

Overexpression of RECQL4 is associated with poor prognosis in patients with gastric cancer

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Abstract. The present study aimed to investigate the expression, clinical association, and prognosis of RecQ protein-like 4 (RECQL4) protein in human gastric cancers (GCs). The expression levels and prognostic value of RECQL4 were initially predicted by using bioinformatics. GC specimens and matched normal gastric tissues were evaluated by immunohistochemistry (IHC), and patient clinicopathological parameters and survival times were analyzed. Multivariate Cox analysis was used to determine the prognostic role of RECQL4 expression. The Oncomine database predicted that RECQL4 mRNA expression levels were significantly increased in GCs as compared with those in normal gastric tissues ($P<0.05$) and that patients with increased RECQL4 mRNA expression levels had significantly lower overall survival (OS) ($P<0.001$). The results of IHC showed that the positive rate of RECQL4 in the GC samples was significantly higher than that in the normal gastric mucosa specimens ($P<0.05$). RECQL4 expression was positively associated with depth of invasion and TNM ($P<0.05$). High RECQL4 expression in GC samples was significantly associated with poor OS ($P=0.024$). Positive RECQL4 expression, depth of invasion, lymphatic invasion, and TNM staging were independent factors for predicting worse OS rates by using multivariate analysis. Compared with expression levels in normal gastric tissues, RECQL4 was significantly overexpressed in GC samples, and increased

RECQL4 expression was an independent predictor of poor prognosis in GC patients.

Introduction

Gastric cancer (GC) is one of the most common malignant tumors in the digestive track and a leading cause of cancer death in the world (1). In China, according to cancer statistics, the estimated incidence rate for GC was 679.1 per 100,000 people per year, and the mortality rate was ~498.0 per 100,000 people per year, while over 60% patients were in the advanced stage (2). Since the prognosis for GC patients is very poor, many molecular markers, including HER2, E-cadherin, EGFR, and KRAS have been evaluated as candidate prognostic factors for GC (3,4).

RecQ helicases are a group of DNA unwinding enzymes that participate in the process of DNA repair (5). RecQ helicases play an important role in maintaining genome stability, replication, recombination, and transcription (5,6). The RecQ helicase family has five members in human cells: RECQL1, WRN, BLM, RECQL4, and RECQL5. Loss of RecQ helicase protein expression can induce genomic instability and predisposition to cancers (7,8). RecQ protein-like 4 (RECQL4) is a key member of the RecQ family and plays an important role in the initiation of DNA replication, progression of stalled replication forks, and telomere maintenance, as well as in the repair of DNA double-strand breaks via the homologous recombination pathway (9,10). Mutations of the RECQL4 gene are associated with the rare type II Rothmund-Thomson syndrome, which has a propensity for osteosarcomas (11,12). Recent studies have shown that RECQL4 acts as a tumor-promotor in some cancers, such as osteosarcomas, prostate cancer, colorectal cancer, and breast cancer (13-17). Moreover, depletion of RECQL4 has been shown to significantly reduce the proliferation of cancer cells, promote apoptosis, and impair tumorigenicity in tumor-bearing mice (13,15). However, the expression of RECQL4 in GC and its clinical and prognostic significance have been rarely mentioned.

Therefore, in this study, we aimed to compare the expression of RECQL4 in GC tissues with matched normal gastric tissues and to evaluate its prognostic significance in GC

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patients, by using bioinformatics prediction methodology combined with immunohistochemical validation.

Materials and methods

Bioinformatics prediction. The expression of RECQL4 in GC was examined via the online Oncomine database (www.oncomine.org). The following filter combination was applied to analyze corresponding datasets to demonstrate the differences between RECQL4 expression in GC and normal tissues. The data type was set as mRNA, and the analysis type was set as cancer vs. normal analysis. Each filtered dataset was analyzed separately. Differences in RECQL4 expression between different types of GC and normal tissues were compared by using datasets including Cho Gastric, D'Errico Gastric, Chen Gastric and Wang Gastric. The log-transformed and normalized expression values of RECQL4 were abstracted, analyzed, and read from a scatter plot. We performed Kaplan-Meier survival analysis of RECQL4 by using an online tool (<http://kmplot.com/analysis/>). Through the Kaplan-Meier plotter database, we were able to assess the effects of 54,675 genes on survival by using 10,188 cancer samples, including breast, lung, ovarian, and gastric cancers (18). RECQL4 expression and survival data from Affymetrix microarray, including 1,065 GC patients (ID:213520_at), were also analyzed. To analyze the prognostic value of the RECQL4 gene, the samples were divided into two patient groups according to the median expression levels of RECQL4 (high and low). Levels of expression between the two patient groups were compared via a Kaplan-Meier survival plot. The hazard ratio (HR) with 95% confidence intervals (CIs) and the log rank P-value were computed.

Tissue samples and clinicopathological data. GC samples and matched normal gastric tissues from 60 patients who had undergone initial surgical resection between August 2008 and January 2009 were selected from the Department of Gastrointestinal Surgery at the Sixth Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). All samples were collected with the respective patients' informed consent after approval from the Institute Research Medical Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-sen University.

Immunohistochemical analysis. All specimens had previously been fixed in 10% buffered formalin and embedded in paraffin wax. Immunohistochemistry staining was performed according to the manufacturer's instructions by using the rabbit polyclonal antibody against human RECQL4 (H00009401-M09; dilution rate: 1:25, Abnova; Taipei City, China). The protein expression level of RECQL4 was then evaluated by microscopic examination of the stained tissue slides. RECQL4 expression level was determined by visual immunoreactive score (IRS), which was generated by staining intensity (SI) \times number of stained cells. The SI was scored as follows: Negative (score 0), weak (score 1), moderate (score 2), and strong (score 3). We scored the staining extent according to the percentage of positively stained tumor cells in the field: Negative (score 0), 0-25% (score 1), 26-50% (score 2), 51-75% (score 3), and >76% (score 4). If the IRS score was ≥ 4 , the expression of RECQL4 was defined as high, and an IRS score of ≤ 4 was defined as low or none.

Table I. Expression of RECQL4 in gastric tumor and normal gastric tissues.

Samples	High (%)	Low or none (%)	P-value
Gastric cancer	33 (55.0)	27 (45.0)	0.0004
Normal gastric tissue	14 (23.3)	46 (76.7)	
RECQL4, RecQ protein-like 4.			

Statistical analysis. All statistical analyses were performed with SPSS 17.0 software (SPSS, Chicago, IL, USA). The association between RECQL4 protein expression and clinicopathological features was analyzed by using the chi square test. Survival rate was calculated by using the Kaplan Meier method, and the difference was determined by the log-rank test. Multivariate analysis by using the Cox proportional hazards regression model was performed to identify the independent prognostic factors. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Overexpression of RECQL4 mRNA and protein levels in GC. By using Oncomine database mining, we examined and analyzed the expression levels of RECQL4 in GC tissues. As shown in Fig. 1, expression of RECQL4 was significantly elevated in GC tissues ($n=19$, $P=3.23E-8$) as compared with that in normal tissues ($n=31$) by using the Cho Gastric dataset (Fig. 1A). It also demonstrated that RECQL4 expression was significantly increased in the Wang Gastric dataset ($n=27$, $P=0.012$, Fig. 1B). The Chen Gastric dataset showed that RECQL4 expression in gastric intestinal type adenocarcinoma ($n=63$, $P=1.89E-13$), gastric mixed adenocarcinoma ($n=8$, $P=0.002$), and diffuse gastric adenocarcinoma ($n=12$, $P=0.040$) was higher than that in normal tissues ($n=26$, $P=0.01$) (Fig. 1C). D'Errico Gastric dataset revealed that RECQL4 was upregulated in gastric intestinal type adenocarcinoma ($n=26$, $P=3.91E-10$) and gastric mixed adenocarcinoma ($n=4$, $P=0.005$) as compared with that in normal colon tissues (Fig. 1D). To verify the above predictions, immunohistochemical analysis showed that RECQL4 positivity was clearly localized in the nuclei of GC cells and some was found in the cytoplasm (Fig. 2). The positive rate of RECQL4 in the GC samples was significantly higher than that of the normal gastric mucosa specimens ($P < 0.05$; Table I).

Association between RECQL4 differential expression and clinicopathological parameters of patients with GC. RECQL4 expression was positively associated with depth of invasion and TNM ($P < 0.05$), but not with the patients' sex or age, tumor size, tumor location, histological differentiation, lymphatic or venous invasion, lymph node metastasis, or distant metastasis ($P > 0.05$; Table II).

Prognostic value of RECQL4 expression in patients with GC. We analyzed the association between RECQL4 mRNA expression levels and overall survival (OS) in GC patients by using the Kaplan-Meier plotter online software

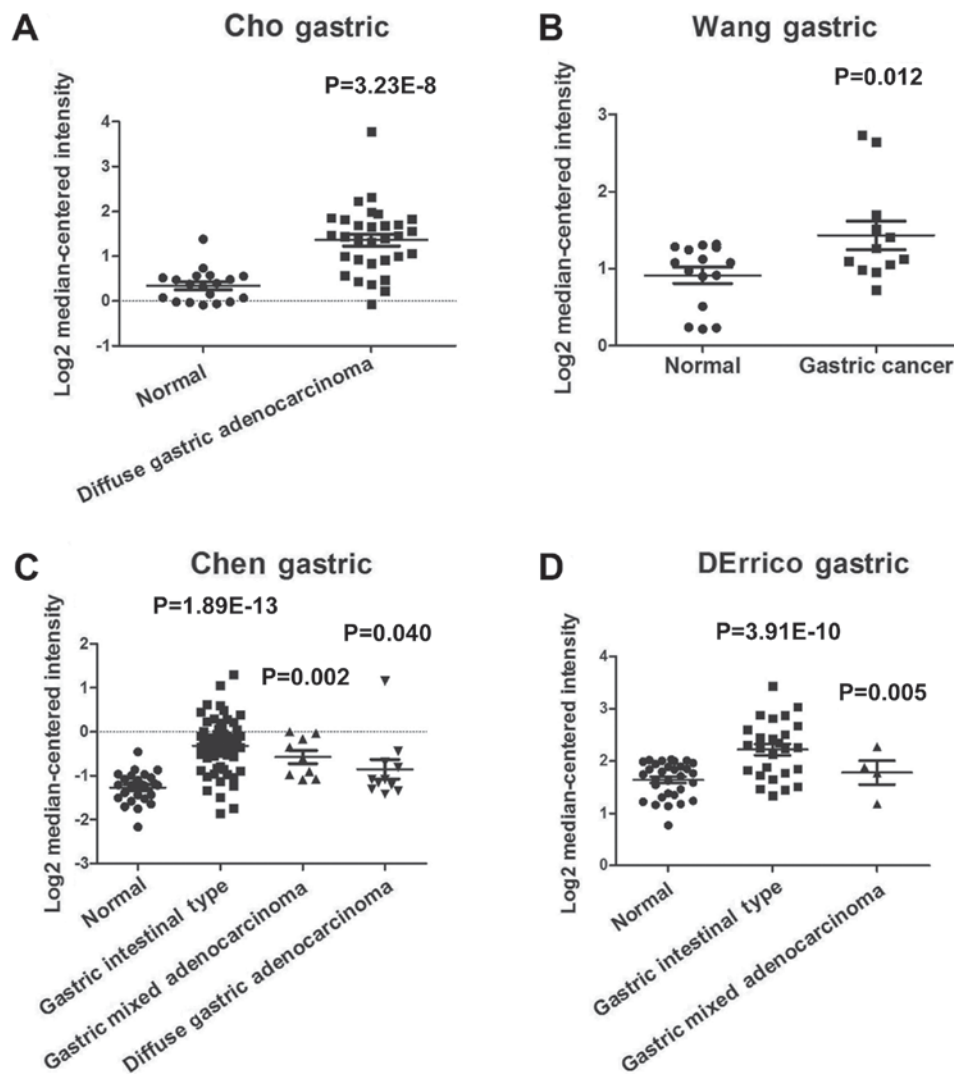


Figure 1. Analysis of RECQL4 mRNA expression in human GC using the Oncomine database. (A) Detection of RECQL4 gene expression in normal and diffuse gastric adenocarcinoma tissues by the Cho Gastric dataset. (B) Differences in RECQL4 gene expression between normal and GC tissues were shown by the Wang Gastric dataset. (C) RECQL4 expression in the normal, gastric intestinal type, gastric mixed adenocarcinoma and diffuse gastric adenocarcinoma tissues as shown by the Chen Gastric dataset. (D) Differences in RECQL4 expression among normal tissues, gastric intestinal type and gastric mixed adenocarcinoma were shown by the DErrico Gastric dataset. P-values were calculated by using two-tailed and Student's t test. RECQL4, RecQ protein-like 4; GC, gastric cancer.

(<http://kmplot.com/analysis/>) based on a public database. We found that the OS of patients with low expression of RECQL4 was remarkably longer than of patients with high expression (HR=1.28, 95% CI=1.06-1.54, $P=0.0093$, Fig. 3A). To verify the above result, the prognostic value of RECQL4 expression in patients with GC was performed by using immunohistochemical data. Follow-up information was available for all of the gastric carcinoma patients for periods ranging between 0.2 months and 10.2 years (mean 65.1 months). The overall 5-year survival rate of the study participants was 53.3% (32/60). The 5-year survival rate of the RECQL4-negative and RECQL4-positive groups was 66.7% (18/27) and 42.4% (14/33), respectively. The OS of patients with GC with high expression of RECQL4 protein was significantly less than that of patients with low expression ($P=0.000$) (Fig. 3B). Univariate analysis using Cox's proportional hazard model indicated that the RECQL4 expression, depth of invasion, lymphatic invasion, and TNM staging were independent prognostic factors for GCs ($P<0.05$; Table III).

Discussion

RECQL4 has been reported to be essential for the maintenance of genomic stability (19). As a key factor of the DNA unwinding helicase, RECQL4 is involved in cell processes (20). A tumor-promoting function of RECQL4 has been widely described (21). For example, Fang *et al* (13) found that overexpression of RecQL4 due to gene amplification plays a critical role in human breast tumor progression, and Arora *et al* (22) demonstrated that shRNA-mediated RECQL4 suppression in MDA-MB453 breast cancer cells significantly inhibited *in vitro* clonogenic survival and *in vivo* tumorigenicity. Su *et al* (15) found that elevation of RECQL4 level was positively associated with the aggressiveness of prostate cancer both *in vitro* and *in vivo*, implying that RECQL4 plays critical roles in prostate-cancer carcinogenesis and is a valuable biomarker for this cancer. However, the expression level of RECQL4 in GC, and its prognostic significance remains controversial.

Table II. Association between RECQL4 expression and clinicopathological features of gastric carcinomas.

Characteristics	No. of patients	RECQL4 protein expression (%)		P-value
		Positive	Negative	
Sex				0.165
Male	39	24 (61.5)	15 (38.5)	
Female	21	9 (42.9)	12 (57.1)	
Age, years				0.706
≥60	37	20 (54.05)	17 (45.95)	
<60	23	13 (56.52)	10 (43.48)	
Location				0.430
Upper third	21	13 (61.9)	8 (38.1)	
Middle and lower third	39	20 (51.3)	19 (48.7)	
Tumor size, cm				0.653
≥5	15	9 (60.0)	6 (40.0)	
<5	45	24 (53.3)	21 (46.7)	
Histological differentiated				0.454
Well/moderate	15	7 (46.7)	8 (53.3)	
Poorly/other	45	26 (57.8)	19 (42.2)	
Depth of invasion				0.035
T1-T2	18	6 (33.3)	12 (66.7)	
T3-T4	43	27 (62.8)	16 (37.2)	
Vascular invasion				0.340
Yes	24	15 (62.5)	9 (37.5)	
No	36	18 (50.0)	18 (50.0)	
Lymphatic invasion				0.306
Yes	22	14 (63.6)	8 (36.4)	
No	38	19 (50.0)	19 (50.0)	
Lymph node metastases				0.172
N0	19	8 (42.1)	11 (57.9)	
N1/N2	41	25 (61.0)	16 (39.0)	
Distant metastasis				0.925
M0	47	26 (55.3)	21 (44.7)	
M1	13	7 (53.8)	6 (46.2)	
TNM				0.028
I-II	20	7 (35.0)	13 (65.0)	
III-IV	40	26 (65.0)	14 (35.0)	

RECQL4, RecQ protein-like 4; TNM, tumor node metastasis stage.

Oncomine is the largest available cancer microarray database. In the present study, we used data mining of the independent microarray datasets (Cho Gastric, D'Errico Gastric, Chen Gastric, Wang Gastric, and Cui Gastric) within the Oncomine database and demonstrated the overexpression of RECQL4 in GC. To verify the above results, we investigated the expression of RECQL4 protein levels in GC tissues. We observed that RECQL4 was localized mainly in the nucleus and some was found in the cytoplasm-results similar to those of other studies (23,24). RECQL4 was expressed in 55.0% of GC samples and 23.3% of matched normal gastric mucosal tissues. Moreover, RECQL4 expression was positively associated with

depth of invasion, TNM staging, and survival times, but not with aggressive parameters such as lymph node metastasis and differentiation. These results are consistent with bioinformatics predictions and suggest that RECQL4 may be a critical factor in promoting the development of GC.

To date, few studies have evaluated the relationship between RECQL4 expression and prognosis of cancer patients. Online Kaplan-Meier plotter analysis proved that RECQL4 predicts a poorer prognosis rate in GC patients. In our present study of GC, the 5-year survival rate of the RECQL4-negative group was higher than that of the RECQL4-positive group. Survival curves showed that cumulative survival rate was significantly

Table III. Multivariate analysis on overall survival (Cox regression model).

Characteristics	Hazard ratio	95% CI	P-value
RECQL4 (+)	1.227	1.062-1.522	0.009
Depth of invasion (T3-T4)	2.956	1.035-8.443	0.043
Lymph node metastases (+)	3.629	1.848-7.126	0.022
TNM (III-IV)	0.309	1.845-6.982	0.007

RECQL4, RecQ protein-like 4; CI, confidence interval; TNM, tumor node metastasis stage.

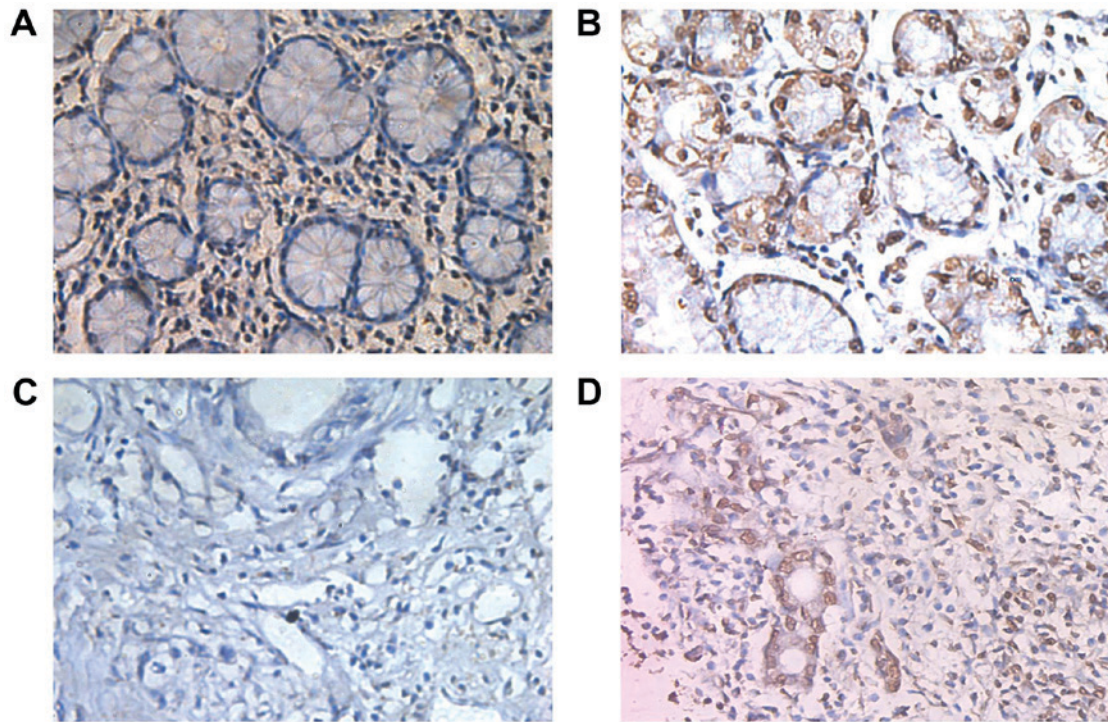


Figure 2. Immunohistochemical examination. Immunohistochemical staining of RECQL4 in the (A) Normal gastric mucosal tissues with negative expression of RECQL4; (B) Normal gastric mucosal tissues with positive expression of RECQL4; (C) GC with negative expression of RECQL4; (D) GC with positive expression of RECQL4. Magnification, x100. RECQL4, RecQ protein-like 4; GC, gastric cancer.

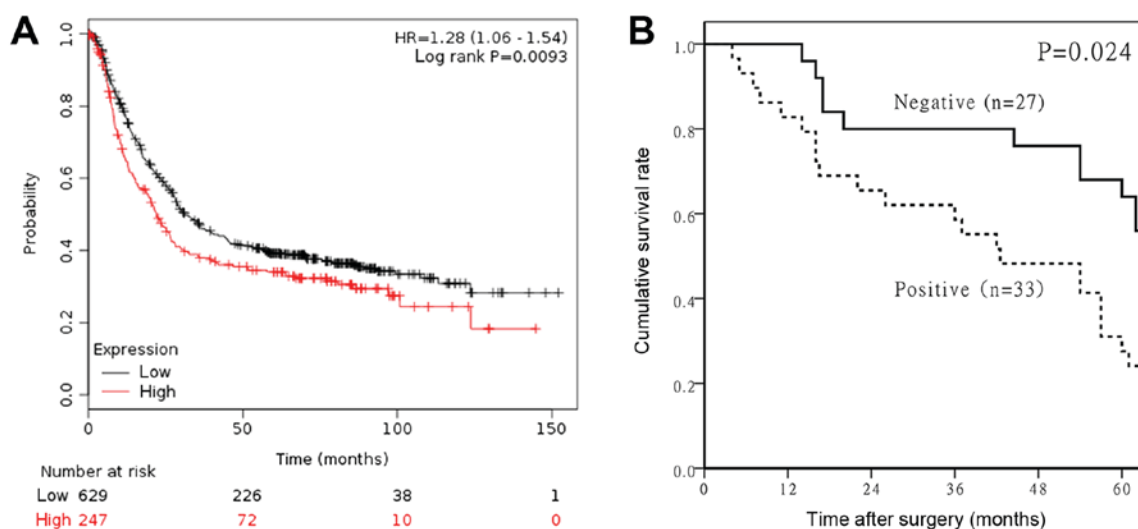


Figure 3. Kaplan-Meier curves of OS of patients with GC based on expression of RECQL4. (A) The relationship between RECQL4 mRNA expression level and OS of patients with GC using the online tool (<http://kmplot.com/analysis/>). (B) The relationship between RECQL4 protein expression levels and OS of patients with GC based on the immunohistochemical data. OS, overall survival; GC, gastric cancer; RECQL4, RecQ protein-like 4.

higher in the RECQL4-negative group. Thus, RECQL4 expression was shown to be a potential prognostic factor in survival analysis. Multivariate analysis also showed that high expression of RECQL4 was an independent factor predicting low OS in GC. Taken together, these observations indicate that RECQL4 may be a predictor of poor prognosis for GC patients.

Several limitations should be mentioned for this study. First, a selection bias may exist due to the non-sequential sample collections. Second, due to the limited sample size, future studies with larger sample sizes are needed to verify these results. Third, further investigations are needed to determine the molecular mechanisms behind the relationship between RECQL4 expression and gastric adenocarcinoma.

In summary, we have shown that RECQL4 is overexpressed in GC samples, and, therefore, suggest that elevated RECQL4 protein expression is an independent factor for poor prognosis in patients with gastric adenocarcinoma and other GCs. The results of this study provide important implications for improving future treatment strategies for these cancers.

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HC, XBW and JP designed this study; HC and KY analyzed and interpreted the patient data, and were major contributors in writing the manuscript. XYW analyzed and interpreted the patient data. HW and QW performed the histological examination of the samples, and were major contributors in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Ethics Committee of the Sixth Affiliated Hospital, Sun Yat-sen University and have been performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all individual participants included in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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