62)	
Angle	11	11.84(11.11, 12.64)		11	19.30(18.47, 21.65)		3	NA	
Antrum	23	12.60(11.24, 13.84)		23	18.26(17.78, 19.67)		10	2.66(1.40, 44.06)	
Entire	11	12.03(11.31, 14.05)		11	19.59(17.00, 20.61)		3	NA	
Macroscopic Type			0.856			0.718			0.565
Protruded Type	3	NA		3	NA		2	NA	
Ulcerative Type	13	12.44(11.75, 12.86)		13	18.60(17.17, 20.06)		6	2.15(0.88, 16.99)	
Ulcerative Infiltrative Type	32	12.37(10.72, 13.30)		32	18.53(17.88, 20.22)		13	5.17(1.57, 16.53)	
Diffuse Infiltrative Type	18	11.87(11.27, 14.15)		18	18.30(16.77, 19.77)		9	2.97(1.18, 32.08)	
Histological Type			0.744			0.287			0.499
Papillary Adenocarcinoma (I)	0	NA		0	NA		0	NA	

Variability	Patient Number	hsa_circ_0001324(ΔCt) p50(p25, p75)	P($2^{-\Delta Ct}$)	Patient Number	hsa_circ_0001483(ΔCt) p50(p25, p75)	P($2^{-\Delta Ct}$)	Patient Number	hsa-let-7c($2^{-\Delta Ct}$) p50(p25, p75)	P($2^{-\Delta Ct}$)
Well Differentiated Type (II)	1	NA		1	NA		1	NA	
Moderately Differentiated Type (III)	8	12.39(11.84, 12.90)		8	18.07(17.53, 20.10)		2	NA	
Poorly Differentiated Type (IV)	39	12.38(11.24, 14.09)		32	18.33(17.42, 19.77)		15	1.69(1.23, 8.51)	
Mucinous Adenocarcinoma (V)	6	12.73(10.91, 13.30)		5	20.04(18.57, 20.52)		4	2.50(0.92, 8.34)	
Signet-ring Cell Cinoma (VI)	10	11.43(10.88, 13.07)		9	17.87(16.85, 19.18)		6	26.63(3.37, 76.49)	
Lauren Classification			0.757			0.915			0.577
Intestinal	9	12.33(11.84, 12.77)		9	18.15(17.62, 19.84)		3	NA	
Diffuse	55	12.22(11.22, 13.84)		55	18.47(17.42, 20.07)		25	2.97(1.28, 13.88)	
TNM stage			0.473			0.160			0.422
	7	12.01(10.71, 12.64)		7	19.30(18.26, 22.69)		4	4.11(0.78, 9.54)	
	23	12.86(11.11, 14.35)		23	18.15(17.34, 20.37)		9	10.13(1.34, 55.27)	
	36	12.22(11.34, 13.30)		36	18.30(17.44, 19.70)		17	2.03(1.18, 6.84)	
	0	NA		0	NA		0	NA	
			0.606			0.575			0.315
+	30	12.54(11.07, 13.95)		30	18.54(17.51, 21.06)		13	6.73(1.24, 29.72)	
III + IV	36	12.22(11.34, 13.30)		36	18.30(17.44, 19.70)		17	2.03(1.18, 6.84)	
Peritumoral Inflammatory Cells			0.685			0.03			0.159
+	18	11.97(11.29, 12.89)		18	17.80(16.85, 18.68)		9	8.51(2.66, 42.26)	
++	26	12.41(11.02, 13.42)		26	19.30(18.15, 20.62)	0.02	11	1.69(0.99, 5.17)	
+++	21	12.60(11.42, 14.15)		21	18.32(17.40, 20.43)		9	1.44(1.00, 10.30)	
Lymphovascular invasion			0.931			0.984			0.485
+	42	12.37(11.26, 13.77)		42	18.33(17.43, 20.15)		18	2.19(1.10, 8.91)	
-	24	12.36(11.35, 13.29)		24	18.43(17.48, 20.20)		12	4.85(1.36, 33.11)	
Ganglion Invasion			0.352			0.375			0.518
+	53	12.09(11.23, 13.74)		53	18.32(17.37, 20.38)		23	2.97(1.34, 22.94)	

Variability	Patient Number	hsa_circ_0001324(ΔCt) p50(p25, p75)	P($2^{-\Delta Ct}$)	Patient Number	hsa_circ_0001483(ΔCt) p50(p25, p75)	P($2^{-\Delta Ct}$)	Patient Number	hsa-let-7c($2^{-\Delta Ct}$) p50(p25, p75)	P($2^{-\Delta Ct}$)
-	12	12.58(12.09, 13.51)		12	18.53(18.06, 19.59)		6	4.11(0.79, 10.22)	
Lymphatic metastasis			0.332			0.044			0.944
+	45	12.22(11.25, 13.39)		45	18.27(17.42, 19.67)		22	2.19(1.21, 12.00)	
-	21	12.64(11.66, 13.76)		21	19.30(17.87, 22.14)		8	4.85(0.78, 19.83)	
Depth of Invasion			0.583			0.264			0.937
Mucous Layer (pT1)	2	NA		2	NA		1	NA	
Submucosal Layer (pT2)	5	11.48(10.34, 12.68)		5	18.03(17.06, 18.10)		3	NA	
Muscular Layer (pT3)	7	12.52(11.11, 12.86)		7	18.88(18.47, 22.69)		3	NA	
Subserosa Layer (pT4)	1	NA		1	NA		0	NA	
Serosal Layer or Invasion Adjacent Organs(pT5)	51	12.52(11.28, 14.09)		43	18.32(17.40, 20.37)		23	2.35(1.23, 22.94)	
			0.340			0.263			0.845
pT1 + pT2	7	1201(10.37, 12.44)		7	18.04(17.45, 18.26)		4	2.23(1.02, 5.79)	
pT3 + pT4	8	12.18(11.19, 12.81)		8	19.09(18.50, 21.86)		3	NA	
pT5	51	12.52(11.28, 14.09)		51	18.32(17.40, 20.37)		23	2.34(1.23, 22.94)	
Growth Pattern			0.904			0.938			0.067
Massive or Nested	11	12.52(10.31, 13.34)		11	18.15(17.78, 20.38)		26	3.63(1.31, 18.96)	
Diffuse Infiltrative or Infiltralive	55	12.22(11.28, 13.67)		55	18.47(17.42, 20.07)		4	1.17(0.45, 1.89)	

Note: CON, control; GC, gastric cancer. The higher ΔCt value indicates a lower expression while the higher $2^{-\Delta Ct}$ value means the higher expression level .

4. Discussion

As we know, PGC is a key gene in the epithelial differentiation process of stomach, and the regulatory mechanism involved in the significant downregulation of PGC expression in GC still needs in-depth investigation. In the present study, we predicted miRNAs with target binding sites to PGC gene as well as the target circRNAs for the miRNA. Then the dual-luciferase reporter experiments were performed to confirm the targeted binding relationships between PGC and miRNAs, miRNAs and circRNAs. And the expression patterns of PGC and PGC-related ncRNAs in GC tissues have also been verified by qRT-PCR. Finally, let-7c, hsa_circ_0001483 and hsa_circ_0001324 were identified as PGC-related ncRNAs in GC and the relationship between these ncRNAs and clinicopathological characteristics of GC was also revealed from the study.

Dysregulation of ncRNAs have been recognized as the novel molecular signatures that indicated the occurrence and development of cancer. As a kind of ncRNAs, miRNAs play an important role in down-regulating the transcription of target mRNAs. Relationship between miRNAs and GC has been reported in the previous studies [25]. For example, miR-92a-1-5p could increase CDX2 by targeting FOXD1 and mediate gastric intestinal metaplasia [26]. MiR-143-3p may inhibit GC growth and be more sensitive to cisplatin by targeting BRD2 [27]. Our present study focused on finding the ncRNAs that regulated the low expression of PGC in GC tissues. The results of dual-luciferase reporter assay showed that 3 of the miRNAs

predicted by the software were verified to bind to PGC (hsa-miR-520a, hsa-let-7c and hsa-miR-98). According to previous studies, hsa-miR-520 was demonstrated to play role in regulating proliferation and migration of lung cancer and modulating progression of renal carcinoma while there was no evidence about this miRNA and GC [28, 29]]. And miR-98 could be served as biomarker for prostate cancer diagnosis and it may up-regulated in GC, which was involved in the development and progression of GC [30, 31]. Let-7 miRNA family played a central role in the regulatory relationships between miRNAs and mRNAs in GC, and hsa-let-7c was associated with the clinicopathological parameters of GC [32]. The above three miRNAs were first reported to be related to PGC tissue expression in the present study. And our preliminary research results have reported that the serum hsa-let-7c expression level was inversely related to PGC [33]. These PGC-related miRNAs may be involved in the development of GC by regulating the PGC expression while the specific mechanisms still need to be illuminated. In addition, these miRNAs may act as potential biomarkers for GC.

CircRNA was another type of ncRNAs, whose roles in cancers remained to be further investigated. CircRNAs have been demonstrated to act as miRNAs sponges in a variety of cancer types and may potentially become new biomarkers for cancers [34–36]. Many researches have reported that circRNAs played regulatory roles in GC. Rong et al have found that circHECTD1 activated β-catenin/c-Myc signaling by adsorption of miR-1256, and facilitated glutaminolysis, and thus promoting GC progression [37]. CircAKT3 was found to enhance cisplatin resistance in GC by suppressing hsa-miR-198 to up-regulate PIK3R1 [38]. In this study, we predicted the target circRNAs for hsa-let-7c by bioinformatics methods and these 12 candidates were also validated by qRT-PCR in 30 pairs of GC tissues. We selected four down-regulated circRNAs (hsa_circ_0001483, hsa_circ_0001324, hsa_circ-0001614 and hsa_circ_0001051) (p0.001) for the dual-luciferase report assay with hsa-let-7c. The results suggested that hsa_circ_0001483 and hsa_circ_0001324 could combine with hsa-let-7c. Considering the results of dual-luciferase reporter assay, we constructed PGC-specific ceRNA network including hsa_circ_0001483/ hsa_circ_0001324, hsa-let-7c and PGC in GC. According to the results, we supposed that hsa_circ_0001483 and hsa_circ_0001324 could down-regulate PGC expression by competitively binding with hsa-let-7c and these two unreported circRNAs may also serve as biomarkers for diagnosis of GC.

In this study, the expression level of hsa-let-7c, hsa_circ_0001483, hsa_circ_0001324 and PGC in GC tissues and distant normal tissues were further investigated by qRT-PCR. We found that hsa_circ_0001483 and hsa_circ_0001324 was significantly down-regulated in GC tissues and have good collinear relationship with PGC expression which indicated that the low expression of the two circRNAs coincides with PGC low expression in GC. In addition, hsa-let-7c was significantly overexpressed in GC tissues. The above results suggested the possible endogenous interaction between these ncRNAs and gene PGC, that is, the abnormally low expression of hsa_circ_0001483 and hsa_circ_0001324 as well as high expression of hsa-let-7c in GC tissues causes more hsa-let-7c bind to PGC-UTR region, thus down-regulating the expression of PGC. Beyond that, hsa_circ_0001483 expression level was associated with peritumoral inflammatory cell infiltration level and lymphatic metastasis. The immune cells infiltration and metastasis are key events that should be considered in evaluating GC prognosis and therapeutic strategy [39–41]. All these results suggested that the dysregulation of hsa_circ_0001483 may influence the immune response, chemosensitivity-related features and metastasis situation of GC patients, which may serve as potential biomarker for GC prognosis. In addition, we found that the higher expression of hsa_circ_0001324 and hsa_circ_0001483 suggested the trend of better OS ($p = 0.070$ and $p = 0.081$, respectively). Combined with the results of previous study, the low PGC expression level was correlated with shorter OS in GC patients [42], we hypothesized that low expression of hsa_circ_0001483 and hsa_circ_0001324 attenuated their ability to absorb hsa-let-7c, thereby down-regulating the expression of PGC, which may affect the prognosis of GC patients.

5. Conclusions

In conclusion, we first identified the PGC-related ncRNAs and their relationship with the clinicopathologic parameters of GC. Hsa_circ_0001483, hsa_circ_0001324 and let-7c were newly identified and validated as PGC-related ncRNAs and they were associated with the clinicopathological features of GC in some ways. The hsa_circ_0001483/hsa_circ_0001324-hsa-let-7c-PGC axis was existed in GC, which may explain the downregulation of PGC in GC tissues. The identification of the underlying molecular mechanisms in GC is of great significance for detecting therapeutic targets for management strategies. More importantly, by further studying the biological functions of PGC, hsa-let-7c as well as hsa_circ_0001324 and hsa_circ_0001483, we could improve our understanding of the mechanisms of GC and improve the diagnosis and treatment of GC.

Abbreviations

PGC
Pepsinogen
GC
Gastric cancer
PG
Pepsinogen
SG
Superficial gastritis
AG
Atrophic gastritis
3’-UTR

3’-Untranslated region
MRE
MiRNA response element
OS
Overall survival

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of the first hospital of China Medical University. And the written informed consent was obtained from all the participants.

Consent for publication

Not applicable

Availability of data and materials

The data that support the conclusion of this manuscript are included within the article (and its supplementary material).

Competing interests

The authors declare that they have no conflict of interests.

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Authors’ contributions

YY and XQ provide direction and guidance throughout the preparation of this manuscript. XQ and DH completed experiments and data statistics. WY repeated the luciferase experiments independently. DH drafted the manuscript. YY reviewed the manuscript and made significance revisions on the drafts. All authors read and approved the final manuscript.

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References

1. Shen S, Jiang J, Yuan Y: **Pepsinogen C expression, regulation and its relationship with cancer**. *Cancer Cell Int* 2017, **17**:57.
2. Ning PF, Liu HJ, Yuan Y: **Dynamic expression of pepsinogen C in gastric cancer, precancerous lesions and Helicobacter pylori associated gastric diseases**. *World J Gastroenterol* 2005, **11**(17):2545-2548.
3. Ishihara T, Ichihara Y, Hayano T, Katsura I, Sogawa K, Fujii-Kuriyama Y, Takahashi K: **Primary structure and transcriptional regulation of rat pepsinogen C gene**. *J Biol Chem* 1989, **264**(17):10193-10199.
4. Dong Y, Kang H, Liu H, Wang J, Guo Q, Song C, Sun Y, Zhang Y, Zhang H, Zhang Z *et al*: **Myoferlin, a Membrane Protein with Emerging Oncogenic Roles**. *Biomed Res Int* 2019, **2019**:7365913.
5. Massarrat S, Haj-Sheykholeslami A, Mohamadkhani A, Zendehdel N, Aliasgari A, Rakhshani N, Stolte M, Shahidi SM: **Pepsinogen II can be a potential surrogate marker of morphological changes in corpus before and after H. pylori eradication**. *Biomed Res Int* 2014, **2014**:481607.
6. Jiang J, Shen S, Dong N, Liu J, Xu Q, Sun L, Yuan Y: **Correlation between negative expression of pepsinogen C and a series of phenotypic markers of gastric cancer in different gastric diseases**. *Cancer Med* 2018, **7**(8):4068-4076.
7. Melle C, Ernst G, Schimmel B, Bleul A, Kaufmann R, Hommann M, Richter KK, Daffner W, Settmacher U, Claussen U *et al*: **Characterization of pepsinogen C as a potential biomarker for gastric cancer using a histo-proteomic approach**. *J Proteome Res* 2005, **4**(5):1799-1804.
8. Jin G, Ma H, Wu C, Dai J, Zhang R, Shi Y, Lu J, Miao X, Wang M, Zhou Y *et al*: **Genetic variants at 6p21.1 and 7p15.3 are associated with risk of multiple cancers in Han Chinese**. *Am J Hum Genet* 2012, **91**(5):928-934.
9. Liu Y, Guo R, Hao G, Xiao J, Bao Y, Zhou J, Chen Q, Wei X: **The expression profiling and ontology analysis of noncoding RNAs in peritoneal fibrosis induced by peritoneal dialysis fluid**. *Gene* 2015, **564**(2):210-219.

10. Li C, Wang Z, Zhang J, Zhao X, Xu P, Liu X, Li M, Lv C, Song X: **Crosstalk of mRNA, miRNA, lncRNA, and circRNA and Their Regulatory Pattern in Pulmonary Fibrosis**. *Mol Ther Nucleic Acids* 2019, **18**:204-218.
11. Gu Y, Zhao S, Wan J, Meng J, Zuo C, Wang S, Zhou Y, Li H, Wang X: **Hsa-miRNA-125b may induce apoptosis of HTR8/SVneo cells by targeting MCL1**. *Reprod Biol* 2019, **19**(4):368-373.
12. Lin S, Gregory RI: **MicroRNA biogenesis pathways in cancer**. *Nat Rev Cancer* 2015, **15**(6):321-333.
13. Liu L, Wang J, Khanabdali R, Kalionis B, Tai X, Xia S: **Circular RNAs: Isolation, characterization and their potential role in diseases**. *RNA Biol* 2017, **14**(12):1715-1721.
14. Zhang Y, Han T, Li J, Cai H, Xu J, Chen L, Zhan X: **Comprehensive analysis of the regulatory network of differentially expressed mRNAs, lncRNAs and circRNAs in gastric cancer**. *Biomed Pharmacother* 2020, **122**:109686.
15. Sibbald IR, Wolynetz MS: **True and apparent metabolisable energy**. *Br Poult Sci* 1987, **28**(4):782-788.
16. Hsu SD, Lin FM, Wu WY, Liang C, Huang WC, Chan WL, Tsai WT, Chen GZ, Lee CJ, Chiu CM *et al*: **miRTarBase: a database curates experimentally validated microRNA-target interactions**. *Nucleic Acids Res* 2011, **39**(Database issue):D163-169.
17. Maragkakis M, Vergoulis T, Alexiou P, Reczko M, Plomaritou K, Gousis M, Kourtis K, Koziris N, Dalamagas T, Hatzigeorgiou AG: **DIANA-microT Web server upgrade supports Fly and Worm miRNA target prediction and bibliographic miRNA to disease association**. *Nucleic Acids Res* 2011, **39**(Web Server issue):W145-148.
18. Betel D, Wilson M, Gabow A, Marks DS, Sander C: **The microRNA.org resource: targets and expression**. *Nucleic Acids Res* 2008, **36**(Database issue):D149-153.
19. Wong N, Wang X: **miRDB: an online resource for microRNA target prediction and functional annotations**. *Nucleic Acids Res* 2015, **43**(Database issue):D146-152.
20. Bordley JE: **The American Rhinologic Society: its foundation and its future responsibilities. Address of the guest of honor**. *Laryngoscope* 1980, **90**(5 Pt 1):876-879.
21. Rela M, Heaton ND: **Split-liver transplantation**. *Br J Surg* 1998, **85**(7):881-883.
22. Chiang HR, Schoenfeld LW, Ruby JG, Auyeung VC, Spies N, Baek D, Johnston WK, Russ C, Luo S, Babiarz JE *et al*: **Mammalian microRNAs: experimental evaluation of novel and previously annotated genes**. *Genes Dev* 2010, **24**(10):992-1009.
23. Yong MS, Marks GS: **Studies of the chemical nature of the alpha-adrenergic receptor. IV. Labeling studies on nerve-free rabbit aortic strips**. *Biochem Pharmacol* 1969, **18**(7):1619-1626.
24. John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS: **Human MicroRNA targets**. *PLoS Biol* 2004, **2**(11):e363.
25. Cho WC: **OncomiRs: the discovery and progress of microRNAs in cancers**. *Mol Cancer* 2007, **6**:60.
26. Li T, Guo H, Li H, Jiang Y, Zhuang K, Lei C, Wu J, Zhou H, Zhu R, Zhao X *et al*: **MicroRNA-92a-1-5p increases CDX2 by targeting FOXD1 in bile acids-induced gastric intestinal metaplasia**. *Gut* 2019, **68**(10):1751-1763.
27. Chen Z, Li Z, Soutto M, Wang W, Piazuelo MB, Zhu S, Guo Y, Maturana MJ, Corvalan AH, Chen X *et al*: **Integrated Analysis of Mouse and Human Gastric Neoplasms Identifies Conserved microRNA Networks in Gastric Carcinogenesis**. *Gastroenterology* 2019, **156**(4):1127-1139 e1128.
28. Moradi Marjaneh R, Khazaei M, Ferns GA, Avan A, Aghaee-Bakhtiari SH: **MicroRNAs as potential therapeutic targets to predict responses to oxaliplatin in colorectal cancer: From basic evidence to therapeutic implication**. *IUBMB Life* 2019, **71**(10):1428-1441.
29. Zhou W, Wu Y, Pan M, Liu D, Liu B: **Proliferation and Migration of Lung Cancer Could be Inhibited by Oxymatrine through the Regulation for miR-520/VEGF**. *Am J Chin Med* 2019, **47**(4):865-878.
30. Moya L, Meijer J, Schubert S, Matin F, Batra J: **Assessment of miR-98-5p, miR-152-3p, miR-326 and miR-4289 Expression as Biomarker for Prostate Cancer Diagnosis**. *Int J Mol Sci* 2019, **20**(5).
31. Yao Y, Suo AL, Li ZF, Liu LY, Tian T, Ni L, Zhang WG, Nan KJ, Song TS, Huang C: **MicroRNA profiling of human gastric cancer**. *Mol Med Rep* 2009, **2**(6):963-970.
32. Yepes S, Lopez R, Andrade RE, Rodriguez-Urrego PA, Lopez-Kleine L, Torres MM: **Co-expressed miRNAs in gastric adenocarcinoma**. *Genomics* 2016, **108**(2):93-101.
33. Liu WJ, Xu Q, Sun LP, Dong QG, He CY, Yuan Y: **Expression of serum let-7c, let-7i, and let-7f microRNA with its target gene, pepsinogen C, in gastric cancer and precancerous disease**. *Tumour Biol* 2015, **36**(5):3337-3343.
34. Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J: **Natural RNA circles function as efficient microRNA sponges**. *Nature* 2013, **495**(7441):384-388.
35. Ashwal-Fluss R, Meyer M, Pamudurti NR, Ivanov A, Bartok O, Hanan M, Evantal N, Memczak S, Rajewsky N, Kadener S: **circRNA biogenesis competes with pre-mRNA splicing**. *Mol Cell* 2014, **56**(1):55-66.
36. Su H, Lin F, Deng X, Shen L, Fang Y, Fei Z, Zhao L, Zhang X, Pan H, Xie D *et al*: **Profiling and bioinformatics analyses reveal differential circular RNA expression in radioresistant esophageal cancer cells**. *J Transl Med* 2016, **14**(1):225.
37. Cai J, Chen Z, Wang J, Wang J, Chen X, Liang L, Huang M, Zhang Z, Zuo X: **circHECTD1 facilitates glutaminolysis to promote gastric cancer progression by targeting miR-1256 and activating beta-catenin/c-Myc signaling**. *Cell Death Dis* 2019, **10**(8):576.

38. Huang X, Li Z, Zhang Q, Wang W, Li B, Wang L, Xu Z, Zeng A, Zhang X, Zhang X *et al*: **Circular RNA AKT3 upregulates PIK3R1 to enhance cisplatin resistance in gastric cancer via miR-198 suppression**. *Mol Cancer* 2019, **18**(1):71.
39. Li L, Ouyang Y, Wang W, Hou D, Zhu Y: **The landscape and prognostic value of tumor-infiltrating immune cells in gastric cancer**. *PeerJ* 2019, **7**:e7993.
40. Jiang Y, Xie J, Huang W, Chen H, Xi S, Han Z, Huang L, Lin T, Zhao LY, Hu YF *et al*: **Tumor Immune Microenvironment and Chemosensitivity Signature for Predicting Response to Chemotherapy in Gastric Cancer**. *Cancer Immunol Res* 2019, **7**(12):2065-2073.
41. Yang Y, Shao Y, Zhu M, Li Q, Yang F, Lu X, Xu C, Xiao B, Sun Y, Guo J: **Using gastric juice lncRNA-ABHD11-AS1 as a novel type of biomarker in the screening of gastric cancer**. *Tumour Biol* 2016, **37**(1):1183-1188.
42. Fernandez R, Vizoso F, Rodriguez JC, Merino AM, Gonzalez LO, Quintela I, Andicoechea A, Truan N, Diez MC: **Expression and prognostic significance of pepsinogen C in gastric carcinoma**. *Ann Surg Oncol* 2000, **7**(7):508-514.

Figures

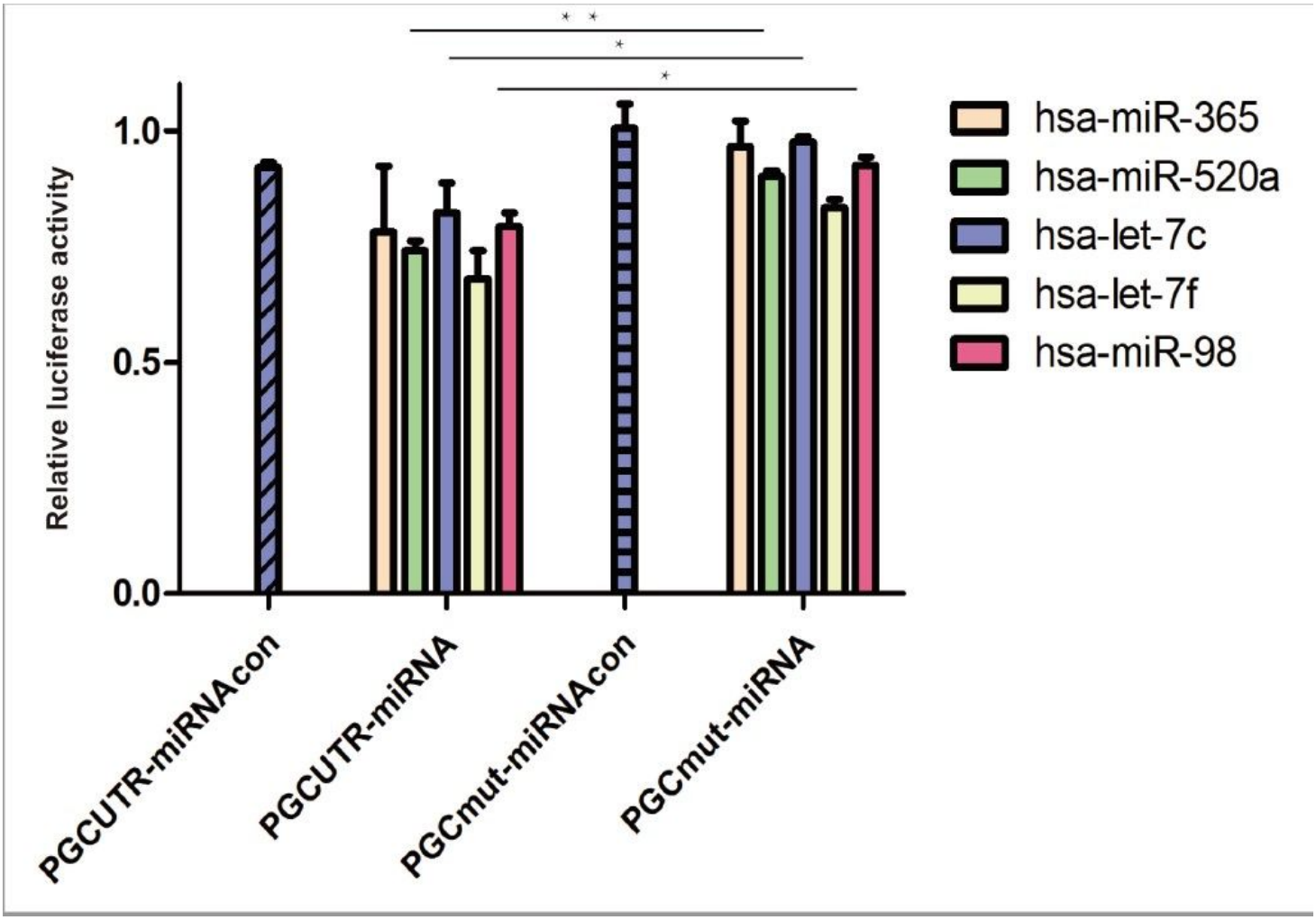

Figure 1

MiRNAs targeted to PGC in dual-luciferase reporter assay. Hsa-miR-520a, hsa-let-7c and hsa-miR-98 was significantly down-regulated in PGC wild-type group when compared with the PGC mutant-type (p=0.002, p=0.050 and p=0.020, respectively).

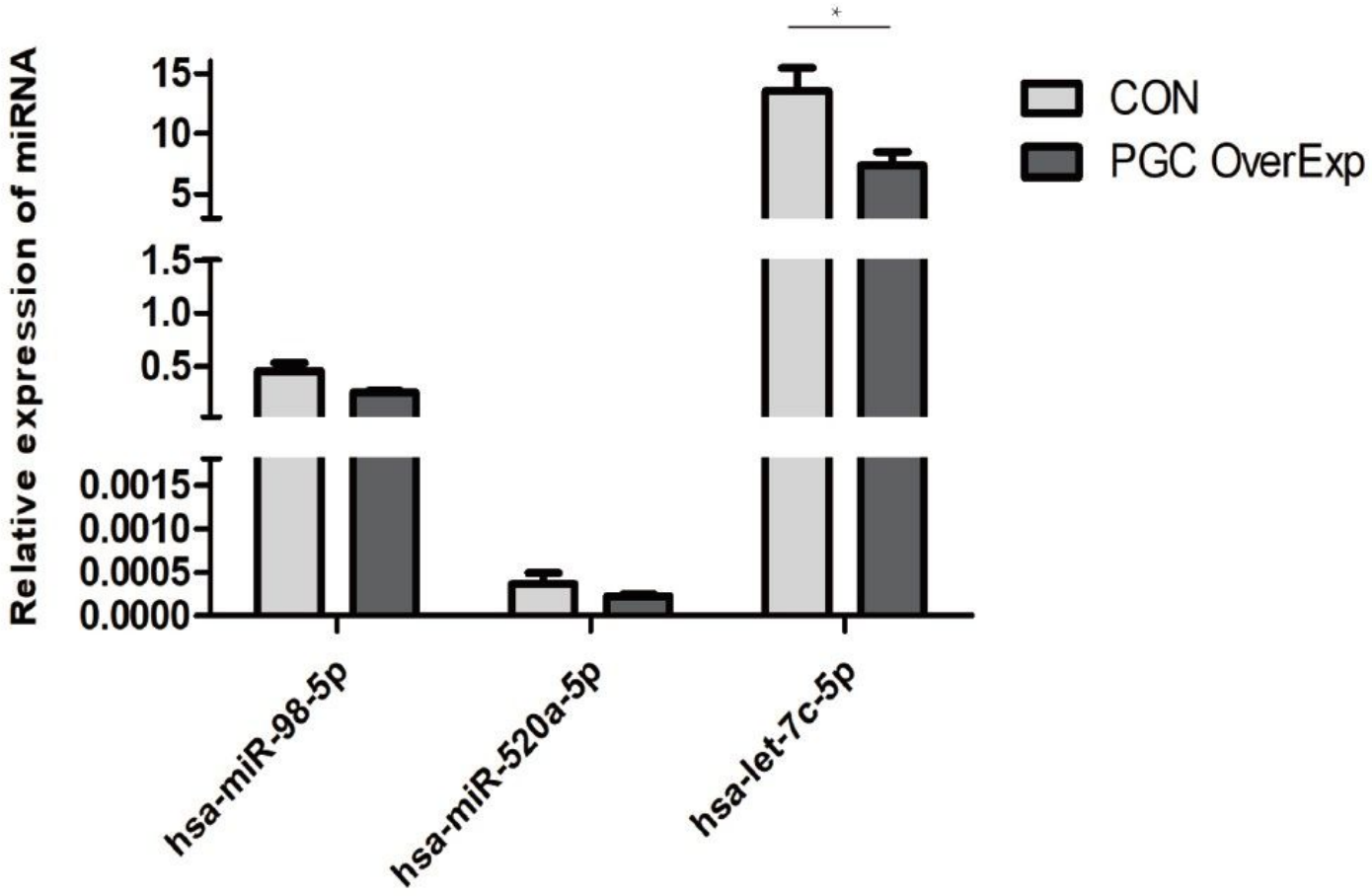

Figure 2

Hsa-let-7c was significantly down-regulated when confected with PGC over-expression plasmid in AGS cells (p=0.050).

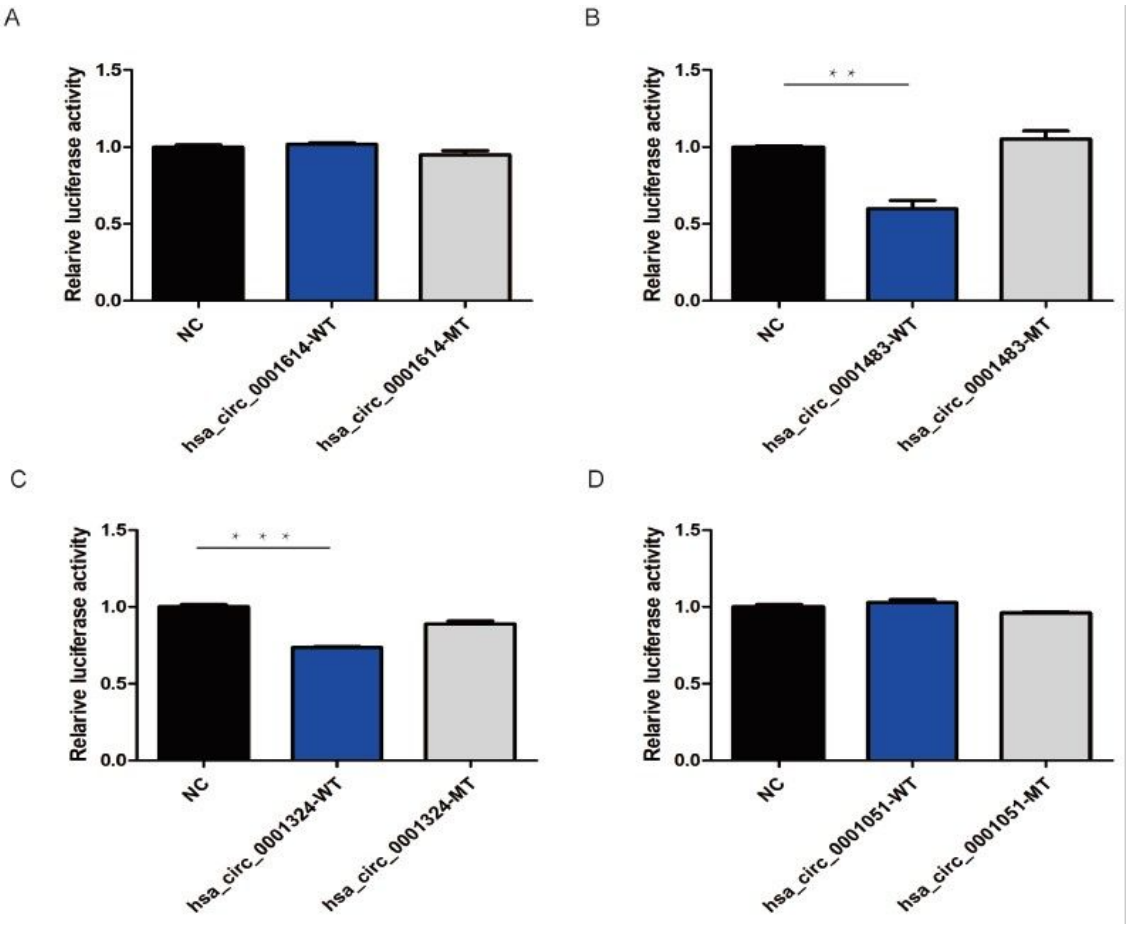

Figure 3

CircRNAs targeted to hsa-let-7c in dual-luciferase reporter assay. A, D. There was no significant reduction in luciferase reporter activity when compared hsa_circ_0001614-WT type and hsa_circ_0001051-WT type with control group. B, C. A significant reduction of luciferase reporter activity was observed in hsa_circ_0001483 and hsa_circ_0001324 wild-type reporter gene group than control group (p=0.002 and p□0.001, respectively).

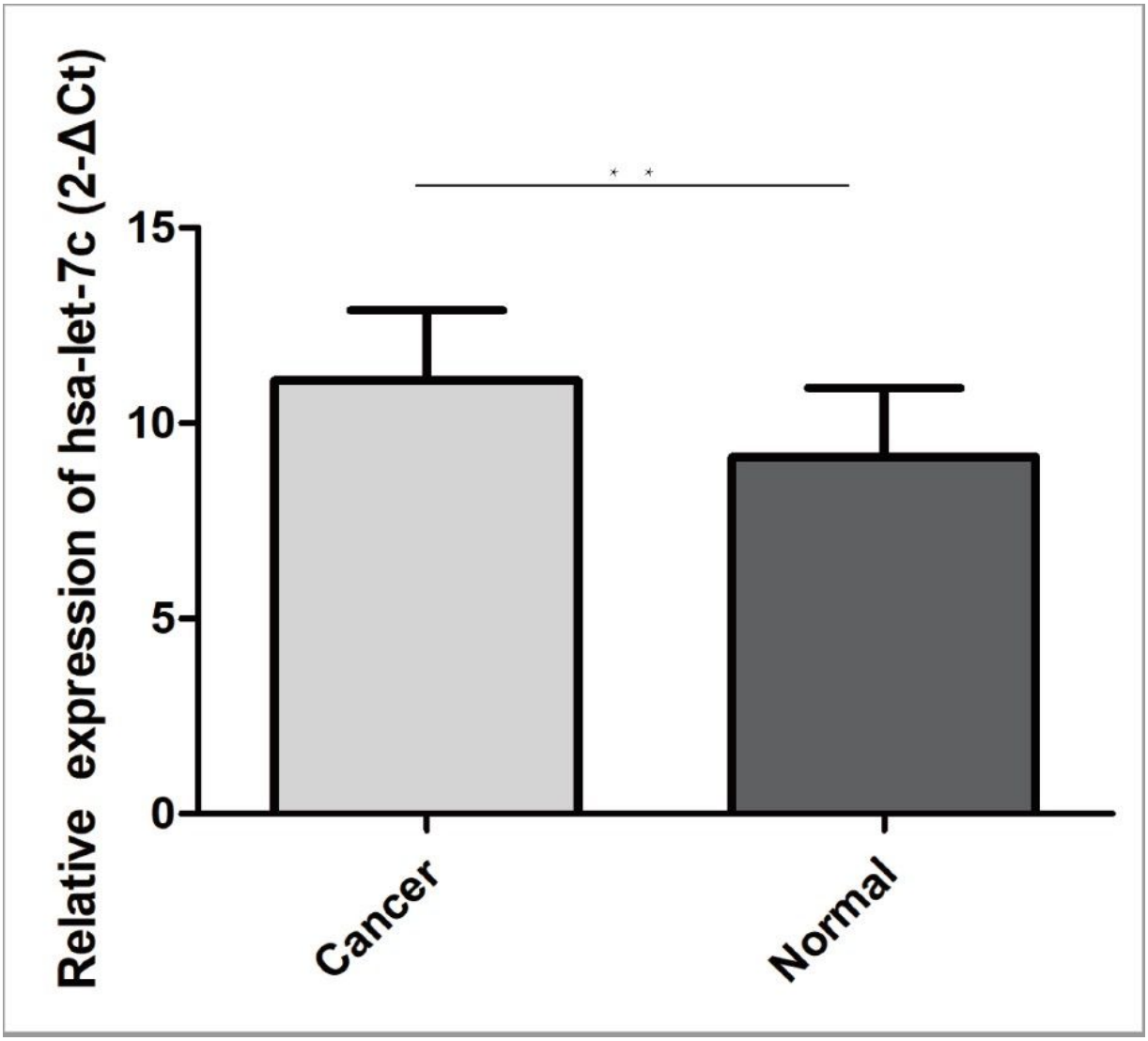

Figure 4

Hsa-let-7c was up-regulated in 30 pairs GC tissues (p=0.003).

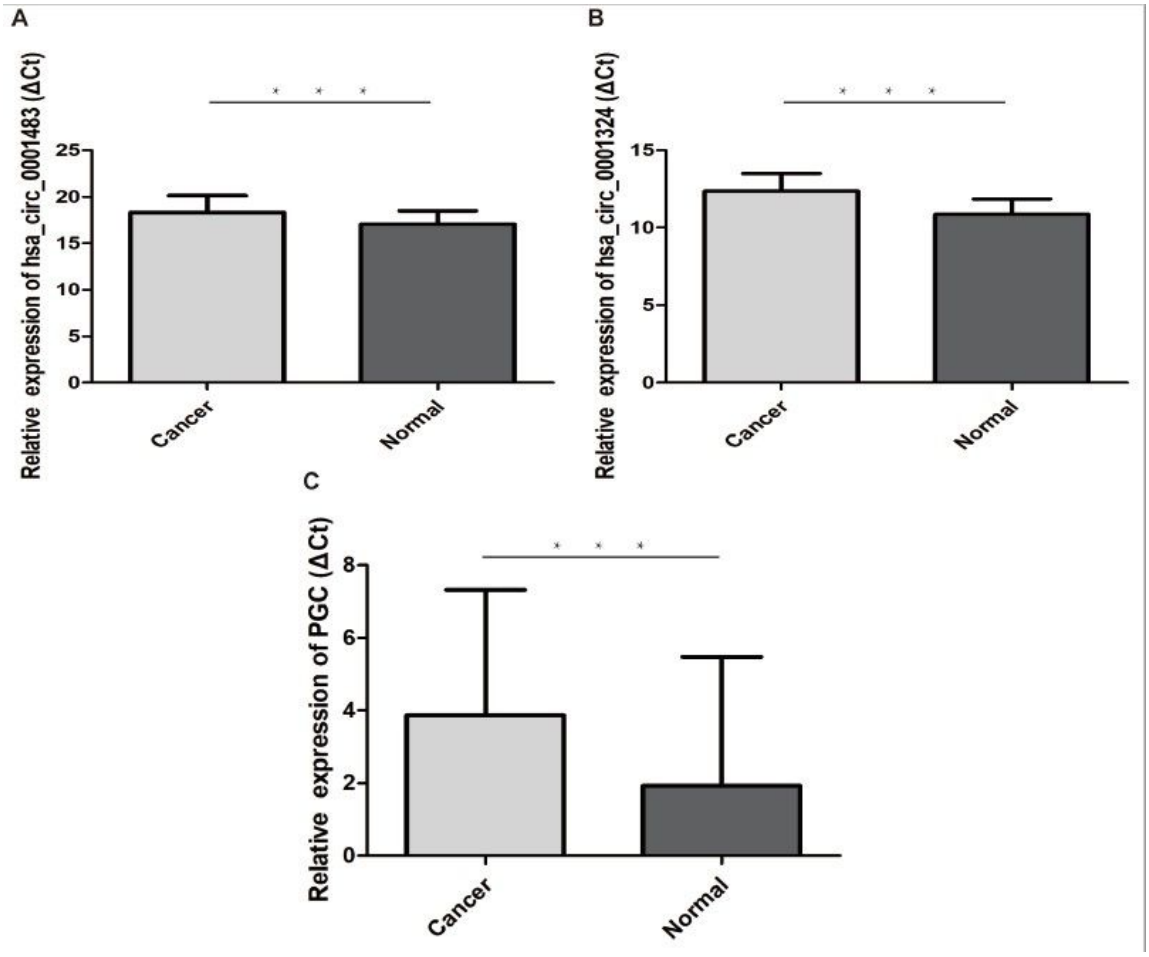

Figure 5

Hsa_circ_0001483, hsa_circ_0001324 and PGC was down-regulated in 66 pairs GC tissues (P 0.001). The higher ΔCt value indicates a lower expression.

Supplementary Files

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