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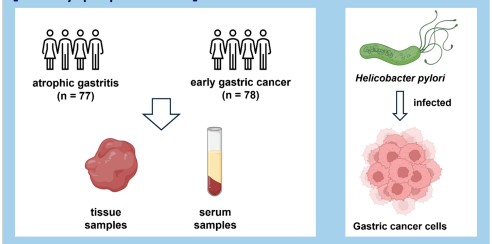
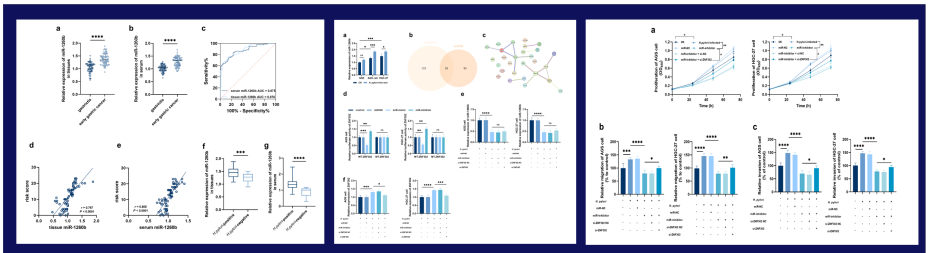
Study population & Methods

[Study population]



[Methods]

Outcomes



- **miR-1260b predicts the risk of gastric cancer in atrophic gastritis and diagnoses gastric cancer**
- **miR-1260b indicates the severity of gastric cancer patients**
- **miR-1260b protects gastric cancer cell from *H. pylori* infection by targeting ZNF302**

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OR 10527

Increasing miR-1260b predicts the risk of gastric cancer in atrophic gastritis patients and regulates cell growth and metastasis of gastric cancer

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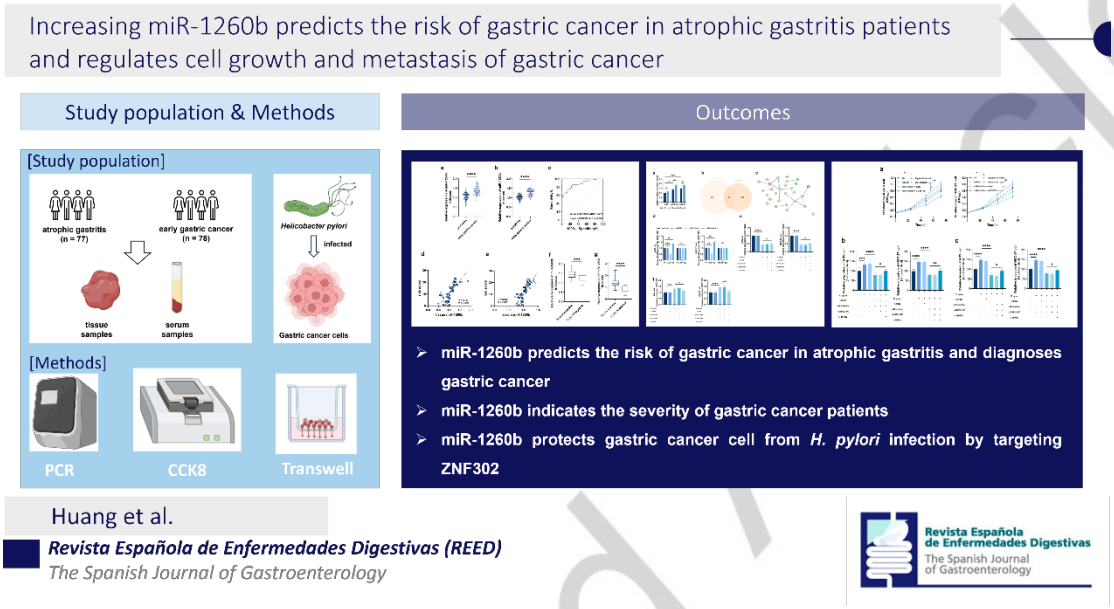
Artificial intelligence: the authors declare that they did not use artificial intelligence (AI) or any AI-assisted technologies in the elaboration of the article.



Lay summary

1. miR-1260b was previously screened of great potential to diagnose gastric cancer, and it was demonstrated to be involved in the development of colorectal cancer and non-small cell lung cancer.

2. This study revealed the predictive value of miR-1260b in early gastric cancer and tumor progression.

3. Silencing miR-1260b could alleviate the promotion of gastric cancer induced by *H. pylori* via negatively modulating ZNF302.

4. Increasing miR-1260b can be considered a diagnostic biomarker for early gastric cancer predicting cancer risk and mediating tumor progression.

ABSTRACT

Background: atrophic gastritis has a high risk of progressing to gastric cancer. Screening early gastric cancer and predicting the risk of atrophic gastritis developing

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into gastric cancer could improve the prognosis.

Objective: this study evaluated the significance of miR-1260b in early gastric cancer and the progression of atrophic gastritis to gastric cancer aiming to explore a reliable biomarker.

Materials and methods: the study enrolled 78 early gastric cancer patients and 77 atrophic gastritis patients. The expression of miR-1260b was detected in serum and tissue samples by PCR. The risk of atrophic gastritis patients progressing to gastric cancer was assessed and correlated with miR-1260b levels. The potential of miR-1260b to distinguish early gastric cancer was evaluated by ROC. *In vitro*, gastric cancer cells were infected with *Helicobacter pylori (H. pylori),* and the regulatory effect of miR-1260b on cell growth and metastasis was evaluated by CCK8 and Transwell assay.

Results: significant upregulation of miR-1260b was observed in early gastric cancer patients relative to atrophic gastritis patients, which distinguishes early gastric cancer patients and showed a positive correlation with the risk of atrophic gastritis patients developing gastric cancer. Early gastric cancer patients with positive *H. pylori* infection had higher miR-1260b levels, and increasing miR-1260b was also observed in *H. pylori*-infected gastric cancer cells. *H. pylori* promoted cell growth and metastasis of gastric cancer while silencing miR-1260b alleviated these effects. miR-1260b negatively regulated ZNF302, and the knockdown of ZNF302 reversed the protective effect of miR-1260 knockdown on gastric cancer cells.

Conclusion: increasing miR-1260b can assist in the early diagnosis of gastric cancer and predict the risk of gastric cancer in atrophic gastritis patients. Silencing miR-1260b may alleviate the promotion of gastric cancer induced by *H. pylori* via negative modulation of ZNF302.

Keywords: Risk assessment. Diagnostic biomarker. ceRNA. *Helicobacter pylori.* Biological function.

INTRODUCTION

Gastric cancer is one of the most lethal malignancies and ranked as one of the top

death-related causes, which has become a serious public health problem. The number of new cases is gradually increasing, particularly in East Asia (1-3). Although chemotherapy, radiotherapy, immune therapy, and targeted therapy have been increasingly developed in the past decades, the mortality rate of gastric cancer is still high, resulting in an unfavorable prognosis. Early detection could help to resect the lesion at an early stage, and therefore effectively suppress the mortality. Endoscopy is the commonly used diagnostic technique for gastric cancer screening (4). However, the clinical application of endoscopy is limited to various factors, such as the risk of complications induced by invasive operations and the antipathy of patients due to discomfort (5). Therefore, non-invasive detection is of urgent need. On the other hand, the inducing factors of gastric cancer have been widely reported, but the etiology has not been conclusively confirmed. *Helicobacter pylori (H. pylori)* infection and its related gastric mucosal lesions have been considered to possess a close association with the onset and development of gastric cancer (6,7). The regulation of *H. pylori*-related gastric mucosal lesions is of great significance for the diagnosis and management of gastric cancer.

Recently, microRNAs (miRNAs) have attracted special attention, and they have been demonstrated to exist in various tissues and body liquids of eukaryotes. Moreover, miRNAs have also been considered as potential biomarkers as non-invasive liquid biopsy biomarkers. Previous studies have reported several miRNAs that regulate gastric cancer progression and predicted unfavorable outcomes, but did not focus on the diagnosis of early gastric cancer (8,9). A recent study explored three circulating miRNAs that diagnosed gastric cancer patients from the online datasets GSE113486 and GSE124158, including miR-320a, miR-1260b, and miR-6515-5p (10). However, there was a lack of confirmation with clinical samples, and their significance in predicting the risk of early gastric cancer in *H. pylori* infection and gastric mucosal lesions also remained unclear. miR-1260b was suggested to regulate non-small cell lung cancer metastasis and drug resistance and therefore affected tumor progression (11-14). Regarding clinical significance, miR-1260b was identified as a biomarker in colorectal cancer to predict an adverse prognosis and in breast cancer screening of tumor occurrence (15,16). The dysregulation of miR-1260b in gastric

cancer was validated in this study. Different from previous studies, this study enrolled atrophic gastritis patients as the control group. Except for evaluating the potential role of miR-1260b to diagnose early gastric cancer, its significance in predicting the risk of atrophic gastritis progressing to gastric cancer was also assessed. Additionally, the infection of *H. pylori* was also considered as an inducing factor in the development of atrophic gastritis and miR-1260b expression, aiming to identify a novel biomarker for screening early gastric cancer and improve prognosis.

MATERIALS AND METHODS

Patients

A retrospective study enrolled a total of 155 patients who underwent endoscopy in The Affiliated Hospital of Panzhihua University and were diagnosed with atrophic gastritis or early gastric cancer. The enrolled patients were composed of 77 atrophic gastritis patients and 78 gastric cancer patients and were not consecutively enrolled. Patients met the diagnostic criteria for atrophic gastritis or gastric cancer and had never received medications that might interfere with study results. Patients with other malignant tumors or gastric diseases were excluded. The age and gender composition of the two groups of patients were matched with no significant differences. All patients knew the research design and signed an informed consent.

Risk assessment of gastric cancer in atrophic gastritis patients

The risk assessment of gastric cancer in atrophic gastritis patients was performed according to a novel gastric cancer scoring system (4). This scoring system was composed of five perspectives including age, serum gastrin, gender, *H. pylori* antibodies, and pepsinogen ratio. The total score of the system was 23 points, where patients scored 17-23 were classified as high-risk, 12-16 were classified as medium-risk, and 0-11 was classified as low-risk.

Sample collection

Tissue and blood samples were collected from all study subjects. For atrophic gastritis patients, tissue samples were collected from the gastroscopic biopsy

sample, and tissues from early gastric cancer patients were collected during the resection surgery. Fasting venous blood samples were collected after fasting water and food for eight hours and centrifugated at 3,000 g for 15 minutes to isolate serum. All samples were stored at -80 °C until the following analyses.

Cell culture and treatments

Two gastric cancer cell lines (AGS and HGC-27 cell) and a human gastric epithelial cell line (GES-1 cell) were obtained from ATCC and cultured with Dulbecco's modified Eagle's medium (DMEM) culture medium with 10 % fetal bovine serum (FBS) at 37 °C with 5 % CO_2 and 95 % air. Cells were infected with *H. pylori* NCTC116347 purchased from ATCC after reaching the logarithmic phase with the incubation ratio of 1: 100 (the number of cells: the number of bacterial colonies). After 24 hours of infection, cells were available for the following experiments.

Cell transfection

Cells were transfected with miR-1260b inhibitor or negative controls synthesized by Ribo Biotechnology Co. (China). Cell transfection was conducted with Lipofectamine® 2000 (Invitrogen, USA) at room temperature, and transfected cells were available after 48 hours of cell transfection. Cells without any infection or transfections were used as the CK group.

Real-time quantitative PCR

Total RNA was extracted from tissues, serum, and cells using TRIzol™ reagent (Invitrogen, USA). Extracted RNA was evaluated using the OD260/280 value, and the ratio ranged from 1.8-2.2 indicating high-quality RNA. cDNA was generated from extracted RNA with a TaqMan™ microRNA reverse-transcription kit or high-capacity reverse transcription kit (Applied Biosystem, USA). Amplification was performed on the ABI 7900 instrument (Applied Biosystem, USA). The relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method with cel-miR-39 and β-actin as internal references.

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Target prediction of miR-1260b

The targets of miR-1260b were predicted from TargetScan (https://www.targetscan.org/vert_72/) and miRDB (https://mirdb.org/) databases. Targets with cumulative weighted context ++ score over -0.5 (from the TargetScan database) and target score over 80 (for miRDB database) were intersected, and the function of obtained targets was evaluated by Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene ontology (GO) function enrichment.

Luciferase reporter assay

The wild-type and mutant-type plasmids were constructed by cloning wild-type binding sites or mutant sites into pGL3 vectors. Cells were co-transfected with established plasmids and miR-1260b mimic or inhibitors with the aid of Lipofectamine® 2000 at room temperature. The luciferase activity of ZNF30 was detected with a Dual-Luciferase® Reporter System (Promega, USA) with Renila as the internal reference.

Cell counting kit-8 assay

Cells were seeded into 96-well plates supplied with FBS-containing culture medium and incubated at 37 °C. Cell incubation was conducted for 0, 24, 48, and 72 hours followed by the addition of CCK8 reagent (Dojindo, Japan). The incubation was continued for another two hours after adding CCK8, and then the plates were analyzed with a microplate reader at 450 nm. Cell proliferation was assessed by the time-OD450 curve.

Transwell assay

Cells were seeded onto the upper chambers of 24-well Transwell® plates supplied with an FBS-free culture medium. The completed culture medium was added to the lower chamber. Cells were permitted to migrate or invade for 24 hours at 37 °C. Then, the upper chamber was wiped with a cotton swab to remove residual cells, and cells on the subsurface were stained with violet and counted under an optical microscope with five random fields.

Statistical analyses

Data were expressed as mean ± SD with at least three independent repeated measurements, and analyzed with SPSS 26.0 or GraphPad Prism 9.0 software. The clinical significance of miR-1260b was evaluated by ROC for the diagnostic value, logistic regression analysis for the predictive value for gastric cancer, and Chi-squared test for correlation with patients' clinicopathological features based on the grouping of gastric cancer patients with the average tissue miR-1260b expression as the cutoff. Comparisons between clinical samples was performed with unpaired Student's t-test and one-way ANOVA followed by Turkey post-hoc test in cell line experiments. $p < 0.05$ indicated statistically significant differences.

RESULTS

Baseline information of study subjects

Atrophic gastritis and early gastric cancer patients were well matched by age and gender composition. The atrophic gastritis group included 54 males and 23 females with an average age of 58.38 ± 8.09 years, and the early gastric cancer group was composed of 53 males and 25 females with an average age of 59.62 ± 10.27 years ($p > 0.05$). Early gastric cancer patients showed a higher proportion of *H. pylori* infection than atrophic gastritis patients. The majority of early gastric cancer patients were diagnosed with concave form lesions, while atrophic gastritis patients mainly had bulge form lesions. Moreover, early gastric cancer patients more frequently experienced white fur than atrophic gastritis patients. There was no significant difference between the two groups of patients between the incidence of corroding and nodules (Table 1).

Expression and significance of miR-126-b in atrophic gastritis and early gastric cancer

Significantly increased expression of miR-1260b was observed in serum (Fig. 1A) and tissues (Fig. 1B) samples from early gastric cancer patients compared with corresponding samples from atrophic gastritis patients. Both abnormal serum

(sensitivity = 75.64 % and specificity = 85.71 %) and tissue (sensitivity = 83.33 % and specificity = 76.62 %) miR-1260b levels could discriminate early gastric cancer patients with an AUC of 0.875 and 0.876 (Fig. 1C). Serum (Fig. 1D) and tissue (Fig. 1E) expression of miR-1260b in atrophic gastritis patients showed a significantly positive correlation with the risk score.

Early gastric cancer patients were grouped as a low-miR-1260b (patients with tissue miR-1260b less than the average expression) and a high-miR-1260b group (patients with tissue miR-1260b over the average expression), according to the average tissue expression of miR-1260b. Higher miR-1260b level showed a close association with *H. pylori* infection (p = 0.006), tumor size (p = 0.013), lesion form (p = 0.023), and the presence of white fur (p = 0.018) (Table 2). Consistently, early gastric cancer patients with positive *H. pylori* infection showed higher serum (Fig. 1F) and tissue (Fig. 1G) miR-1260b level.

Regulatory effect and molecular mechanism of miR-1260b in *H. pylori*-infected gastric cancer cells

Compared with normal gastric epithelial cells, miR-1260b was significantly upregulated in gastric cancer cells, AGS, and HGC-27 cells. Additionally, the expression of miR-1260b significantly increased in *H. pylori*-infected AGS and HGC-27 cells but showed no significant changes in GSE cells compared with uninfected cells (Fig. 2A).

From the TargetScan and miRDB databases, the downstream targets of miR-1260b were predicted, and a total of 28 target genes were screened from both databases (Fig. 2B). From the results of KEGG and GO enrichment (Table 3) and the protein-protein-interaction network (Fig. 2C), ZNF302 was considered as a hub gene among 28 predicted targets. The target relationship between miR-1260b and ZNF302 was confirmed by luciferase reporter assay. It was demonstrated that the overexpression of miR-1260b significantly suppressed the luciferase activity of ZNF302 in AGS and HGC-27 cells while silencing miR-1260b showed an opposite effect (Fig. 2D). Moreover, the expression of miR-1260b in *H. pylori*-infected AGS and HGC-27 cells was knocked down by the transfection of miR-1260b inhibitor (Fig. 2E), which

dramatically enhanced the expression of ZNF302 (Fig. 2F). Silencing ZNF302 could reverse the effect of miR-1260b but showed no significant effect on the expression of miR-1260b.

The infection of *H. pylori* induced increased proliferation (Fig. 3A), migration (Fig. 3B), and invasion (Fig. 3C) of AGS and HGC-27 cells. Silencing miR-1260b alleviated the induced effect of *H. pylori* infection, which was reversed by the knockdown of ZNF302.

DISCUSSION

A previous study reported the abnormal expression of miR-1260b in gastric cancer and revealed its significance to discriminating gastric cancer patients from non-cancer individuals (10). Atrophic gastritis is one of the major subtypes of chronic gastritis, which shows a high risk of developing gastric cancer (17,18). Gastroscopy and gastric mucosal biopsy could assist in predicting the risk of gastric cancer, but these two examinations are limited to the high cost and the relatively painful process (19,20). In contrast, blood examination has the advantages of being easy to perform, with a good reproducibility, and it is non-invasive. Therefore, this study focused on serum miR-1260b, evaluating its significance to distinguish early gastric cancer and predict risk in atrophic gastritis. Serum miR-1260b showed significantly increased expression in early gastric cancer patients, distinguishing between early gastric cancer patients and atrophic gastritis. Tissue expression of miRNAs was closer to the patients' disease development (21). The expression and diagnostic significance of tissue miR-1260b was consistent with serum miR-1260b. The diagnostic efficiency, sensitivity, and specificity of tissue and serum miR-1260b levels were also similar. This study used a risk assessment score that evaluated the risk of atrophic gastritis patients developing gastric cancer. Increasing serum and tissue miR-1260b levels showed a positive correlation with the risk of gastric cancer in atrophic gastritis patients. Therefore, serum miR-1260b levels are consistent with its expression in lesions of early gastric cancer patients, which could diagnose early gastric cancer and predict the risk of gastric cancer in atrophic gastritis patients. Additionally, early gastric cancer showed a larger number of patients with white fur in the lesion, and

the expression of miR-1260b also showed a close association with the presence of white fur and larger tumor size in early gastric cancer patients. Although the presence of white fur cannot represent the severity of early gastric cancer patients, it is also correlated with the gastric lesion (22,23). Tumor size could also indirectly indicate patients' disease conditions. Therefore, in addition to the diagnostic significance, miR-1260b has the potential to indicate the severity of early gastric cancer patients.

H. pylori has been considered as a class I carcinogen of gastric cancer, and the infection of *H. pylori* has also been considered as an infectious disease (24). As shown in the present study, the infection rate of early gastric cancer patients is higher than that of atrophic gastritis patients, which is consistent with previous reports (25). miR-1260b was closely associated with the infection of *H. pylori* in early gastric cancer patients, and the tissue and serum expression level of miR-1260b is higher in early gastric cancer patients with positive infection of *H. pylori*. The infection of *H. pylori* is a controllable risk factor, which could be of benefit for the prevention of gastric cancer. The regulatory effect of miR-1260b on *H. pylori* is meaningful to suggest its significance (26). On the cellular level, significant upregulation of miR-1260b was shown in gastric cancer cells relative to normal cells, and the expression of miR-1260b was enhanced after the infection of *H. pylori* in gastric cancer cells, but the expression in normal cells was not significantly affected. *H. pylori* significantly promoted gastric cancer cell growth and metastasis, which further caused the severe development of gastric cancer. Silencing miR-1260b alleviated the enhancement of gastric cancer cell growth and metastasis, indicating its regulatory effect on *H. pylori* infection. Interestingly, the knockdown of miR-1260b suppressed proliferation, migration, and invasion of gastric cancer cells infected with *H. pylori* to lower levels than uninfected cells. Previously, miR-1260b was demonstrated to regulate tumor progression of non-small cell lung cancer, colorectal cancer, breast cancer, and prostate cancer via modulating cellular processes (11,15,16,27). Therefore, miR-1260b was also hypothesized as a tumor regulator of gastric cancer, and inhibiting miR-1260b might be a potential therapeutic strategy for gastric cancer.

Regarding the mechanism, targeting downstream genes has been accepted as the major modulating mechanism underlying miRNAs. For example, the promotion effect of miR-1260b on non-small cell lung cancer was revealed to be mediated by PTPRK and HIPK2 (11,14). CASP8 was identified as the direct target of miR-1260b in regulating breast cancer cells (16). The targets of miR-1260b were predicted from public databases, and ZNF302 was screened due to its functional enrichment and interaction prediction with other mRNAs. Moreover, ZNF302 was identified as a potential functional gene in human diseases, including nasopharyngeal carcinoma, congenital heart disease, and frontotemporal dementia (28-30). The regulation of ZNF302 by miR-1260b was demonstrated in the present study. Silencing ZNF302 could reverse its increase by miR-1260b knockdown. Knockdown of ZNF302 also reversed the protective effect of silencing miR-1260b on *H. pylori* infection in gastric cancer cells. Therefore, miR-1260b was hypothesized to regulate *H. pylori* infection, cell growth, and metastasis via modulating ZNF302.

In conclusion, increasing miR-1260b can be considered to be a predictive indicator for early gastric cancer, especially in atrophic gastritis patients. miR-1260b was associated with *H. pylori* infection and disease severity and regulated *H. pylori* induced tumor progression via negatively modulating ZNF302.

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Table 1. Basic clinicopathological features of study subjects

	Atrophic gastritis	**Early gastric cancer**	***p*-value**
Age (mean ± SD, years)	58.38 ± 8.09	59.62 ± 10.27	0.404
Gender (male/female)	54/23	53/25	0.769
Infection of *H. pylori* (*n*, %)	35, 45.5 %	57, 73.1 %	< 0.0001
Lesion form (n, %)			< 0.0001
Bulge	52, 67.53 %	22, 28.21 %	
Concave	4, 5.19 %	54, 69.23 %	
Flat	21, 27.28 %	2, 2.56 %	
Corrode (*n*, %)	8	9	0.819
Nodule (*n*, %)	4	11	0.061
White fur (*n*, %)	7	22	0.002

H. pylori: Helicobacter pylori.

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Table 2. Association of miR-1260b with the clinicopathological features of early gastric cancer patients

	Total	Low-miR-1260b	High-miR-1260b	*p*-value
Age (years)				0.658
< 60	37	19	18	
≥ 60	41	19	22	
Gender				0.567
Male	53	27	26	
Female	25	11	14	
Infection of H. pylori				0.006
Negative	20	15	5	
Positive	58	23	35	
Tumor size (cm)				0.013
< 1	9	8	1	
1-2	13	8	5	
> 2	56	22	34	
Lesion form				0.023
Bulge	22	15	7	
Concave	54	21	33	
Flat	2	2	0	
Corrode				0.252
Absent	69	32	37	
Present	9	6	3	
Nodule				0.086
Absent	67	30	37	
Present	11	8	3	
White fur				0.018
Absent	56	32	24	
Present	22	6	16	

H. pylori: Helicobacter pylori.

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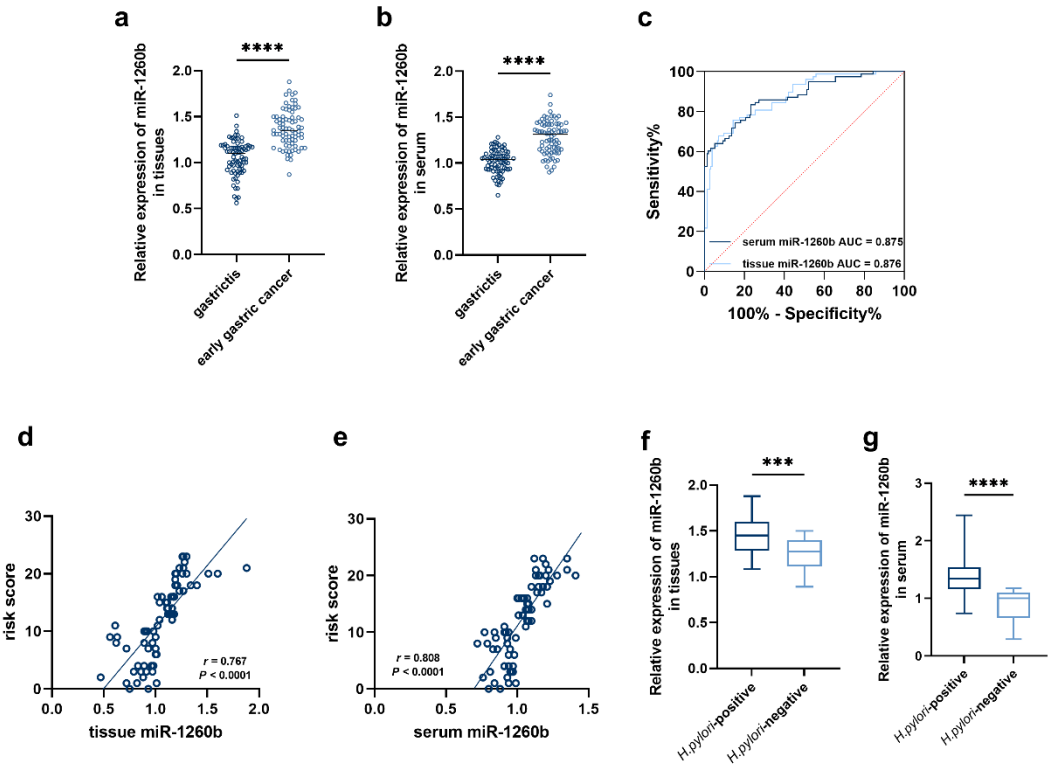

Fig. 1. Expression and significance of miR-1260b in early gastric cancer and atrophic gastritis. A and B. Expression of miR-1260b in tissue (B) and serum (B) of the study subjects. C. ROC evaluating the significance of miR-1260b to distinguish early gastric cancer patients. D and E. Correlation of tissue (D) and serum (E) miR-1260b levels with risk scores of atrophic gastritis patients. F and G. Expression of miR-1260b in tissue (F) and serum (G) of early gastric cancer patients based on the infection of *Helicobacter pylori (H. pylori)*. ***$p < 0.001$, ****$p < 0.0001$.

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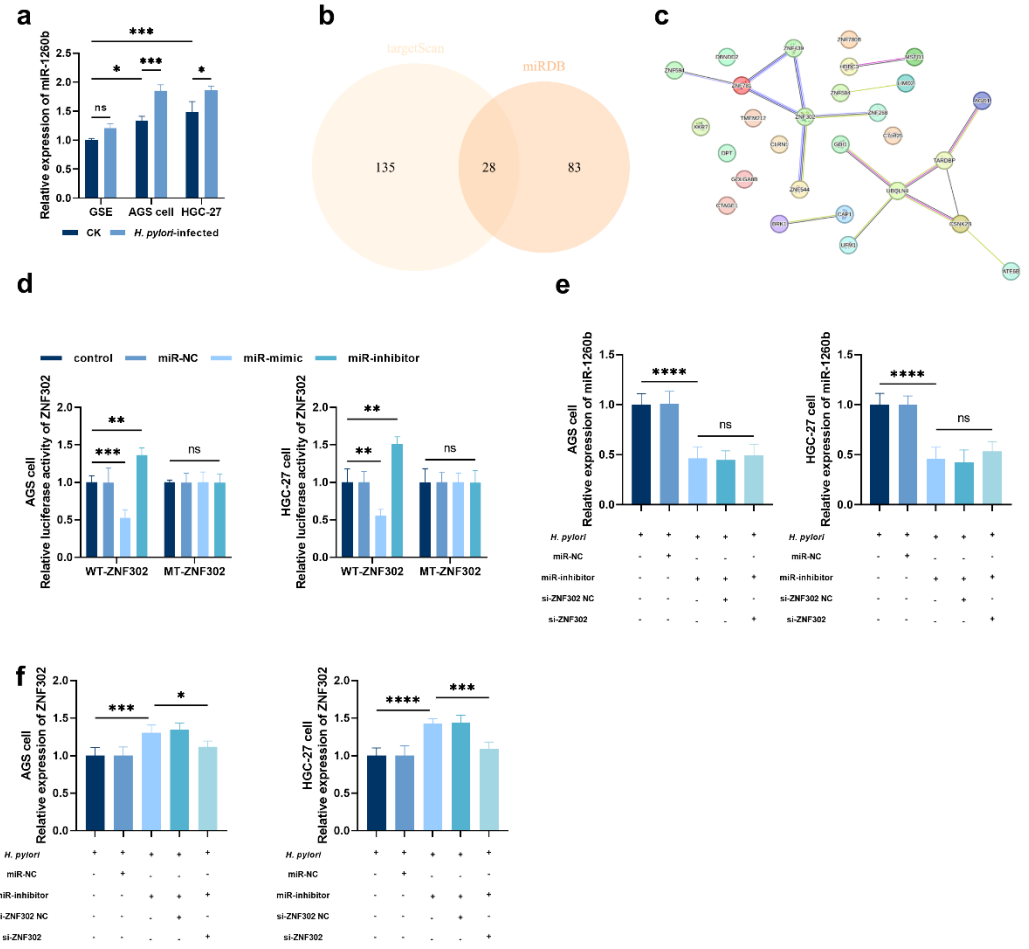

Fig. 2. Target interaction between miR-1260b and ZNF302. A. Expression of miR-1260b in GSE, AGS, and HGC-27 cells with or without infection of *Helicobacter pylori (H. pylori).* B and C. Prediction (B) and protein-protein interaction network (C) of miR-1260b targets. D. Luciferase reporter assay. E and F. Expression of miR-1260b (E) and ZNF302 (F) in AGS and HGC-27 cells with different transfections. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. miR-NC: miR-1260b negative controls; miR-mimic: miR-1260b mimic; WT-ZNF302: luciferase reporter vectors with wild-type binding sites of ZNF302; MT-ZNF302: luciferase reporter vectors with mutant-type binding sites of ZNF302; miR-inhibitor: miR-1260b inhibitor; si-ZNF302 NC: small interference RNA negative control of ZNF302; si-ZNF302: small interference RNA of ZNF302.

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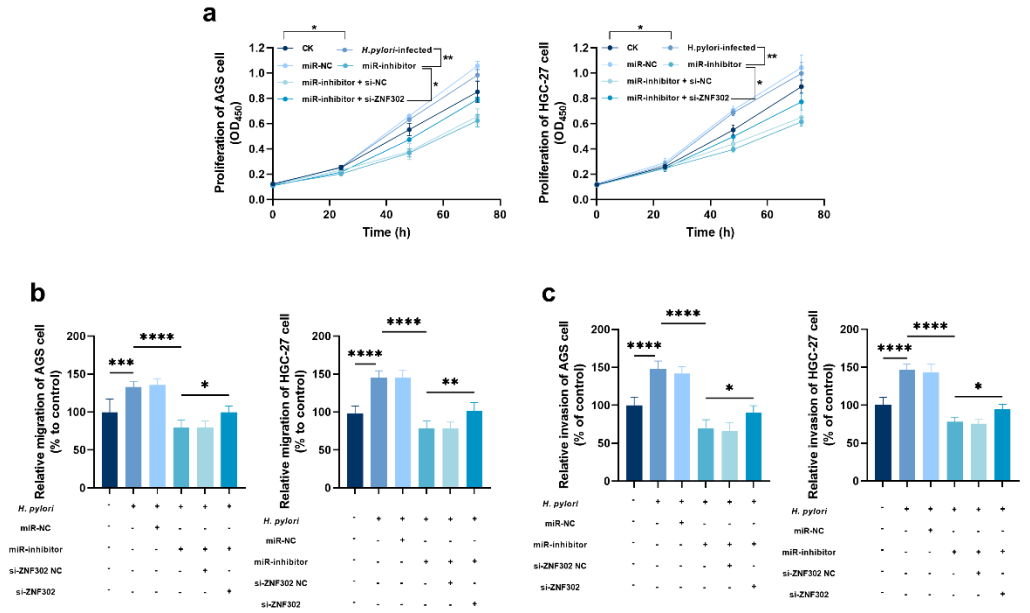

Fig. 3. Regulatory effect of miR-1260b/ZNF302 on *Helicobacter pylori (H. pylori)* infection and biological function of gastric cancer cells. A. Cell proliferation. B. Cell migration. C. Cell invasion. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. miR-NC: miR-1260b inhibitor negative control; miR-inhibitor: miR-1260b inhibitor; si-ZNF302 NC: small interference RNA negative control of ZNF302; si-ZNF302: small interference RNA of ZNF302.