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Helicobacter pylori infection promotes methylation of WWOX gene in human gastric cancer

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ABSTRACT

WW domain-containing oxidoreductase (WWOX), a tumor suppressor gene, was reported to be down-regulated in gastric cancer and other tumors. However, the mechanism by which WWOX is inactivated remains unclear. In our study, methylation status of WWOX was determined by MSP and sequencing. Our results showed that WWOX hypermethylation was frequently detected in gastric cancer, and also significantly correlated with *Helicobacter pylori* (*H. pylori*) infection. Promoter methylation of WWOX was induced in BCG823 and AGS cells co-cultured with *H. pylori*. Finally, we found that expression of DNMT1 and DNMT3A were enhanced when cells were co-cultured with *H. pylori*. Our study indicated that *H. pylori* infection promoted methylation of WWOX gene in gastric cancer.

1. Introduction

WW domain-containing oxidoreductase (WWOX) locates at chromosome 16q23.3–24.1, an area also recognized as the common fragile site FRA16D, and encodes a 414-amino acid protein which contains two WW domains in the NH2 terminus and a short chain dehydrogenase domain in the central portion of the protein [1,2]. WW motifs are known to be involved in protein–protein interactions [3]. Fragile sites are genomic regions that show more frequent gaps and breaks following DNA replication stress or exposure to carcinogens. In gastric cancer, a growing number of genes have been identified as undergoing aberrant promoter hypermethylation, suggesting that promoter hypermethylation is an important mechanism involved in gastric cancer [4,5]. Loss of heterozygosity, hypermethylation of the WWOX regulatory site and resultant reduction of WWOX expression have been reported in various human malignancies, such as esophageal cancer, gastric cancer, prostate cancer and pancreatic cancer [6].

Helicobacter pylori plays an important role in the pathogenesis of chronic superficial gastritis, peptic ulcer disease, gastric cancer, and mucus-associated lymphoid tissue lymphomas. *H. pylori* induced CpG island methylation has been reported in non-neoplastic gastric mucosa. For example, *H. pylori* infection could induce methylation of TFF2 and E-cadherin in gastric cancer [7]. In addition, eradication of *H. pylori* infection in dyspeptic patients was associated with disappearance of methylation at the E-cadherin gene in non-neoplastic gastric mucosa [8].

It was reported that the expression of WWOX was reduced in gastric cancer exposed to *H. pylori* for 72 h [9]. The mechanism through which *H. pylori* suppresses WWOX expression is unknown at present. In this study, we examined if *H. pylori* could induce promoter methylation of WWOX in human gastric cancer cell lines.

2. Materials and methods

2.1. Patients and cell line

Human gastric cancer samples were collected from surgical specimens from 50 patients with gastric cancer at Department of Pathology, The First Affiliated Hospital, Zhengzhou University, China. Tissue samples were collected, immediately snap frozen in liquid nitrogen, and stored at −80 °C until DNA extraction, all samples were obtained their informed consent and with institutional review board approval of the hospital. All patients obtained a confirmed diagnosis of gastric carcinoma after resection. *H. pylori* infection status was analyzed by rapid urease test (Otsuka, Tokushima, Japan), or pathological identification. Two gastric cancer cell lines (AGS and BCG823) were all preserved in our laboratory and maintained in RPMI 1640 with 10% FBS.

2.2. DNA methylation analysis of the WWOX gene

Genomic DNA (2 μg) was modified with sodium bisulfite using EpiTect Bisulfite kit (Qiagen). Methylation status was analyzed by

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bisulfite genomic sequencing of the CpG islands. The fragment covering 59 CpG sites from WWOX promoter region was amplified from bisulfite-modified DNA. The primers used were 5′-GTTTTTGTAGGATTGGTTAGAA-3′ (sense) and 5′-TAAACTATACAAAATCCCAAAT-3′ (antisense). Amplified bisulfite-sequencing PCR products were cloned into pMD18-T simple vector (Takara). Methylation status of human gastric tumor samples was examined by methylation-specific PCR (MSP) analysis. Primers for methylated reaction were 5′-AGCGGTCGGGGTTTCGACGCGCGCG-3′ (sense), and 5′-TATCCGTATCGTCCAACCCCGCG-3′ (antisense), and for the unmethylated reaction were 5′-AGTGGTTGGGGTTTTGATGTGTGTG-3′ (sense), and 5′-TATCCATATCATCCAACCCCACA-3′ (antisense).

2.3. H. pylori infection of gastric cancer cells

H. pylori strain NCTC 11638 was cultured routinely on brain heart infusion (BHI) agar plates with 5% sheep blood in mixed air containing 10% CO_2, 5% O_2 and 85% N_2 at 37 °C. *H. pylori* was cultured in BHI broth with 10% FBS in mixed air at 37 °C with agitation (200 rpm) for 48 h, harvested and resuspended in sterile phosphate buffered saline (PBS), and counted by absorbance at 660 nm (1 OD 660 nm = 1×10^8 colony forming units/ml). The gastric cancer cells were maintained in DMEM (Gibco-BRL) with 10% FBS. The cells grown to 90% confluency were infected with *H. pylori*. Live *H. pylori* (at *H. pylori*/cell ratios of 0, 10/1 and 50/1) were added to 6-well cell culture plates. The cells were co-cultured with *H. pylori* at 37 °C in a humidified atmosphere before being collected.

2.4. Real time RT-PCR

To test WWOX, DNMT1 and DNMT3A expression in gastric cancer cells, real time RT-PCR was carried out. Primers used in the RT-PCR: W-F: 5′-TCGCAGCTGGTGGGTGTAC-3′; W-R: 5′-AGCTCCCTGTTGCATGGACTT-3′. D1F: 5′-AGCAAGTCCGATGGAGAGGC-3′; D1R:5′-AAATGAGATGTGATGGTGGT-3′. D3AF: 5′-TTGCCTCGGAGGTGTGTGAG-3′; D3AR: 5′-ATGGGCCCCACTCCTGGATA-3′. GAPDH-F: 5′-AACTTTGGCATTGTGGAAGG-3′; GAPDH-R: 5′-ACAC ATTGGGGGTAGGAACA-3′. A 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) was used for testing. All the experiments were performed triplicate. The expression of WWOX, DNMT1 and DNMT3A was normalized to GAPDH and was given by: 2^{-dCt}.

2.5. Statistical analysis

Pearson Chi-Square test was used for statistical analysis of group differences. p Values less than 0.05 are considered significant.

3. Results

3.1. Hypermethylation of WWOX is associated with H. pylori infection in gastric cancer

To test whether the promoter of WWOX is hypermethylated in gastric cancer, we examined the methylation status using MSP in 50 patients with gastric cancer. We found that in 35 of the 50 (70%) gastric carcinomas analyzed, the WWOX promoter was hypermethylated (Table 1). The representative result of MSP is shown in Fig. 1. To characterize the correlation between hypermethylation of WWOX promoter and clinical features of gastric cancer, several clinicopathological characteristics including age, gender, gross type, cell differentiation, *H. pylori* infection and nodal metastases were compared between patients with normal and hypermethylation of WWOX promoter (Table 1). The result found that hypermethylation WWOX promoter was not associated with nodal metastasis ($p = 0.358$), cell differentiation ($p = 0.165$), gross type of gastric cancer ($p = 0.760$), patient's age and gender (data not shown). However, hypermethylation of WWOX was significantly correlated with *H. pylori* infection ($p = 0.001$).

Table 1
Clinical characteristics of gastric cancer patients according to hypermethylation status of WWOX.

		WWOX methylation		*p* Value
		U	M	
Differentiation	Well	4	4	
	Moderate	3	16	
	Poor	8	15	$p = 0.165$
Nodal status	Yes	6	20	
	No	9	15	$p = 0.358$
Gross type	Early	8	16	
	Advanced	7	19	$p = 0.760$
H. pylori	Yes	4	27	
	No	11	8	$p = 0.001$

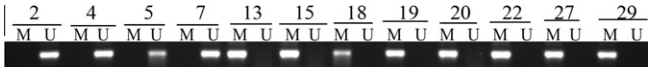

Fig. 1. Representative MSP results of WWOX hypermethylation in primary gastric cancer tumors. Case numbers are shown on top. M: Methylated primers; U: unmethylated primers.

3.2. Influence of H. pylori on WWOX methylation

To determine the effect of *H. pylori* on WWOX methylation, the gastric cancer cell lines AGS and BCG823 were co-cultured with *H. pylori* 11638 at a *H. pylori*/cell ratio of 1/1, 10/1 or 50/1 for 2 d. MSP analysis showed promoter methylation of WWOX was detected when AGS and BCG823 cells were co-cultured with *H. pylori* at a *H. pylori*/cells ratio of 10–50/1. BSP assay was also performed to verify the result of MSP.

The area of the CpG-rich region around the transcription initiation site of WWOX gene between the nucleotides −295 and +282 which spanned 59 CpG sites was sequenced. Most CpG dinucleotides were methylated in AGS and BCG823 cells when they were treated with *H. pylori*/cells ratio of 10–50/1. The percentage of methylation on methylated CpG sites of a total of 59 CpGs was calculated in AGS and BCG823 cells. Forty percent and sixty-five percent of 59 CpGs were methylated in BCG823 cells treated with *H. pylori*/cells ratio 10/1 and 50/1, respectively. Twenty percent and eighteen percent of 59 CpGs were methylated in AGS cells treated with *H. pylori*/cells ratio 10/1 and 50/1, respectively. As shown in Fig. 2B, the density of methylated CpG dinucleotides is increased in a *H. pylori*/cells ratio-dependent manner in BCG823 cells, similar result was not detected in AGS cells.

To confirm that CpG methylation is indeed responsible for the silencing of WWOX, the expression of WWOX at mRNA level is investigated by realtime-PCR and normalized to GAPDH level. The expression of WWOX in AGS and BCG823 cells is suppressed after exposure to *H. pylori*/cells ratio of 10–50/1 (Fig. 3), which keeps good accordance with the result of MSP.

3.3. Effect of H. pylori on expression of DNMTs

As we all know, the DNA methyltransferases (DNMTs) play important roles in DNA methylation, a covalent chemical modification resulting in addition of a methyl (CH3) group at the carbon 5 position of the cytosine ring in CpG dinucleotides. The major and best known of DNMTs are DNMT1 and DNMT3A, they transfer

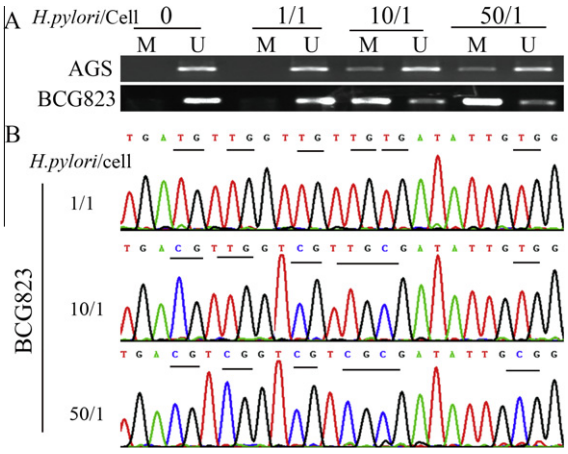

Fig. 2. (A) Promoter methylation status at WWOX in cell lines co-cultured with *H. pylori* at different *H. pylori*/cell ratios was investigated by MSP. (B) Demonstration of WWOX promoter methylation by sequencing of sodium bisulfite-modified DNA from the indicated gastric cancer cells co-cultured with *H. pylori* at different *H. pylori*/cell ratios. Methylated cytosine residues within WWOX promoter region that are unchanged are shown as underlined.

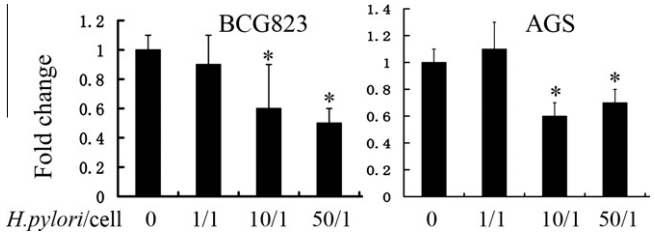

Fig. 3. WWOX mRNA expression levels in BCG823 and AGS cells co-cultured with *H. pylori* at different *H. pylori*/cell ratios were detected by realtime RT-PCR. GADPH was coamplified as an internal control. Data shown are means ± SD of triplicates and have been repeated at least three times. $^{*}p < 0.001$ compared with control.

methyl groups from S-adenosylmethionine to cytosines. Next we asked whether DNMT1 and DNMT3A mRNA expression were correlated with levels of *H. pylori*/cells ratio in AGS and BCG823 cells. A statistically significant correlation was observed between DNMT1 and DNMT3A mRNA with levels of *H. pylori*/cells ratio in BCG823 cells ($p < 0.05$), and AGS cell ($p < 0.05$) (Fig. 4).

4. Discussion

Epigenetic gene silencing represents an important mechanism of inhibition of tumor suppressor gene expression in various cancers. Aberrant methylation of the promoter DNA regions rich in CpG islands is the key step in the epigenetic gene silencing. Etiologic backgrounds of carcinogenesis and/or persistent infection with viruses or other pathogenic microorganisms, such as hepatitis B virus or hepatitis C virus infection in chronic hepatitis and Epstein–Barr virus or *H. pylori* infection in stomach are significantly associated with marked accumulation of DNA hypermethylation of CpG islands [10,11]. Currently, an increasing numbers of hypermthylated genes which were associated with the *H. pylori* infection in stomach are being found and the aberrant DNA hypermethylation caused by *H. pylori*-associated gastritis can be persistent even after the disappearance of *H. pylori*. For example, active *H. pylori* infection significantly increased methylation levels in TFF2, E-cadherin, THBD, LOX, and HAND1 [7,11,12].

WWOX is a tumor suppressor gene that spans a common chromosomal fragile site, FRA16D [13]. Loss of heterozygosity,

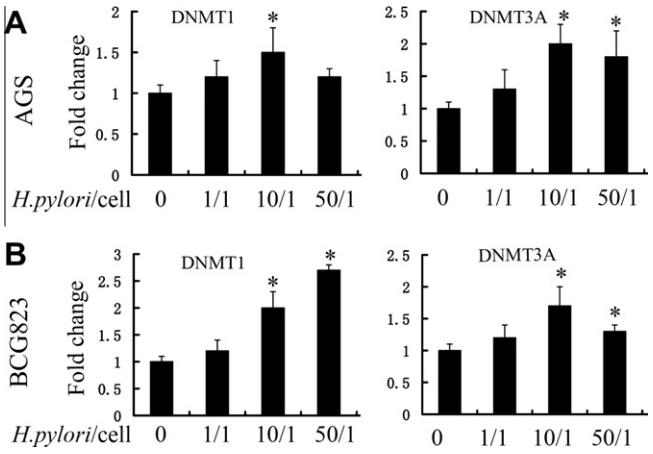

Fig. 4. DNMT1 and DNMT3A mRNA expression levels in BCG823 and AGS cells co-cultured with *H. pylori* at different *H. pylori*/cell ratios were detected by realtime RT-PCR, respectively. GADPH was coamplified as an internal control. Data shown are means ± SD of triplicates and have been repeated at least three times. $^{*}p < 0.05$ compared with control.

hypermethylation of the WWOX regulatory site and resultant reduction of WWOX expression have been reported in various human malignancies, such as esophageal cancer, lung cancer, gastric cancer, prostate cancer and liver cancer [14–16]. For example, 37% of human gastric carcinoma samples exhibited loss of heterozygosity, whereas 65% of stomach cancer samples had reduced or lost WWOX protein expression. Exogenous expression of WWOX strongly inhibits anchorage-independent growth in soft agar of breast cancer cell lines and that WWXO dramatically inhibits tumorigenicity of breast cancer cells *in vivo* [17].

It was reported that the expression of WWOX was reduced in gastric cancer exposed to *H. pylori* for 72 h [9]. We postulated that *H. pylori* infection promoted hypermthylation of WWOX promoter in gastric cancer. To test this hypostasis, we assessed the methylation status of WWOX gene in gastric cancer samples, and we found that in 35 of the 50 (70%) gastric carcinomas analyzed, the WWOX promoter was hypermethylated. We also found that the hypermethylation of WWOX was significantly correlated with *H. pylori* infection ($p = 0.001$).

To further study the effect of *H. pylori* on WWOX methylation, the gastric cancer cell lines AGS and BCG823 were directly co-cultured with *H. pylori* 11638. The WWOX methylation was initiated by *H. pylori* infection in AGS and BCG823 cells. At present, the molecular mechanisms through which *H. pylori* infection alters DNA methylation are still unclear. It was reported that *H. pylori* induced E-cadherin methylation through the action of IL-1b mediated by NO production [12,18]. *H. pylori* infection has been shown to be associated with up-regulation of inducible nitric oxide synthase (iNOS) both *in vivo* and in vitro. The fact that IL-1b can increase the activity of DNA methyltransferase via nitric oxide (NO) production convince us that *H. pylori* induces E-cadherin methylation through the action of IL-1b mediated by NO production [12].

For the sake of limited condition, the levels of NO and IL-1b in AGS and BCG823 cells were not assessed, we detected the altered expression of DNMT1 and DNMT3A in *H. pylori*-infection cells. DNMT1 and DNMT3A play important roles in DNA methylation [19]. In concert with the findings obtained by other groups, our results showed that the hypermethylated WWOX promoter was associated with DNMT1 and DNMT3A. However, the underlying mechanism of the altered expression of DNMT1 and DNMT3A in our present study will require further study.

In conclusion, our data presented here clearly demonstrate that hypermethylation of promoter CpG island of WWOX gene is significantly associated with *H. pylori* infection. Additional studies

are required to elucidate the exact mechanism by which WWOX is hypermethylated in gastric tumorigenesis.

Conflict of interest statement

The authors disclose no conflict.

Acknowledgment

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