**Helicobacter pylori** Induces a Novel NF-kB/LIN28A/let-7a/hTERT Axis to Promote Gastric Carcinogenesis

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**ABSTRACT**

Reactivated telomerase is a crucial event in the development and progression of a variety of tumors. However, how telomerase is activated in gastric carcinogenesis has not been fully uncovered yet. Here, we identified a key role of the NF-kB/LIN28A/let-7a axis to promote human telomerase reverse transcriptase (hTERT) expression for gastric cancer initiation. Mechanistically, LIN28A expression was upregulated by *H. pylori*-induced NF-kB activation. And LIN28A, in turn, suppressed let-7a expression, forming the NF-kB/LIN28A/let-7a axis to regulate gene expression upon *H. pylori* infection. Of note, we first discovered hTERT as a direct target of let-7a, which inhibited hTERT expression by binding to its 3′UTR of mRNA. Therefore, *H. pylori*-triggered let-7a downregulation enhanced hTERT protein translation, resulting in telomerase reactivation. Furthermore, hTERT enhanced LIN28A expression, forming the positive feedback regulation between hTERT and NF-kB/LIN28A/let-7a axis to maintain the sustained overexpression of hTERT in gastric cancer.

**Implications:** The NF-kB/LIN28A/let-7a axis was crucial for the overexpression of hTERT upon *H. pylori* infection during gastric cancer development and may serve as a potential target to suppress hTERT expression for gastric cancer prevention and treatment.

**Introduction**

Gastric cancer is a major threat to healthy people for the absence of effective early diagnostic methods, making it a leading cause of cancer-related mortality worldwide (1). As one of the known inflammation-associated cancers, gastric cancer has been demonstrated to derive from chronic progressive gastritis (2–4). *Helicobacter pylori* (*H. pylori*) is a major risk factor of gastric malignancy, as its long-term colonization in gastric mucosa leads to chronic inflammation. *H. pylori* has been found to inject its virulence factor CagA into host gastric epithelial cells via the bacterial type IV secretion system (T4SS), triggering signal cascades to promote gastric carcinogenesis (5–8). CagA also activated NF-kB in gastric epithelium (9, 10). Intriguingly, Iliopoulos and colleagues reported that an epigenetic switch involving NF-kB, LIN28, let-7, and IL6 linked inflammation to cell malignant transformation in breast cancer (11). However, the molecular mechanisms and key proteins participating in the process of chronic gastritis–gastric cancer transformation still need to be fully elucidated.

Telomerase is an RNA-dependent DNA polymerase composed of a reverse transcriptase (TERT) and an RNA template TERC (telomerase RNA component), maintaining telomere length and preventing replicative senescence (12). Upregulated TERT protein level to reactivate telomerase is observed in up to 90% of all human cancers (13). Although reactivated telomerase in cancer mainly serves to elongate telomere length, mounting evidence suggests that telomere-independent roles of TERT are vital as well for oncogenesis (14, 15). For instance, Artandi and colleagues reported that TERT promoted epithelial cell proliferation through transcriptional control of Wnt signaling (16). Previous study showed that telomerase directly promoted transcription of NF-kB (17). However, the distinct role of hTERT in the process of inflammation-cancer transformation remains elusive. Hence, whether hTERT is critical for *H. pylori*-induced gastric malignant transformation and its specific role is worthwhile to explore.

MicroRNAs (miRNA) are a class of small, noncoding RNAs that posttranslationally regulate gene expression through inhibiting mRNA translation or promoting mRNA degradation (18). MiRNAs participate in various biological processes, ranging from cell proliferation, differentiation, migration, to apoptosis (19–22). Altered miRNA expression has been reported in a variety of human malignancies. Let-7 was initially discovered in *Caenorhabditis elegans* as an important developmental-timing controlling regulator, and its function as tumor suppressor was elucidated in multiple cancers (23–26). Emerging evidence suggested that decreased mature let-7 expression in cancers was caused by the conserved RNA-binding protein LIN28, which was a key regulator for let-7 biogenesis (27, 28). LIN28 was upregulated in diverse human cancers, including breast, lung, and liver cancers (29–31). Recent studies have focused on the role of the LIN28/let-7 axis in the process of oncogenesis (32, 33). The RNA-binding protein LIN28 contains two paralogs, LIN28A and LIN28B, and both repress the maturation of the microRNA let-7 family. Furthermore, LIN28 enhanced many gene expression through direct binding to the target mRNA to promote its translation (34–36). However, whether the LIN28/let-7 axis is crucial for *H. pylori*-induced gastric malignant transformation remains unknown.

In this study, we clarified the regulation of hTERT by the NF-kB/LIN28A/let-7a axis during *H. pylori* induced gastric malignant transformation. *H. pylori* upregulated LIN28A expression by activating NF-kB, leading to let-7a downregulation. Suppressed let-7a lost its inhibitory effect on hTERT expression. The elevated hTERT protein...
level as well as the telomerase activity contributed to gastric carcinogenesis induced by H. pylori. And hTERT in turn promoted LIN28A expression, forming the positive feedback regulation between the NF-κB/LIN28A/let-7a axis and hTERT.

**Materials and Methods**

**Patients and tissue specimens**

All gastric species were collected at Qilu Hospital, Shandong University (Jinan, P.R. China) from 25 patients with primary gastric cancer who underwent gastrectomy and 32 patients with gastritis who underwent gastroscopy. Patients with a history of receiving adjuvant chemotherapy were excluded from this study. The diagnosis of all patients was histopathologically confirmed. General information about all patients is in **Table 1**. Biopsy collection was approved by the Ethics Committee of Shandong University School of Medicine (Jinan, P.R. China and the Ethics Committee approval number is LL-201501032).

**Cell lines and cell culture**

Human gastric cell lines (AGS, BGC-823, HGC-27, MGC-803, and SGC-7901) were obtained from Shanghai Institute of Biochemistry and Cell Biology at the Chinese Academy of Science and were authenticated by the institute. All the cells used were Mycoplasma negative detected by Zhongqiaoxinzhou Biological Company (website: www.zqxzbio.com) using traditional PCR method. In addition, all the cells used were maintained in F12 medium supplemented with 10% fetal bovine serum (FBS). BGC-823, HGC-27, MGC-803, and SGC-7901 cells were obtained from Shanghai Institute of Biochemistry and Cell Biology. These cell lines were cultured for 12 hours. Then cells were incubated with the primary antibodies against p65 at a dilution of 1:200 overnight at 4°C after blocking. The cells were then washed and cultured gastric cell lines at different multiplicity of infection (MOI) and time points.

**Plasmid construction and luciferase assay**

Complementary DNA encoding let-7a precursor (~100 bp), which was amplified from human genomic DNA, was cloned into the pSilencer3.1-H1 (Ambion) to construct the pSilencer-let-7a plasmid, the miR let-7a complementary sequence was synthesized as DNA oligonucleotides and cloned into the pSuper vector (Omegaigo) after annealing. The wild-type hTERT 3’UTR of miRNA with the intact putative let-7a recognition sequence was cloned into an Ambion pMIR-REPORT vector to generate pMIRluc-hTERT wild-type 3’UTR. All these recombined vectors were confirmed by sequencing. Luciferase activity was assayed by use of the Dual Luciferase Reporter Assay system (Promega). To validate miRNA target, pSilencer-let-7a, wild-type 3’UTR of hTERT mRNA, and pRl-TK plasmids were cotransfected into AGS and BGC-823 cells. Cells were lysed after 48 hours of transfection, and then luciferase activity was determined.

**Let-7a mimics and inhibitors**

Exogenous miRNA let-7a mimics and inhibitors were purchased from RIBO BIO (China), and transfection of those miR-let-7a mimics upregulated mature let-7a expression, whereas transfection of miR-let-7a inhibitor antagonized mature let-7a expression.

**RNA extraction, reverse transcriptase PCR, and qRT-PCR**

Total cellular RNA was extracted using TRIzol reagent according to the manufacturer’s instructions (Invitrogen). For detection of mature let-7a, RNA (1 μg) was converted into cDNA using the TaqMan MicroRNA Reverse Transcription Kit, and the real-time PCR (RT-PCR) was conducted with specific RT primers (TaqMan MicroRNA Assay) and Taq-Man Universal PCR Master Mix (Applied Biosystems). For detecting hTERT and LIN28A expression, RNA (1 μg) was reversely transcribed into cDNA by the MMLV Reverse Transcription System (Invitrogen), and RT-PCR was performed using the SYBR Premix Ex Taq system (TaKaRa). All PCR reactions were conducted in triplicates using an ABI 7500 sequence detector (Applied Biosystems). U6 snRNA, β2-microglobulin, and β-actin were used as endogenous controls.

**Immunofluorescence staining**

Cells were transfected with CagA expression plasmids for 12 hours. Then cells were incubated with the primary antibodies against p65 at a dilution of 1:200 overnight at 4°C after blocking. The cells were then incubated with appropriate Alexa Fluor 488 secondary antibodies diluted 1:1,000 in blocking solution for 1 hour at room temperature. The nuclei were counterstained with DAPI. The cells were mounted on slides with Antifade Mounting Medium (Beyotime).

**Western blot and IHC staining**

Total cellular proteins were extracted from cells with RIPA lysis buffer. Equal quantities of proteins were subjected to 10% SDS-polycrylamide gel electrophoresis and then transferred to PVDF membrane (Bio-Rad). The membranes were blocked in 5% non-fat milk, probed with the specific antibodies against hTERT (Abcam), LIN28A (Abcam), β-ACTIN (Sigma-Aldrich) and then with secondary anti-mouse or rabbit

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**Table 1.** The relevance between hTERT expression and clinicopathologic parameters in human gastric tissues.

<table>
<thead>
<tr>
<th>Staging</th>
<th>Number</th>
<th>Age (years)</th>
<th>Sex (M/F)</th>
<th>hTERT</th>
<th>qRT-PCRa</th>
<th>hTERT</th>
<th>IHCb</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG</td>
<td>19</td>
<td>45.8 ± 16.2</td>
<td>9/10</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>n = 15</td>
</tr>
<tr>
<td>AG/M</td>
<td>32</td>
<td>49.7 ± 19.1</td>
<td>20/12</td>
<td>n = 26</td>
<td>16.58 ± 1.91</td>
<td>n = 15</td>
<td>20.06 ± 5.89</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>19</td>
<td>57.8 ± 14.8</td>
<td>12/7</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>n = 16</td>
</tr>
<tr>
<td>Cancer</td>
<td>27</td>
<td>59.1 ± 15.3</td>
<td>19/8</td>
<td>n = 20</td>
<td>29.81 ± 2.58</td>
<td>n = 25</td>
<td>58.82 ± 7.32</td>
</tr>
</tbody>
</table>

aRelative levels of real-time RT-PCR analyses: % based on CT values and normalization to SG 19 45.8

bImmunohistochemical staining: % based on positive cells to the whole.

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**H. pylori and bacterial culture**

The standard strains of H. pylori (26695, SS1) were kindly provided by Dr. Jianzhong Zhang (Chinese Disease Control and Prevention Center, Beijing, China). The strains were cultured in Brucella broth containing 5% FBS under microaerobic conditions (3% O2,10% CO2, and 85% N2) at 37°C. The strains were harvested at exponential amplification periods by centrifugation and added to cultured gastric cell lines at different multiplicity of infection (MOI) and time points.

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**Mol Cancer Res; 19(1) January 2021**

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Figure 1. Augmented hTERT expression in the specimens of progressive gastric diseases (from chronic inflammation to gastric cancer; GC) and H. pylori–infected gastric cancer cell lines, and high expression of hTERT predicted poor prognosis of gastric cancer patients. A, Expression of hTERT in primary gastric cancer and normal tissues from TCGA database. B, Expression of hTERT in different grades of primary gastric cancer and normal tissues from TCGA database. C, IHC staining of hTERT protein expression in superficial gastritis (SG), atrophic gastritis with intestinal metaplasia (AG/IM), and cancerous gastric tissues (GC). Scale bars, 50 mm. D, hTERT mRNA expression in H. pylori–negative and –positive gastric epithelial cell line GES-1 from public database (GSE74577). E and F, Augmented expression of hTERT in H. pylori–infected AGS, BGC-823, and GES-1 cells. G, hTERT mRNA expression increased with the rising of H. pylori strains 26695 [MOI 1:25–1:100 (cell:H. pylori)] for 6 hours. H, hTERT mRNA expression in AGS cells infected by CagA-negative H. pylori at different time points. I, AGS and BGC-823 cells were infected with H. pylori strains 26695 (MOI 1:100) for 2 and 4 hours. hTERT protein expression was detected using western blot, respectively. J, hTERT protein was induced in AGS cells by H. pylori 26695 in a dose-dependent way. K, H. pylori CagA induced hTERT expression. AGS and BGC-823 cells were transfected with expression vectors encoding CagA for 72 hours and then analyzed hTERT expression using western blot. L, High expression of two different hTERT transcripts predicted poor prognosis of gastric cancer patients from public database. Error bars indicate mean ± SD; Student t test, *, P < 0.05; ***, P < 0.001.
horseradish peroxidase–conjugated immunoglobulin G antibody (Santa Cruz Biotechnology). hTERT and LIN28A levels in gastric specimens were determined by IHC staining and incubated with primary antibodies at 4°C overnight.

**Telomerase activity assay**

Telomerase activity assay was performed with a Telomerase PCR enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics) according to the instructions. For each assay, 1 μg protein was used, and 30 PCR cycles were performed.

**Telomere length assay**

Telomere length assay was performed with a Telomerase PCR enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics) according to the instructions. For each assay, 1 μg protein was used, and 30 PCR cycles were performed.

**Genomic DNA extraction and southern blot**

Total genomic DNA were isolated from cells with E.Z.N.A. Genomic DNA kit (Omega bio-tek, D3396–02). Digestion of genomic DNA into terminal restriction fragments (TRF) generated by Hinf I/Rsa I.

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**Figure 2.**

NF-κB/LIN28A/let-7 axis was activated upon *H. pylori* infection. 

A, miR-let-7a expression in *H. pylori*–positive and *H. pylori*–negative patients from GEO database (GSE19769). B and C, qRT–PCR detection of miR-let-7a expression in AGS and BGC-823 cells infected with *H. pylori* strains 26695 and transfected by CagA expression plasmid, respectively. D, miR-let-7a downregulation by *H. pylori* strains 26695 in a dose-dependent way in AGS cells. E, Let-7a expression in BGC-823 cells infected by *H. pylori* 26695 at different time points. F, Let-7a expression in AGS cells transfected with DMSO or specific pathway inhibitors for 1 hour, and then transfected with or without CagA for 48 hours (10 μmol/L), (BAY 11-7082, 5 μmol/L), (LY294002, 10 μmol/L). G, Let-7a expression in AGS cells transfected with DMSO or specific pathway inhibitors for 1 hour, and then transfected with or without CagA for 48 hours (10 μmol/L), (BAY 11-7082, 5 μmol/L), (LY294002, 10 μmol/L). H, Let-7a expression in AGS cells transfected with DMSO or specific pathway inhibitors for 1 hour, and then transfected with or without CagA for 48 hours (10 μmol/L), (BAY 11-7082, 5 μmol/L), (LY294002, 10 μmol/L). I, CagA induction of LIN28A expression in AGS and BGC-823 cells. LIN28A protein expression was analyzed using qRT–PCR and western blot, respectively. J, Immuno-fluorescence (IF) assay of NF-κB nuclear translocation in gastric cancer (GC) cells after CagA transfection. K, CagA-induced LIN28A expression was blocked by BAY 11-7082. Shown is one representative of two independent experiments.
Let-7a directly targeted hTERT to inhibit its expression. **A**, Schematic diagram of the predicted let-7a target site in 3’-UTR of hTERT mRNA. **B** and **D**, hTERT mRNA expression was determined in control or let-7a–overexpressed or –suppressed AGS and BGC-823 cells. **C** and **E**, hTERT protein expression was determined in control mimics/inhibitors or miR-let-7a mimics/inhibitor-transfected AGS and BGC-823 cells. **F**, Telomerase activity in let-7a–overexpressed or –suppressed BGC-823 cells was analyzed using telomerase ELISA kit. **G**, Telomere length assay in let-7a–upregulated/downregulated BGC-823 cells was analyzed using TeloTAGGG telomere length assay kit. Shown is one representative of two independent experiments. (Continued on the following page.)
digestion using Telomerase TagGFP telereader length assay. DNA fragments were separated by gel electrophoresis and transferred to a nylon membrane (Amersham Hybond-N, 0.45 μm) by southern blot.

**Colonies formation assay**

AGS and BGC-823 cells were transfected with plasmids for 48 hours, and then seeded into 6-well plates (300 cells/well), then incubated at 37°C for 10 to 14 days. Plates were rinsed with PBS and then stained with Giemsa. Colonies containing more than 50 cells were counted for analysis.

**EdU incorporation assay**

AGS and BGC-823 cells were transfected with plasmids and siRNAs for 48 to 72 hours, and then seeded into 96-well plates, then incubated at 37°C for 24 hours. Plates were rinsed with PBS and then stained with Cell-Light EdU Apollo567 in vitro kit (RIBOBIO).

**Mouse model**

BGC-823 cell suspension (100 μL) with hTERT knocking down or control cells at a concentration of 2 × 10^5 cells/mL was subcutaneously injected into nude mice. Mice were sacrificed after 3 weeks, and tumors were removed for further study. Animal experiment was approved by the Ethics Committee of Shandong University School of Medicine (Jinan, P.R. China).

**Statistical analysis**

Data are expressed as mean ± SD. Statistical analysis was performed with SPSS 13.0 software, with two-tailed Student’s t test in two groups or one-way ANOVA for analysis of multiple groups. Statistical significance was set at *P* < 0.05.

**Results**

**Augmented hTERT expression (with prognostic value) in the specimens of progressive gastric diseases (from chronic inflammation to gastric cancer) and H. pylori–infected gastric cancer cell lines**

Although hTERT overexpression has been confirmed in almost all human cancers, the level of hTERT protein in premalignant lesions remains unclear. To investigate the differential expression of hTERT during gastric cancer progression course, we analyzed hTERT expression from the public The Cancer Genome Atlas (TCGA) database. We found the upregulation of hTERT mRNA level in Hp 26695–infected gastric epithelial cell line GES-1 from the public microarray GEO database (GSE74577; Fig. 1D). Upon H. pylori standard strain Hp 26695 infection in gastric epithelial cell lines (AGS, GE-1, and BGC-823), both mRNA and protein levels of hTERT showed upregulation in a time- and dose-dependent pattern by using quantitative RT-PCR (qRT-PCR) and western blot assays, respectively (Fig. 1E–I, J, and K). However, when using CagA-negative H. pylori to infect AGS cells, no increase of hTERT can be observed at all (Fig. 1H), indicating the indispensable role of CagA for H. pylori to induce hTERT expression. Indeed, H. pylori virulence factor CagA also promoted the expression of hTERT when overexpressing CagA in gastric cancer cells (Fig. 1K). These data suggested that CagA mediated the upregulation of hTERT induced by H. pylori.

To evaluate whether hTERT expression was correlated with gastric cancer prognosis, we took advantage of the Kaplan–Meier plotter database to investigate the roles of hTERT on the survival of gastric cancer patients. High expression of the two transcripts of hTERT predicted poor prognosis of gastric cancer patients (Fig. 1L). The above results implied the oncogenic role of hTERT in H. pylori–related gastric cancer, as well as its prognostic value in the clinic.

**NF-κB/LIN28A/let-7 axis was activated upon H. pylori infection**

Upon previous whole microRNA microarray gene-expression analysis from the GEO database (GSE19769), we distinguished the differential miRNA expression between the biopsy of 10 H. pylori–negative and 9 H. pylori–positive patients. Among these miRNAs, let-7a was found to be significantly decreased in H. pylori–positive patients (Fig. 2A). Next, qRT-PCR was conducted to confirm the results of microarray profiling. Both H. pylori infection and CagA overexpression led to significant decline of let-7a in gastric cancer cells in a dose-dependent way (Fig. 2B–D). And let-7a was also downregulated by H. pylori infection at different time points in BGC-823 and GES-1 cells (Fig. 2E and F). However, when using CagA-negative H. pylori to infect GES-1 cells at different time points, no decrease of let-7a expression was observed at all (Fig. 2H), indicating the indispensable role of CagA for H. pylori to suppress let-7a expression, which was similar to the effects of CagA on hTERT (Fig. 1H). It has been reported that the NF-κB/LIN28A/let-7 axis was activated in the process from inflammation to cell malignant transformation (11). As CagA promoted NF-κB nuclear translocation directly, we aimed to know whether H. pylori–mediated let-7a downregulation was through CagA-induced NF-κB activation and subsequent LIN28A upregulation. To identify the pathway involved in H. pylori–mediated let-7a downregulation, cells were pretreated with a series of signaling pathway inhibitors before CagA transfection. These included the inhibitors
Let-7a directly targeted hTERT to inhibit its expression

Both hTERT protein and let-7a are closely associated with NF-xB signaling (11, 27). MiRNAs posttranscriptionally regulated gene expression through binding to the 3′-UTR of mRNA of target genes. By using several bioinformatic programs—miRanda, PicTar, and miRBase—we found that hTERT was a potential target of let-7a (Fig. 3A). To confirm this, AGS and BGC-823 cells were transfected with psilencer-let-7a expression plasmid, and let-7a expression was significantly upregulated accordingly, whereas hTERT expression was reduced (Fig. 3B). Correspondingly, depletion of let-7a expression showed the opposite effects on hTERT (Fig. 3D). Furthermore, introduction of exogenous miR-let-7a mimics and inhibitors achieves a similar regulation relationship between let-7a and hTERT as confirmed by the western blot assay (Fig. 3C and E). To further prove that let-7a directly affected the 3′-UTR of hTERT mRNA, we cloned hTERT mRNA 3′-UTR, which contained the predicted let-7a binding site into a luciferase reporter plasmid. Luciferase activity was significantly reduced by cotransfection of let-7a and hTERT mRNA 3′UTR plasmid (Fig. 3H), whereas inhibition of endogenous let-7a expression showed the opposite effect (Fig. 3I), supporting the interaction between let-7a and 3′UTR of hTERT mRNA. Importantly, we used colony formation assay to evaluate the effect of let-7a on cell proliferation in AGS and BGC-823 cells. Let-7a overexpression remarkably inhibited gastric cancer proliferation, whereas let-7a inhibition promoted gastric cancer proliferation (Fig. 3J). For further revealing the effect of let-7a on hTERT functionality, PCR-ELISA assay was carried out and showed that decreased hTERT expression in BGC-823 cells by upregulation of let-7a caused about 50% decrease of the telomerase activity, whereas downregulation of let-7a led to increased telomerase activity (Fig. 3F), indicating the key functional hTERT change mediated by let-7a inside gastric cancer cells. To evaluate whether the change of hTERT regulated by let-7a mediated telomere length variation in gastric cancer cells, the Telo TAGGG telomere length assay was performed and revealed that upregulation of let-7a in BGC-823 cells caused the mean length of TRF decrease from 3.6 to 2.8 kb (Fig. 3G). The TRFs obtained DNA with uniform telomeric (TTAGGG) repeats may suggest the telomere length of corresponding samples. As expected, inhibition of let-7a in BGC-823 cells caused mean length of TRF increase from 3.0 to 3.6 kb (Fig. 3G). To further verify the negative regulation of hTERT by let-7a in vivo, clinical samples of gastric diseases were evaluated for their mRNA expression, which showed obvious negative correlation between the two molecules. The level of hTERT was elevated along with the deterioration of the diseases, whereas let-7a exhibited the opposite change (Fig. 3K). Collectively, these data indicated hTERT was a direct target of let-7a and hTERT mediated the functional change (telomerase activity and telomere length) exerted by let-7a inside the cells.

To address whether the induction of hTERT by CagA was mediated by let-7a, we detected hTERT expression by cotransfecting psiI-let-7a and CagA expression plasmid in gastric cancer cells. Let-7a overexpression significantly relieved CagA-mediated hTERT upregulation (Fig. 3L and M). Furthermore, CagA-promoted cell proliferation was attenuated by let-7a overexpression as well (Fig. 3N). Therefore, combining previous results, the downregulation of let-7a by H. pylori-mediated activation of the NF-κB/LIN28A/let-7a axis was responsible for the overexpression of hTERT in gastric cancer.

hTERT mediated the effects of let-7a on gastric cancer cell proliferation

To test the direct effect of hTERT on gastric cancer cell proliferation, we used two specific siRNAs to inhibit hTERT expression (Fig. 4A). Colony formation and EdU incorporation assay indicated inhibition of hTERT significantly decreased gastric cancer cell proliferation (Fig. 4B and C). Because let-7a overexpression or inhibition also decreased or increased gastric cancer cell proliferation, respectively (Fig. 3), and hTERT was a direct target of let-7a (Fig. 3J and H), we wondered to testify if hTERT mediated the effects of let-7a on gastric cancer cell proliferation. Indeed, the inhibition of gastric cancer proliferation by downregulation of hTERT could be reversed by let-7a suppression (Fig. 4D). Moreover, colony formation assay and EdU incorporation assay revealed that the promotion of let-7a inhibitors on gastric cancer proliferation could be obviously reversed by hTERT inhibition (Fig. 4E and F), whereas the suppression of gastric cancer proliferation by let-7a mimics could be reversed by hTERT overexpression (transfection of hTERT overexpression vector, pBabe-hTERT; Fig. 4G and H). Taken together, hTERT mediated the effects of let-7a on gastric cancer cell proliferation, further confirming hTERT was a direct target of let-7a.

hTERT positively regulated LIN28A expression and downregulated let-7a expression

As mentioned previously, telomerase directly regulated NF-xB target gene transcription (27). Hence, we wondered whether hTERT may directly regulate LIN28A expression, which was transactivated by NF-xB. We knocked down hTERT expression by using hTERT-specific siRNA. LIN28A expression was decreased, whereas let-7a was increased upon hTERT depletion (Fig. 5A; Supplementary Fig. S2A). Furthermore, hTERT overexpression in BGC-823 cells transfected with hTERT overexpression vector led to enhanced expression of LIN28A (Fig. 5B). And this regulation was NF-xB pathway independent because NF-xB inhibitor BAY 11-7082 showed no effect on hTERT-induced LIN28A upregulation (Fig. 5B). What is more, the endogenous expression of hTERT and LIN28A was detected in several gastric epithelial cell lines. The protein expression of hTERT was in line with that of LIN28A in most of the cell lines, especially for the lowest expression of both in GE-1 cell line, which was nonmalignant, compared with other cancer cell lines, implying the oncogenic role and mutual regulation between these two molecules (Supplementary Fig. S2B). Interestingly, let-7a inhibition significantly relieved the suppression of LIN28A by hTERT depletion, confirming the negative regulation of hTERT by let-7a as well as the positive regulation of LIN28A by hTERT (Fig. 5C). Furthermore, after subcutaneously injecting BGC-823 cells with hTERT suppression into nude mice, LIN28A was downregulated, whereas let-7a was upregulated in the formed tumors (Fig. 5D and E), which supported our assumption.
hTERT positively regulated LIN28A expression and downregulated let-7a expression in vivo. Therefore, the activated NF-κB/LIN28A/let-7a axis enhanced hTERT expression and hTERT in turn promoted LIN28A expression, forming this positive feedback regulation between hTERT and the axis in gastric cancer. This explained well the reason for sustained hTERT overexpression during gastric carcinogenesis.
hTERT positively regulated LIN28A expression and downregulated let-7a expression. A, Let-7a, hTERT, and Lin28A expression were determined using qRT-PCR in hTERT-depleted gastric cancer (GC) cells. B, LIN28A and hTERT expression were determined by western blot in differently treated gastric cancer cells. AGS cells were pretreated with DMSO or BAY 11-7082 (5 μM) for 1 hour and then transfected with control or hTERT overexpression plasmids for 48 hours. C, LIN28A and hTERT expression was determined by western blot in differently treated BGC-823 cells. The cells were transfected with control or hTERT siRNA for 24 hours, and then were transfected with control or anti-let-7a plasmids for another 24 hours. D and E, LIN28A and let-7a expression levels in tumors derived from control- or hTERT-depleted BGC-823 cell injected mice (n = 7). Error bars, mean ± SD; Student t test, **, P < 0.01; ***, P < 0.001.

Confirmation of positive correlation between hTERT and LIN28A, and negative correlation between hTERT and let-7a in mice and human tissues

We conducted the mouse model of gastritis by administration of H. pylori (SS1) for 8 months. Both mice TERT (mTERT) and LIN28A protein levels were elevated in the gastritis group compared with that in the control group (Fig. 6A). In addition, we examined the expression of hTERT, LIN28A, and let-7a in superficial gastritis, AG/IM, and dysplasia samples. As expected, hTERT and LIN28A protein expression was gradually increased along with the deterioration of gastric diseases by IHC staining (Fig. 6B). The mRNA level of hTERT and LIN28A and the expression level of let-7a were evaluated in AG/IM and gastric cancer samples, respectively. We found a significant increase of hTERT and LIN28A expression in gastric cancer samples, whereas let-7a expression showed the opposite change (Fig. 6C–E). Of note, hTERT and LIN28A showed colocalization expression in the diseased gastric samples by using serial sections of the tissues, indicating the potential mutual regulation between them in vivo (Fig. 6B). Collectively, these results confirmed the regulation between different above-mentioned molecules during the progression from gastric chronic inflammation to gastric cancer.

Discussion

As Rudolf Virchow first proposed that chronic inflammation may lead to cancer initiation and progression, there is accumulating evidence to show that tumor-promoting inflammation mediated oncogenesis and tumor progression (37–40). However, as a known inflammation-related cancer, molecular pathways involved in gastritis transforming to gastric cancer are obscure. In the current study, we uncovered a circuit involving hTERT and NF-κB/LIN28A/let-7a axis in human gastric cells. H. pylori and its key virulent factor CagA induced hTERT expression through NF-κB mediated let-7a downregulation, and hTERT in turn directly transactivated lin28A expression to reduce let-7a expression. This positive loop promoted gastric cell proliferation.

Although reactivated telomerase in almost all human malignant cancers has been certainly confirmed (13), whether and how hTERT was involved in tumor-promoting inflammation and tumor initiation remains ambiguous. In the present study, our findings showed that increased hTERT expression occurred in the early phase of gastric malignancy, such as AG/IM, thereby indicating a novel role of hTERT in cancer initiation. Collectively, our findings further supported emerging evidence for a broader role of telomerase or hTERT as the central regulator of all of the hallmarks of cancer (41).
pharmacologic approaches to deliver miRNAs to cells have been reported, which reminded us that the delivery of let-7a to target hTERT may be an effective strategy for treatments of gastric cancer (46).

It has been reported that H. pylori CagA induced aberrant epigenetic silencing of let-7 expression via enhanced c-myc, EZH2, and DNMT3B expression (47). Our current study demonstrated a novel mechanism of let-7 dysregulation under H. pylori infection. H. pylori directly activated NF-κB signaling and then lin28A was transactivated. They form a LIN28-let-7-IL-6 epigenetic switch, which had been proved to be key for the maintenance of cancer stem cells (CSC; ref. 48). Recent studies revealed that hTERT stimulated EMT program and CSC self-renewal in gastric cancer progression (49). What is more, HCC progenitor cells extracted from premalignant lesions may evolve into CSC and lead to hepatocellular carcinogenesis (50). Thus, our novel findings of the LIN28A-let-7a-hTERT axis, which has been proved in clinical gastric specimens, may be closely related to gastric CSC maintenance and gastric carcinogenesis.

There is increasing evidence to demonstrate that hTERT exerts telomere-independent functions in malignant transformation and tumorigenesis (51–53). For instance, it has been reported that the complex consisting of hTERT and Wnt/β-catenin could bind to its target genes, such as c-myc and cyclin D1, to promote their transcription. Furthermore, hTERT can directly regulate NF-κB-dependent gene transcription. Intriguingly, as mentioned above, NF-κB-dependent LIN28A transactivation and let-7a repression mediated hTERT upregulation under H. pylori infection, and we further showed...
that hTERT in turn activated LIN28A expression and inhibited let-7a expression in gastric cancer cells. Hence, a positive feedback loop existed during gastric carcinogenesis. In addition, LIN28A was found to block the maturation of miR-370 in hepatocellular carcinoma (47). This implied that LIN28A upregulation mediated by hTERT overexpression in gastric cancer cells could possibly regulate various microRNAs maturation, and thus played a crucial role in gastric malignant transformation.

In summary, our current study defined that a positive feedback circuit involving NF-κB, LIN28A, let-7a, and hTERT promoted gastric carcinogenesis (Fig. 6F). We also highlighted the possible contribution of this circuit in very early stage of gastric carcinogenesis. Therefore, disruption of this positive feedback loop may be useful for gastric cancer prevention and effective treatments.

**Authors’ Disclosures**

No disclosures were reported.

**References**

LIN28A/let-7a/hTERT Axis in Gastric Carcinogenesis


Molecular Cancer Research

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