



Expression of three essential antioxidants of *Helicobacter pylori* in clinical isolates^{*}

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Abstract: Objective: *Helicobacter pylori* maintains long-term persistence in the host and combats oxidative stress via many antioxidant proteins, which are expected to be relevant to bacterial-associated gastric diseases. We aimed to investigate the expression of three essential antioxidants in *H. pylori* strains isolated from patients with different clinical outcomes. Methods: Forty *H. pylori* strains were isolated from endoscopic biopsy specimens of gastric mucosa from 13 patients with gastric cancer, 13 with peptic ulcer, and 14 with gastritis. The expression of thioredoxin 1 (*Trx1*), arginase (*RocF*), and alkyl hydroperoxide reductase (*AhpC*) in *H. pylori* was measured by real-time PCR. Comparisons among multiple sample sets were analyzed using a one-way ANOVA test. Pearson's correlation test was used to assess relationships among multiple continuous variables. Results: *Trx1* expression of *H. pylori* in gastric cancer and peptic ulcer tissues was higher than that in tissues with gastritis. *RocF* expression of *H. pylori* in gastric cancer tissues was higher than that in tissues exhibiting peptic ulcer and gastritis. However, we did not find any differences in *AhpC* expression in samples from patients with different clinical outcomes. The expression of *Trx1* and *RocF* had a positive, linear correlation. The expression of *Trx1* and *AhpC* had a positive correlation without a linear trend. We found no correlation between the expression of *RocF* and *AhpC*. Conclusions: Our observations indicate that the expression of *Trx1* and *RocF* in *H. pylori* might be related to gastric carcinogenesis. In *H. pylori*, the expression of members of the antioxidant system may be correlated and relevant to gastric cancer.

Key words: Antioxidant, Gastric cancer, *Helicobacter pylori*, Oxidative stress

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1 Introduction

Helicobacter pylori is a microaerophilic bacterium and a gastric pathogen that colonizes about 50% of the world's population. *H. pylori* infection causes chronic inflammation and significantly increases the risk of developing duodenal and gastric ulcer diseases and gastric cancer. Chronic *H. pylori* infection is now considered to be the strongest risk factor for gastric cancer (Wroblewski *et al.*, 2010). Recent reports have suggested that exacerbated DNA damage of gastric cells results from inflammation-related oxidative

stress induced by reactive oxygen species (ROS) *in vivo* (Coussens and Werb, 2002).

During the process of colonizing and infiltrating the host cells by *H. pylori*, innate and adaptive inflammatory responses are activated. The activated neutrophils of the host can produce ROS and reactive nitrogen species (RNS), and it has been reported that *H. pylori*-induced ROS and RNS production in gastric epithelial cells might affect signal transduction and damage DNA in gastric cells, resulting in gastric carcinogenesis (Handa *et al.*, 2010). The inflammatory host response can not only lead to gastric cancer, but also threaten the survival of *H. pylori*. The suppressing effect of oxidative stress species has also been reported *in vitro* (Qu *et al.*, 2011). Nevertheless, *H. pylori* successfully survives these conditions and persistently colonizes the gastric mucosa. Therefore,

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mechanisms for detoxification of redox compounds and repair of damaged cell components in *H. pylori* are particularly important in understanding *H. pylori* pathogenesis and persistence (Wang *et al.*, 2006).

The antioxidant systems in *H. pylori* appear to be essential for its survival in the host. Studies have indicated that these systems have many components. Thioredoxin-1 (Trx1) is one of the antioxidants of *H. pylori* (Comtois *et al.*, 2003). *H. pylori* Trx1 is similar to a eukaryotic peroxidase reduction system. Trx1 contains a redox-active site, Cys-Gly-Pro-Cys, and serves as a ubiquitous enzyme that catalyzes the reduction of disulfide bonds (Alamuri and Maier, 2006). Using the purified *H. pylori* Trx1 and alkyl hydroperoxide reductase (AhpC), Baker *et al.* (2001) found that Trx1 can transfer electrons to AhpC. AhpC is an enzyme that reduces peroxides and can protect bacterial and human cells against reactive nitrogen intermediates (Chen *et al.*, 1998). *H. pylori* Trx1 has also been reported as an arginase (RocF) chaperone. Trx1 and arginase equip *H. pylori* with a “redox guardian” to overcome oxidative and nitrosative stress (McGee *et al.*, 2006). The functions and relationships of these antioxidants have been reported. However, there is little direct evidence showing the expression of these antioxidants in clinical isolates and their associations with clinical gastric diseases.

In this study, we investigated *Trx1*, *RocF*, and *AhpC* expression in *H. pylori* strains isolated from tissues exhibiting gastritis, peptic ulcer, and gastric cancer. The relationships among the expression of these antioxidants in *H. pylori* were also assessed using Pearson’s correlation test.

2 Materials and methods

2.1 Patient samples

Gastric biopsies were obtained from a total of 61 patients with gastric cancer, peptic ulcer, and gastritis, who had undergone gastroscopic examination at Peking University Third Hospital (Beijing, China) from 2010 to 2012. All collected tissues had positive results from a rapid urease test, which were confirmed by Warthin-Starry staining. Written informed consent with a signature was obtained from all patients. All tissues were assessed by hematoxylin-eosin (HE) staining. Diagnoses of all the samples were confirmed

histologically by two independent pathologists.

2.2 Culturing of bacterial strains

Two antral biopsies used for *H. pylori* culture were collected in tubes containing brucella broth. After homogenizing immediately, the samples were plated on 5% (v/v) sheep blood agar plates containing amphotericin B 4 µg/ml, trimethoprim 4 µg/ml, and vancomycin 4 µg/ml (Life Tech, Carlsbad, CA, USA). The plates were incubated for 5 to 7 d at 37 °C under microaerophilic conditions using a microaerobic pack (Mitsubishi Gas Chemical Co., Inc., Japan). *H. pylori* cultures were examined using urease tests and Gram staining. Oxidase tests and catalase tests were used to ensure that the cultures were not contaminated. Bacterial culture was successful in samples from 40 out of 61 infected patients. The 40 samples were isolated from 14 tissues displaying gastritis (including 9 superficial gastritis tissues and 5 atrophic gastritis tissues), 13 peptic ulcer tissues (including 8 gastric ulcer tissues and 5 duodenal ulcer tissues), and 13 gastric cancer tissues (including 2 tissues at an early stage and 11 tissues at an advanced stage; 6 intestinal type tissues, 4 diffuse type tissues, and 3 mixed type tissues).

2.3 RNA isolation

RNA extraction from *H. pylori* was performed as described previously (Shi *et al.*, 2013). Briefly, *H. pylori* strains were resuscitated on blood agar plates and harvested to precooled sterile phosphate buffered saline (PBS). After washing with PBS twice, total RNA was isolated from *H. pylori* strains using TRIzol (Invitrogen) following the manufacturer’s instructions. RNAs were treated with RNase-free DNase I (Fermentas). The quality of RNA was determined by agarose gel electrophoresis and the quantity by spectrophotometry at 260 nm. RNA was stored at –80 °C until needed.

2.4 Reverse transcription and real-time polymerase chain reaction (PCR)

Reverse transcription and real-time PCR procedures were performed as described previously (Shi *et al.*, 2013; Zhang *et al.*, 2013). Total RNA (2 µg) was reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas). Real-time PCR was performed using SYBR Green on a Lightcycler 480II

Real-Time PCR Detection System (Roche, Indianapolis, IN, USA). All results were normalized to 16S rRNA amplification. Each reaction was carried out in technical triplicates. Primers were as follows: *H. pylori Trx1*: 5'-GGGGTTGCGTTAGTGGATTTTTG-3' (forward primer) and 5'-GACGACTTCGCCATCTTTGTGA-3' (reverse primer); *H. pylori RocF*: 5'-TTTACCTTAGCCTGGATTTAGACA-3' (forward primer) and 5'-GTTGTATTTCGGTTACTTCAAGTGC-3' (reverse primer); *H. pylori AhpC*: 5'-CCCTACAGAAATCATTGCGTTTG-3' (forward primer) and 5'-GGAAAGACACTTGACCAATACCG-3' (reverse primer); 16S rRNA: 5'-CCGCCTACGCGCTCTTTAC-3' (forward primer) and 5'-CTAACGAATAAGCACCGGCTAAC-3' (reverse primer). The relative expression of the target genes was calculated based on cycle threshold (C_T) measurements. The ΔC_T values of each sample were calculated as $C_{T, \text{target gene}} - C_{T, 16S \text{ rRNA}}$ (Shen et al., 2010).

2.5 Statistical analysis

A one-way analysis of variance (ANOVA) test followed by a Student-Newman-Keuls (SNK) test was used to analyze the significant differences among multiple sample sets. Pearson's correlation test was used to assess the relationships between multiple continuous variables. Data were presented as mean \pm standard deviation (SD) of three independent experiments. All statistical analyses were performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). P -values less than 0.05 were considered statistically significant. GraphPad Prism 5 was used to create the artwork.

3 Results

3.1 Expression of *Trx1*, *RocF*, and *AhpC* in *H. pylori* strains isolated from tissues displaying gastric cancer, peptic ulcer, and gastritis

H. pylori was isolated from 14 gastritis samples, 13 peptic ulcer samples, and 13 gastric cancer samples, and the expression of *Trx1*, *RocF*, and *AhpC* was evaluated. *Trx1* and *AhpC* expression was successfully measured in all 40 strains. For the expression of *RocF*, 33 strains out of 40 were successfully evaluated. The mRNA expression level of *Trx1* in *H. pylori* isolated from the gastric cancer and peptic ulcer tis-

ues was higher ($P < 0.05$) than that in gastritis tissues (Fig. 1a). No significant difference in *Trx1* expression was found between the gastric cancer and peptic ulcer samples. The mRNA expression level of *RocF* in *H. pylori* isolated from the gastric cancer samples was significantly higher ($P < 0.05$) than the level observed for tissues exhibiting gastritis and peptic ulcer (Fig. 1b). However, no significant difference in *RocF* expression was found between the gastritis and peptic ulcer samples. The mRNA expression levels of *AhpC* in *H. pylori* isolated from the gastritis, peptic ulcer, and gastric cancer samples showed no significant differences (Fig. 1c).

3.2 Correlation among the expression of *RocF*, *Trx1*, and *AhpC* in *H. pylori* strains

The relationships between *Trx1*, *RocF*, and *AhpC* expression in *H. pylori* strains were assessed using Pearson's correlation test. *Trx1* expression had a positive, linear correlation with *RocF* expression ($r = 0.411$, $P < 0.05$; Fig. 2a). *Trx1* expression had a low positive correlation with *AhpC* expression ($r = 0.178$), but did not present a linear trend ($P > 0.05$; Fig. 2b). However, there was no significant correlation ($r = 0.009$; $P > 0.05$) between the expression of *RocF* and *AhpC* (Fig. 2c).

4 Discussion

It is well known that *H. pylori* infection induces progressive inflammatory changes in the gastric mucosa and increases the risk of peptic ulcer disease and gastric cancer. The antioxidant systems in *H. pylori* were expected to be relevant to bacterial-associated, and especially inflammation-associated, infectious diseases (Wang et al., 2006). In a previous study, we investigated the comparative proteome of clinical *H. pylori* strains by two-dimensional gel electrophoresis. We reported differential proteins of *H. pylori* strains isolated from gastritis and gastric cancer, including antioxidants (Zhang et al., 2011). In this study, we investigated the expression of three essential antioxidants of *H. pylori* in clinical isolates to show the relationship between the expression of these antioxidants and different gastric diseases. Correlations between the expression of these antioxidants were also assessed.

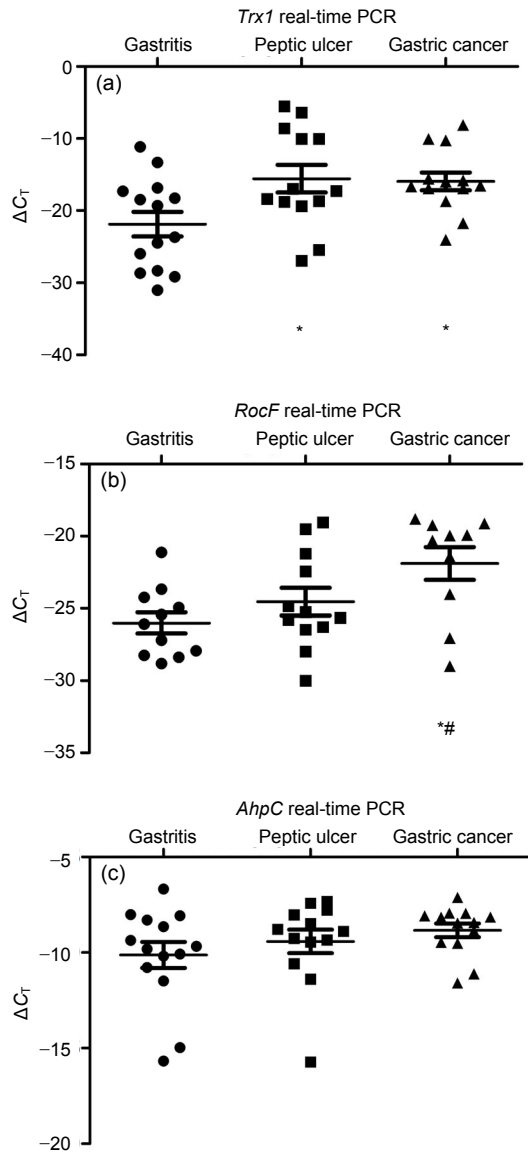


Fig. 1 *Trx1* (a), *RocF* (b), and *AhpC* (c) expression of *H. pylori* in gastric diseases (gastritis, peptic ulcer, and gastric cancer) evaluated by real-time PCR

The mRNA was extracted from *H. pylori* strains isolated from 14 gastritis samples, 13 peptic ulcer samples, and 13 gastric cancer samples. The *Trx1* (a), *RocF* (b), and *AhpC* (c) expression of *H. pylori* was evaluated in these tissues by real-time PCR and normalized to the control gene 16S rRNA. The ΔC_T value of each sample was calculated as $C_{T, \text{target gene}} - C_{T, 16S \text{ rRNA}}$. We successfully detected *Trx1* and *AhpC* in all 40 strains and *RocF* in 33 strains out of 40. *When compared with tissues displaying gastritis, $P < 0.05$ was considered statistically significant. #When compared with peptic ulcer samples, $P < 0.05$ was considered statistically significant.

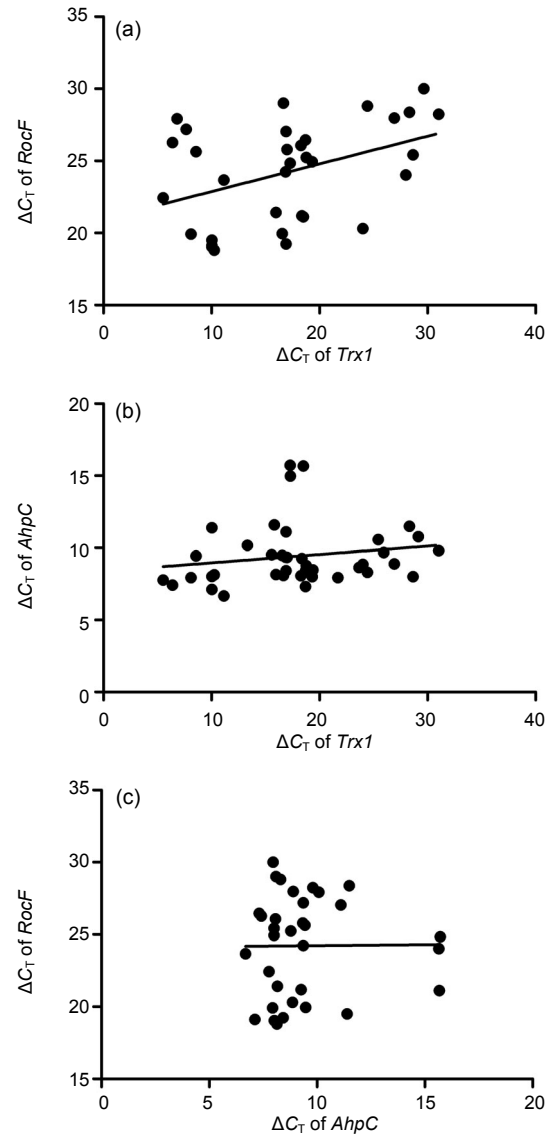


Fig. 2 Correlation analysis of *Trx1*, *RocF*, and *AhpC* expression in clinical *H. pylori* isolates

(a) The expression of *Trx1* in *H. pylori* strains had a positive, linear correlation with the expression of *RocF*; (b) The expression of *Trx1* in *H. pylori* strains had a weakly positive correlation with the expression of *AhpC*, but there was no linear trend; (c) The expression of *AhpC* in *H. pylori* strains was not significantly correlated with the expression of *RocF*.

Our results showed that *Trx1* expression of *H. pylori* in clinical gastric cancer and peptic ulcer tissues was higher than that in gastritis tissues. *RocF* expression of *H. pylori* in gastric cancer tissues was

higher than that in tissues exhibiting peptic ulcer and gastritis. Furthermore, Pearson's correlation test showed that *Trx1* and *RocF* expression had a positive, linear correlation. Trx1 is a key protein in many crucial cellular functions, including oxidative stress management (Ritz and Beckwith, 2001). Studies have shown that the expression of intracellular *H. pylori* Trx1 is altered in response to a variety of applied extracellular stresses, suggesting that Trx1 behaves as a stress response element in *H. pylori* (Windle et al., 2000). Moreover, Trx1 of *H. pylori* is considered to be an arginase chaperone because of its active site dithiol/disulfide (Berndt et al., 2008). The RocF protein arginase hydrolyzes arginine to ornithine and urea, and urease hydrolyzes urea to carbon dioxide and ammonium, which can neutralize acid. Several reports have shown that the enzymatic pathway of arginase used by *H. pylori* for the production of ornithine and urea can also serve as a mechanism responsible for the lack of protective effect of the immune response and the chronicity of *H. pylori* infection by inhibiting T cell proliferation and by down-regulating eukaryotic NO production (Gobert et al., 2001; Zabaleta et al., 2004). There is a critical disulphide bond for the stability of arginase, which is modulated at the post-translational level by Trx1. Trx1 can convert denatured or suboptimally folded arginase into an optimal three-dimensional catalytically active structure (McGee et al., 2006). In this study, we investigated the relationship between *Trx1* and *RocF* expression in clinical isolates of *H. pylori*, based on the supposition that the expression of *Trx1* and *RocF* might be correlated and might relate to gastric carcinogenesis.

In this study, we isolated 40 strains from gastric tissues. It has been reported that *H. pylori* *RocF* in clinical isolates has genetic microheterogeneity and phenotypic variation (Hovey et al., 2007). To avoid genetic variation in *RocF* affecting our PCR performance, we designed real-time PCR primers for *RocF* in its conserved region suggested by gene sequencing (Hovey et al., 2007). For the expression of *RocF*, only 33 strains out of 40 were successfully evaluated, probably because of the generally low expression of *RocF* shown by the PCR results in this work.

In addition to *RocF*, we investigated the expression of *AhpC*, one of the most abundant proteins expressed in *H. pylori* and an antioxidant enzyme whose

function is essential for reducing different peroxides (Baker et al., 2001). Huang and Chiou (2011) reported the upregulation of *H. pylori* AhpC after treatment with hydrogen peroxide, which suggested that AhpC might play a part in protecting organisms from damage by ROS. *AhpC* mutants are hypersensitive to oxygen and organic peroxides and are restricted to atmospheres of less than 2% oxygen for growth (Olczak et al., 2002). AhpC might assume an important role in combating exogenous peroxides arising from lifelong chronic inflammation (Croxen et al., 2007). Further study using 2-dimensional gel electrophoresis (2-DE) showed that AhpC was expressed in higher amounts in *H. pylori* strains isolated from three patients with gastric cancer than in strains from three patients with gastritis (Huang et al., 2011). In this study, we assessed the expression of *AhpC* using real-time PCR in 40 clinical strains from patients, including 13 displaying gastric cancer, 13 displaying peptic ulcer, and 14 displaying gastritis. However, our data did not show any differences in *AhpC* expression in *H. pylori* isolated from gastric cancer, peptic ulcer, and gastritis tissues. Although it has been reported that purified Trx1 can transfer electrons to AhpC (Baker et al., 2001), we did not find any relationship between the expression of *AhpC* and *Trx1* in clinical *H. pylori* isolates. Some previous studies indicated that AhpC was essential for both *in vitro* growth and gastric colonization (Chalker et al., 2001). However, Croxen et al. (2007) showed that high levels of AhpC were not required for *in vitro* growth or for primary gastric colonization. The functions of AhpC remain unclear and are worthy of further study.

In conclusion, our findings demonstrate that the expression of *H. pylori* *Trx1* and *RocF* is higher in gastric cancer tissues than in gastritis tissues. *H. pylori* infection can induce inflammatory responses followed by ROS/RNS and DNA damage in tissues. Nevertheless, *H. pylori* successfully survives these conditions by means of antioxidant enzymes, chronically infects gastric epithelium, and induces severe gastric outcomes. This study lends support to the supposition that the antioxidant enzymes in *H. pylori* may be related to the development of gastric cancer from *H. pylori* infection. *Trx1* and *RocF* of *H. pylori* may prove to be prognostic for monitoring varied clinical manifestations of gastrointestinal patients

infected with *H. pylori*. Further studies should be performed to confirm the relationship between *H. pylori* Trx1 and RocF, and to gain more insight into the functions and mechanisms of action of *H. pylori* Trx1 and RocF on gastric mucosa, using cell experiments and animal models. Research related to clinical patients and diseases should facilitate a better understanding of the involvement of *H. pylori* antioxidants in pathogenic mechanisms.

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Compliance with ethics guidelines

Yan-yan SHI, Mo CHEN, Yue-xia ZHANG, Jing ZHANG, and Shi-gang DING declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

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中文概要:

本文题目: 幽门螺杆菌临床分离菌株中三个重要抗氧化物的表达水平及相关性分析

Expression of three essential antioxidants of *Helicobacter pylori* in clinical isolates

研究目的: 幽门螺杆菌 (*Helicobacter pylori*) 长期在宿主体内定植, 并通过多种抗氧化蛋白对抗氧化应激, 目前的研究认为该过程与其所致的胃粘膜疾病相关。我们拟临床研究不同胃粘膜疾病来源的 *H. pylori* 菌株中三个关键抗氧化物的表达。

创新要点: 首次分析 *H. pylori* 临床分离菌株中氧化应激相关因子与胃粘膜疾病类型的关系, 并对三种因子的表达水平进行相关性分析。突出临床病人感染 *H. pylori* 中氧化应激相关分子的表达特点, 对研究 *H. pylori* 各氧化应激相关分子与临床胃粘膜疾病的关系, 以及临床病人感染的 *H. pylori* 中各个氧化应激相关分子之间的关系具有重要的意义。

研究方法: 在胃镜检查时, 取患者胃粘膜组织进行 *H. pylori* 培养, 共分离培养出 40 例临床菌株, 其中 13 例来自胃癌患者, 13 例来自消化性溃疡患者, 14 例来自胃炎患者。用荧光实时定量聚合酶链式反应 (PCR) 的方法测定 *H. pylori* 临床分离菌株中硫氧还蛋白-1 (*Trx1*)、精氨酸酶 (*RocF*) 以及烷基过氧化物酶 (*AhpC*) 的表达水平。多组样本间比较用单因素方差分析法, 并用 Pearson 相关性分析法评价多组连续变量的相关性。

重要结论: *H. pylori Trx1* mRNA 表达水平在胃癌和消化性溃疡分离菌株中显著高于胃炎分离株, *H. pylori RocF* mRNA 表达水平在胃癌患者分离菌株中显著高于胃炎和消化性溃疡分离株, 我们未发现胃炎、消化性溃疡和胃癌患者分离的 *H. pylori* 菌株中 *AhpC* 的表达差异。*H. pylori RocF* 的表达水平与 *Trx1* 呈显著线性正相关, *AhpC* 的 mRNA 表达水平与 *Trx1* 呈非线性正相关, *RocF* 与 *AhpC* 的 mRNA 表达水平无相关性。结果表明, *H. pylori Trx1* 和 *RocF* 的表达水平可能与胃癌相关, 在 *H. pylori* 菌株中, 抗氧化系统的各个因子可能相互联系, 在胃癌发病中发挥作用。

关键词组: 抗氧化物; 胃癌; 幽门螺杆菌; 氧化应激