Microbial Pathogenesis 108 (2017) 129–136

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

H. pylori modifies methylation of global genomic DNA and the gastrin gene promoter in gastric mucosal cells and gastric cancer cells

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ARTICLE INFO

Article history:
Received 3 March 2017
Received in revised form
2 May 2017
Accepted 2 May 2017
Available online 3 May 2017

Keywords:
Helicobacter pylori
CagA
Gastrin
Methylation
Gene expression

ABSTRACT

Aims: The aim of this study was to evaluate the correlation between *H. pylori* infection and global DNA methylation, as well as the methylation levels of the gastrin promoters.
Materials and methods: We constructed a eukaryotic expression vector, pcDNA3.1::cagA, and transfected it into GES-1 gastric mucosal cells and SGC-7901 gastric cancer cells. Both cell lines were infected with the *H. pylori*/CagA$^+$ strain NCTC11637. Then, we detected global DNA methylation by capture and detection antibodies, followed by colorimetric quantification. The methylation levels of the gastrin promoter were evaluated by base-specific cleavage and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.
Results: In *H. pylori*/CagA$^+$-infected GES-1 and SGC-7901 cells, the methylation levels of genomic DNA decreased by 49.4% and 18.8%, and in GES-1 and SGC-7901 cells transfected with pcDNA3.1::cagA, the methylation levels of genomic DNA decreased by 17.05% and 25.6%, respectively. Among 24 methylation sites detected in the gastrin promoter region, the methylation levels of 9 CpG sites were significantly decreased in *H. pylori*/CagA+-infected and pcDNA3.1:: cagA-transfected cells in comparison to corresponding control cells.
Conclusion: These results indicate that *H. pylori*/CagA$^+$ decreases the methylation of the genome and the gastrin promoter at some CpG sites in gastric mucosal and gastric cancer cells.

1. Introduction

Helicobacter pylori is a class I carcinogen in gastric cancer [6]. *H. pylori*-cytotoxin-associated protein A (CagA) is a virulence factor that is encoded by the *cag* pathogenicity island (cag PAI) of *H. pylori*. After the human stomach is infected with *H. pylori*, CagA is translocated into gastric epithelial cells by a bacterial type IV secretion system, and induces responses in many cells in a tyrosine phosphorylation-dependent and -independent manner [20]. Gastrin, a trophic factor, has been identified as the principal effector of gastric secretion, although several studies have demonstrated its role as a biomarker of cancer risk and as a growth factor for colorectal, stomach, liver, and pancreatic cancers [12]. Recently, some studies have examined the relationship between serum gastrin levels and CagA in patients with gastric cancer. Their results show that infection with *H. pylori* can lead to the secretion of gastrin and can significantly increase the risk of gastric cancer, and positive rates of serum gastrin and serum antibodies to CagA are all increased in patients with gastric cancer, demonstrating that these entities may play a role in the pathogenesis of gastric cancer [10]. Furthermore, it has been confirmed that *H. pylori* can induce serum hypergastrinemia, and high concentrations of gastrin have been detected in patients infected with *H. pylori* [9]. In a previous study, we found that either *H. pylori* infection or forced CagA expression significantly enhanced the transcription activity of the gastrin promoter and increased gastrin mRNA levels in gastric cancer cells. However, to date, the relationship between *H. pylori* infection and gastrin has been unclear.

DNA methylation is an epigenetic mechanism for gene regulation. *H. pylori* is known to induce chronic inflammation that may lead to severe outcomes [3,5,14] and aberrant methylation in gastric epithelial cells. Several studies have demonstrated that *H. pylori* infection is associated with gene promoter

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http://dx.doi.org/10.1016/j.micpath.2017.05.003
0882-4010/

hypermethylation and gene type-specific methylation profiles involved in the multistep process of carcinogenesis [15,21]. It has been found that *H. pylori* infection may induce the methylation of the Trefoil factor family 2 and E-cadherin promoters in gastric cancer [19]. Therefore, the purpose of this study was to elucidate whether CagA-positive *H. pylori* (*H. pylori*/CagA$^+$) can affect the methylation levels of the gastrin promoter, as well as global methylation levels, in gastric mucosal cells and gastric cancer cells.

2. Materials and methods

2.1. Cell culture

The human gastric mucosal GES-1 cell line and gastric cancer SGC-7901 cell line (from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were incubated in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in a humidified incubator (NSE, Brunswick, NJ, USA) containing 5% CO_2.

2.2. H. pylori culture

H. pylori strain NCTC11637 (ATCC 43504, CagA-positive, from the Chinese Center of *Helicobacter pylori* Strain Management and Preservation) was cultured on a Columbia agar plate (Oxoid Ltd, Basingstoke, UK) containing 10% sheep blood for 48–72 h at 37 °C in a microaerophilic atmosphere. Other *H. pylori* strains were boiled for 15 min to serve as a negative control, which was termed D-Hp.

2.3. H. pylori NCTC11637 infection

After gastric mucosal GES-1 cells and gastric cancer SGC-7901 cells were incubated in six-well plates containing antibiotic-free medium for 40 h, they were infected with *H. pylori* NCTC11637 with a multiplicity of infection (MOI) of 100. Six hours later, these cells were harvested to investigate gastrin mRNA.

2.4. Transfection of pcDNA3.1::cagA and pcDNA3.1::GFP

GES-1 and SGC-7901 cells were incubated in six-well plates for 24 h, at which point they were 80% confluent. These cells were transfected with pcDNA3.1::cagA, pcDNA3.1::GFP, or the negative control pcDNA3.1/Zeo(−), which we constructed previously. Six hours later, the medium was replaced by antibiotic-free medium containing 10% fetal bovine serum and incubated for 18 h. Finally, pcDNA3.1::cagA and pcDNA3.1/Zeo(−) transfected cells were harvested to isolate gastrin mRNAs; pcDNA3.1::GFP served to evaluate the transfection efficiency.

2.5. Analysis of the gastrin mRNA by real-time PCR

One microgram of total cellular RNA (extracted with Trizol Reagent, Invitrogen, Carlsbad, CA, USA), was reverse transcribed (RT) with MMLV reverse transcriptase and oligo (dT)15 primers (Promega, San Luis Obispo, CA, USA). cDNA was submitted to real-time quantitative PCR with a TaqMan_Gastrin Gene Expression Assay kit (Scoresby, Victoria, Australia); Assay ID Hs00174945_m1) and human beta-actin served as the endogenous controls, which were performed utilizing the ABI StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Crossing threshold (Ct) values of the gastrin gene were normalized to beta-actin, and then the data were calculated and analyzed by a comparative Ct method [7]. Gastrin mRNA expression was expressed as a percentage relative to the control.

2.6. Global methylation analyses

The harvested cells were washed with phosphate-buffered saline, and then genomic DNA was extracted according to the standard phenol/chloroform method. The percentage 5-methylcytosine (5-mC) levels of genomic DNA were measured by the Methylated DNA Quantification Kit (Colorimetric) (Epigentek New York, NY, USA) according to the manufacturer's instructions. In this assay, the methylated fractions of DNA are recognized by an anti–5-methylcytosine antibody and quantified using an enzyme-linked immunosorbent assay-like reaction. For each assay, the input DNA amount was 100 ng. Meanwhile, triplicate tests were performed on each sample. Test and control samples were measured simultaneously to avoid systemic bias.

2.7. Gastrin methylation analyses

Quantitative methylation analysis of the gastrin promoter was performed using the Sequenom MassARRAY platform (Capital Bio, Beijing, China). This system uses matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry in combination with RNA base-specific cleavage (MassCLEAVE). A detectable pattern is then analyzed to determine its methylation status. 1-mg sample of DNA was converted with sodium bisulfite using an EZ DNA methylation kit and the modified DNA was amplified by PCR. Gastrin promoter regions were amplified using three primer pairs, as shown in Table 1. Each forward primer was tagged with a 10-mer (5′-AGGAAGAGAG-3′) to balance the PCR, and each reverse primer had a T7-promoter tag (5′-CAGTAATACGACTCACTATAGGGAGAAGGCT-3′) for in vitro transcription. After PCR amplification and Shrimp alkaline phosphatase treatment, the PCR products were used as a template for in vitro transcription, and RNase A cleavage was used for the reverse reaction, following the manufacturer's instructions (Sequenom, San Diego, CA, USA). Then, the samples were conditioned and spotted on a 384-pad Spectro-CHIP (Sequenom) using a MassARRAY nanodispenser (Samsung, Irvine, CA, USA), followed by spectral acquisition on a MassARRAY analyzer compact MALDI-TOF MS (Sequenom). The distinct signal pair pattern results of MALDI-TOF MS were analyzed with EpiTyper software v1.0 (Sequenom) to generate quantitative results for each CpG site, or an aggregate of multiple CpG sites. This method permits the high-throughput

Table 1
Gastrin promoter amplificating primers.

Name	Primer sequence	Target length	Number of CpGs
Gastrin-1F	aggaagagagTTTTGTTTTTTGGTTTATTTTTGGT	447	11
Gastrin-1R	cagtaatacgactcactatagggagaaggctAATCCTACCTTTAAAACCTCCTTCA		
Gastrin-2F	aggaagagagGGTGGTGTATGGGTAGTTGATTTAG	477	8
Gastrin-2R	cagtaatacgactcactatagggagaaggctAATACTCCCCACCATATCCTAAAAA		
Gastrin-3F	aggaagagagTTTTTAGGATATGGTGGGGAGTATT	296	5
Gastrin-3R	cagtaatacgactcactatagggagaaggctTCCAAACCTACCTTATAAAACCCTC		