Reduced Kiss-1 expression is associated with clinical aggressive feature of gastric cancer patients and promotes migration and invasion in gastric cancer cells

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Abstract. Gastric cancer (GC) causes high morbidity and mortality in patients largely due to its invasion and metastasis. Kiss-1 has been shown to be a metastasis suppressor in various malignancies. However, its clinical significance and biological functions in GC have not been thoroughly investigated. The present study investigated the association between Kiss-1 expression and its methylation status and clinicopathological features in GC. Kiss-1 expression was reduced in GC and its low expression was associated with poor histological grade, lymph node metastasis and TNM III+IV stage. Kiss-1 overexpression in AGS GC cells significantly inhibited cell proliferation, migration and invasion in vitro. Kiss-1 knockdown promoted the proliferation, migration and invasion of HGC-27 cells. In summary, the data demonstrated that a low expression of Kiss-1 played a suppressive role for the proliferation, migration and invasion of GC cells. Its expression and methylation levels were associated with the clinical progression of GC. Thus, Kiss-1 is a potential diagnostic and prognostic marker as well as a new target for the treatment of GC.

Introduction

Gastric cancer (GC) is one of the most common malignant tumors in the digestive system. According to the latest statistics of GLOBOCAN, there were approximately 1,033,000 new cases of GC worldwide in 2018, with approximately 783,000 fatalities (1). Based on this evidence, GC ranks 5th in the incidence of malignant tumors and 2nd in mortality worldwide (1). Although various comprehensive treatments including surgical resection, radiotherapy and chemotherapy have been used at different stages of the disease, the incidence, mortality and impaired quality of life thereof are on the increase (2). Therefore, it is necessary to explore new biomarkers and therapeutic targets that may aid the development of targeted therapies for GC.

Metastasis suppressor genes play a key role in tumor metastasis. A previous study demonstrated that the effects of metastasis suppressor genes on tumor metastasis were more critical than those of metastasis promoter genes and that the reduction in the expression levels of metastasis suppressor genes or their loss of expression may induce the invasion and metastasis of tumor cells (3). Kiss-1 was initially identified as an important tumor metastasis suppressor gene in human melanoma cells (4). Kiss-1 is located on the long arm of human chromosome-1. The protein-encoding gene acts as an endogenous ligand for G-protein coupled receptor 54 and produces a variety of physiological effects, including inhibition of tumor cell proliferation, metastasis, invasion and induction of tumor cell differentiation and apoptosis (5,6). A decrease in Kiss-1 levels and the role of this protein in tumor invasion and metastasis have been evaluated in various tumors, such as those of the bladder (7), colorectum (8) and breast (9). However, the expression levels of Kiss-1 and its pathogenesis in GC remain to be elucidated.

The present study examined the methylation status and expression levels of Kiss-1 in GC tissues and subsequently assessed the association between Kiss-1 methylation, Kiss-1 expression and clinicopathological features. The effects of Kiss-1 on the biological function of specific GC cell lines were also studied. The primary aim of the study was to investigate the role of Kiss-1 in the development and progression of GC and whether it could be used for the prevention or treatment of this disease. The data demonstrated that a low expression of Kiss-1 played a suppressive role for the proliferation, migration and invasion of GC cells, rendering kiss-1 a potential diagnostic and prognostic marker.

Materials and methods

Patients and specimens. Samples from GC and non-tumor tissues were collected at the time of surgical resection at the...
First Hospital of Hebei Medical University from June 2014 to June 2016. The samples were snap-frozen in liquid nitrogen and stored at -80°C. Paraffin-embedded tissues were prepared at the Department of Pathology at the same hospital. All diagnoses of GC and gastritis were confirmed by histopathological examination. The relevant information regarding patient history and disease characteristics was extracted from a review of the patients' medical records. The present study was approved by the Institutional Ethical Review Committee of the First Hospital of Hebei Medical University and adhered to the principles of the Declaration of Helsinki. Informed consent was obtained from each patient prior to the collection of the tissues.

Cell culture. Human GC cells (AGS and HGC-27) were purchased from the Culture Collection of the Chinese Academy of Sciences and cultured in F-12k (AGS) and RPMI-1640 (HGC-27) (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml penicillin and 50 mg/ml streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. The cells used were as follows: Methylated Kiss-1 from other cell lines was found._authenticated by STR analysis and no cross-contamination was observed.

The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. The cells were used were as follows: Methylated Kiss-1 forward, 5'-AAAGTT TCGTTTGGAGGTTT-3' and reverse, 5'-CTT TTA TAA AAC CCG AAA TAA CG-3', unmethylated Kiss-1 forward, 5'-AAAGTT TTTTTGGGTTT-3' and reverse, 5'-CCT TTTTAAAACCCAAATAACA-3' (10). The specific location of methylation sites in Kiss-1 promoter region is shown in Fig. 1A. Genomic DNA from GC patient tissue was extracted and modified with sulftle using Universal Genomic DNA Kit and DNA Methylation Kit (CWDBIO, Inc.). Methylation-specific PCR (MSP) with GoldStar Master Mix (CWDBIO, Inc.) was also employed according to the manufacturer's protocol. The thermocycling conditions were: Pre-denaturation at 95°C for 10 min, denaturation at 95°C for 45 sec, annealing for 45 sec (methylation-specific primer amplification annealing temperature 59°C, non-methylation specific primer amplification annealing temperature 55°C) and extension at 72°C for 30 sec. A total of 34 cycles were performed and the final extension was conducted at 72°C for 7 min. The reaction products were separated by 2.0% agarose gel electrophoresis and detected with Ethidium Bromide staining. During electrophoresis, the methylated positive control (CpG methylation enzyme modification of DNA extracted from fresh placental tissues used as a template), the unmethylated positive control (DNA extracted from fresh placental tissues used as a template) and the negative control (H₂O₂) were established. The data were collected using a UV transilluminator (Alpha Innotech Corporation; ProteinSimple) and subsequently analyzed by AlphaView 3.4 (Alpha Innotech Corporation; ProteinSimple).

RNA extraction and reverse transcription PCR (RT-qPCR). Total RNA was extracted from cell or from GC tissues using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was synthesized with the PrimeScript RT reagent kit (Takara Bio, Inc.) according to the manufacturer's instructions. Subsequently, Kiss-1 expression levels were quantified by RT-qPCR using the AceQ qPCR SYBR-Green Master Mix (Vazyme Biotech, Co. Ltd.) in an ABI-7500 quantitative PCR instrument. Gene expression was defined based on Cq values and the gene expression levels were normalized compared with those of the housekeeping gene GAPDH. The 2^(-ΔΔCq) method (11) was used to calculate the relative changes in the expression levels. The thermal cycling conditions used were: 95°C for 5 min, followed by 40 cycles at 95°C for 10 sec, 60°C for 30 sec and 95°C for 15 sec, 60°C for 60 sec and 95°C for 15 sec. The primers used for Kiss-1 were the following: Forward: 5'-CTC ACTGTGTTTTGGGACG-3'; reverse: 5'-CTGCGTCTCCT CCGTGCC-3'. GAPDH was used as an internal reference control and its detection was performed with the following primers: Forward: 5'-GAGTCACCGGATTGTCGT-3' and reverse: 5'-CATGGGTGGAATCATATTGGA-3'.

Detection of the expression levels of Kiss-1 protein by immunohistochemistry. The GC tissue was fixed in 10% neutral formalin for 24-48 h at room temperature and then cut into paraffin sections (4 μm). The sections were placed into a 60-65°C box overnight and deparaffinized in green parent agent, rehydrated with an alcohol gradient and washed briefly in distilled water. For antigen retrieval, the sections were boiled in 0.01 M citrate buffer (pH 6.0) for 20 min at 100°C and washed in PBS three times prior to cooling to room temperature. Endogenous peroxidase activity was blocked with 3% H₂O₂ (Bohai) for 30 min and the sections were washed in PBS three times. Subsequently, the sections were incubated with normal sheep serum for 30 min at room temperature and at 4°C overnight with rat Anti-Kiss peptide monoclonal antibody (1:100 dilution; cat. no. ab55384; Abcam). The following day, the sections were rinsed with fresh PBS and incubated with biotinylated secondary antibody working solution and horseradish enzyme-labeled streptavidin (cat. no. SP-9002; ZSGB Biotech) working solution at room temperature for 30 min. Finally, the sections were stained with 3,3'-diaminobenzidine (cat. no. ZLI-9032; ZSGB Biotech) for visualization. Five fields were randomly selected from the slices and scored according to the percentage of positive cells in the field. The scoring system was as follows: ≤5% corresponded to 0, 6-25% to 1, 26-50% to 2 and ≥51% to 3. The staining intensity score used was as follows: None corresponded to 0, light yellow to 1, yellow to 2 and brown to 3. When the two aspects were added together, a score of ≤3 indicated negative expression, while a score >3 positive expression.

Vectors and transfection of the target genes. Kiss-1 m98 and the control m98 vectors were transfected into AGS GC cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Kiss-1 sh and NC sh were also transfected into HGC-27 GC cells. The transfection efficacy was evaluated by RT-qPCR and western blot analyses.

Western blot analysis. Total protein was extracted from cell using RIPA lysis buffer containing proteinase inhibitor (Solarbio Science & Technology Co. Ltd.), and quantified using the bicinchoninic acid protein assay (Solarbio Science & Technology Co. Ltd.) as recommended by the manufacturers. Approximately 20 μg of total protein was separated by 10% SDS-PAGE and transferred to a polyvinylidene
Anti-mouse IgG (1:2,500 dilution; cat. no. A23910; Abbkine) overnight at 4 °C. The Cell Counting Kit-8 (MedChemExpress) was added to each well and the cells were incubated at 37°C for 3 h. Absorbance was measured at 450 nm using a microplate reader (Promega Corporation).

Cell proliferation assay. The transfected cells were independently seeded in 96-well plates and cultured for 24, 48, 72 or 96 h. The Cell Counting Kit-8 (MedChemExpress) was added to each well and the cells were incubated at 37°C for 3 h. Absorbance was measured at 450 nm using a microplate reader (Promega Corporation).

Transwell migration and Matrigel invasion assays. Cell migration and invasion were measured using 8 µm Transwell chambers (Corning, Inc.). To measure migration, 3.5x10^4 transfected cells were resuspended in 300 µl serum-free F-12K or RPMI-1640 medium and added to the upper chamber, whereas 800 µl F-12K or RPMI-1640 medium containing 10% FBS was added to the lower chamber. To measure invasion, a chamber containing Matrigel (Corning, Inc.) was used and the assay was performed as stated before. Following 24 h of incubation, the chamber was stained by diff-quick staining and the cells were counted in five random fields under a light microscope with a magnification of x200. The number of cells was expressed as an average.

Wound healing assay. When the transfected cells reached a growth density of 85%, the confluent monolayers were scratched with a pipette tip in order to create a gap to simulate a wound and the non-viable cells were washed with PBS. The transfected cells were cultured in RPMI-1640. The images of the plates were obtained under a microscope (Olympus Corporation) at 0, 24 and 48 h.

Statistical analysis. All results are shown as mean ± SD and were analyzed using GraphPad Prism 7 (GraphPad Software Inc.). The differences determined in the in vitro experiments were analyzed using the unpaired two-tailed t-test and the cellular experiments were repeated three times. The differences determined in the clinical tissue experiments were analyzed using the Pearson's Chi-square test. P<0.05 indicated significant differences.

Results

Methylation-specific PCR (MSP) analysis of Kiss-1 gene promoter methylation. The MSP data indicated that hypermethylation of the Kiss-1 gene promoter was observed in 78.43% (40/51) of the GC tissues, whereas this process was present only in 53.49% (23/43) of the non-tumor tissues (Fig. 1B). Chi-square analysis of the patient data collected indicated that the hypermethylation of the Kiss-1 promoter in GC was significantly associated with TNM III+IV stage and lymph node metastasis (P<0.05). However, no significant correlation was noted between the Kiss-1 promoter hypermethylation and other clinicopathological variables such as age, sex and histological grade (P>0.05; Table I).

Kiss-1 mRNA and protein expression in GC. The mRNA expression levels of Kiss-1 were evaluated in 53 GC tissue samples and 53 non-tumor tissue samples. The data indicated that the levels of Kiss-1 in the GC tissues were significantly downregulated compared with those of the normal tissues (Fig. 2A). The expression levels of the Kiss-1 protein were assessed in 56 paired GC and adjacent non-tumor tissues by immunohistochemistry. The data demonstrated that Kiss-1 was expressed in the cytoplasm of gastric carcinoma cells (Fig. 2B). Kiss-1 staining was detected in 48.21% (27/56) of GC tissues and its positive expression was significantly lower in adjacent non-tumor tissues 82.14% (46/56). The correlation between Kiss-1 expression and clinicopathological features was also analyzed in patients with GC. Low expression levels of Kiss-1 in GC tissues were significantly associated with poorly histological grade, lymph node metastasis and TNM III+IV stage (P<0.05). No significant association was observed with the remaining variables including age, sex, tumor size, or depth of invasion (P>0.05; Table II).

Regulation of the Kiss-1 gene in GC cells. The AGS and HGC-27 cell lines were selected to investigate the biological function of Kiss-1 in GC. Transfection of Kiss-1 m98 vector into AGS cells resulted in a significant upregulation of the expression of Kiss-1 at both the mRNA and protein levels (Fig. 3A and B), whereas transfection of Kiss-1 sh significantly downregulated the transcription and synthesis of Kiss-1 in HGC-27 cells (Fig. 3C and D).

Effects of Kiss-1 overexpression on migration, invasion and proliferation of AGS cells. The number of cells migrating through the chamber in Kiss-1 m98 was significantly lower than that noted in the m98 and control groups (P<0.05).
Similarly, the number of cells invading through the chamber in Kiss-1 m98 was significantly lower than that of the m98 and control groups (P<0.05) (Fig. 4A and B). The scratch healing rate of Kiss-1 m98-transfected cells was significantly lower than that of the m98 and control groups (P<0.05) (Fig. 4C and D). In addition, the cell OD values were significantly reduced at 48, 72 and 96 h in the Kiss-1 m98 group (Fig. 4E). The results indicated that overexpression of Kiss-1 inhibited the migration, invasion and proliferation of GC cells.

Effects of Kiss-1 knockdown on migration, invasion and proliferation of HGC-27 cells. Transwell migration, matrigel invasion and wound healing assays were performed following transfection of HGC-27 cells with Kiss-1 sh in order to assess migration and invasion in GC cells devoid of Kiss-1.
The number of cells migrating through the chamber in Kiss-1 sh cells was significantly higher than that in the NC sh and control groups (Fig. 5, \( P<0.05 \)). Similarly, the number of cells invading through the chamber in the Kiss-1 sh group was significantly higher than that noted in the NC sh and control groups (\( P<0.05 \)). The scratch healing ability of Kiss-1 sh-transfected HGC-27 cells was significantly higher than that of the NC sh and control groups (\( P<0.05 \)). In addition, the OD values were significantly increased at 48, 72, and 96 h in the sh group (Fig. 5E). It was deduced that knockdown of Kiss-1 enhanced cell migration, invasion and proliferation.

**Discussion**

GC is the fifth most common cancer in the world and the second most lethal cancer (1). The vast majority of GC fatalities are caused by complications caused by tumor cell metastasis. The signaling pathways involved in the initial control of the tumor cells are activated and the primary tumor cells migrate into adjacent tissues (12). Following contact of the tumor cells with blood and lymphatic vessels, the basement membrane and the endothelial wall are penetrated and the cells are dispersed through the lumen of blood vessels to reach distant organs, facilitating the progression of metastasis (13,14). Therefore, investigation of the expression of tumor metastasis suppressor genes that interfere with specific points in these steps and block the metastatic cascade is critical for early diagnosis, treatment and improved clinical outcomes of GC patients. The Kiss-1 gene was initially reported as a novel metastasis suppressor gene in human melanoma and breast cancer cells (15,16). The translation product of Kiss-1 is a protein containing 145 amino acids, which is further cleaved into Kisspeptin-10, -13, -14 and -54 proteins (17,18). Kiss-1 proteins bind specifically to GPR54 (AXOR12 or hOT7T175) and induce the release of the secondary messenger inositol trisphosphate (IP3) and of the diglycerides, which play a role in cell proliferation, differentiation and apoptosis (5,6).

However, the expression levels of Kiss-1 and its pathogenesis in GC remain unclear. To investigate the mechanism of action of Kiss-1 in GC, the association of promoter methylation with the clinicopathological data was investigated with regard to GC progression. In the present study, hypermethylation of Kiss-1 was present in GC tissues compared with the corresponding levels noted in adjacent tissues, indicating that methylation of Kiss-1 may contribute to the progression of GC. Statistical analysis of the levels of the Kiss-1 promoter methylation and

**Table II. Relationship between expression of Kiss-1 and clinicopathological variables in 56 patients with GC.**

| Clinicopathological features | No. of patients | Strong | Weak | Expression rate (%) | \( \chi^2 \) | P-value |
|-----------------------------|----------------|-------|------|---------------------|-----------|--------|
| Adjacent tissues            | 56             | 46    | 10   | 82.14               | 14.202    | <0.001* |
| Gastric carcinoma           | 56             | 27    | 29   | 48.21               |           |        |
| Sex                         |                |       |      |                     |           |        |
| Male                        | 38             | 20    | 18   | 52.63               | 0.924     | 0.336  |
| Female                      | 18             | 7     | 11   | 38.89               |           |        |
| Age (years)                 |                |       |      |                     |           |        |
| \( \leq 62 \)               | 28             | 13    | 15   | 46.43               | 0.072     | 0.789  |
| >62                         | 28             | 14    | 14   | 50.00               |           |        |
| Tumor size (cm)             |                |       |      |                     |           |        |
| \( \leq 4 \)                | 36             | 19    | 17   | 52.78               | 0.841     | 0.359  |
| >4                          | 20             | 8     | 12   | 40.00               |           |        |
| Histological grade          |                |       |      |                     |           |        |
| Poorly                      | 23             | 7     | 16   | 30.43               | 4.941     | 0.026* |
| Well, Moderately            | 33             | 20    | 13   | 60.61               |           |        |
| Depth of infiltration       |                |       |      |                     |           |        |
| Soaked in serosa            | 35             | 13    | 22   | 37.14               | 4.582     | 0.032  |
| Not soaked in the film      | 21             | 14    | 7    | 66.67               |           |        |
| Lymph node metastasis       |                |       |      |                     |           |        |
| Presence                    | 32             | 8     | 24   | 25.00               | 16.116    | <0.001* |
| Absence                     | 24             | 19    | 5    | 79.17               |           |        |
| TNM stage                   |                |       |      |                     |           |        |
| I+II                        | 31             | 23    | 8    | 74.19               | 18.771    | <0.001* |
| III+IV                      | 25             | 4     | 21   | 16.00               |           |        |

\*Statistically significant result.
the pathological parameters of the GC patients indicated association among lymph node metastasis, TNM III+IV stage and the higher methylation positive rate of the Kiss-1 promoter. This demonstrated that the complexity of tumor pathogenesis was, not only a reflection of genetic change by mutation or deletion, but also a reflection of epigenetic alterations, such
as DNA methylation. In addition to the deletion and mutation of the associated genes, aberrant changes in DNA methylation were considered as the third mechanism leading to anti-oncogenic inactivation (19,20), which played an essential role in tumor development. A previous study suggested that hypermethylation of Kiss-1 occurred frequently in colorectal cancer (CRC) (21). Moreover, it has been confirmed that Kiss-1 methylation is associated with tumor differentiation, depth of invasion, distant lymph node metastasis and predictive recurrence (8,21,22). These data indicated that Kiss-1 methylation may be associated with invasion, metastasis and poor prognosis in GC. Results of those studies are similar to those presented in this study.

Kiss-1 mRNA and protein expression levels are substantially downregulated in GC tissues compared with the corresponding levels noted in non-tumor tissues. This is consistent with the findings of Kostakis et al demonstrating that Kiss-1 expression in adjacent gastric mucosa was considerably higher than that noted in malignant mucosa (23). Subsequently, we performed statistical analysis of the pathological features of the GC tissues and confirmed that low protein expression levels of Kiss-1 were significantly associated with poor histological grade, lymph node metastasis and TNM III+IV stage. This is consistent with other studies reporting that Kiss-1 exhibited low expression levels in CRC and that it may be considered a putative metastasis suppressor in human CRC (24-26). It was hypothesized that Kiss-1 promoter methylation resulted in loss of Kiss-1 expression and metastasis of GC. Previous findings have demonstrated that Kiss-1 hypermethylation is associated with loss of transcription and protein expression in CRC (8). Furthermore, promoter CpG island methylation has been shown to reduce the expression levels of related tumor suppressor genes and is considered the main tumor suppressor-inactivation mechanism in GC (27). Therefore, it was essential to examine whether Kiss-1 expression in GC tissues is also directly affected by the methylation levels of its promoter. Based on results of the present study, we suggest that the Kiss-1 protein plays a role in inhibiting tumor metastasis during the development of GC, further confirming that the Kiss-1 gene is a metastasis suppressor gene in GC and that the downregulation of its expression exhibits considerable significance for clinical development of individualized treatment and disease prognosis.

The biological function of Kiss-1 on AGS and HGC-27 cells was further examined. The data indicated that overexpression of the Kiss-1 gene significantly inhibited migration and invasion of AGS in the GC cell lines used. Its decreased expression was able to promote migration and invasion of HGC-27 cells. These results are similar to those reported by Lee and Kim demonstrating that Kiss-1 may inhibit the invasion of NUGC-3 and MKN-28 GC cells (28). Chen et al reported that Kiss-1 overexpression significantly decreased the invasiveness of CRC cells (29). Previous reports have also suggested that the reduction of Kiss-1 expression promotes cell migration and invasion in pancreatic (30), ovarian (31), prostate (32) and endometrial cancer (33) as well as in nasopharyngeal carcinoma (34). Considering the importance of migration and invasion as two key processes required for tumor progression and metastasis, the results demonstrated the therapeutic potential of Kiss-1 by reducing tumor cell metastatic activity.
The data from the proliferation experiments indicated that the overexpression of Kiss-1 exhibited an inhibitory effect in AGS cells, whereas its knockdown exhibited a promoting effect in the proliferation of HGC-27 cells. These effects appeared 48 h following treatment mainly because Kiss-1 required a longer time to exert its effect on proliferation. Notably, the role of Kiss-1 in the proliferation of various tumors has been well established. Chen et al (29) demonstrated that silencing of the Kiss-1 gene did not influence proliferation of HCT-116 CRC cells, whereas its overexpression resulted in the opposite effects. Knockdown of Kiss-1 in HT115 and HRT18 CRC cells did not have an effect on their proliferation (25). Certain inconsistent proliferation results may be associated with the characteristics of different tumor cells. It has been reported that Kiss-1 inhibits growth of matrix-independent tumors but not of matrix-dependent tumors (34). Therefore, the regulation of Kiss-1 in different tumor phenotypes is more complex than expected and requires further investigation. Some previous studies have only examined Kiss-1 expression in GC tissues or part of its biological role in GC cells (23,28). By contrast, our research systematically investigated the role of Kiss-1 in GC, including mRNA expression, protein expression, methylation status, and clinicopathological data in GC tissues, as well as biological functions after upregulation and downregulation of Kiss-1 in GC cells. We hypothesized that Kiss-1 promoter methylation would lead to loss of Kiss-1 expression, thereby promoting the metastasis of GC. This makes our research content more diverse and the results clearer.

In conclusion, the experiments demonstrated that Kiss-1 may be considered a tumor metastasis suppressor gene closely associated with the development of GC. Kiss-1 was able to inhibit migration and invasion of GC to a certain extent. Consequently, Kiss-1 can be used as a new target for clinical treatment, which may not only eliminate local disease, but also inhibit the systemic spread of GC cells. Additional future studies should be performed to confirm these findings.

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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

WY designed the study. CL, MX and DL performed the experiments. LY, SH and BT analyzed the data. CL and LY wrote the manuscript together. WY helped to revise the manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The study was approved by the Institutional Ethical Review Committee of the First Hospital of Hebei Medical University and adhered to the principles of the Declaration of Helsinki. Informed consent was obtained from each patient before collection of tissues.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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