

RESEARCH ARTICLE

MicroRNA-146a Enhances *Helicobacter pylori* Induced Cell Apoptosis in Human Gastric Cancer Epithelial Cells

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Abstract

Helicobacter pylori (*H. pylori*) infection induces apoptosis in gastric epithelial cells, and this occurrence may link to gastric carcinogenesis. However, the regulatory mechanism of *H. pylori*-induced apoptosis is not clear. MicroRNA-146a has been implicated as a key regulator of the immune system. This report describes our discovery of molecular mechanisms of microRNA-146a regulation of apoptosis in human gastric cancer cells. We found that overexpression of microRNA-146a by transfecting microRNA-146a mimics could significantly enhance apoptosis, and this up-regulation was triggered by COX-2 inhibition. Furthermore, we found that microRNA-146a density was positively correlated with apoptosis rates in *H. pylori*-positive gastric cancer tissues and intratumoral microRNA-146a density was negatively correlated with lymph node metastasis among *H. pylori*-positive gastric cancer patients. Understanding the important roles of microRNA-146a in regulating cell apoptosis in *H. pylori* infected human gastric cancer cells will contribute to the development of microRNA targeted therapy in the future.

Keywords: *Helicobacter pylori* - apoptosis - microRNA - COX-2

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Introduction

About 50% of the world's population is infected with *Helicobacter pylori* (*H. pylori*), which is a causative agent for gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer (Salama et al., 2013). It is estimated that individuals infected with *H. pylori* have more than two-fold increased risk of developing gastric cancer compared with non-infected ones (Brenner et al., 2000) and nearly all gastric cancer is related to *H. pylori* (Uemura et al., 2000). Why only 1 to 5% of *H. pylori* infected persons develop gastric cancer remains unknown and it seems to depend on the relationship between environmental, bacterial virulence factors and host genetics.

As a type of cellular suicide, apoptosis occurs in single cells, in response to the expression of specific cellular genes. Tissue integrity is maintained when the rate of cell loss by apoptosis is matched by the rate of new cell production by proliferation. For this reason, apoptosis was considered as a mechanism to block tumor formation. It has been demonstrated that *H. pylori* infection is crucial in disturbed regulation of epithelial cell apoptosis and may therefore link the multistep process of carcinogenesis (Targosz et al., 2012). However, the regulatory mechanism of *H. pylori*-induced apoptosis in gastric cancer cells is still not well understood.

The human transcriptome comprises not only large numbers of protein-coding messenger RNAs (mRNAs),

but also a large set of non-protein coding transcripts that have structural, regulatory, or unknown functions. Noncoding RNAs are divided into long noncoding RNAs (lincRNAs) and short noncoding RNAs (microRNAs) according to their length. MicroRNAs are recently discovered class of small noncoding RNAs that are implicated in many physiological and pathological responses as post-transcriptional repressors of gene expression. Mature microRNAs can specifically bind to the 3' untranslated regions (3'UTRs) of target cellular mRNA, in turn triggering mRNA degradation or inhibition of translation (Bartel, 2004). Abnormal expressions of certain microRNAs are identified to regulate the immune response against *H. pylori* infection (Matsushima et al., 2011). Previous work has reported that microRNA-146a negatively regulated the release of proinflammatory cytokines (Liu et al., 2013) during *H. pylori* infection. Recently, we focused on the regulatory role of microRNA-146a in cell apoptosis triggered by *H. pylori* infection in gastric cancer cell.

Materials and Methods

Cell and bacteria culture

The MKN7 human gastric carcinoma cells were routinely cultured in RPMI 1640 medium (Invitrogen, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS) and 100U/ml penicillin in a humidified incubator containing 5% CO₂ at 37°C. The

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wild-type *H. pylori* strain 26695 was obtained from ATCC and grown as previously described (8). Subsequently, cells were seeded to the wells of a 12-well plate and grown to 80% concentration. Then, the medium was replaced with antibiotic-free medium. *H. pylori* was added to cells at a multiplicity of infection of 300:1. The infection model was monitored by the release of interleukin-8 (IL-8) and growth-related oncogene-alpha (GRO-alpha), as measured by DuoSet ELISA Development System (R&D).

Cell transfection

pcDNA3.1-myc-COX-2 were purchased from Biogot technology, co, Ltd, China. MicroRNA mimics, inhibitors, or scrambled microRNA-control were synthesized from GenePharma (Shanghai, P.R. China) products. Transfections were performed using Lipofectamine 2000 (Invitrogen) with 50 nM pcDNA3.1-myc-COX-2, microRNA mimics, inhibitors, or scrambled microRNA-control for certain time.

Clinical samples

For clinical samples, totally 38 *H. pylori*-positive gastric cancer patients together with 10 *H. pylori*-negative, gastric cancer subjects were included in the study. The *H. pylori* infection status was confirmed by bacterial culture, C 13-urea breath test, and histologic testing. Patients were regarded as being *H. pylori* positive if one of the tests yielded positive results. Patients with history of gastric surgery, active gastrointestinal bleeding, use of steroids, immunosuppressive drugs, NSAIDs, proton pump inhibitors or who were treated for *H. pylori* eradication were excluded from the study. Gastric fragments were obtained during endoscopy from five different sites as recommended by the Updated Sydney System for classification of gastritis (Dixon et al., 1996). A number of clinicopathological variables such as gender, age, tumor location, histological type, tumor-node-metastasis (TNM) stage, depth of tumor invasion, lymph node metastasis, distant metastasis and vascular invasion were obtained from the histopathological records and included for survival analysis.

RNA extraction

Total RNA was extracted from cells using Trizol reagent (Invitrogen) according to the manufacturer's protocol. qRT-PCR analyses for microRNAs were performed by using TaqMan microRNA assays (Ambion) in an iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). U6 small nuclear RNA was used as endogenous control for data normalization. Relative expression was calculated using the comparative threshold cycle (Ct) method. To identify apoptotic nuclei, DAPI (4, 6-diamidino-2-phenylindole) (Roche-Boehringer, Mannheim, Germany) staining was performed according to the manufacturer's protocol.

Cell apoptosis assay

An annexin V-Fluos staining kit (Roche-Boehringer) was used to detect early stages of apoptosis, as represented by phosphatidylinositol flip to the outer membrane. The cells were washed with PBS and stained according to

the manufacturer's protocol. Slides were mounted with Permafluor mounting medium (Immunotech, Marseille, France) and viewed under a fluorescence microscope (Axiophot Olympus).

Statistical analyses.

The results are expressed as means±SD from at least 3 separate experiments performed in triplicate. The differences between groups were determined using two-tailed Student's t-test, using SPSS software (Armonk, NY, USA). P values of less than 0.05 were considered significant. Correlations between microRNA-146a expression and apoptosis in clinical samples were calculated by means of the Pearson correlation test if data showed Gaussian distribution; otherwise, they were calculated by means of the Spearman rank correlation test. The Chi-square test or Fisher's exact test was used to analyze the relationship between microRNA-146a expression and the clinicopathological features.

Results

Using the *H. pylori* infected MKN7 cell model, we found that expression of microRNA-146a (Figure 1A) and cell apoptosis (Figure 1B) were enhanced simultaneously under *H. pylori* infection ($p < 0.05$), which is in accordance with previous report. We next generated MKN7 cells in which microRNA-146a mimics, inhibitors, and control were respectively transfected for 24h, followed by *H. pylori* infection. MicroRNA-146a mimics significantly enhanced the apoptosis rate, while microRNA-146a inhibitors had little influence (Figure 2A). Meanwhile, expression of Bax (an apoptotic gene) and Bcl-2 (an anti-apoptotic gene) were determined by RT-PCR over different periods of time starting from 3h up to 48h. MicroRNA-146a mimics obviously up-regulated the mRNA levels of Bax in 24h and 48h after infection (Figure 2B) and decreased the mRNA levels of Bcl-2 48h after infection (Figure 2C). These data indicated that microRNA-146a participate an important role in apoptosis development.

Previous publications have reported that COX-2 is a potential target of microRNA-146a (Liu et al., 2013), and COX-2 was closely related to cancer cell apoptosis, so we designed the next series of work to explore whether microRNA-146a promote apoptosis by suppressing COX-2. We transient transfection with plasmids pcDNA3.1-

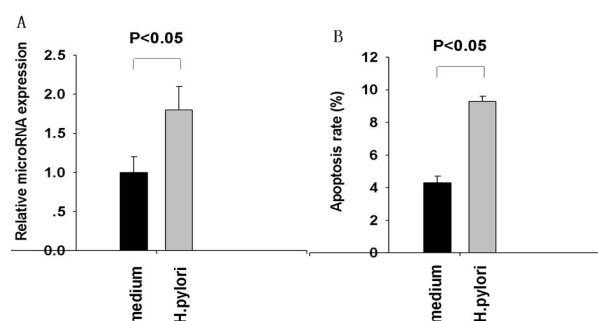


Figure 1. Helicobacter Pylori Infection in MKN7 Cells: (A) microRNA-146a Expression was Determined by RT-PCR (B) Cell Apoptosis was Determined by Annexin V-Fluos Staining Kit

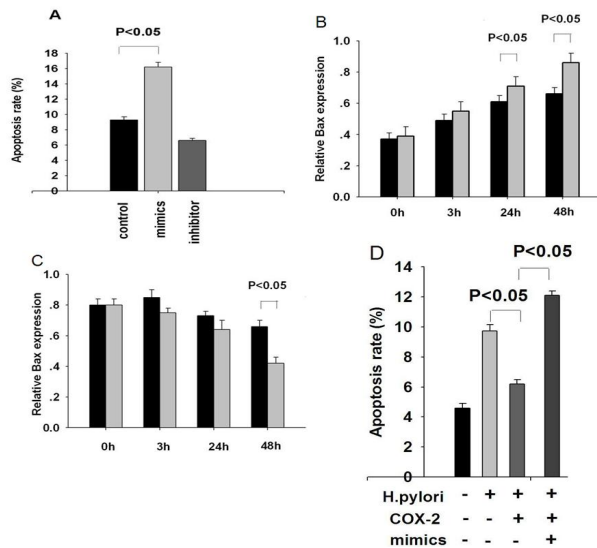


Figure 2. MicroRNA-146a Attenuates *Helicobacter Pylori* Induced Apoptosis in MKN7 Cells by Inhibition of COX-2. (A) cells were transfected with microRNA-146a mimics, inhibitors, or microRNA-control for 24 h, followed by *H. pylori* infection and apoptosis was determined. (B, C) cells were transfected with microRNA-146a mimics (grey) and control (black), and then apoptosis related genes were determined by RT-PCR in time dependent manner. (D) cells were co-transfected with microRNA-146a mimics and COX-2, compared with the transfection of COX-2 only and control

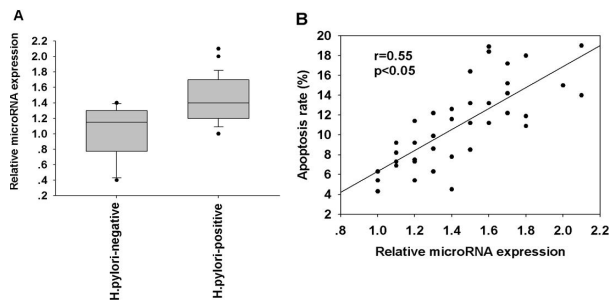


Figure 3. Correlation Between MicroRNA-146a and Cell Apoptosis in Clinical Samples. (A) Expression of microRNA-146a in gastric cancer tissues from *H. pylori*-positive patients (38) and *H. pylori*-negative gastric cancer individuals (10). (B) correlations between microRNA-146a and cell apoptosis in gastric cancer tissues from *H. pylori*-positive patients

myc-COX-2, together with microRNA-146a mimics/control for 24 h followed by *H. pylori* infection. As shown in (Figure 2D), apoptosis was sharply reduced in COX-2 overexpressed group, indicating that COX-2 weakened the apoptosis development. Then cotransfection with microRNA-146a mimics could block this apoptosis reduction, suggesting that microRNA-146a up-regulated apoptosis by suppressing COX-2.

Subsequently, the mRNA expression of microRNA-146a was evaluated in 38 *H. pylori*-positive gastric cancer and 10 *H. pylori*-negative gastric cancer. Almost all the *H. pylori*-positive gastric cancer samples were observed to have higher microRNA-146a mRNA levels compared with the control (Figure 3A). In *H. pylori*-positive patients,

Table 1. Association of microRNA-146a Expression with Clinicopathological Variables of *H. pylori*-Positive Gastric Cancer Patients

Variables	Cases	miR-146a		p value
		high	low	
Gender				0.22
Male	21	13	8	
Female	17	9	8	
Age				0.2
<55	16	10	6	
>55	22	11	11	
Location				0.32
Upper	6	2	4	
Lower	32	14	18	
TNM stage				0.15
I+II	11	8	3	
III+IV	27	14	13	
Lymph node metastasis				0.04
Negative	20	8	12	
Positive	18	13	5	
Distant metastasis				0.06
M0	32	11	21	
M1	6	2	4	

microRNA-146a density was positively correlated with apoptosis rates (Figure 3B, $r=0.55$, $p<0.05$). We also analyzed the relationship between microRNA-146a mRNA expression and clinical features of *H. pylori*-positive gastric cancer in the high and low microRNA-146a expression groups based on the results of RT-PCR analysis. As shown in Table 1, intratumoral microRNA-146a density was negatively correlated with lymph node metastasis. Meanwhile, there was no significant association between microRNA-146a expression and the other clinical features.

Discussion

Because of the cellular migration and metastasis, the mortality rate of gastric cancer remains high. The development of gastric cancer is a comprehensive action associated with multiple factors, such as inhibition of tumor suppressor genes, overexpression of related genes and a failure to regulate cell proliferation. Therefore, it is urgently needed to find a sensitive biomarker for the detection of gastric cancer at the curative stage. Given the documented importance of apoptosis in regulating cell proliferation, many efforts have been done to reveal the apoptosis-related molecules expression in *H. pylori* infection, in order to better understand the process of carcinogenesis and metastasis. Previous work has demonstrated that NF- κ B can suppress apoptosis by inducing anti-apoptotic proteins, such as GADD45 β , A1/B11, and cIAP1. Bax protein expression was up-regulated in patient's gastric precancerous lesions after *H. pylori* infection and is highly expressed in intestinal metaplasia regions nearby to tumors and related with induction of apoptosis (Cheng et al., 2013).

COX-2 is a type of oncogenic protein, which is involved in many signal pathway in inflammation, proliferation, and apoptosis. In MKN-45 cells, inhibition of COX-2 led to reduced proliferation and induction of

apoptosis, connected with down-regulation of Bcl-2 and up-regulation of Bax (Sun et al., 2008). Furthermore, COX-2 was identified as a regulatory factor in the Bcl-2 upstream sequences, which up-regulated the expression of Mcl-1, a member of the Bcl-2 family, through the phosphatidylinositol 3-kinase (PI3K) signal pathway, and eventually inhibited the apoptosis of cancer cells (Chen et al., 2005). Some studies confirmed that COX-2 could inhibit the apoptosis of cancer cells by inducing the mutation of P53 (Han et al., 2002). Other researchers indicated that COX-2 weakened the apoptotic signal mediated by Fas protein (Casado., 2007). Above findings showed that COX-2 was a negative regulator of apoptosis. However, in contrast, some studies also demonstrated an opposite effect of COX-2 in apoptosis. For example, Li et al. also showed that the selective COX-2 inhibitor attenuated the cell proliferation and apoptosis of the human gastric cancer cell line BGC-823, which may be attributed to the inhibition of cell cycle progress (Li et al., 2008). This suggested that COX-2 may play different role in different internal environment, cell type or under different infected periods. Modulation of COX-2 levels might act as an important factor influencing the overall cell apoptosis.

Many microRNAs were confirmed to target COX-2. For example, Lin et al. have found that MicroRNA-101 play a critical role in Cervical Cancer in Uygur Women by targeting COX-2, and might be biomarker for early diagnosis (Lin et al., 2014). MicroRNA-146a has been confirmed to target COX-2, and regulate the complex network of the downstream signal transduction. In this study, examinations indicated that microRNA-146a up-regulation of apoptosis rate via COX-2 inhibition in MKN7 cells. To our best knowledge, this is the very first report to discuss the relationship between microRNA-146a and apoptosis under *H. pylori* infection. Aberrant expression of microRNA-146a has been reported to be involved in development and progression in various types of cancers and our results may partly elucidate the molecular mechanism of *H. pylori* induced gastric cancer.

Another important point of this work is that we observed a significant correlation between microRNA-146a and apoptosis rate in clinical samples, which is compatible with the *in vitro* data. Furthermore, the clinical significance of microRNA-146a expression was also studied. The result reveals that expression of microRNA-146a in gastric cancer is negatively correlated with lymph node metastasis ($p=0.04$), but has no obvious association with other clinicopathological variables. As many reports have demonstrated that promoting apoptosis can reduce cell growth and lymph node metastasis (Lu et al., 2012; Ding et al., 2014), we can draw the conclusion that microRNA-146a up-regulate apoptosis by suppressing COX-2, and therefore prevent the lymph node metastasis of gastric cancer. Thus, microRNA-146a may be a novel biological marker and potential therapeutic target for the treatment of gastric cancer.

References

Bartel DP (2004). MicroRNAs: genomics, biogenesis,

- mechanism, and function. *Cell*, **116**, 281-97.
- Brenner H, Arndt V, Stürmer T, et al (2000). Individual and joint contribution of family history and infection to the risk of gastric carcinoma. *Cancer*, **88**, 274-9.
- Casado M, Mollá B, Roy R, et al (2007). Protection against Fas-induced liver apoptosis in transgenic mice expressing cyclooxygenase 2 in hepatocytes. *Hepatology*, **45**, 631-8.
- Chen XL, Su BS, Sun RQ, et al (2005). Relationship between expression and distribution of cyclooxygenase-2 and Bcl-2 in human gastric adenocarcinoma. *World J Gastroenterol*, **11**, 1228-31.
- Cheng J, Fan XM (2013). Role of cyclooxygenase-2 in gastric cancer development and progression. *World J Gastroenterol*, **19**, 7361-8.
- Ding H, Wu YL, Wang YX, et al (2014). Characterization of the microRNA expression profile of cervical squamous cell carcinoma metastases. *Asian Pac J Cancer Prev*, **15**, 1675-9.
- Dixon MF, Genta RM, Yardley JH, et al (1996). Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol*, **20**, 1161-81.
- Han JA, Kim JI, Ongusaha PP, et al (2002). P53-mediated induction of Cox-2 counteracts p53 - or genotoxic stress-induced apoptosis. *EMBO J*, **21**, 5635-44.
- Li Q, Peng J, Zhang GY (2008). Effect of a selective COX-2 inhibitor on cell proliferation and apoptosis in human gastric cancer cell line BGC-823. *Zhong Nan Da Xue Xue Bao Yi Xue Ban*, **33**, 1123-8.
- Lin C, Huang F, Zhang YJ, et al (2014). Roles of MiR-101 and its target gene COX-2 in early diagnosis of cervical cancer in Uygur women. *Asian Pac J Cancer Prev*, **15**, 45-8.
- Liu Z, Wang D, Hu YL, et al (2013). MicroRNA-146a negatively regulates PTGS2 expression induced by *Helicobacter pylori* in human gastric epithelial cells. *J Gastroenterol*, **48**, 86-92.
- Lu H, Sun HZ, Li H, et al (2012). The clinicopathological significance of Bmi-1 expression in pathogenesis and progression of gastric carcinomas. *Asian Pac J Cancer Prev*, **13**, 3437-41.
- Matsushima K, Isomoto H, Inoue N, et al (2011). MicroRNA signatures in *Helicobacter pylori*-infected gastric mucosa. *Int J Cancer*, **128**, 361-70.
- Salama NR, Hartung ML, Muller A (2013). Life in the human stomach: persistence strategies of the bacterial pathogen *Helicobacter pylori*. *Nat Rev Microbiol*, **11**, 385-99.
- Sun WH, Zhu F, Chen GS, et al (2008). Blockade of cholecystokinin-2 receptor and cyclooxygenase-2 synergistically induces cell apoptosis, and inhibits the proliferation of human gastric cancer cells *in vitro*. *Cancer Lett*, **263**, 302-11.
- Targosz A, Brzozowski T, Pierzchalski P, et al (2012). *Helicobacter pylori* promotes apoptosis, activates cyclooxygenase (COX)-2 and inhibits heat shock protein HSP70 in gastric cancer epithelial cells. *Inflamm Res*, **61**, 955-66.
- Uemura N, Okamoto S (2000). Effect of eradication on subsequent development of cancer after endoscopic resection of early gastric cancer in Japan. *Gastroenterol Clin North Am*, **29**, 819-27.