

## Original Article

# Helicobacter pylori infection associated miR-451 suppressed the proliferation of gastric cancer by targeting CASC4

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**Abstract:** Aberrant microRNA (miRNA) expression has been observed in gastric cancer (GC) but fragmentary information is available on the miRNA dysregulation occurring with each phenotypic change involved in gastric carcinogenesis with *Helicobacter pylori* (*H.pylori*). In this study we mainly aimed to investigate the potential function of miRNA in GC patients with *H.pylori*. Based on the microarray screening, we found miR-451 was suppressed in the tumor tissues GC patients with *H.pylori* and was further confirmed in a larger sample size and GC cell lines. Ectopic expression of miR-451 dramatically suppressed cell proliferation in vitro. Bioinformatical analysis revealed that CASC4 might be the potential target gene of miR-451. We also found that miR-451 strongly reduced the expression of CASC4 oncogene in GC cells. In clinical specimens, CASC4 was over-expressed in tumors and *H.pylori* positive tissues. Univariate analysis indicated that low miR-451 expression in GC patients with *H.pylori* were significantly negative prognostic predictors for overall survival in patients. Taken together, our results indicated that miR-451 functions as a growth-suppressive miRNA in *H.pylori* related GC mediated mainly by repressing CASK expression and might act as a poor fingerprint for the prognosis.

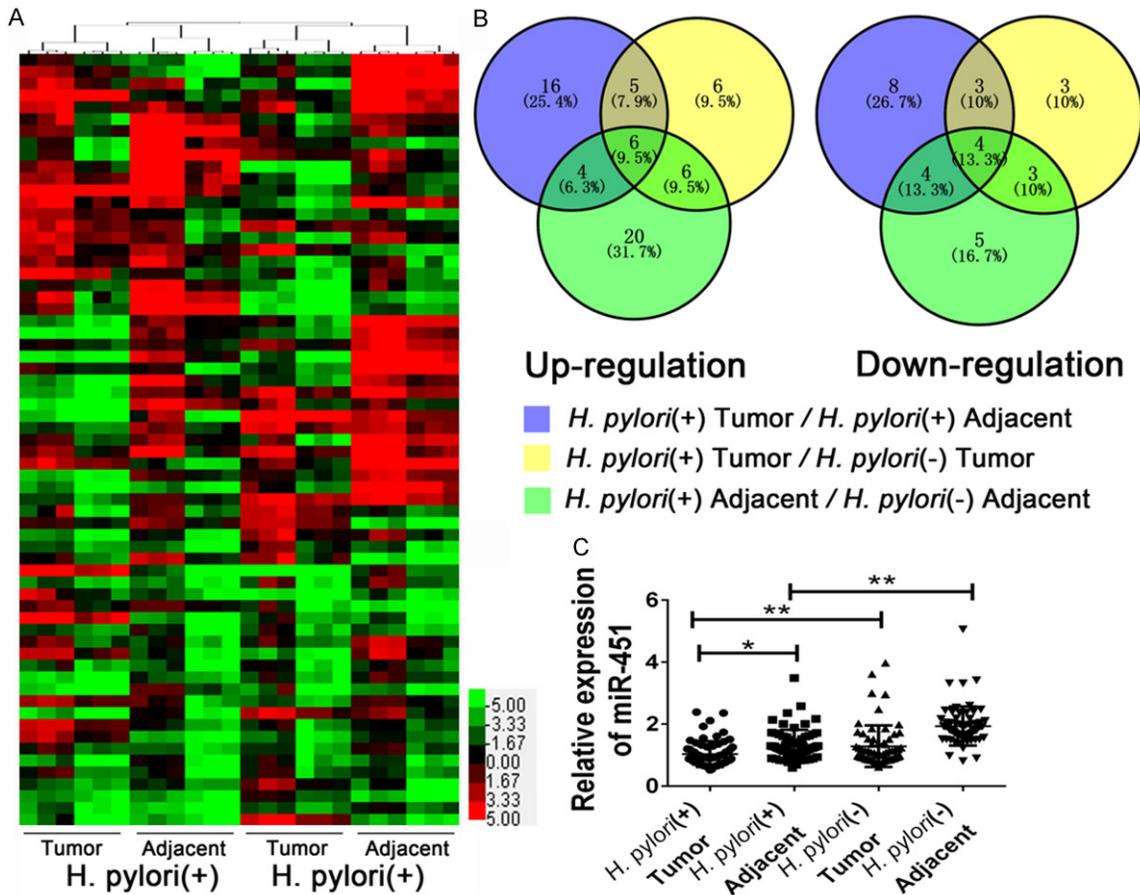
**Keywords:** miRNA, CASC4, proliferation, prognosis, *H.pylori*

## Introduction

Gastric cancer is the fourth most common cancer and second leading cause of cancer-related death worldwide, and about 90% of non-cardia GCs are the ultimate consequence of long-standing *Helicobacter pylori* (*H.pylori*) infection [1, 2]. Chronic *H.pylori* infection of the gastric epithelium is strongly associated with the development of gastritis, peptic ulcers, mucosa-associated lymphoid tissue lymphoma and gastric cancer. Despite recent extensive investigations on the molecular landscape of GC [3, 4], the molecular grounds for the various steps in *H.pylori*-related carcinogenesis have yet to be fully elucidated, and no consistent and reliable biomarkers have become available for use in GC secondary prevention strategies [5-7].

MicroRNAs (miRNAs) are short, non-coding RNA molecules that regulate gene expression

by directly binding to the 3'-untranslated region (UTR) of their target gene mRNA [8, 10]. Cumulative evidence suggests that microRNAs (miRNAs) might play important roles in the initiation and progression of various human diseases [11, 13]. Among other miRNAs, the let-7 family members have reliably been found down-regulated in association with both *H.pylori*-associated gastritis and *H.pylori*-related gastric cancer [14]. To date, several human and *H.pylori* associated miRNAs have been shown to be dysregulated in GC, such as let-7 and miR-141, which contribute to the development and progression of GC. These findings suggest the involvement of miRNAs in GC tumorigenesis. However, the systematical analysis of aberrant expressed miRNAs in the *H.pylori*-related gastric cancer was few. In this context, we sought to identify miRNAs that are causally involved in gastric malignancies associated with *H.pylori* infection by using several systematic and bioin-



**Figure 1.** The clinicopathological sample analysis of miR-451 expression in GC patients. A: Cluster analysis of aberrant expressed miRNAs in tissues samples. B: Venny analysis in four groups. C: Relative expression of miR-451 in GC patients with different *H.pylori* infection status. Data was presented as mean  $\pm$  SEM. \*indicates  $P < 0.05$  while \*\*indicated  $P < 0.01$ .

formatic approaches and studying gastric epithelial cell lines, stomach tissue based on the high throughput screening. In this comprehensive study, we demonstrate that miR-451 is a critical miRNA, which regulates gastric epithelial cell proliferation by targeting potential oncogenes. Furthermore, our work provides substantial evidence for the causal involvement of miR-451 in predicting the prognosis of patients with gastric malignancies induced by chronic Hp infection.

**Materials and methods**

*Study subjects*

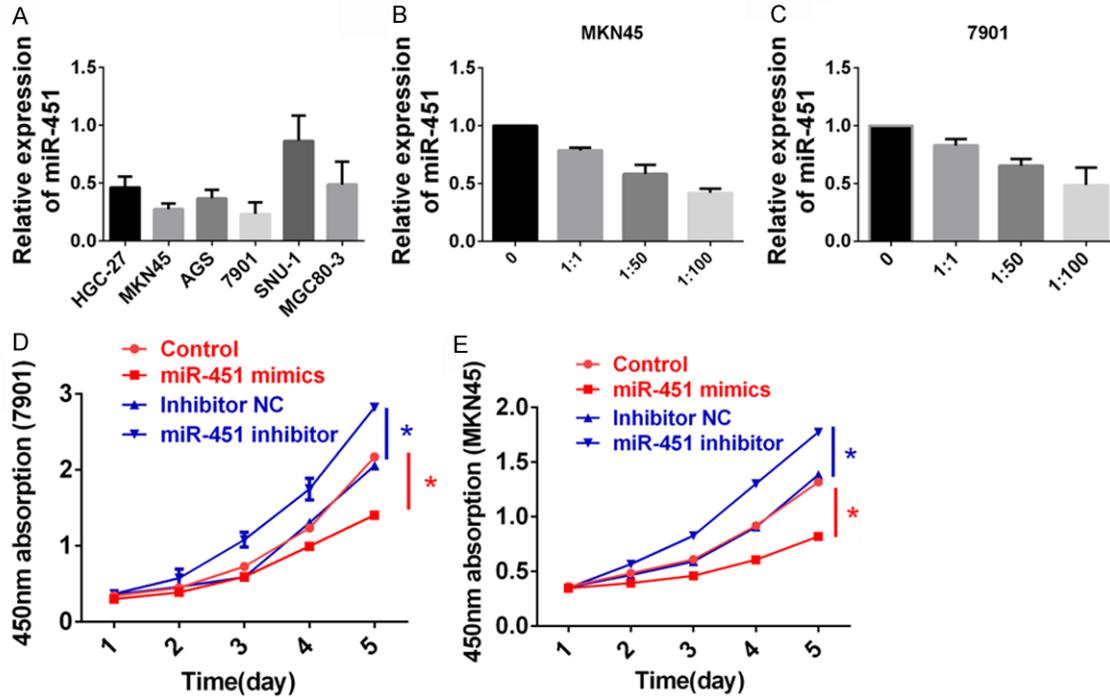
A total of 130 GC cases from The 359<sup>th</sup> Hospital of PLA and Wujin Hospital Affiliated to Jiangsu University were enrolled in this study. Patients were consecutively recruited between July

2012 and August 2015 at The 359<sup>th</sup> Hospital of PLA and Wujin Hospital Affiliated to Jiangsu University. All cases are incident ones during enrollment of the current case-control study. The diagnosis of all patients was histological confirmed. All participants have provided their written informed consents to participate in this study. This study was approved by the institutional Review Board of the 359<sup>th</sup> Hospital of PLA and Wujin Hospital Affiliated to Jiangsu University.

*Cell culture and reagents*

Gastric cancer cell lines including HGC-27, MKN45, AGS, 7901, SNU-1 and MGC80-3 were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). All cells were cultured in Dulbecco modified Eagle medium (DMEM) purchased from Gibco (CA, USA)

## miR-451 in *H.pylori* associated GC



**Figure 2.** *H.pylori* infection associated miR-451 suppressed cell proliferation. A: Relative expression of miR-451 in different GC cell lines. B and C: miR-451 expression in MKN45 and 7901 cells infected with different MOIs of *H.pylori*. D and E: The absorption under 450 nm of cells treated with miR-451 mimics, inhibitor or controls. Data was presented as the mean  $\pm$  SEM. \*indicates  $P < 0.05$ .

supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, USA) and grown in humidified 5% CO<sub>2</sub> at 37°C. The miR mimics, inhibitor and normal control were obtained from Genepharma (Shanghai, China). The transfection was conducted by using Lipofectamine 2000 (Invitrogen Corp, CA, USA).

### TaqMan low density array (TLDA)

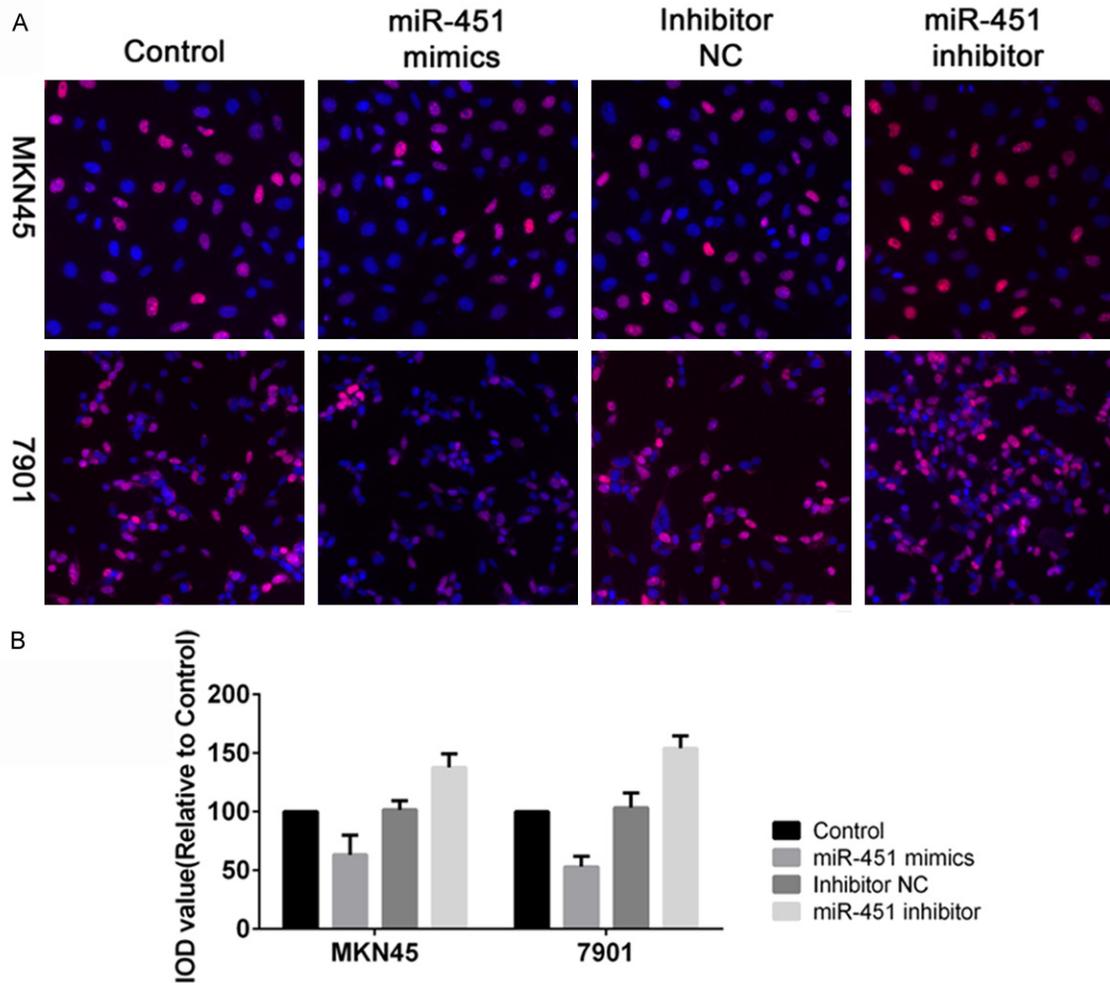
In the screening stage, TLDA Chips (Life Technologies) was used to screen differentially expressed miRNAs from the four grouped samples (five samples in each group). Total RNA was extracted from the tissues samples using the TRIzol Reagent. Megaplex RT reactions and pre-amplification reactions were run according to the manufacture's protocol, in which 75- $\mu$ l 0.16 \* TE was added to PreAmp product, and 9- $\mu$ l diluted PreAmp product was used to run the Real time Polymerase Chain Reaction (RT-PCR) reactions by dispensing 100  $\mu$ l of the PCR reaction mix into each port of the TaqMan MicroRNA Array. The default PCR procedure was used, and the analysis was performed by using RQ manager software (Life Technologies).

### Quantitative RT-PCR and Western blot analysis

Total RNA was extracted using Trizol (TAKARA, Japan). Levels of mature miR-451 were measured using TaqMan MicroRNA Assay (Applied Biosystems, CA, USA) by normalizing to the levels of U6. SYBR Green PCR Kit (TAKARA, Japan) was used to quantify the mRNA levels of CASC4 by normalizing to GAPDH. The PCR reactions were performed and analyzed using ABI Step-one system. The relative expression ratio of miR-451 in paired tissues and cells was calculated by the 2<sup>- $\Delta\Delta$ CT</sup> method. Western blots were performed as described previously. Briefly, total protein was separated on a precast 10% polyacrylamide gel and blotted with antibodies for CASC4 (diluted 1:1000, cell signaling technology) and  $\beta$ -action (diluted 1:1000, cell signaling technology). Densitometric analysis of protein bands was performed via Image J software.

### Cell proliferation assays

The ability of cells proliferation was assayed using CCK8 (Beyotime, Nantong, China) and EDU (Millipore, Massachusetts, America)



**Figure 3.** miR-451 suppressed cell growth. A: Cells proliferation was measured by EDU assay. Cells were treated with miR-451 overexpression lentivirus (mimics group) and suppression lentivirus (inhibitor group). The proliferative cells were stained with red. B: Proliferative cells were counted and normalized with DAPI stain. Data was presented as the mean  $\pm$  SEM.

according to the manufacturer's instructions. The mock and infected cells were seeded at a density of  $1 \times 10^4$  cells/well in 96-well flat-bottom and respectively cultured for EDU assays and CCK8 assay.

*Construction of luciferase-based reporter plasmids*

The total fragment of the CASC4 3'UTR was amplified. The PCR production was cloned into the pGL3-promoter luciferase-based plasmid (Promega, CA, USA). The amplified fragment was verified by DNA sequencing.

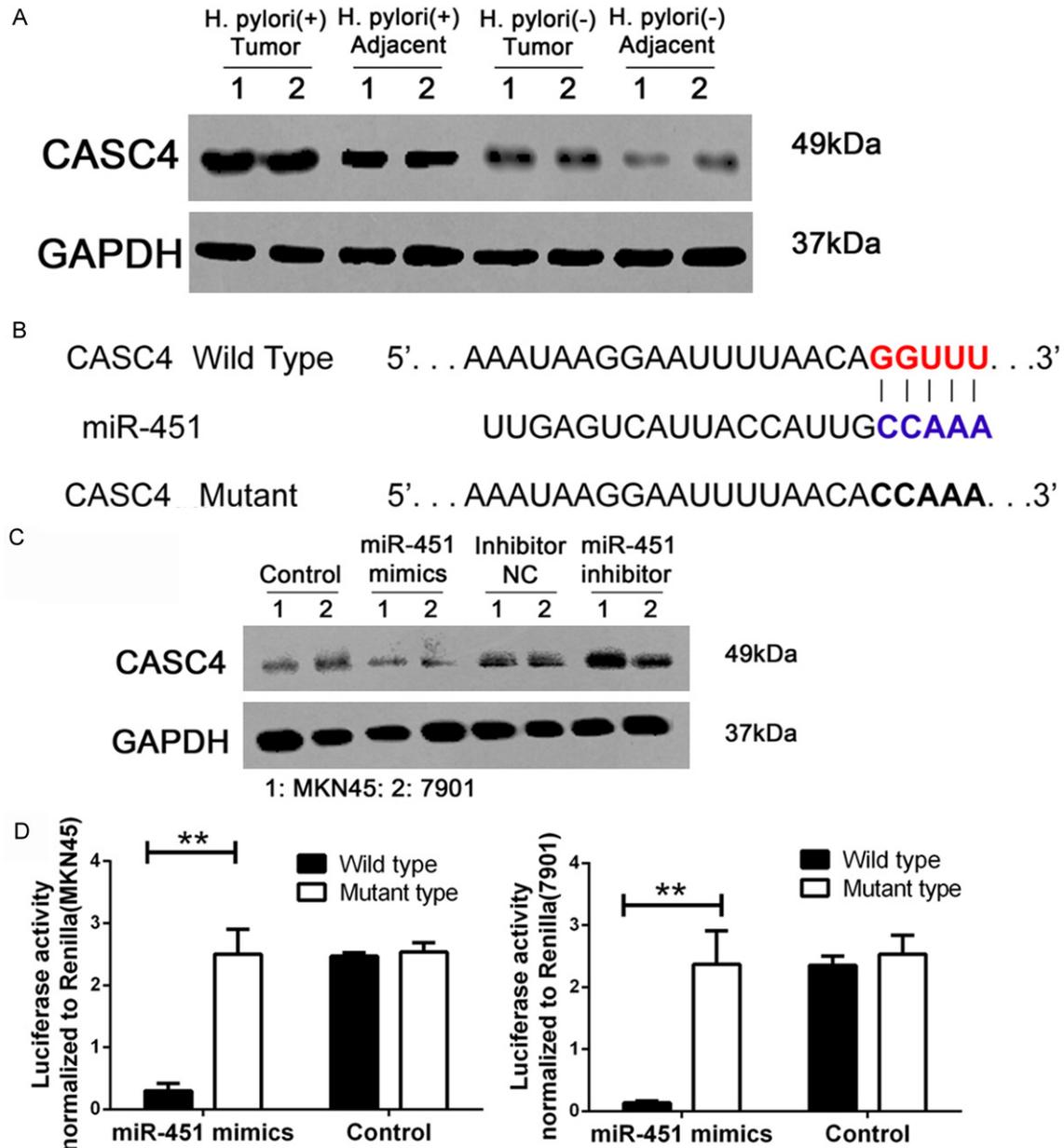
*Dual-luciferase reporter assay*

For luciferase activity analysis, HGC-27 and SGC-7901 cells were co-transfected with 100

ng of luciferase reporter constructs 5 ng control plasmid and 10 pmol of miRNAs with 1  $\mu$ l lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, NY, USA). After incubation for 48 h, we carried out the luciferase assay using the luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer's protocol. Measurements of luminescence and absorbance of control plasmid were performed on a luminometer (Glomax 20/20; Promega). Three independent experiments were performed in triplicate.

*Statistical analysis*

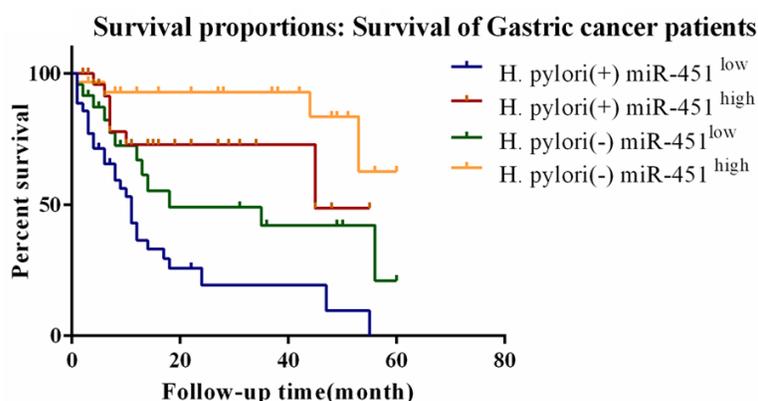
The Mann-Whitney U-test or Fisher's exact test was used for between-group comparisons where appropriate, and correlation between



**Figure 4.** miR-451 suppressed CASC4 expression by targeting the 3'UTR region. A: Relative expression of CASC4 GC patients detecting by western blot. GAPDH were used as internal reference. B: Detailed sequence information of plasmid construction of CASC4 3'UTR. C: Relative expression of CASC4 in cells treated with either miR-451 mimics or inhibitor detecting by western blot. GAPDH were used as internal reference. D: Cells were co-transfected with miR-451 with different types of the 3'UTR of CASC4 and Renilla luciferase vector pRL-SV40 for 48 h. Both firefly and Renilla luciferase activities were measured in the same sample. Firefly luciferase signals were normalized with Renilla luciferase signals. Data was presented with mean  $\pm$  SEM. \*\*indicated  $P < 0.01$ .

the results obtained with the two different analyses determined with Spearman's test. Follow-up studies of patients were performed until the time of writing or patient death. Cancer-specific survival outcomes were evaluated by applying the Kaplan-Meier method for all patients, except those who died from surgical complica-

tions. The log-rank test was used to compare the prognostic significance of individual variables on survival. Cox's proportional hazards model was applied in multivariate analysis to identify independent predictors of survival.  $P$ -values  $< 0.05$  were considered statistically significant. Statistical analysis was performed



**Figure 5.** *H.pylori* infection induced miR-451 expression might be an ideal prognostic indicator of GC. The Kaplan-Meier curve for the overall survival of patients was presented by using different indicators as miR-451 expression combined with *H.pylori* infection.

with SPSS 13.0 and SAS software (version 9.1.3; SAS Institute, Cary, NC, USA). The graphs were generated by Graphpad Prism 5.0 (Graphpad Software, Inc.).

## Results

*miR-451 was down-regulated in tumor tissues of H.pylori-positive gastric cancer patients.*

The TLDA microarray was applied to screen the dysregulated miRNAs in GC patients with *H.pylori*-positive or *H.pylori*-negative. As presented in **Figure 1A**, each group contained five GC tumor tissues or corresponding adjacent tumor tissues. Clustering analysis revealed an aberrant different expression miRNA profile in these four groups. We further analyzed the different expressed miRNAs in four groups by using Venny diagram by pairwise comparison. We found that only six miRNAs were upregulated and four miRNAs were downregulated with the following characteristics: 1) upregulated/downregulated in *H.pylori*-positive GC tumor tissues comparing with *H.pylori*-negative GC tumor tissues; 2) upregulated/downregulated in *H.pylori*-positive GC adjacent tumor tissues comparing with *H.pylori*-negative GC adjacent tumor tissues; 3) upregulated/downregulated in *H.pylori*-positive GC tumor tissues comparing with *H.pylori*-positive adjacent GC tumor tissues; 4) with the cutoff 4/0.25 (**Figure 1B**). Among the ten candidate miRNAs, miR-451 presented the most significant, we next detected the expression of miR-451 in a larger sample size with 65 samples in each group, as pre-

sented in **Figure 1C**, and we found that miR-451 was aberrant decreased in *H.pylori*-positive GC tumor tissues comparing with either *H.pylori*-negative GC tumor tissues or *H.pylori*-positive GC adjacent tumor tissues.

*MiR-451 was decreased in H.pylori positive cells*

In order to investigate the detailed function of miR-451, we first analyzed the expression of miR-451 in multiple GC cell lines. As presented in **Figure 2A**, MKN45 and 7901 cells displayed lowest miR-

451. Consequently, we chose MKN45 and 7901 cells for further functional studies. We then infected 7901 and MKN45 cells with different MOIs of *H.pylori* (0, 1:1, 1:50, 1:100) and we found that miR-451 expression gradually decreased with increased MOIs (**Figure 2B and 2C**,  $P < 0.05$ ) indicating that miR-451 was down-regulated in *H.pylori* infected state and its down-regulated was significantly associated with GC progression.

*Ectopic miR-451 inhibits growth GC cells in vitro*

To explore the effect of miR-451 on cell growth, MKN45 and 7901 cells were transiently transfected with miR-451 mimic or inhibitor, respectively. The expression of miR-451 validated after mimic or inhibitor transfection was confirmed. The results of CCK8 assay displayed that miR-451 significantly inhibited cell growth in 7901 cells and in MKN45 cells ( $P < 0.05$ ), whereas miR-451 inhibitor promoted cell growth in these two cells ( $P < 0.05$ ). By contrast, negative control (NC) or inhibitor NC had no effect on cell growth, indicating that the effect caused by miR-451 was specific (**Figure 2D and 2E**).

We next used lentiviral vectors to stably restore the expression of miR-451 in MKN45 and 7901 cells and examined cell growth rate. We showed that the expression levels of miR-451 were increased in 7901 and MKN45 cells respectively in a dose-dependent manner and reached a very high level at MOI 100. Therefore, the

same condition (MOI=100) was applied for further experiments. The growth inhibition induced by miR-451 overexpression was similar to that induced by miR-451 mimic transfection (**Figure 3A**). As demonstrated in EDU assay (**Figure 2E**), miR-451 overexpressed 7901 and MKN45 cells displayed much fewer and smaller stain compared with control groups (**Figure 3B**).

### *CASC4* was the direct target of miR-451 in GC cells

To explore the mechanism of growth inhibition induced by miR-451, we predicted the potential target genes by using bioinformatical analysis. The following tools were employed including TargetScan, PicTar, miRTarget and miRBase. After screened by ranking index in different databases, we found that *CASC4* might be the target gene of miR-451. We first detected the expression of *CASC4* in GC patients, we found that *CASC4* expression were significant inversely with miR-451 with the highest expression in tumor tissues of *H.pylori*-positive GC patients (**Figure 4A**). We further performed luciferase reporter assay to determine whether miR-451 could directly target the 3'UTR of *CASC4* in GC cells. The target sequence of *CASC4* 3'UTR (wt 3'UTR) or the mutant sequence (mut 3'UTR) was cloned into a luciferase reporter vector (**Figure 4B**). 7901 cells were then transfected with wt or mut 3'UTR vector and miR-451 mimic. Ectopic expression of miR-451 led to a dose-dependent decrease in *CASC4* protein levels. Moreover, inhibition of endogenous miR-451 resulted in up-regulated expression of *CASC4* in 7901 cells (**Figure 4C**). The luciferase reporter assay showed a significant decrease of luciferase activity when compared with miRNA control (**Figure 4D**). The activity of mut 3'UTR vector was unaffected by a simultaneous transfection with miR-451. Moreover, co-transfection with miR-451 inhibitor and wt 3'UTR vector in 7901 cells led to a 2-fold increase of luciferase activity. Taken together, all these results strongly suggested that *CASC4* was a direct target of miR-451 in GC cells.

### *miR-451* predicted poor prognosis of GC patients

Since miRNA was reported to be indicator of various diseases recently, we also accessed miR-451 in GC patients with different *H.pylori* infection status. Assessment of the 95% confi-

dence interval (CI) in the healthy control group was the threshold from discriminating normal from suppressed systemic levels. The GC patients were divided into four groups accordingly: *H.pylori*-positive with miR-451 low (39 patients); *H.pylori*-positive with miR-451 high (32 patients); *H.pylori*-negative with miR-451 low (29 patients); *H.pylori*-negative with miR-451 high (30 patients). We assessed the 5-year survival rate in the four groups. The 5-year survival rate in the *H.pylori*-positive with miR-451 low group was significantly lower than in the other three groups. The 5-year survival rate in the *H.pylori*-negative with miR-451 high was significantly higher than in the other three groups (**Figure 5**) indicating that miR-451 might be a poor predictor for the prognosis of GC patients together with *H.pylori* infection.

## Discussion

GC is defined as cancer that forms in the tissues lining the stomach. Globally, GC is the fifth leading cause of cancer and the third leading cause of cancer mortality, comprising 7% of cases and 9% of deaths. In 2012 GC occurred in 950,000 people and caused 723,000 deaths [15, 16]. The most common cause is infection by *H.pylori*, which accounts for >60% of cases [17]. Most clinical evidence suggested that *H.pylori* infection is related to GC, but the underlying molecular mechanism remained largely unknown.

MiRNAs could negatively regulate their target genes expression at the post-transcription level through binding to 3' untranslated regions (UTRs) of their targets message RNAs [18, 19]. MiR-451 is mapped to chromosome 17q11.2 in human, and was first identified in the human pituitary glands in 2005. It has been found that miR-451 may either regulate the expression of tumor suppressor genes and oncogenes or function as oncogenes or tumor suppressor in the progression of CRC [20, 21]. The clinicopathological analysis showed that miR-451 was associated with a poor prognosis of gastric cancer [22]. Nerea and his colleagues found that miR-451 expression was down-regulated in paraffin-embedded human CRC sections via in-situ hybridization and miR-451 was able to suppress the tumor formation by regulating the expression of macrophage migration inhibitory factor (MIF). They also found this in colon cancer stem cells tumor sphere in vitro [23].

However, miRNAs may function according to a combinatorial circuit model, in which a single miRNA may target multiple mRNAs, and several co-expressed miRNAs may target a single mRNA. Recent studies have suggested that the biological concept of “one hit-multiple targets” could be used in clinical therapeutics.

In addition to the oncogenic effects of CASC4 in GC cells, CASC4 (cancer susceptibility candidate 4) encodes a transmembrane protein predicted to localize to the Golgi apparatus. Researchers have found that overexpressing CASC4 could increase acinar size and proliferation, and decreased apoptosis, partially recapitulating SRSF1's oncogenic effects in breast cancer [24, 25]. Since no evidence has been identified in human gastric cancer, additional work needs to be conducted in the future focusing on the detailed mechanism of CASC4 in human gastric cancer.

In conclusion, we mainly focused on the screened miR-451 in GC patients with different *H.pylori* infection status. We identified that suppressed miR-451 was highly associated with *H.pylori* infection. Overexpressed miR-451 could cause an inhibition of cell proliferation via targeting CASC4 in vitro which was also associated with the *H.pylori* lower expression of miR-451 in *H.pylori* positive GC patients indicated the worst prognosis.

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#### Disclosure of conflict of interest

None.

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