


title: Helicobacter pylori infection impairs gastric epithelial barrier function via microRNA-100-mediated mTOR signaling inhibition

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abstract: Helicobacter pylori (H. pylori) infection has an important effect on human health as it is an established cause of gastric carcinoma. microRNAs (miRNAs/miRs) are a family of small RNAs with various functions in the control of cellular profiles. However, the effect of miR-100 in H. pylori infection remains unknown. Healthy volunteers (n=100) and patients with H. pylori infection (n=98) were included in the present study. H. pylori infection was confirmed by urea breath tests. The levels of miR-100 in gastroscopic biopsy samples and cultured GES-1 cells were measured by reverse transcription-quantitative polymerase chain reaction. Furthermore, miR-100 was overexpressed or inhibited in GES-1 cells by an miR-100 mimic or inhibitor, respectively. The expression of cell-junction proteins and members of the mechanistic target of rapamycin kinase (mTOR) signaling pathway was investigated by western blotting. The results demonstrated that miR-100 levels were upregulated in infected patients and cultured gastric epithelial cells, compared with the respective controls. Additionally, the expression of epithelial (E)-cadherin and zona occludens-1 in the gastric mucosa of infected patients and GES-1 cells was downregulated. Furthermore, infected gastric epithelial cells exhibited impaired barrier functions, as measured by resistance and permeability tests. Overexpression of miR-100 inhibited junction protein expression, as well as the activation of the mTOR signaling pathway, while suppression of miR-100 restored E-cadherin expression and mTOR signaling. The results of the present study indicate that H. pylori infection may cause dysfunction of the gastric epithelial barrier by increasing miR-100 levels, which subsequently inhibit mTOR signaling. These results may have potential applications affecting miR-100 in H. pylori-related diseases.

introduction: Helicobacter pylori (H. pylori), which was first isolated and discovered on the surface of the gastric epithelium by Warren and Marshall in 1983 (1), is a gram-negative pathogenic bacterium. H. pylori induces chronic inflammation and epithelial damage in the stomach and duodenum. Infection induced by H. pylori usually occurs early in life and persists if left untreated (2). The prevalence of H. pylori infection has increased worldwide in the past two decades, affecting ~50% of the global population (2). H. pylori infection has been associated with a number of upper gastrointestinal disorders, including peptic ulcers in the stomach and duodenum, gastric carcinoma and gastric mucosa-associated lymphoid tissue lymphoma (3).

The precise pathophysiological process of H. pylori-induced chronic inflammation and mucosal damage, and the consequences of this chronic inflammation, remain elusive. Virulence factors of H. pylori, including the cytotoxin-associated gene A (CagA), Cag pathogenicity island (Cag PAI) and vacuolating cytotoxin A (VacA), have been widely investigated in past decades. The Cag PAI, composed of a series of genes encoding members of a type IV secretion system, is essential in the transfer of the bacterial products of H. pylori, including CagA, into host cells to mediate host cell damage (4). The portion of CagA which is translocated into host cells is phosphorylated. Phosphorylated CagA induces alterations in cell motility and proliferation (5-8), whereas secreted unphosphorylated CagA results in the impairment of epithelial barrier function (9). VacA is essential for the binding of H. pylori toxins to host cells, which disrupt the autophagy of epithelial cells, impair tight junctions between epithelial cells and enhance apoptosis (10-13). The responses of host cells to H. pylori infection, particularly the molecular mechanisms of infected cells, remain unclear.

Members of a newly discovered class of non-coding RNAs, microRNAs (miRNAs/miRs), have been demonstrated to exhibit numerous functions in health and disease (14,15). Mature miRNAs, which are composed of 19-25 nucleotides, are cleaved from precursors of 60-110 nucleotide hairpin miRNAs by the RNase III enzyme Dicer in the cytoplasm (16). Single-stranded miRNAs bind target mRNAs at the 3'-untranslated region with
imperfect complementarity, which results in mRNA degradation and subsequent inhibition of translation. miRNAs have been demonstrated to exhibit numerous roles in physiological and pathological processes, including the pathogenesis of cancer.

It is well established that miR-100 is involved in the pathophysiological process of various types of cancer, including ovarian (17), cervical (18) and prostate (19) cancer. However, the effects of miR-100 in the infection of *H. pylori* in gastric epithelial cells remain unclear. The results of the present study demonstrated that miR-100 was upregulated during *H. pylori* infection, and upregulation of miR-100 may mediate gastric epithelial barrier impairment via inhibition of mechanistic target of rapamycin kinase (mTOR) signaling.

**Materials and methods**

**Ethics consideration.** The Ethics Committee of The Affiliated Hospital of Medical School of Ningbo University (Ningbo, China) reviewed, approved and supervised the proposal for the present study (approval number: 2016-07-01). All participants involved in the present study gave written informed consent.

** Patients and volunteers.** Individuals with epigastric complaints visiting the outpatient clinic of The Affiliated Hospital of Medical School of Ningbo University were recruited in the present study. Between July 2016 and January 2017, a total of 100 age- and sex-matched volunteers and 98 patients with *H. pylori* infection were enrolled. The clinical features of individuals in the present study are summarized in Table I. The diagnosis of *H. pylori* infection was confirmed by the typical endoscopic appearance of the stomach and a positive urea breath test (20). Healthy volunteers were defined as those with a normal endoscopic appearance of the stomach and a negative urea breath test. Two double-biopsies were obtained during gastroscopy from each individual.

**Cell culture.** The GES-1 gastric epithelial cell line was purchased from the Cell Resource Center, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). GES-1 cells were cultured in RPMI 1640 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.; without any antibiotics) in an incubator at 37°C with 5% CO₂. Measurements for fluorescein isothiocyanate (FITC)-dextran permeability and resistance were performed in Transwell plates according to previous publications (21,22). Briefly, GES-1 cells were seeded on the upper compartment of a Transwell chamber. At 80-90% confluency, FITC-dextran (0.5 mg/ml; purchased from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added into the upper compartment. The concentration of FITC in the bottom compartment was read by an infrared fluorescence laser reader (LI-COR Biosciences, Lincoln, NE, USA). The electrical resistance between the upper and lower compartments of the Transwell chambers was measured in ohms.

**miR-100 mimic and inhibitor.** Inhibitors for miR-100 (single-stranded chemically modified oligonucleotide) and miR-100 mimic (double-stranded oligonucleotides) were purchased from Thermo Fisher Scientific, Inc. GES-1 cells (5x10°) were seeded in 75 mm² flasks. The next day, transfection with miR-100 mimic (5’-CAACGUUGAUACCUAAGG UAUG-3’) or miR-100 inhibitor (5’-CAACGUUGAUACCUAAGGUAUG-3’) was performed using DharmaFECT 1 reagents (50 nM, GE Healthcare Dharmacon, Inc., Lafayette, CO, USA) according to the manufacturer's protocol. Control cells were treated by a mock transfection using a non-targeting miRNA sequence (5’-UCACAACCUCCUAGAAAGAGUAGA-3’). The levels of miR-100 in transfected cells were confirmed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) 24 h after transfection.

**Infection of H. pylori.** The *H. pylori* strain 7.13 (generously provided by Dr. D. Scott Mereel from Uniformed Services University of Health Services, Bethesda, MD, USA) was cultured in Brucella broth (purchased from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) with 10% FBS for 16 h at 37°C. Subsequently, *H. pylori* was collected by centrifugation (400 x g for 10 min at 4°C) and used to infect GES-1 cells at a bacterium-to-cell ratio of 100:11 for 24 h, as described previously (23). Control cells (referred to as Ctrl) were treated with PBS.

**RT-qPCR for mature miR-100.** Quantification of miRs was performed to measure the levels of mature miR-100, as described previously (24). Total RNA was extracted using a commercial RNeasy Micro kit (Qiagen, Inc., Valencia, CA, USA). RNA was reverse-transcribed to cDNA with an miR-100-specific stem-loop-like reverse transcription primer (5’-GTCGATTCAGTGCTCGGGAGGTTATCCGC AACTGATACGGACGTTGA-3’) using a commercial kit (High Capacity cDNA Reverse Transcription kit purchased from Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. qPCR was subsequently performed with SYBR Green Master Mix (Thermo Fisher Scientific, Inc.) using the following primers: Forward primer, 5’-GAGGCA ACCCCTGATCCCGT-3’; and reverse primer, 5’-GTCGAG GGTCCCGAGGTT-3’. The accuracy of the PCR amplification of the mature miR-100 sequences was verified by sequencing using Applied Biosystems 3730xl 96 capillary DNA Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). Small nuclear RNA U6 was used as a housekeeping gene. Relative expression of miR-100 was normalized with the 2^ΔΔCt method (25).

Table I. Characteristics of study participants.

<table>
<thead>
<tr>
<th></th>
<th>Volunteers (n=100)</th>
<th><em>H. pylori</em> infection (n=98)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>64.55±5.70</td>
<td>63.17±63.2</td>
<td>0.378</td>
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<tr>
<td>Gender</td>
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<td>Male</td>
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<td>57</td>
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</tr>
<tr>
<td>Female</td>
<td>42</td>
<td>41</td>
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</tbody>
</table>

**H. pylori, Helicobacter pylori.**
For qPCR analyses of mRNAs of interest, total RNA (2 µg) was reverse-transcribed to cDNA using an RT-PCR kit (Verso 1-step RT-PCR kit ReadyMix; Thermo Fisher Scientific, Inc.) at 55°C according to the manufacturer's protocol. The expression of the genes of interest and the housekeeping gene hypoxanthine phosphoribosyltransferase was measured using a SYBR Green Master Mix (94°C for 2 min, 40 cycles of 94°C for 15 sec and 60°C for 1 min), according to the manufacturer's protocol. The sequences of primers are listed in Table II. Quantification of each mRNA was performed using the 2^ΔΔCq method.

Western blotting. Whole-cell lysates from biopsies or cultured cells were lysed in 30 µl lysis buffer [1% Triton X-100, 0.5% Nonidet P-40, 10 mM Tris-HCl, 150 mM NaCl, (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride]. A total of 50 µg protein measured by Lowry assays was resolved by SDS-PAGE and subsequently transferred to nitrocellulose membranes. Membranes were blocked in 5% fat-free milk for 1 h at room temperature. Membranes were then incubated with primary antibodies against epithelial (E)-cadherin (1:10,000; cat. no. ab40772; Abcam, Cambridge, MA, USA), zona occludens-1 (ZO-1; 1:1,000; cat. no. ab59720; Abcam), mTOR (1:1,000; cat. no. 2983; Cell Signaling Technology, Inc., Danvers, MA, USA), phosphorylated-eukaryotic translation initiation factor (P-4EBP-1; 1:1,000; cat. no. 9459, Cell Signaling Technology, Inc.), 4EBP-1 (1:1,000; cat. no. 9452, Cell Signaling Technology, Inc.), p-P70S6K (1:1,000; cat. no. 9204; Cell Signaling Technology, Inc.) and P70S6K (1:1,000; cat. no. 9202; Cell Signaling Technology, Inc.) at the optimized titrations at 4°C overnight. Following incubation with corresponding horseradish peroxidase-conjugated secondary antibodies (anti-rabbit secondary antibody, 1:10,000; cat. no. ab6721); anti-goat secondary antibody, 1:5,000; cat. no. ab6885; both were purchased from Abcam) at room temperature for 30 min, the bands were visualized with an enhancing chemiluminescence system (Amershan ECL Western Blotting Detection kit, GE Healthcare, Chicago, IL, USA). Densitometric analysis was performed with ImageJ software (Windows Edition, 1.35j, National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data are presented as the mean ± the standard error of the mean. The difference between two groups was analyzed using a two-tailed student's t-test. The statistical significance between >2 groups was measured by one-way analysis of variance followed by Bonferroni's post-hoc tests. Statistical analysis was performed by GraphPad Prism 5 Windows Edition (GraphPad Software, Inc., La Jolla, CA, USA). All experiments were performed with a minimum of three times. P<0.05 was considered to indicate a statistically significant difference.

Results

Patients with H. pylori infection exhibit increased miR-100 levels and reduced expression of junction proteins. The present study initially investigated whether miR-100 may be involved in the pathophysiological process of H. pylori-mediated gastric epithelial infection. Individuals with epigastric complaints visiting the outpatient clinic of The Affiliated Hospital of Medical School of Ningbo University, who underwent upper gastrointestinal endoscopy, were recruited to the present study. H. pylori infection was confirmed or excluded by a urea breath test and the endoscopic appearance of the gastric mucosa. A total of 100 healthy controls without H. pylori infection and 98 patients infected with H. pylori were analyzed. Two double-biopsies under endoscopy were obtained from each individual. The level of miR-100 was assessed by RT-qPCR, as described above. The expression of miR-100 in the gastric mucosa of patients with H. pylori infection was significantly upregulated compared with infection-free individuals (P<0.0001; Fig. 1A). As the adherens junctions and tight junctions have an important role as a barrier to prevent the influx of luminal contents into the lamina propria (26), the mRNA levels of E-cadherin and ZO-1, important components of the adherens junctions and tight junctions, respectively, were also measured. As illustrated in Fig. 1B and C, in patients with H. pylori infections, the mRNA levels of E-cadherin and ZO-1 significantly decreased (P<0.0001), indicating an impaired barrier function of the gastric epithelium compared with infection-free controls. The protein expression levels of E-cadherin and ZO-1 in patients with H. pylori infection were also measured using western blotting, which demonstrated that patients with H. pylori infection exhibited decreased levels of the proteins of the adherens and tight junctions (Fig. 1D and E).

H. pylori infection increases gastric epithelial monolayer permeability in vitro. As H. pylori infection decreased the

<table>
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<tr>
<th>Target gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>E-cadherin</td>
<td>CTGAGAACGAGGCTAAGG</td>
<td>TTCACATCCAGACATCC</td>
</tr>
<tr>
<td>ZO-1</td>
<td>GTTGTGGATACGTTTGT</td>
<td>GATGATGCCCTGGTTCTAC</td>
</tr>
<tr>
<td>HPRT</td>
<td>GCAGACTTTGCTTCTTTGG</td>
<td>AAGCAGATGGCCACAGAATC</td>
</tr>
<tr>
<td>U6</td>
<td>GCCATGCTAATCTCTCTGTATC</td>
<td>CGGCCAGCACATAACTAAAAATTGG</td>
</tr>
</tbody>
</table>

E-cadherin, epithelial-cadherin; ZO-1, zona occludens-1; HPRT, hypoxanthine phosphoribosyltransferase 1.
expression of E-cadherin and ZO-1 in the gastric epithelium, which are vital for maintaining normal gastric epithelial barrier function, the permeability of the cultured gastric epithelial monolayer was assessed. The resistance of cultured GES-1 cells treated with a PBS vehicle or *H. pylori* for 1 h was checked. After 12 and 24 h of infection, the resistance of the cultured cell monolayer was measured. The resistance prior to infection was used as the baseline value. As demonstrated in Fig. 2A, cultured GES-1 gastric epithelial cells had a significantly decreased level of resistance in the *H. pylori* group compared with the vehicle (*P*<0.001). FITC-dextran in the lower chamber of Transwells used for the culture of GES-1

Figure 2. Cultured gastric epithelial cells infected with HP exhibited impaired permeability. Gastric epithelial cells were cultured as a monolayer in Transwells. The permeability of cultured gastric epithelial cells was determined by measuring (A) resistance and (B) the concentration of FITC-dextran in the basal compartment. Results are representative of three experimental repeats. ***P*<0.001 vs. Ctrl. HP, *Helicobacter pylori*; FITC, fluorescein isothiocyanate. Ctrl, control.

Figure 3. Gastric epithelial cells infected with HP exhibited increased levels of miR-100 and decreased expression of E-cadherin and ZO-1. Cultured epithelial cells were infected with HP. The relative levels of (A) miR-100, (B) E-cadherin mRNA and (C) ZO-1 mRNA were measured by reverse transcription-quantitative polymerase chain reaction. Results are representative of three experimental repeats. ***P*<0.001 vs. Ctrl. HP, *Helicobacter pylori*; miR, microRNA; E-cadherin, epithelial-cadherin; ZO-1, zona occludens-1; Ctrl, control.
cells was also measured. As illustrated in Fig. 2B, the lower chamber of the Transwell in which gastric epithelial cells were infected with \textit{H. pylori} had significantly increased levels of FITC-dextran after 12 and 24 h of infection (P<0.001). Results from both the resistance and FITC-dextran assays indicated that gastric epithelial cells infected with \textit{H. pylori} had increased permeability compared with controls. \textit{H. pylori} infection increases the levels of miR-100 and decreases the expression of E-cadherin and ZO-1 in vitro. The present study further investigated whether impaired barrier function of infected gastric epithelial cells in vitro may be a result of miR-100-mediated reduction of E-cadherin and ZO-1 expression. The levels of miR-100, as well as the mRNA and protein expression of E-cadherin and ZO-1 junction proteins, was assessed in cultured GES-1 gastric epithelial cells infected with \textit{H. pylori}. As demonstrated in Fig. 3A, the levels of miR-100 in cells infected with \textit{H. pylori} were significantly elevated compared with control cells (P<0.001). However, the mRNA levels of E-cadherin and ZO-1 were significantly downregulated in infected cells (P<0.001; Fig. 3B and C). Notably, the protein levels of these two junction proteins were also decreased in infected cells (data not shown). These data indicate the possibility that increased miR-100 levels induced by \textit{H. pylori} infection may mediate the downregulation of E-cadherin and ZO-1 expression.

\textit{miR-100 mediates the inhibition of E-cadherin and ZO-1 expression.} In order to investigate the effects of miR-100 on the expression of E-cadherin and ZO-1 in vitro, 'loss-of-function' and 'gain-of-function' protocols were used to inhibit or overexpress miR-100 in GES-1 gastric epithelial cells. As demonstrated in Fig. 4, E-cadherin protein was significantly downregulated in gastric epithelial cells transfected with miR-100 mimic (P<0.05), while the protein expression of E-cadherin was significantly increased in cells transfected with the miR-100 inhibitor (P<0.01), compared with the mock group, as measured by western blotting. A similar effect of miR-100 on the expression of ZO-1 in gastric epithelial cells was also observed in the present study (data not shown).

\textit{mTOR is the downstream signaling molecule involved in miR-100-mediated regulation of E-cadherin and ZO-1.} It is well established that the mTOR signaling pathway regulates the epithelial barrier function, including the expression of the adherens junctions and tight junctions (27,28). The activation status of mTOR signaling in gastric epithelial cells infected with \textit{H. pylori} was therefore assessed in the current study. As demonstrated in Fig. 5A, in contrast to the levels of miR-100 in infected gastric epithelial cells, mTOR and the downstream active forms of 4EBP-1 and PFS6K were downregulated (Fig. 5A and B). In order to assess the effects of miR-100 on the activation of mTOR signaling, the same protocols of overexpression and suppression of miR-100 in GES-1 cells were employed. The mimic of miR-100 significantly inhibited the mTOR signaling pathway in infected gastric epithelial cells; however, suppression of miR-100 significantly increased the activation of this pathway, compared with mock-treated cells (P<0.05; Fig. 5C and D). These data indicated that miR-100 may be a negative regulator of mTOR signaling in tuning the barrier functions.
Discussion

In the present study, the role of miR-100 in the impaired functioning of gastric epithelial cells infected with *H. pylori* was described. The results of the present study indicated that patients, as well as cultured cells in *vitro*, infected with *H. pylori* exhibited increased levels of miR-100 and decreased expression of junction-proteins. Furthermore, cultured gastric epithelial cells infected with *H. pylori* exhibited impaired barrier functions and mTOR signaling was demonstrated to be controlled by an upregulation of miR-100.

Infections induced by *H. pylori* are a huge public health burden. *H. pylori* infection has been reported to be implicated in gastritis, precancerous lesions and gastric...
carcinoma (29). The eventual consequence of *H. pylori* infection, gastric carcinoma, is the third leading cause of cancer-associated mortality worldwide. As the association between *H. pylori* infection and gastric carcinoma has become clear, *H. pylori* has been demonstrated to be the most common pathogen that is associated with malignancy (30). The process from chronic *H. pylori* infection to gastric carcinoma is complex and involves a number of molecular and cellular events, some of which remains inclusive (31). Although the pathogenesis of *H. pylori* has been investigated thoroughly, the responses of host epithelial cells and intracellular molecular events require further attention.

Based on the results of the present study, part of the host gastric epithelial cell response to *H. pylori* infection may be described as follows. Following infection, levels of miR-100 are upregulated, this miR-100 upregulation may suppress the activation of the mTOR signaling pathway, which subsequently downregulates the expression of the adherens and tight junction proteins E-cadherin and ZO-1, respectively. Decreased expression of junction proteins leads to an impaired barrier function, which was indicated in the present study as decreased resistance and an increased permeability level were observed in monolayer gastric epithelial cells infected with *H. pylori*. It may be hypothesized that an impaired gastric epithelial barrier function will result in the influx of gastric contents to the lamina propria of the stomach, subsequently inducing chronic and persistent inflammation. Chronic inflammation has been positively associated with the onset of cancer (32), therefore, miR-100 may be involved in gastric oncogenesis. However, the mechanism by which miR-100 is upregulated in gastric epithelial cells infected with *H. pylori* remains unknown at present, and the regulation of miR-100 in *H. pylori* infection requires investigation in the near future.

miRNAs are single-stranded RNA molecules that are 20-23 nucleotides in length. miRNAs control gene expression in numerous cellular processes, including inflammation, cell cycle regulation, stress differentiation, apoptosis, proliferation and tumorigenesis (33). As a member of the miRNA family, miR-100 has gained interest from biologists and oncologists. miR-100 has been demonstrated to be associated with ovarian (17), cervical (18) and prostate (19) cancer. To the best of our knowledge, the present study is the first to demonstrate that upregulation of miR-100, as part of the host epithelial cell response to *H. pylori* infection, may mediate impaired gastric barrier functions by inhibiting mTOR signaling. These data expand on the current understanding of host responses to *H. pylori* infection.

However, there are a number of limitations associated with the present study. As mentioned above, the mechanism by which miR-100 is upregulated during *H. pylori* infection was not investigated. In addition, the mechanism by which miR-100 regulates E-cadherin and ZO-1 expression is yet to be established. When the ‘loss-of-function’ and ‘gain-of-function’ assays were performed for miR-100, the mRNA levels of E-cadherin and ZO-1 were also determined in addition to the protein levels, which indicated lower levels of mRNA in ‘gain-of-function’ assays and increased levels of mRNA in ‘loss-of-function’ assays (data not shown). Although these data indicate that miR-100 may affect the transcription of E-cadherin and ZO-1, the precise underlying mechanisms of this process require investigation in the future.

In conclusion, the results of the current study indicated that miR-100 may impair the gastric epithelial barrier function by affecting mTOR signaling in *H. pylori* infection.

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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

GH designed this study, performed most experiments, and prepared the first draft; LG performed some of repeated experiments, and performed data analysis. GY designed this study, supervised this study, and reviewed the draft.

### Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The Affiliated Hospital of Medical School of Ningbo University, China (approval number: 2016-07-01). All participants involved in the present study gave written informed consent.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

### References