Introduction

Colorectal cancer, including colon cancer (CC) and rectal cancer (RC), remains the third highest cancer-related incidence and mortality rate (1). At present, surgical resection and chemotherapy, which are the primary strategies for treating CC, have made substantial progress in recent years (2). However, limited advance has been made in cure rates and long-term survival of CC patients in the past several decades. Therefore, more research should be done to understand the progress of CC and discover effective therapeutic strategies for the treatment of CC.

Long non-coding RNAs (lncRNAs) are important regulatory factors in tumor development and progression (3-5). LncRNAs have complicated functions with multiple mechanisms (6). Mounting evidence indicates the existence of a network of lncRNA-microRNA (miRNA) pathways, and the lncRNA-miRNA-gene interaction...
patterns have been systematically characterized in cancers. A comprehensive sponge regulatory network mediated by lncRNA has also shown in prostate cancer (7,8) and hepatocellular carcinoma (9). In CRC, lncRNA-PNUTS serves as a competitive sponge for miR-205, suppressing cell growth through destabilizing HuR protein (10). LINC00336 inhibits ferroptosis in lung cancer cells by acting as a competing miR6852 (11). LncRNA Cancer Susceptibility Candidate 9 (CASC9) is known to be an oncogene and is significantly up-regulated in various cancers (12-15). In hepatocellular carcinoma cells, CASC9 interacts with RNA binding protein heterogeneous nuclear ribonucleoprotein L (HNRNPL) and regulates AKT signaling and DNA damage sensing (16,17). In addition, CASC9 is a potential prognosis marker and therapeutic target in esophageal cancer (18). Studies show that the up-regulation of CASC9 promotes tumorigenesis (19) and metastasis (20) of esophageal squamous, doxorubicin-resistant in breast cancer (21). However, the role of CASC9 in CC has been less reported.

One of main the mechanisms through which lncRNAs function in tumor is acting as microRNA sponges. MicroRNAs are short non-coding RNAs that affect gene expression through targeting and regulating mRNAs. MiR-145-5p is a tumor suppressor and closely related to the prognosis of cancer patients (22-25). MiR-145-5p was also identified significantly associated with overall survival and disease-free survival of patients with glioblastoma or gastric cancer (26-28). In CC, miR-145-5p is down-regulated (29), however, population-based data show that miR-145-5p is high-expressed at advanced stage of CC (30). Therefore, we are interested in investigating the role of miR-145-5p in CC.

In this study, we aim to explore the role of CASC9 as a microRNA sponge for miR-145-5p in CC and to provide a new understanding on CASC9 and miR-145-5p in cancers.

Methods

Patients and tissue sample

Thirty samples and normal adjacent tissues were collected from patients [stage I/II (n=17) and III/IV (n=13)] after surgery in the First Affiliated Hospital of Hebei North University, and stored at −80 °C. This study was authorized by the Ethics Committee of the First Affiliated Hospital of Hebei North University (NO. HNU20150703324), and all patients signed an informed consent.

Cell culture

Human intestinal epithelial cells (HIEC, CRL-3266) and human CC cell lines [(LoVo (CCL-229), HCT116 (CCL-247), SW480 (CCL-228), HT29 (HTB-38)] were obtained from the American Type Culture Collection. The HIEC, LoVo, HCT116 and SW480 cells were grown in RPMI-1640 (61870044, Gibco, ThermoFisher, USA) containing 10% FBS (16140044, Gibco, ThermoFisher, USA), 1% penicillin/streptomycin (15070063, Gibco, ThermoFisher, USA) at 37 °C with 5% CO2. HT29 cells were grown in McCoy's 5A (Modified) Medium (16600108, Invitrogen, ThermoFisher, USA) supplemented with 20% FBS (16140071, Invitrogen, ThermoFisher, USA), 1% penicillin/streptomycin (15070063, Gibco, ThermoFisher, USA).

Cell transfection

The siRNA targeted toward human CASC9 (30) (siCASC9: 5'-GCCUGUGAUAGCAGAACAATT-3') and a non-specific negative control siRNA (siNC: 5'-UUCUUCGAACGUGUCACGUTT-3'), miRNAs inhibitor control and miR-145-5p inhibitor were purchased from Gene Pharma Co., Ltd. (Shanghai, China). 100 pmol of siRNA or miRNA inhibitor were respectively transfected into the cells (4×105 cells/well) by using 500 µL Opti-MEM (11058021, Invitrogen, USA) containing 5µL lipofectamine® 2000 (11668019, Invitrogen, USA) at room temperature for 5min. After incubation at 37 °C for 24 h, subsequent functional detections were performed.

RNA isolation and qualitative real time PCR analysis

Tumor specimens and corresponding adjacent normal tissues were disrupted using liquid nitrogen and homogenized using Trizol reagent (15596018, Invitrogen, Thermofisher, USA) at 4 °C. Qiagen RNeasy Plus Mini Kit (74134, Qiagen, Germany) was used to isolate the total RNA from the tissue samples at 4 °C. For isolation of RNA in the cells, the cells were homogenized using TRIZol reagent and isolated by chloroform and isopropanol at 4 °C. The concentration of RNA was measured by using NanoDrop 8000 (ND-8000-GL, Thermo Scientific, USA).

For the quantification of mRNA, cDNA was prepared by using PrimeScript™ II 1st Strand cDNA Synthesis Kit (6210B, Takara, Japan). SYBR® Green PCR Master Mix (4312704, ABL, USA) and Bio-Rad CFX 96 Touch Real-Time PCR Detection System (1855196, Bio-Rad,
China) were used for qRT-PCR, with GAPDH served as endogenous control. QRT-PCR parameters were as follows: pre-denaturation at 95 °C for 5 min, then 40 cycles of denaturation (at 95 °C for 30 s), annealing (at 60 °C for 30 s), and extension (at 72 °C for 30 s). For the quantification of microRNA, the cDNA was prepared by One step miRNA RT kit (D1801, HaiGene, China). SYBR Green qPCR kit for miR-145-5p (AP01411, HaiGene, China) and for U6 snRNA (AP02055, HaiGene, China) were then used for qRT-PCR, with U6 snRNA served as an endogenous control. QRT-PCR parameters were as follows: at 95 °C for 5 min, 40 cycles of denaturation (at 95 °C for 15 s), annealing (at 60 °C for 30 s), and extension (at 70 °C for 10 s). 2-ΔΔCT was used to calculate the relative expression levels. All primers used in qRT-PCR were shown in Table 1. The primers used in qRT-PCR for miR-145-5p were designed according to one previous study (31).

### CCK-8 assay

The cells (5×10^3 cells/well) were cultured in 96-well culture dishes. After culturing at 37 °C for 24, 48 and 72 h, CCK-8 solution (70-CCK801, MultiSciences, China) was separately added into the dishes (10 µL/well) to continue the incubation of the cells at 37 °C for 4 h in the dark. Absorbance values were measured using a microplate reader (1681135, Bio-rad, China) at 450 nm.

### Cell colony formation assay

The cells (1,000 cells/well) were seeded into 60 mm plates and cultured for 7 d. Afterwards, colonies were fixed by 4% paraformaldehyde (30525-89-4, Aladdin, China) for 20 min and stained by 0.5% crystal violet (548-62-9, Aladdin, China) at room temperature for 1 h. The picture of colonies was directly took by a camera. The colonies (diameter >0.5 mm) were counted by Image J software v.1.48 (National Institutes of Health, USA). The experiments were conducted in triplicate.

### Western blotting

The cells were gently washed by 1× PBS twice and treated by 1% NP-40 (FNN0021, Invitrogen, ThermoFisher, USA) supplemented with halt protease inhibitor cocktail (78429, Thermo Scientific, ThermoFisher, USA) on ice and proteins were collected by centrifugation at 1,000 g, 4 °C for 30 min. The concentration of the proteins was measured by Pierce™ BCA Protein Assay Kit (23225, Thermo Scientific, ThermoFisher, USA). Next, 25 µg protein extraction were separated on 10% SDS-PAGE gel and then transferred to PVDF membranes (LC2002, Invitrogen, Thermofisher, USA). The membranes were washed by 1xTBST (50 mM Tris, 150 mM NaCl and 2% Tween-20; pH 7.5) for three times at room temperature and then incubated with primary antibodies (Anti-Ki-67 (ab15580, 1:2,000, Abcam, UK), Anti-PCNA (ab92552, 1:2,000, Abcam, UK) and Anti-GAPDH (ab181602, 1:5,000, Abcam, UK)) at 4 °C overnight. Next, the membranes were further incubated with anti-rabbit IgG antibody (1:2,000, 7074, Cell Signaling Technology, USA). The membranes were then exposed to X-ray. Images were captured by ImageQuant ECL Imager (28-9605-63, GE Healthcare, USA) and analyzed by using Image J software v.1.48. GAPDH was used for normalization.

### Flow cytometry

For evaluation of cell-cycle arrest, the cells (1×10^6–5×10^6 cells) were digested by trypsin (25300054, Gibco, Thermofisher, USA) for 2min at 37 °C and collected by centrifugation at 450 g, 4 °C for 5 min. After removing the supernatant, the cells were fixed by 70% ethanol (64-17-5, Aladdin, China) at 4 °C for 2 h and then treated by 1 µg/ml propidium iodide (PI, 25535-16-4, Aladdin, China) in 1 mL PBS at 4 °C for 30 min. Then, FACSCalibur flow cytometer (342973, BD Biosciences)

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**Table 1** Primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>CASC9</td>
<td>TTGGTCAGCCACATTGTTGTTTGTCATAAAGGTCCTTAGG</td>
<td>AGTGCCATGACTCTGCCAGC</td>
</tr>
<tr>
<td>MiR-145-5p</td>
<td>TCTTGTCAAAAGTCTCTTGGAACTCTTGG</td>
<td>GTGCAGGGTCCGAGGT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGAGCGAGATCCCTCCAAAT</td>
<td>GGCTGTTGCTACATTCTCATGG</td>
</tr>
<tr>
<td>U6</td>
<td>CTCGCTTCGGCAGCACAAAC</td>
<td>AACGCTTCAGAATTGGCATCTGGT</td>
</tr>
</tbody>
</table>
USA) was used for determining cell cycle, and data were analyzed by BD FACSCanto™ system software v2.4 (646602, BD Biosciences, USA).

Annexin-V kit (70-AP101-100-AVF, MultiSciences, China) was used to detect cell apoptosis. After trypsinization, the cells were washed twice by cold 1× PBS. After centrifugation at 450 g, 4 °C for 5 min, the cells (1×10^5–5×10^6 cells) were resuspended with 5 µL Annexin-V-FITC solution and incubated for 15 min at room temperature in the dark. The nuclei were stained by 5 µL PI (25535-16-4, Aladdin, China) for 5 min, followed by adding of 200 µL binding buffer. After that, FACSCalibur flow cytometer (342973, BD Biosciences, USA) were used to determine cell apoptosis. BD FACSCanto™ system software v2.4 (646602, BD Biosciences, USA) was used for data analysis.

**Dual-luciferase reporter assay**

The binding sites for miR-145-5p in lncRNA CASC9 was predicted by the StarBase Software (http://starbase.sysu.edu.cn/). CASC9 wild type sequence and mutation sequence containing miR-145-5p target site were purchased from Tsingke Co., Ltd. and respectively cloned into luciferase reporter gene vector (pmirGLO, E1330, Promega, USA) as CASC9-WT reporter plasmid and CASC9-MUT luciferase reporter plasmid. Briefly, reporter plasmids CASC9-WT and CASC9-MUT were separately transfected into HCT116 cells or SW480 cells. MiRNA-145-5p inhibitor was also transfected into cells by luciferase reporter system. After incubation for 48 h, the luciferase activity was determined by dual-luciferase assay system (E1910, Promega, USA) and Microplate Luminometer (11300010, Berthold, Germany). The firefly luciferase activity was normalized against renilla luciferase activity and presented as mean ± SD.

**Statistical analysis**

SPSS 16.0 was used for statistical analyses. The data were shown as mean ± standard deviation (SD). Student’s t-test (two-tailed) and one-way ANOVA, followed by Dunnett’ s post hoc test, were performed for statistical analyses. The difference was considered to be significant if P<0.05.

**Results**

**LncRNA CASC9 is up-regulated in CC tissues and cells**

The expression of IncRNA CASC9 in tumor-adjacent CC tissues and normal colonic tissues from CC patients was determined. The results showed that CASC9 was obviously up-regulated in CC tissues (P<0.001), and that the level of CASC9 was higher in CC tissues derived from patients at III/IV stages than those at I/II stages (P<0.001, Figure 1A,B). In addition, the level of IncRNA CASC9 was also evaluated in HIEC cells and LoVo, HCT116, SW480 and HT29 cells. The results demonstrated that CASC9 was up-regulated in CC cell lines than in HIEC cells (P<0.001, Figure 1C), with CASC9 level higher in HCT116 and SW480 cells than that in other CC cell lines, thus, HCT116 and SW480 cells were used for further research.

**Knockdown of CASC9 inhibits cell growth, promotes cell apoptosis and arrests cell cycle in CC cells**

The expression level of CASC9 in HCT116 and SW480 was significantly down-regulated by siCASC9 (both P<0.001, Figure 1D,E). CCK-8 assays showed that the cell viabilities of HCT116 and SW480 cells were decreased by knocking down CASC9 (both P<0.001, Figure 1F and G). Cell colony formation assays in HCT116 and SW480 cells also confirmed that silencing CASC9 suppressed cell growth (both P<0.001, Figure 2A,B). The protein expressions of Ki67 and PCNA protein were determined by western blotting, the result of which showed that the levels of Ki-67 and PCNA in HCT116 and SW480 cells were significantly down-regulated by siCASC9 (both P<0.001, Figure 2C,D).

**CASC9 is identified as a molecular sponge for miR-145-5p**

Bioinformatics analysis revealed the binding site for miR-145-5p in CASC9 and indicated a potential link between IncRNA CASC9 and miR-145-5p (Figure 4A). The luciferase activity in HCT116 cells transfected with CASC9-WT was observably increased by miR-145-5p inhibitor (P<0.001), while there was no significant alteration in HCT116 cells transfected with CASC9-MUT reporter (Figure 4B) was observed, moreover, similar results were shown in SW480 cells (Figure 4C). Next, the expression level of miR-145-5p was measured by qRT-PCR, and we
Figure 1  The expression of lncRNA CASC9 in patients’ tissues and the effect of CASC9 knockdown on cell viability in CC cells. (A) The levels of CASC9 in tumor-adjacent normal colonic tissues (n=30) and colon cancer tissues (n=30) determined by qRT-PCR. **P<0.001 vs. Normal. (B) The level of CASC9 in colonic tissues with I/II TNM stage (n=17) and III/IV stage (n=13) measured by qRT-PCR. **P<0.001 vs. I/II. (C) The level of CASC9 in different cell lines, including in HIEC, LoVo, HCT116, SW480 and HT29 measured by qRT-PCR. **P<0.001 vs. HIEC. (D,E) The knockdown efficiency of CASC9 in CC cells HCT116 (D) and SW480 (E) determined by qRT-PCR. (F,G) The proliferation capacity of HCT116 (F) and SW480 (G) cells measured by CCK-8 assays after interference with nothing (blank), siNC (negative control) or siCASC9 at 0, 24, 48 and 72 h. **P<0.001 vs. Blank; ##P<0.001 vs. siNC.

observed that knockdown of CASC9 up-regulated the level of miR-145-5p in HCT116 and SW480 cells (both P<0.001, Figure 4D,E). Comparison on the expression level of miR-145-5p in tumor-adjacent normal colonic tissues with that in CC tissues confirmed that miR-145-5p was significantly down-regulated in CC tissues (P<0.001, Figure 4F). Further experiments in HCT116 and SW480 cells showed that the down-regulated level of miR-145-5p by miR-145-5p inhibitor could be rescued by knocking down CASC9 (both P<0.001, Figure 4G,H).

CASC9 regulates cell growth, cell apoptosis and cell cycle arrest via modulating miR-145-5p in CC cells

To further verify the effects of CASC9 on CC cells through miR-145-5p, we evaluated the cell proliferation ability, cell apoptosis and cell cycle arrest in HCT116 and SW480 cells after transfection with miR-145-5p inhibitor or co-transfection with miR-145-5p inhibitor and siCASC9. The results showed that miR-145-5p inhibitor increased the cell viability, while siCASC9 abrogated the effect of miR-145-5p inhibitor on the cells (both P<0.001, Figure 4I,J). Additionally, knockdown of CASC9 also abrogated the increase of cell colony formation rates caused by miR-145-5p inhibitor in HCT116 and SW480 cells (both P<0.001, Figure 5A,B). Flow cytometry results confirmed that the increase of cell apoptosis rates caused by the inhibition of miR-145-5p was also rescued by siCASC9 (both P<0.001, Figure 5C,D). Moreover, miR-145-5p inhibitor increased the cells at S phase but was compromised by siCASC9 in
Figure 2 The effects of lncRNA CASC9 knockdown on cell proliferation in CC cells. (A,B) Images of cells colonies in HCT116 cells (A) and SW480 cells (B) captured by camera in the left. The relative colony (diameter >0.5 mm) formation rates counted by Image J software v.1.48 is shown in the right charts. (C,D) Western blotting analysis of expression levels of Ki-67, PCNA in HCT116 cells. **P<0.001 vs. Blank; ##P<0.001 vs. siNC. Blank, transfection with nothing; siNC, non-specific negative control siRNA; siCASC9, siRNA for CASC9.

Discussion

LncRNAs are dysregulated in various cancers, and they promote tumor initiation and progression (32). In this study, we found that lncRNA CASC9 acts as an oncogene to regulate cell proliferation, apoptosis and cycle through modulating miR-145-5p in CC cells.

In this study, the level of CASC9 was found higher in CC tissues and cell lines. Moreover, CASC9 was high-expressed in CC tissues at advanced stage, indicating that CASC9...
Figure 3 The effect of lncRNA CASC9 knockdown on cell apoptosis and cycle in CC cells. (A,B) Flow cytometry analysis shows the cell apoptosis of HCT116 cells (A) and SW480 cells (B). PI+/Annexin V+ are considered as apoptotic cells. The apoptosis rates are shown in the right. (C,D) The cell cycle of HCT116 cells (C) and SW480 cells (D) measured by flow cytometry. The proportions of cells with G0/G1, S and G2/M were counted in the right. **P<0.001 vs Blank, ##P<0.001 vs siNC. Blank, transfection with nothing; siNC, non-specific negative control siRNA; siCASC9, siRNA for CASC9.

may be related to the prognosis of CC, which is consistent with a previous study, in which higher CASC9 expression is shown to be related to shorter survival in OC patients (33). We also found that knockdown of CASC9 inhibits cell growth, promotes cell apoptosis and cell cycle arrest in CC cells, which is consistent with the previous study that CASC9 is high-expressed in esophageal squamous (34). Generally, increased cell proliferation and inhibition of cell apoptosis cause tumor formation, while inhibition of cell proliferation and induction of cell apoptosis could suppress the development of tumor. Thus, we consider lncRNA CASC9 to be an oncogene in CC.
Figure 4 LncRNA CASC9 regulates the cell proliferation through targeting miR-145-5p in CC cells. (A) A target site for miR-145-5p in the CASC9 mRNA predicted by starBase. (B-C) The relative luciferase activities in HCT116 cells (B) and SW480 cells (C). **P<0.001 vs. Blank. (D,E) The level of miR-145-5p in HCT116 cells (D) and SW480 cells (E) after interference with nothing (blank), siNC or siCASC9. **P<0.001 vs. Blank. ##P<0.001 vs. siNC. (F) The levels of miR-145-5p in tumor-adjacent normal colonic tissues and CC tissues measured by qPCR (n=30). **P<0.001 vs. Normal. (G,H) The levels of miR-145-5p in HCT116 cells (G) and SW480 cells (H) measured by qPCR. (I,J) The cell proliferation of HCT116 cells (I) and SW480 cells (J) measured by CCK-8 assays. **P<0.001 vs. IC, ##P<0.001 vs. I. Blank, transfection with nothing; siNC, non-specific negative control siRNA; siCASC9, siRNA for CASC9; CASC9-WT, cells transfected with CASC9-WT luciferase reporter; CASC9-MUT, cells transfected with CASC9-MUT luciferase reporter; IC, inhibitor control; I, miR-145-5p inhibitor; siCASC9+I, co-transfection with siCASC9 and miR-145-5p inhibitor.
Figure 5 LncRNA CASC9 regulates the cell colony formation ability, cell apoptosis and cell cycle through targeting miR-145-5p in CC cells. (A,B) Images of cells colonies in HCT116 cells (A) and SW480 cells (B). The relative colony formation rates counted by Image J software is shown in the right. The pictures were took by a camera. Colonies (diameter >0.5 mm) were counted by Image J software v1.48. (C,D) The cell apoptosis of HCT116 cells (C) and SW480 cells (D) measured by flow cytometry. The apoptosis rates are shown in the right. (E,F) Flow cytometry analysis for cell cycle of HCT116 cells (E) and SW480 cells (F). **P<0.001 vs. IC, ***P<0.001 vs. I. Blank, transfection with nothing; siNC, non-specific negative control siRNA; siCASC9, siRNA for CASC9. CASC9-WT, cells transfected with CASC9-WT luciferase reporter; CASC9-MUT, cells transfected with CASC9-MUT luciferase reporter; IC, inhibitor control; I, miR-145-5p inhibitor; siCASC9+i, co-transfection with siCASC9 and miR-145-5p inhibitor.
LncRNA-miRNA-gene interactions contribute to the development of cancers. As a miRNAs sponge, CASC9 affects the development of various cancers through targeting multiple miRNAs, for example, in hemangioma endothelial cells, lncRNA CASC9 regulates cell migration and invasion by targeting miR-125a-3p/Nrg1 (35), while in breast cancer cells, lncRNA CASC9 positively affects the expression of CHK1 through sponging the miR-195/497 cluster (36). Research also showed that in glioma, CASC9, miR-519b and STAT3 form a positive feedback loop, facilitating tumourigenesis (37). In ovarian cancer, CASC9 regulates LIN7A expression via targeting miR-758-3p, contributing to the malignancy of ovarian cancer cells (38). Therefore, we suspected that CASC9 targets different miRNAs in different cancer types, or has multiple miRNA targets, suggesting that the role of CASC9 is complicated in cancer cells. By performing bioinformatics analysis and dual-luciferase reporter assay, we found that miR-145-5p was the functional target for CASC9 in CC cells, which has not been found in other studies, RNA pull-down analysis still needs to be further performed to verify the interaction between CASC9 and miR-145-5p. Previous studies demonstrated that miR-145-5p was the target for multiple lncRNAs. For example, miR-145-5p is sponged by lncRNA TUG1 in laryngocarcinoma (39), in prostate carcinoma, lncRNA BRE-AS1 interacts with miR-145-5p to regulate cancer cell proliferation (40). LncRNA MALAT1 could also up-regulate the level of NEDD9 through targeting miR145-5p (41,42). Apart from lncRNAs, miR-145-5p can also be sponged by circular RNA in bladder cancer (31). Thus, we consider that miR-145-5p can be affected by different lncRNAs. In this study, we confirmed that lncRNA CASC9 targets miR-145-5p in CC cells, and that the level of miR-145-5p in CC tissues was obviously lower than that in tumor-adjacent normal colonic tissues, which was opposite to the expression pattern of CASC9.

Further experiments also confirmed that CASC9 regulates cell growth, apoptosis and cell cycle arrest via modulating miR-145-5p in CC cells. MiR-145-5p is a tumor suppressor in cancer cells, and it inhibits cell proliferation and migration in bladder cancer (43), sensitizes prolatinoma to bromocriptine (44), affects the differentiation of gastric cancer (45), and inhibits gastric cancer invasiveness through suppressing epithelial-mesenchymal transition (46). Moreover, miR-145-5p is also found to be related to different signaling pathways, such as JNK signaling pathway (47), spl/NF-κB signaling pathway (48), MAPK and PI3K/AKT pathways (49). In our study, we confirm that miR-145-5p is a tumor suppressor in CC.

**Conclusions**

In conclusion, lncRNA CASC9 is high-expressed in CC and regulates the cell growth, apoptosis and cycle through sponging miR-145-5p. Our research provides a new understanding on the roles of CASC9 and miR-145-5p in CC.

**Acknowledgments**

*Funding:* This work was supported by the Health Commission of Hebei Province 2019 Medical Research approved project (20190913).

**Footnote**

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was authorized by the Ethics Committee of the First Affiliated Hospital of Hebei North University (NO. HNU20150703324), and all patients signed an informed consent.

**References**


