MUC4 is a novel mediator in \textit{H. pylori} infection-related pancreatic cancer

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Received June 1, 2020; Accepted November 24, 2020

DOI: 10.3892/ol.2020.12384

Abstract. Pancreatic cancer (PC) is a common malignant disease worldwide. Among the potential pathogenic factors, \textit{Helicobacter pylori} (\textit{H. pylori}) infection has been associated with the tumorigenesis of PC. The present study aimed to identify the differentially expressed genes (DEGs) of \textit{H. pylori} infection-associated PC and to investigate the key factors involved in PC tumorigenesis. Using bioinformatics methods, overlapping DEGs and key gene were identified from \textit{H. pylori}-infected gastric mucosa (GM) and \textit{H. pylori} infection-associated PC. Survival and tumor stage analyses were performed to assess the clinical associations. In addition, mucin 4 (MUC4) mRNA expression levels were measured in patient blood and tumor samples. According to the correlation analyses of four genes co-expressed, potential biological processes were identified. MUC4 was identified to be associated with \textit{H. pylori} infection, and its levels were significantly upregulated in PC samples compared with those in normal samples in TCGA dataset, the PC cell line and patient tissue samples. \textit{H. pylori} infection was also associated with MUC4 expression in patients' blood and tissue samples. In conclusion, the results of the present study revealed a potentially pathogenic role of MUC4 in \textit{H. pylori} infection-associated PC. Thus, the tumorigenesis and metastasis of PC may be prevented by treating the \textit{H. pylori} infection or using MUC4 antagonists.

Introduction

Pancreatic cancer (PC) is one of most lethal types of malignancy worldwide, with equal incidence rates in men and women (~2.5%) (1). PC is a significant health burden worldwide, affecting >411,600 individuals and exhibiting a 5-year survival rate of <5% (2,3). Although medical technologies have improved in recent decades, no reliable diagnostic biomarkers are currently available for the detection of asymptomatic PC at an early stage. Consequently, PC is usually diagnosed at a late stage, when the majority of patients exhibit distant metastasis. Therefore, >80% of patients with PC are not eligible for surgical resection, which is currently considered to be the most effective treatment (4).

In humans, the incidence of PC is complex, and risk factors include a high-protein diet, high-fat diet, smoking and susceptibility to genetic mutations (5). However, another risk factor is gastric mucosal injury following \textit{Helicobacter pylori} colonization (HP) infection (6). Previous studies have reported that the pathophysiological actions of \textit{H. pylori} colonization, alongside the gastric acidity, potentially modulate pancreatic tumorigenesis via N-nitrosamine intake-mediated hyperchlorhydria (6,7). Due to the crucial role that the tumor microenvironment serves in cancer progression, increasing attention has been paid to the extracellular matrix, stromal, immune and stem cells that predominate the PC microenvironment (8).

According to previous studies, \textit{H. pylori} has developed mechanisms to co-exist in the harsh gastric microenvironment by inducing mucosal inflammation and immune activation (9-11). \textit{H. pylori} may induce the secretion of inflammatory cytokines or neuroendocrine mediators by the infected gastric mucosa (GM) cells (12). In contrast to the direct carcinogenic impact of metabolites on GM or esophageal...
mucosa, *H. pylori* infection may promote neoplasia and metastasis of distant organs such as the pancreas by mediating the cellular microenvironment (13,14).

The results of the aforementioned studies have provided initial evidence of the incidence of *H. pylori* infection-associated PC; however, the biological mechanisms and key factors involved in this process remain poorly understood. In order to improve the current understanding, the present study aimed to identify the factors associated with the potential pathogenicity of *H. pylori* in PC tumorigenesis and metastasis.

**Materials and methods**

**Cell culture.** The human PC cell line PANC-1 and the human normal ductal epithelial cell line HPDE6-C7 were purchased from the Cell Resource Center of Shanghai Institute of Biochemistry and Cell Biology, The Chinese Academy of Sciences, The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and maintained at 37°C with 5% CO₂.

**Patient studies.** Between June and December 2019, a total of 32 inpatient volunteers, among whom 20 patients had chronic gastritis without PC (10 HP⁺ and 10 HP⁻) and 12 had PC (6 HP⁺ and 6 HP⁻), were recruited at The Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University (Changzhou, China) (Table I). The patients were diagnosed with *H. pylori* infection, PC or both. *H. pylori* infection was diagnosed by carbon 13 breath test and gastroscopy. GM infection was determined by gastroscopy. The diagnosis and tumor stage of PC were systemically assessed using imaging, surgery and biopsy. The study was approved by the Ethics Committee of the Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University [approval no: (2019) KYO 073-01], and all patients provided informed consent. PC and adjacent tissue samples (>5 cm from the tumor) were obtained during surgery. Blood samples were obtained after diagnosis. All samples (20 GM, 12 tumor, 12 adjacent tissue and 12 blood samples) were stored at −80°C and preprocessed for further research.

**Microarrays.** Microarray datasets of *H. pylori*-infected GM [dataset nos. GSE6143 (15), GSE47797 (16), GSE70394 (17) and GSE5081 (18)] and PC [dataset nos. GSE85991 (19), GSE27890 and GSE55643 (20)] were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo). By referring to the annotation information, the probe IDs were converted into the corresponding gene symbols. Basic information of the microarray datasets is displayed in Table II.

**Identification and overlap of differentially expressed genes (DEGs).** The DEGs between the tumor and control groups were identified using the GEO2R web tool (http://www.ncbi.nlm.nih.gov/geo/geo2r) by comparing the GEO microarray datasets. Probes without corresponding gene symbols or genes with redundant probe sets were eliminated or processed using the DAVID online tool (https://david.ncifcrf.gov/), respectively. A log (fold-change) ≥1 and P<0.01 were selected to identify statistically significant differences.

The overlap analysis of DEGs from the *H. pylori*-infected GM and PC datasets was conducted and displayed in Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/). In addition, the mRNA expression levels of the DEGs were presented in heatmaps.

**Data acquisition from The Cancer Genome Atlas (TCGA) database.** Gene expression data of PC were acquired from TCGA (https://portal.gdc.cancer.gov) (23). Overall survival (OS) and disease-free survival (DFS) analyses of patients grouped into high/low mucin 4 (MUC4) mRNA expression groups based on the median expression levels were performed using the Gene Expression Profiling Interactive Analysis (GEPIA) online database (http://gepia.cancer-pku.cn) (24). The associations between the expression levels and tumor grades and the meta-analysis of the data from three previous studies (25-27) were analyzed using the Oncomine database (http://www.oncomine.com) (28).

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from the PC cell line and tissues, as well as the corresponding controls, using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The total RNA was reverse-transcribed into cDNA using a PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd.). qPCR was subsequently performed using a SYBR® Premix Ex Taq kit (Takara Biotechnology Co., Ltd.) on a 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reverse transcription was performed at 42°C for 1 h, followed by 95°C for 5 min. The thermostating conditions were as follows: Initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and at 60°C for 1 min. The expression levels were calculated using the 2−ΔΔCq method (29). The primer pairs used were as follows: MUC4 forward, 5'-GAC TTG GAG CTC TTT GAG AAT GG-3' and reverse, 5'-TGCAATGGCCAGACCAGTGCC-3'; GAPDH forward, 5'-ATCATCCCCGTGCCTACTGG-3' and reverse, 5'-GTCCAGGTCCACCACATGAC-3'.

**Multilevel logistic regression analysis.** Basic information of 22 patients (12 PC⁺HP⁺ and 10 PC⁻HP⁻) was obtained from the medical records. The patients were grouped by age (>50 years), sex (male/female), MUC4 expression, HP infection (HP⁺/HP⁻) and PC diagnosis (PC⁺/PC⁻). Multilevel logistic regression analysis was made with grouped patients.

**Statistical analysis.** Data are presented as the mean ± SEM. SPSS 17.0 (SPSS statistics, IBM Inc.) was used for statistical
analysis. GraphPad Prism 6.0 software (GraphPad Software, Inc.) was used to generate the receiver operating characteristic (ROC) curve and perform multilevel logistic regression analysis. Statistical differences were determined using one-way ANOVA with the Bonferroni post hoc test for multiple comparisons. Correlation analysis was performed using the Spearman correlation coefficient. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of overlapping DEGs in H. pylori-infected GM and PC. A total of 32 patients and seven microarray datasets were included in the present study (Fig. 1). Following standardization and processing on the GEO database, DEGs were selected from three H. pylori-infected GM and three PC datasets. Using overlap analysis, six genes were selected among the 44 and 49 DEGs from H. pylori-infected GM and PC datasets, respectively, which were 6-phosphofructo-2-kinase (PFKFB3), sodium-dependent serotonin transporter (SLC6A4), claudin-1 (CLDN1), MUC4, prominin-2 (PROM2) and matrix metalloproteinase 9 (MMP9) (Fig. 2A). The functional annotation of these six genes is presented in Table III. The relative mRNA expression levels of the genes that were significantly upregulated are presented in heat maps in Fig. 2B. For further verification, gene expression data from patients with PC were downloaded from TCGA database. As presented in Fig. 2C, TCGA data analysis revealed that the expression levels PFKFB3, CLDN1, MUC4, PROM2 and MMP9 were significantly upregulated in the tumor tissues compared with those in the normal tissues (P<0.05). However, the expression levels of SLC6A4 were not significantly different between the tumor and normal tissues (P>0.05). These enriched genes were associated with a number of GO functional terms, such as ‘biological regulation’, ‘regulation of biological process’ and ‘regulation of cellular process’, and KEGG signaling pathways, including ‘cytokine-cytokine receptor interaction’,

| First author, year | Platform | Sample type | Sample character | n (Refs.) |
|--------------------|----------|-------------|------------------|----------|
| Wen et al, 2007    | GPL193   | Gastric mucosa | H. pylori⁺ | 9 (15)  |
| Hanada et al, 2014 | GPL13497 | Gastric mucosa | H. pylori⁺ | 4 (57)  |
| Costa et al, 2016  | GPL6480  | Gastric mucosa | H. pylori⁺ | 3 (17)  |
| Galamb et al, 2008 | GPL570   | Injured gastric mucosa | H. pylori⁺ | 8 (18)  |
| Koutsoumpa et al, 2019 | GPL570 | Pancreatic cancer | Tumor | 2 (19)  |
| Chinaranagari et al, 2015 | GPL570 | Pancreatic cancer | Tumor | 4 (58)  |
| Lunardi et al, 2014 | GPL6480 | Pancreatic cancer | Tumor | 45 (20) |

Table II. Microarray datasets of patients with Helicobacter pylori infection and pancreatic cancer.

| First author, year | Platform | Sample type | Sample character | n (Refs.) |
|--------------------|----------|-------------|------------------|----------|
| Wen et al, 2007    | GPL193   | Gastric mucosa | H. pylori⁺ | 9 (15)  |
| Hanada et al, 2014 | GPL13497 | Gastric mucosa | H. pylori⁺ | 4 (57)  |
| Costa et al, 2016  | GPL6480  | Gastric mucosa | H. pylori⁺ | 3 (17)  |
| Galamb et al, 2008 | GPL570   | Injured gastric mucosa | H. pylori⁺ | 8 (18)  |
| Koutsoumpa et al, 2019 | GPL570 | Pancreatic cancer | Tumor | 2 (19)  |
| Chinaranagari et al, 2015 | GPL570 | Pancreatic cancer | Tumor | 4 (58)  |
| Lunardi et al, 2014 | GPL6480 | Pancreatic cancer | Tumor | 45 (20) |

Table I. Clinicopathological characteristics of patients included in the present study.
Table III. Full names and functions of the six key differentially expressed genes identified in the present study.

| Gene symbol | Gene name                      | Functions                                                                                                                                 |
|-------------|--------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------|
| PFKFB3      | 6-Phosphofructo-2-kinase/      | Required for cell cycle progression and prevention of apoptosis; functions as a regulator of cyclin-dependent kinase 1, linking glucose metabolism to tumor cell proliferation and survival. |
|             | fructose-2,6-biphosphatase 3   |                                                                             |
| SLC6A4      | Solute carrier family 6 member 4| A target of psychomotor stimulants, such as amphetamines and cocaine; a member of the sodium: neurotransmitter symporter family; a repeat length polymorphism in the promoter of this gene has been demonstrated to affect the rate of serotonin uptake. |
| CLDN1       | Claudin 1                       | A member of the claudin family; an integral membrane protein; a component of tight junction strands, serving as a physical barrier to prevent solutes and water from passing freely through the paracellular space. |
| MUC4        | Mucin 4, cell surface associated | A major constituent of mucus; serves important roles in the protection of the epithelial cells; has been implicated in epithelial renewal and differentiation. |
| PROM2       | Prominin 2                      | A member of the prominin family of pentaspan membrane glycoproteins; may be involved in the organization of plasma membrane microdomains. |
| MMP9        | Matrix metalloproteinase 9      | Involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction and tissue remodeling, as well as in disease processes, such as arthritis and tumor metastasis. |
‘viral protein interaction with cytokine’ and ‘NF-kappa B signaling pathway’ (Fig. 2D and E).

**MUC4 contributes to H. pylori infection-associated PC.**

The GSE5081 dataset was used to compare the whole genome gene expression profile between patients with HP+ and HP- GM injury. Among the six selected genes, MUC4, MMP9 and CLDN1 were differentially expressed in the two groups. Notably, compared with normal GM tissues, MUC4 expression levels were upregulated in HP+ and downregulated in HP GM injured tissues (Fig. 3A and B). These results indicated that MUC4 may be a potential factor involved in the biological processes of the *H. pylori*-infected GM. For further analysis, the expression profile of MUC4 in human tissues and the associated clinical information were produced using the GEPIA webtool. The human body maps illustrated that MUC4 expression levels were upregulated in pancreatic adenocarcinoma, cervical squamous cell carcinoma and endocervical adenocarcinoma, lung adenocarcinoma (LUAD) and stomach adenocarcinoma (STAD), but downregulated in prostate adenocarcinoma and head & neck squamous cell carcinoma compared with those the corresponding normal tissues (Fig. 3C and D). In addition, Oncomine-based meta-analysis indicated that the expression levels of MUC4 were significantly upregulated in cancer tissues compared with those in normal tissues in the three PC datasets (Fig. 3E). However, as presented in Fig. 3F, MUC4 was not associated with patient OS rates in PC. A weak association was identified...
between low MUC4 levels and DFS rates; however, this result was not significant (P>0.05; Fig. 3G). In addition, although MUC4 expression levels were upregulated at each tumor stage, no significant differences were identified among the four stages (P>0.05; Fig. 3H).

The human PC cell line PANC-1, 20 patients with chronic gastritis without PC and 12 patients with PC with or without \textit{H. pylori} infection were used to verify the trends observed in MUC4 expression. Blood and tissue samples (GM, tumor and adjacent tissues) were collected and analyzed by RT-qPCR. The expression levels of MUC4 were upregulated in HP+ GM compared with those in HP - GM (P<0.05; Fig. 4A). In addition, compared with the normal tissues and cell lines, the expression levels of MUC4 were upregulated in both PC tissues and PANC-1 cells (P<0.05; Fig. 4B). The results of the blood tests revealed that patients in the HP+PC- group exhibited markedly upregulated MUC4 expression levels compared with those in the other groups. (P<0.05; Fig. 4C). These results indicated that the expression levels of MUC4 were significantly upregulated in \textit{H. pylori}-infected GM and PC.

To assess the sensitivity of MUC4, 26 patients were divided into very low-risk (HP-PC-), low-risk (HP+PC-) and high-risk (HP+PC+) groups. A ROC curve was generated, and the results revealed that MUC4 exhibited moderate sensitivity for the identification of \textit{H. pylori}-related PC (Fig. 4D). Multilevel logistic regression analysis of the effects of patient age, sex, MUC4 expression levels and \textit{H. pylori} infection in PC was conducted, which revealed that high MUC4 expression levels and the presence of an \textit{H. pylori} infection were positively associated with PC (Fig. 4E). Correlation analyses of multiple genes co-expressed with MUC4 were conducted using TCGA data, and four tumorigenic factors of PC, including receptor tyrosine-protein kinase erbB-2 (ErbB2), K-Ras, EGFR and SMAD2, were identified to be moderately positively correlated with MUC4 (Fig. 5A).
Figure 4. Verification of MUC4 expression levels in the GM, PC and blood. (A-C) mRNA expression levels of MUC4 in (A) injured GMs with or without 
H. pylori infection, (B) tumor tissues (relative to normal tissues) and PC cell lines (relative to a human normal ductal epithelial cell line), and (C) blood samples 
from patients with H. pylori infection or PC. (D) In a total of 26 patients, a ROC curve was generated to assess the sensitivity and specificity in HP-infection 
related PC of MUC4. (E) Multilevel logistic regression analysis of age, sex, high MUC4 levels and HP infection vs. PC status was conducted. *P<0.01. MUC4, 
mucin 4; GM, gastric mucosa; PC, pancreatic cancer; HP/H. pylori, Helicobacter pylori; ROC, receiver operating characteristic.

Figure 5. Correlation analyses and hypothetical mechanisms of the role of MUC4 in PC. (A) Correlation analyses between the expression levels of ErbB2, 
K-Ras, EGFR or SMAD2 and MUC4 were performed using The Cancer Genome Atlas. (B and C) Hypothetical mechanisms of (B) the secretion and trans-
portation of MUC4 and (C) biological processes of MUC4 in PC. MUC4, mucin 4; PC, pancreatic cancer; ErbB2, receptor tyrosine-protein kinase erbB-2; 
HP, Helicobacter pylori.
Discussion

The pancreas is an important retroperitoneal organ with exocrine and endocrine functions (30). Non-endocrine pancreatic tumors are usually malignant and have different histological features that can be classified as a ductal adenocarcinoma, cystadenocarcinoma or another type of malignant tumor, such as sarcoma and small cell carcinoma (31). In recent decades, the prognosis of PC has remained dismal, with limited improvements achieved for the diagnosis and treatment of the disease. Despite advances in surgery and comprehensive treatment regimens, the poor outcome of patients with PC remains unchanged (32). The results of epidemiological studies have indicated that age, obesity, smoking, long-term alcohol use, chronic pancreatitis and family history are all risk factors for PC (31). With the development of genomics and bioinformatics tools, several genetic mutations have been identified to be involved in PC development; for example, BRCA2 and K-Ras mutations, and loss of p16, SMAD4 or p53 function occur in the epithelium of precursor pancreatic diseases, which markedly accelerate the progression of tumorigenesis (33-35).

The stomach serves several exocrine functions that are similar to the pancreas in the gastrointestinal system, such as producing and secreting various polypeptides and proteins (36-38). *H. pylori*, recognized as the most common gastroduodenal infection, typically colonizes the human stomach (15). Chronic *H. pylori* infection has been associated with an increased risk of several types of disease or pathological lesions, including gastritis, peptic ulcers, dysplasia, neoplasia, mucosa-associated lymphoid tissue lymphoma and invasive gastric adenocarcinoma (39-41). In addition, a potential role of *H. pylori* infection in several extragastric diseases, such as neurodegenerative, cardiovascular, hepatobiliary, pancreatic and colorectal diseases, has been reported (7). Epidemiological and cohort studies in large numbers of PC cases have verified that *H. pylori* infection serves a role in the pathogenesis of chronic and autoimmune pancreatitis, diabetes and PC. Subsequent studies have suggested that *H. pylori* infection may affect the pancreatic physiology and contribute to the tumorigenesis of PC (17). A number of factors produced in response to infection, including ammonia, lipopolysaccharides and inflammatory cytokines, have been demonstrated to induce progressive damage in the pancreas (8). Maisonneuve and Lowenfels and Yadav and Lowenfels (12,42) have reported that similar pathological manifestations to *H. pylori*-infected gastric tissues are also observed in PC. Other previous studies have indicated that increases in gastrin levels and decreases in somatostatin levels are involved in the mechanisms associated with *H. pylori* infection and PC (43). In addition, tissue inflammation, genomic DNA damage and various cytokines, including NF-xB, activator protein-1 and serum response elements, have been reported to contribute to the malignant transformation of pancreatic cells (5,44).

MUC4 has been identified to be associated with *H. pylori* infection. MUC4 is a major constituent of the mucus, the viscous secretion that covers epithelial surfaces, such as those in the trachea, colon and cervix, composed of highly glycosylated proteins called mucins (45). Multiple previous studies have reported a role of MUC4 in the aggressiveness of PC through its ability to enhance tumor growth and metastasis (46-49). The results of the present study demonstrated that the expression levels of MUC4 were also upregulated in LUAD and STAD compared with those in normal tissues, suggesting that the role of MUC4 may depend on the type of cancer.

Although MUC4 levels were upregulated in *H. pylori* infection-associated GM and PC samples compared with those in normal tissues, there is currently lack of evidence of an association between the two in existing studies. Following the analysis of MUC4 expression levels in patient blood and tissues, cytokine transportation and biological networks were hypothetically constructed and visualized in the present study (Fig. 5B and C). The results of the present study revealed that MUC4 expression levels were upregulated in patients with *H. pylori* infection regardless of GM damage compared with those in normal tissues. Thus, *H. pylori* infection potentially promotes the intracellular transcription and the extracellular secretion of MUC4 in GM cells. The circulatory and immune systems and the digestive tract appear to be main routes for MUC4 transportation (46,50). Generally, the microenvironment of PC cells consists of vessels, immune cells, stroma, stem cells, stellate cells and fibroblasts (8,51,52). MUC4 is transported to the surrounding microenvironment, where it potentially participates in the cellular biological processes of PC. MUC4 has been reported to serve as an intramembrane ligand and co-mediate with ErbB2 (53,54). Correlation analyses in the present study revealed that the expression levels of PC tumorigenic factors (ErbB2, K-Ras, EGFR and SMAD2) were associated with those of MUC4 in PC tissues. A previous study has concluded that the MUC4/ErbB2 complex contributes to processes such as differentiation, proliferation, cell cycle, angiogenesis and migration by regulating the EGF, K-Ras, PI3K/AKT and TGF-β signaling pathways (48,55). Therefore, MUC4 produced by the *H. pylori*-infected GM may be involved in mediating cell survival and migration by regulating the PC cell microenvironment.

MUC4 is considered to serve a crucial role in the tumorigenesis and metastasis of PC (47,56). In the present study, MUC4 was found to be upregulated in *H. pylori*-infected GM. Nevertheless, the underlying mechanisms of MUC4 between *H. pylori* infection and PC have received limited attention, especially regarding the cellular transport mechanism. Thus, further studies are required. However, limited samples of *H. pylori*-infected GM are currently available in public databases, which is a limitation of the present study. Additional representative datasets with large samples are needed in future studies.

In conclusion, the results of the present study revealed that MUC4 may be a cytokine involved in the pathogenesis of PC and may represent a novel treatment target. Based on these results, it may be speculated that silencing MUC4 may reduce the tumorigenic risk of *H. pylori* infection in PC.

Acknowledgements

Not applicable.

Funding

This study was supported by the Jiangsu Natural Science Foundation (grant no. BK20181155), the Nanjing Medical
University (grant no. 2017NJMU043), the Changzhou Department of Health (grant no. QN201711) and the Changzhou No. 2 People's Hospital (grant no. 2018K003).

Availability of data and materials

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

Authors' contributions

YG, LT and HL designed the study. HY and YF collected tissue samples and basic information from the patients and carried out statistical analyses. YL and YW carried out bioinformatics analysis. SC performed the experiments. HL wrote and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University [No: (2019) KYO 073-01]. All blood samples and tissues involved in this study were collected with informed consent from the patients and basic information from the patients and carried out statistical analyses. YL and YW carried out bioinformatics analysis. SC performed the experiments. HL wrote and edited the manuscript. All authors read and approved the final manuscript.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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