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Beta-defensins and analogs in *Helicobacter pylori* infections: mRNA expression levels, DNA methylation, and antibacterial activity

Raffaela Pero[1,2]*, Tiziana Angrisano[3], Mariarita Brancaccio[4], Annarita Falanga[5], Lucia Lombardi[6], Francesco Natale[3], Sonia Laneri[5], Barbara Lombardo[1,7], Stefania Galdiero[5], Olga Scudiero[1,2,7]*

1 Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli "Federico II", Napoli, Italy, **2** Task Force sugli Studi del Microbioma, Università degli Studi di Napoli "Federico II", Napoli, Italy, **3** Dipartimento di Biologia, Università degli Studi di Napoli "Federico II", Napoli, Italy, **4** Dipartimento di Biologia ed Evoluzione degli Organismi Marini, Stazione Zoologica Anton Dohrn, Napoli, Italy, **5** Dipartimento di Farmacia, Università degli Studi di Napoli "Federico II", Napoli, Italy, **6** Dipartimento di Agraria, Università degli Studi di Napoli "Federico II", Napoli, Italy, **7** CEINGE-Biotecnologie Avanzate Scarl, Napoli, Italy

* pero@unina.it (RP); olga.scudiero@unina.it (OS)

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Abstract

Antimicrobial peptides can protect the gastric mucosa from bacteria, but *Helicobacter pylori (H. pylori)* can equally colonize the gastric apparatus. To understand beta-defensin function in *H. pylori*-associated chronic gastritis, we investigated susceptibility, human beta-defensin mRNA expression, and DNA methylation changes to promoters in the gastric mucosa with or without *H. pylori* infection. We studied the expression of HBD2 (gene name *DEFB4A*), HBD3 (*DEFB103A*), and HBD4 (*DEFB104*) using real-time PCR in 15 control and 10 *H. pylori* infection patient gastric specimens. This study demonstrates that *H. pylori* infection is related to gastric enhancement of inducible HBD2, but inducible HBD3 and HBD4 expression levels remained unchanged. HBD2 gene methylation levels were overall higher in *H. pylori*-negative samples than in *H. pylori*-positive samples. We also assessed antimicrobial susceptibility using growth on blood agar. The *H. pylori strain Tox+* was susceptible to all defensins tested and their analogs (3N, 3NI). These results show that HBD2 is involved in gastritis development driven by *H. pylori*, which facilitates the creation of an epigenetic field during *H. pylori*-associated gastric tumorigenesis.

Introduction

Chronic inflammation in specific organs is associated with increased cancer risk, including ulcerative colitis (UC)-associated colon cancer, liver cancer, and gastric cancer. Storage of epigenetic modifications caused by chronic inflammation seems to correlate with tumor progression [1–6]. Chronic inflammation is correlated with the incidence of cancer development [7–9]. Chronic inflammation is triggered by infection of *Helicobacter pylori (H. pylori)* in the stomach, and it causes a predisposition in gastric cancer development [10,11].

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DNA methylation is involved in chronic inflammation-mediated carcinogenesis [12,13]. Niwa et al. shown that DNA methylation modifications in gastrointestinal mucosae subsequent to *H. pylori* infection can be transient or permanent, and it is the infection-associated inflammatory response rather than *H. pylori* itself that can cause DNA methylation [14].

Previous studies demonstrated that [15,16] DNA methylation modifications are present in *H. pylori*-infected stomachs, in livers infected with viruses, or in the rectum or colon in the case of UC [17,18]. DNA methylation alterations in non-cancerous inflammatory tissues represent one of the preliminary stages of tumor transformation [19]. Gastritis activity is linked to the evolution of widespread stomach cancer [20]. Many onco-suppressors were detected both in gastric cancer and chronic gastritis [21]. Inactivation of p53, E-cadherin, hMSH2, hMLH1, and microsatellite instability (MSI) are well-recognized examples. However, disparate studies have showed that mutation or deletion is an uncommon process of inactivating these well-established suppressor genes. DNA methylation represents a key mechanism for suppressor gene inactivation [22] and a critical element for the early events of gastric carcinogenesis [23]. Methylation of the E-cadherin DNA promoter is related to *H. pylori* status [24] independent of gastritis type and patient age.

Also, antibiotic eradication of *H. pylori* infection reverses DNA methylation of the E-cadherin promoter [24]. Similarly, the analysis of *H. pylori's* effect on DNA methylation of different genes (HAND-1, p16, LOX, HRASLS, THBD, and P41ARC) in healthy patients with or without *H. pylori* infection and patients affected by gastric cancer revealed that DNA methylation levels were higher in patients with *H. pylori* infection compared to healthy patients. These data suggest that *H. pylori* infection causes DNA methylation especially in premalignant lesions rather than gastric cancer [15, 25].

Antimicrobial peptides (AMPs) are significantly involved in native immune reactions against diverse microbes, and they constitute a promising substitute for antibiotics to resolve the problem of microbial resistance [26]. Defensins are a remarkable set of antimicrobial peptides. They are cationic peptides that have similar folding and an identical six-cysteine motif to disulfide bonds. They are synthesized either constitutively (HBD1) or inducibly by microbe components or inflammation (HBD2, HBD3, HBD4) [26].

Boughan et al., [27] found that gene expression of HBD2 demanded *cag*PAI and was NOD1-dependent. In contrast, HBD3 expression was NOD1-independent but relied on epidermal growth factor receptor (EGFR)-mediated ERK activation. In contrast to a study in AGS cells [27] that established the involvement of NOD1 in HBD2 expression only, other data suggest that NOD1 mediates both HBD2 and HBD3 expression in HEK293 cells in the presence of *H. pylori* [28]. Both HBD2 and HBD3 are differentially present in gastric mucosa in connection to *H. pylori* status, and *H. pylori* infection is related to hBD3 expression reduction in chronic gastritis [29]. In addition, HBD3 release from *H. pylori*-infected gastric epithelial cells happens via a new EGFR-activating pathway during a premature step of *H. pylori* infection [30]. Otherwise, HBD1 is constitutively expressed in non-inflamed normal tissue [31], which calls attention to its significance in defense versus microbial infection. Decreased HBD1 expression was found in *H. pylori* infected humans [32]. In addition, Patel et al., [33] found that *H. pylori* downregulates HBD1 expression via NF-κB signaling, suggesting that this may prolong survival and persistence of bacteria in the stomach niche. These studies, although conflicting, suggest that *H. pylori* may regulate HBD1 expression.

Defensins exert their killing activity against bacteria, yeasts, fungi, and viruses. The activity of antimicrobial peptides involves the initial electrostatic binding to the cell membrane of microorganisms and subsequent incorporation into the membrane. This antimicrobial action induces damage or even destruction of the microbial cell membrane via pore formation [34]. The antimicrobial activity of HBD1, HBD2 and HBD4 is reduced by high NaCl

concentrations, while HBD3 is the only defensin still active up to 150 mM NaCl concentrations. We previously reported the killing activity of β-defensin analogs designed from HBD1 and HBD3, showing that they have augmented potency or decreased sensitivity to high ionic strength. We examined their antibacterial, antiviral, and chemotactic actions, including salt resistance. Based on our analysis, we demonstrated that the C-terminal domain (RRKK) of HBD3 and the inner domain of HBD1 (PIFTKIQGT) are essential for antimicrobial activity. The removal of six ends at the N-terminus of HBD3 did not decrease the activity. Consequently, we developed an analog that maintained the HBD1 killing activity and was resistant to high NaCl concentrations like HBD3 [35–38]. The activity of this analog (3NI) was not evaluated against *H. pylori*, and it may represent a promising tool against *H. pylori* that can be used to combat infection and reverse DNA methylation.

Here, we hypothesized that the degree of altered DNA methylation in gastric apparatus is correlated with the activity of gastritis as methylation observed in the inflamed stomach would elicit diffuse-type cancer development. This study aimed to clarify whether DNA methylation modifications and human beta-defensins levels are related to inflammation activity in the *H. pylori*-infected stomach mucosa. We evaluated methylation levels of promoter CGIs of beta-defensin genes (HBD2-4) in gastric biopsy specimens from 15 controls and 10 patients colonized by *H. pylori*. *H. pylori* can cause the induction of HBD2 but several studies also report the overexpression of HBD3 [39–42], so we also evaluated the mRNA induction pattern of HBD2-4 by *H. pylori*. Finally, we tested the susceptibility of *H. pylori* to treatment by synthetic ß-defensin analogs.

Materials and methods

Tissue sample

The protocol was created based on the Declaration of Helsinki and approved by the Ethics Committee of the University of Naples "Federico II". Appropriate written informed consent was collected before all procedures.

Non-tumoral mucosa specimens with or without chronic gastritis were acquired from 25 patients recruited at the University of Naples "Federico II", Italy. After tissue removal, all samples were immediately frozen and fixed in 100 mL/L formalin.

***H. pylori* infection diagnosis.** Sections were stained with Giemsa, and the rapid urease test (CLO test) was carried out with fresh samples collected from the gastric corpus and antrum. *H. pylori* infection was assumed positive when *H. pylori* was detected or the CLO test was positive.

Patient characteristics are summarized in Table 1.

Real-time qPCR analysis

Total RNA was extracted as previously stated [43]. Total RNA was reverse-transcribed with Quanti-Tect® Reverse Transcription (Qiagen) using oligo-dT and random primers according to the manufacturer's instructions as previously described [44]. Quantitative PCR was performed with Quanti-Tect SYBR Green (Qiagen) using a Chromo 4 Real-Time thermocycler (BIORAD). The following primers were used for HBD2-4 cDNA amplification: (HBD2F) 5′-ATCAGCCATCAGGGTCTTGT-3′ and (HBD2R) 5′- GAGACCACAGGTGCCAATTT-3′, HBD3fw 5′- TGAAGCCTAGCAGCTATGAGGATC-3′ and HBD3rv 5′- CCGCCTCTGACTCT GCAATAA-3′, HBD4F 5′-AGATCTTCCAGTGAGAAGCGA-3′ and HBD4R 5′-GACATTTCT TCCGGCAACGG-3′. G6PD and 18S rRNA genes were old as house-keeping genes for PCR reaction: G6F (forward) 5′-ACAGAGTGAGCCCTTCTTCAA-3′ and G6R (reverse) 5′-GGAGGCTGCATCATCGTACT-3′, and 18SF: (forward) 5′-GCGCTACACTGACTGGCTC-3′

Table 1. Patient characteristics.

Case	Sex	Age (y)	Biopsy site	Urease activity	Severity of gastritis*	Symptom or diagnosis
1	F	45	Antrum	Negative	Mild	No symptom
2	F	46	Antrum	Negative	Normal	No symptom
3	F	33	Antrum	Negative	Normal	No symptom
4	F	37	Antrum	Negative	Normal	Epigastric discomfort
5	M	43	Antrum	Negative	Normal	Epigastric discomfort
6	M	48	Corpus	Negative	Mild	Epigastric discomfort
7	F	35	Antrum	Negative	Mild	Epigastric discomfort
8	F	41	Corpus	Negative	Mild	Chronic gastritis
9	F	40	Antrum	Negative	Mild	Chronic gastritis
10	F	37	Antrum	Negative	Normal	Chronic gastritis
11	F	39	Antrum	Negative	Mild	Chronic gastritis
12	F	45	Antrum	Negative	Mild	Chronic gastritis
13	F	50	Antrum	Negative	Normal	Chronic gastritis
14	F	33	Corpus	Negative	Mild	Chronic gastritis
15	F	44	Antrum	Negative	Normal	Chronic gastritis
16	M	42	Antrum	Positive	Moderate	Chronic gastritis
17	M	45	Antrum	Positive	Moderate	Chronic gastritis
18	M	34	Antrum	Positive	Moderate	No symptom
19	M	39	Corpus	Positive	Severe	No symptom
20	F	40	Corpus	Positive	Severe	No symptom
21	F	57	Antrum	Positive	Moderate	Epigastric discomfort
22	F	65	Antrum	Positive	Moderate	Epigastric discomfort
23	M	53	Antrum	Positive	Severe	Epigastric discomfort
24	M	66	Corpus	Positive	Severe	Epigastric discomfort
25	M	23	Corpus	Positive	Severe	Chronic gastritis

*According to the updated Sydney system

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and 18SR (reverse) 5′- CATCCAATCGGTAGTAGCGAC-3′. The quantitative PCR conditions were 95˚C for 15 min, 40 cycles of 95˚C for 15 s, 59˚C for 30 s, and 72˚C for 30 s. Calculations of relative expression levels were performed using the $2^{-\Delta\Delta Ct}$ method [45], taking into account at least 3 independent experiments.

DNA methylation analysis

Genomic DNA was isolated using the DNeasy extraction kit (Qiagen) according to the manufacturer's instructions. Sodium bisulfite conversion was performed using the EZ DNA Methylation Kit (Zymo Research). The manufacturer's protocol was followed using 1 μg of genomic DNA with elution into 30 mL of H2O.

Bisulfite genomic sequencing. 50 ng of each sample was used as template in PCR reactions using the following primers to analyze the HBD2 promoter region: HBD2/BsF 5′-GGAAGGATAGGGTTTTGAGAGATAT -3′ (position from nucleotides -944 to -920) and HBD2/BsR 5′-AACCAAAACTTTCTCTACTTTCCAC -3′ (nucleotides -781 to -757), HBD3/BsF 5′-GGTAGGTTTTAGATAATGATGAAG -3′ (nucleotides -732 to -709) and HBD3/BsR 5′- ACCCCCTAAATAACTAAAACC -3′ (nucleotides -518 to -498), HBD4/BsF 5′- TAGGT TAGGAGGGTTTTATGGATTT -3′ (nucleotides -1907 to -1883) and HBD4/BsR 5′- CCAAC AAACATAACCCAACTCTAAT -3′ (nucleotides -1738 to -1714). The Fully Human DNA

Methylated (ZYMO RESEARCH) was used as a completely methylated DNA control. Amplification was performed using Hot-Star Taq DNA polymerase (QIAGEN) under the following conditions: 15 min at 95˚C, 40 cycles of 30 sec at 95˚C, 30 sec at 54˚C, 1 min at 72˚C, and a final elongation of 10 min at 72˚C before holding at 4˚C in a final volume of 25 μl. PCR product quality and contamination was assessed using a 1.5% agarose gel with ethidium bromide staining. PCR products were cloned into the pGEM®-T Easy vector (Promega). Plasmid DNA was purified using the Qiagen Plasmid Mini Kit. Plasmids were purified and sequenced in either direction with T7 and Sp6 primers. At least 10 independent clones were sequenced to calculate the DNA methylation pattern of single molecules.

The methylation level of CG cytosines at a given position within the sequence can be calculated as percent methylation as follows: $\%M = [C^M/ (C^M + C^U)] \times 100$, where C^M is the number of clones with methylation at that cytosine and C^U is the number of clones unmethylated at that cytosine. Precision is improved by increasing the number of analyzed clones, unless there is bias in the cloning or amplification steps.

Peptide synthesis

The peptides HBD2-4 were purchased from Peptides International, Inc.-USA. The analogs (3N and 3NI) were synthesized on a Rink Amide Resin using Fmoc. The first pairing of individual amino acids was performed in the presence of 4 equivalents of Fmoc-protected amino acid, four equivalents of OXIMA, and four equivalents of DIC. The second coupling was done with four equivalents of HATU and eight equivalents of DIPEA. The Fmoc deprotection was achieved with two cycles of a piperidine solution (30% v/v in DMF). The crude peptides were cleaved from the resins with a TFA acid solution in the presence of scavengers. RP-HPLC was used to purify the crude peptides, which were identified using LC-MS.

The sequence of the peptides used in this study is indicated in Table 2.

Antimicrobial assay

CFU assays of the antimicrobial activities of HBDs against *H. pylori* (ATCC49503 (tox+ strain 60190); American Type Culture Collection, ATCC) were performed. The toxin-producing *H. pylori* strain was revived from frozen stocks by seeding on a blood agar plate (7% horse blood) at 37˚C for 3 days in microaerophilic conditions (O_2:10% and CO:10%) generated with Anaeropack Campylo (Mitsubishi Gas Chemicals Corp.). Bacteria harvested from the plates were suspended in 200 ml of brain heart infusion broth (10% fetal calf serum) and grown in liquid culture at 37˚C for three days while shaking in a monitored microaerophilic atmosphere.

H. pylori was incubated with HBDs for two hours at 37˚C. Two concentrations of peptides (2.5 μM and 12.5 μM) were used. For the salt dependence assay, 0, 50, 100, and 200 mM concentrations of NaCl were added in the incubation buffer as previously described. Each assay was done in triplicate.

Table 2. The sequence of the peptides used in this study.

HBD2	GIGDPVTC^{1}LKSGAIC^{2}HPVFC^{3}PRRYKQIGTC^{4}GLPGTKC^{5}C^{6}KKP
HBD3	GIINTLQKYYC^{1}RVRGGRC^{2}AVLSC^{3}LPKEEQIGKC^{4}STRGRKC^{5}C^{6}RRKK
HBD4	ELDRIC^{1}GYGTARC^{2}RKK-C^{3}RSQEYRIGRC^{4}PNTYAC^{5}C^{6}LRK
3N	QKYYC^{1}RVRGGRC^{2}AVLSC^{3}LPKEEQIGKC^{4}STRGRKC^{5}C^{6}RRKK
3NI	KYYC^{1}RVRGGRC^{2}AVLSC^{3}PIFTKIQGTC^{4}STRGRKC^{5}C^{6}RRKK

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Statistical analysis

Student's t-test assessed statistical significance between groups. Data are expressed as means S ± standard deviation (SD). Each experiment was repeated at least three times. Results were evaluated to be statistically significant at a p-value < 0.01 or 0.05.

Databases

The Ensembl database retrieved gene sequences: HBD-2, accession number ENST00000318157; HBD-3 accession number ENST0000031435; HBD4, accession number ENSG00000176782.

Results

HBD2 expression and DNA methylation analysis in *H. pylori*-positive patients

To evaluate the influence of *H. pylori* colonization in gastric tissues, we evaluated the *HBD2* gene expression in mucosal samples gastritis from 10 *H. pylori*-positive and 15 *H. pylori* negative patients, by qPCR analysis. *H. pylori* positive samples exhibited a moderate chronic active gastritis, whereas the *H. pylori*-negative specimens displayed no or minimal chronic inflammation.

Consistent with precedent reports, HBD2 gene expression was 53.6-fold higher in *H. pylori*-positive (G) patients than in *H. pylori*-negative (N) patients (Fig 1A). Moreover, we have also examined the HBD1 (gene name *DEFB1)* expression levels in the same gastric biopsy samples and we found that HBD1 transcripts do not differ significantly between *H. pylori*-negative and positive patients. This is consistent with most previous studies in this field [41, 46, 47] (S1 Fig).

Methylation analysis of the HBD2 promoter regions was performed on genomic DNA extracted from human tissue gastric samples in 15 controls and 10 patients colonized by *H. pylori*, obtained from surgical specimens. A diagram of the *HBD2* gene, including the relative positions of the analyzed CpG sites, is shown in Fig 1B. To examine the level of methylation of HBD2 promoter region, a DNA methylation analysis was performed. We analyzed an HBD2 promoter region with 4 CpG sites at nucleotide positions -786, -792, -825, and -848 with respect to the transcription start site (TSS) (Fig 1B). The primers used for DNA methylation analysis, targeting the region from -944 to -757, showed the HBD2 methylation degree for each CpG site as indicated in a histogram of a tissue sample (Fig 1C). Marked differential methylation was detected between the *H. pylori*-positive gastritis (G) and *H. pylori*-negative (N) patients. A high methylation degree (up to 85%) at the 4 CpG sites was observed in all *H. pylori*-negative samples, while a decrease in methylation degree (20–50%) was observed in at least 2 of the 4 CpG sites in the *H. pylori*-positive gastritis. The specific demethylation observed in two CpG sites (-825 and -786) on the HBD2 promoter region could be related to the active transcription pattern of the gene in *H. pylori*-positive gastritis (Fig 1A).

Identification of putative DEFB4B (HBD2) transcription factor binding sites

Since DNA demethylation of HBD2 promoter region is associated with gene expression, we wondered if the region between -825 and -786 nucleotides contains specific transcription factor binding sites (TFBS). To investigate whether this sequence includes some transcription factor binding sites, we performed sequence analysis with the TFBIND program [48]. TFBIND estimates transcription factor DNA binding ability. Putative transcription factor binding sites are indicated in Table 3. These results reveal that the examined genomic DNA fragment

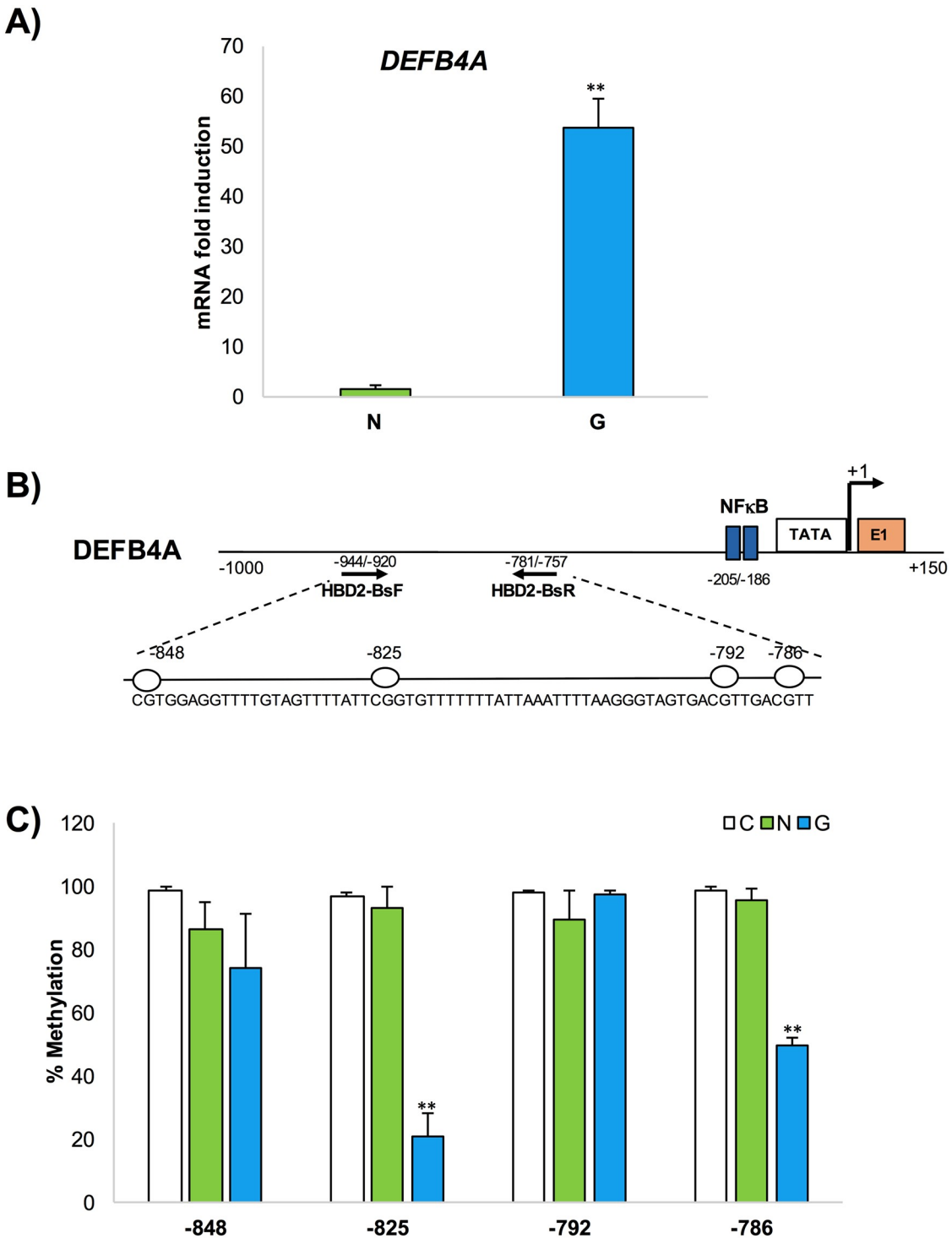

Fig 1. HBD2 gene expression analysis and DNA methylation assay in gastric mucosal tissues. mRNA and genomic DNA was extracted from 15 *H. pylori*-negative patients (N) and -positive (G) patients. (A) A qPCR assay measured HBD2 mRNA expression. (B) Schematic representation of HBD2 sequence: TTS (+1), NFKB sites and primers positions used for methylation analysis are indicated. The white lollipop represents CpG dinucleotide positions.

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Table 3. Identification of DEFB4A transcription factor binding sites using TFBIND.

ID*1	Score*2	Strand*3	Consensus sequence*4	Identified sequence*5
V$GATA1_04	0.820159	+	NNCWGATARNNNN	AGCAGAGAAAGCC
V$NFKAPPAB_01	0.784270	+	GGGAMTTYCC	GAGAAAGCCC
V$P53_02	0.791032	-	NGRCWTGYCY	GAGAAAGCCC
V$SP1_01	0.844281	-	GRGGCRGGGW	AGAAAGCCCT
V$NFKAPPAB_01	0.786267	-	GGGAMTTYCC	AGAAAGCCCT
V$HSF1_01	0.795195	+	RGAANRTTCN	AGAAAGCCCT
V$GC_01	0.822391	-	NRGGGGCGGGGCNK	AAGCCCTGGCTCCC
V$SP1_Q6	0.762756	-	NGGGGGCGGGGYN	AGCCCTGGCTCCC
V$SP1_01	0.801246	-	GRGGCRGGGW	GCCCTGGCTC
V$AHRARNT_02	0.775531	-	GRGKATYGCGTGMSWNSCC	GCCCTGGCTCCCAAAGCCC
V$ER_Q6	0.740494	-	NNARGNNANNNTGACCYNN	CTGGCTCCCAAAGCCCTGA
V$IK2_01	0.854058	-	NNNYGGGAWNNN	TGGCTCCCAAAG
V$GRE_C	0.802055	+	GGTACAANNTGTYCTK	GGCTCCCAAAGCCCTG
V$E2F_02	0.743405	-	TTTSGCGC	GCTCCCAA
V$LYF1_01	0.897266	-	TTTGGGAGR	GCTCCCAAA
V$AP2_Q6	0.875230	+	MKCCCSCNGGCG	CTCCCAAAGCCC
V$CETS1P54_01	0.836230	+	NCMGGAWGYN	CCCTGAAGTC
V$NRF2_01	0.824128	+	ACCGGAAGNS	CCCTGAAGTC
V$CREB_01	0.789243	+	TGACGTMA	TGAAGTCC

*1 ID indicates transcription factor matrix from the transcription factor database TRANSFAC R. 3.3.

*2 Score shows similarity (0.0–1.0) between a registered sequence for the transcription factor binding sites and the identified sequence.

*3 DNA strand.

*4 Consensus sequence of the transcription factor binding sites. S = C or G, W = A or T, R = A or G, Y = C or T, K = G or T, M = A or C, N = any base pair.

*5 Sequence identified by TFBIND from the input sequence (DEFB4A sequence analyzed: from -825 to -786 nucleotides).

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containing CpG sites could be involved in a chromatin conformation change induced by specific DNA demethylation in a region including transcription factor binding sites like NFKB, GATA1, or HSF1 (Table 3).

HBD3-4 expression and DNA methylation analysis in *H. pylori*-positive patients

In contrast to gene expression levels observed for HBD2 in *H. pylori*-positive patients, HBD3 and HBD4 gene expression was only marginally detected in the human gastric mucosa independent of *H. pylori* infection (Figs 2A and 3A).

Taken together, these data indicate that HBD2 gene expression was induced in *H. pylori* positive samples by inflammation. In contrast, HBD3 and HBD4 expression levels were barely detected. Even for the HBD3 and HBD4 genes we evaluated the methylation state of 2 (nucleotide positions -606, and -525) and 4 CpG sites (nucleotide positions -1836/1839, -1827, -1818, and -1805) targeting the regions from -732 to -498 (Fig 2B) and from -1907 to -1714 (Fig 3B), respectively. The results were plotted on a histogram displaying the methylation degree of each CpG site in each tissue sample (Figs 2C and 3C). For both genes HBD3 and HBD4, no differential methylation was observed between *H. pylori*-positive gastritis and *H. pylori*-negative patients in all analyzed CpG sites.

The results of triplicate experiments indicated differential methylation in *H. pylori*-positive gastritis and *H. pylori* negative patients only in the HBD2 genomic region.

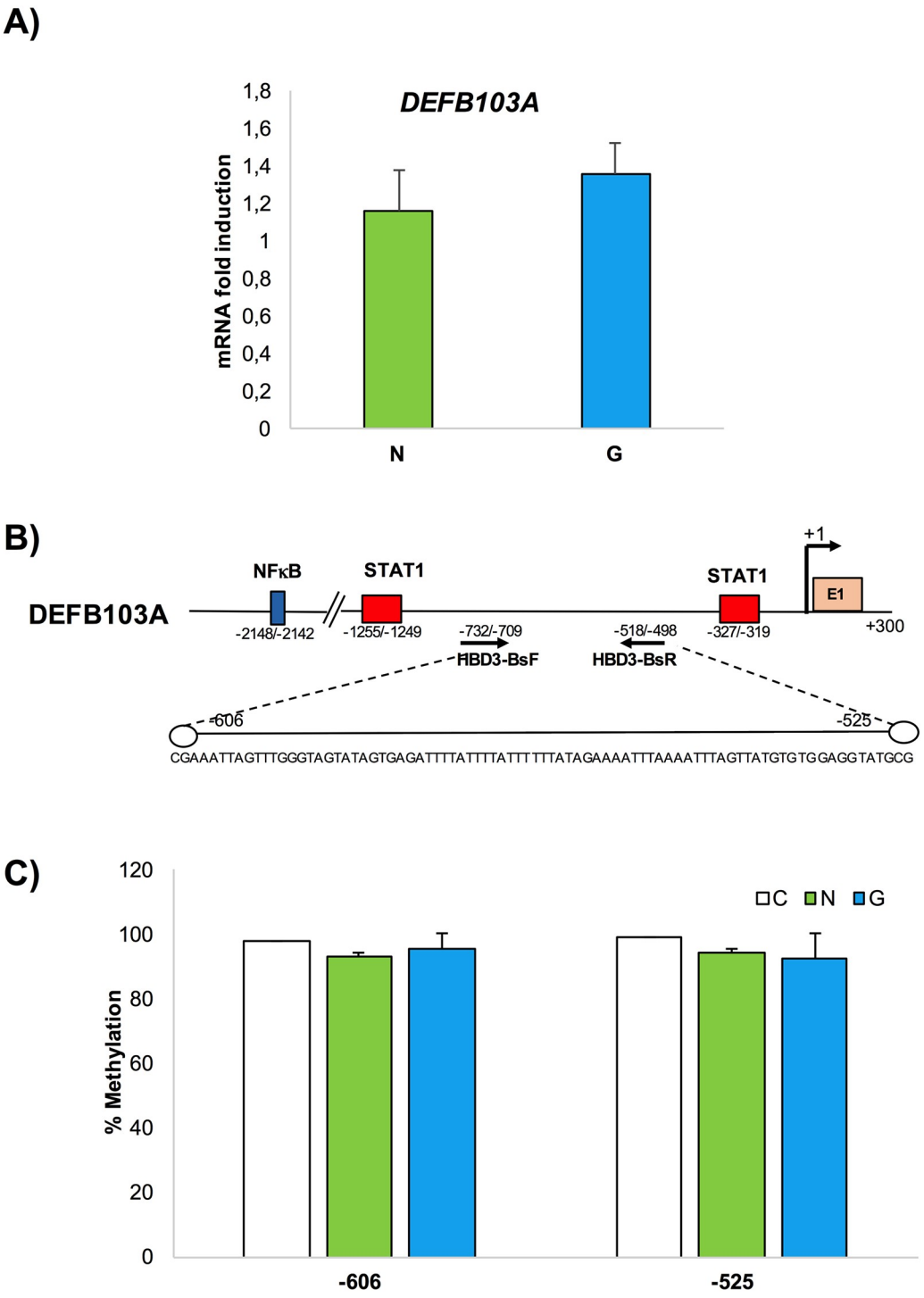

Fig 2. HBD3 gene expression analysis and DNA methylation assay in gastric mucosal tissues. mRNA and genomic DNA was extracted from 15 *H. pylori*-negative patients (N) and -positive (G) patients. (A) qPCR measured HBD3 mRNA expression. (**B**) Schematic representation of HBD3 sequence: TTS (+1), STAT1, NFKB sites, and primer positions used for methylation analysis are indicated. The white lollipop represents CpG dinucleotide positions.

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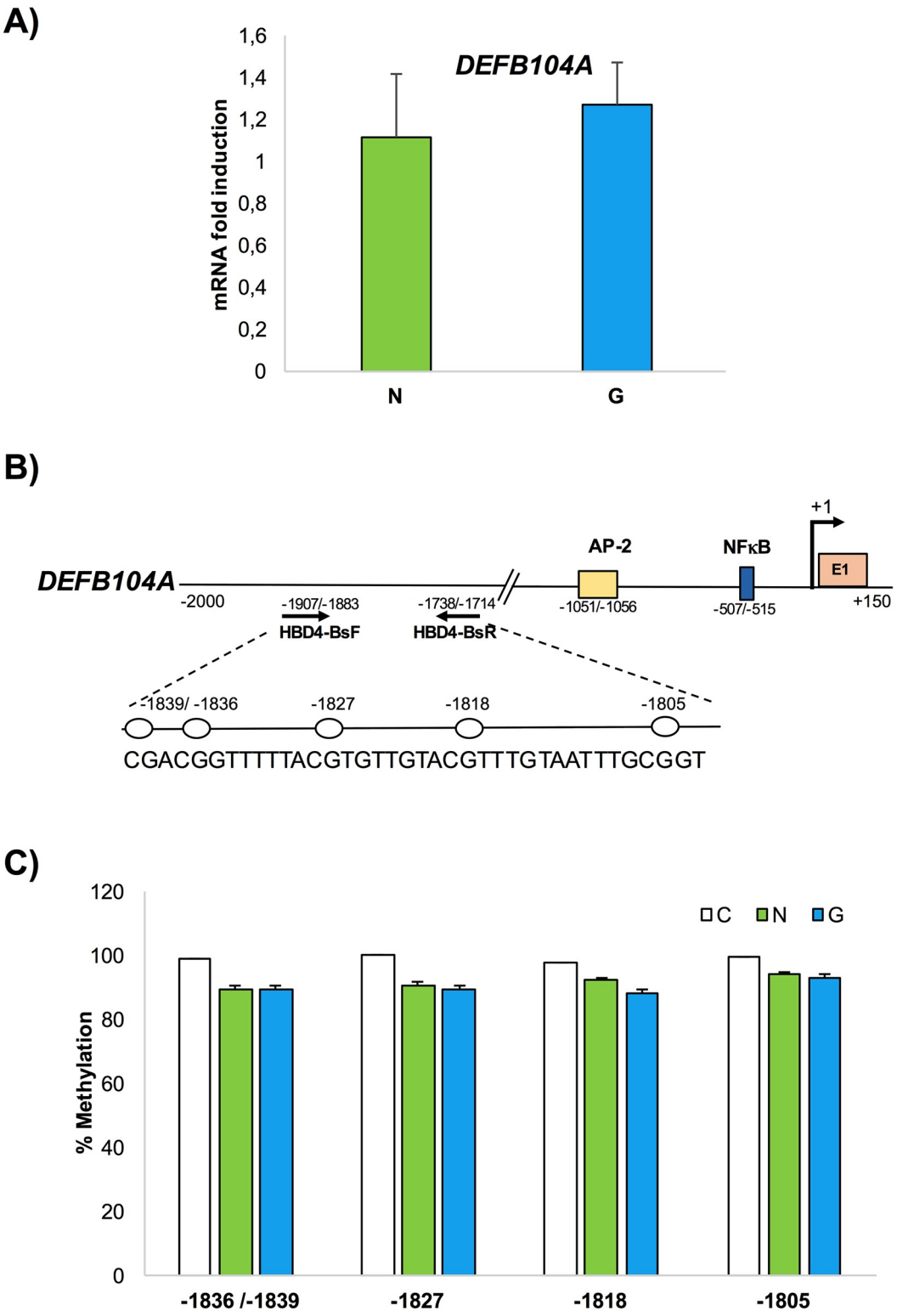

Fig 3. HBD4 gene expression analysis and DNA methylation assay in gastric mucosal tissues. mRNA and genomic DNA was extracted from 15 *H. pylori*-negative patients (N) and positive (G) patients. **(A)** qPCR measured HBD4 mRNA expression. **(B)** Schematic representation of HBD4 sequence: TTS (+1), AP-2, NFKB sites, and primer positions used for methylation analysis are indicated. The white lollipop represents CpG dinucleotide positions.

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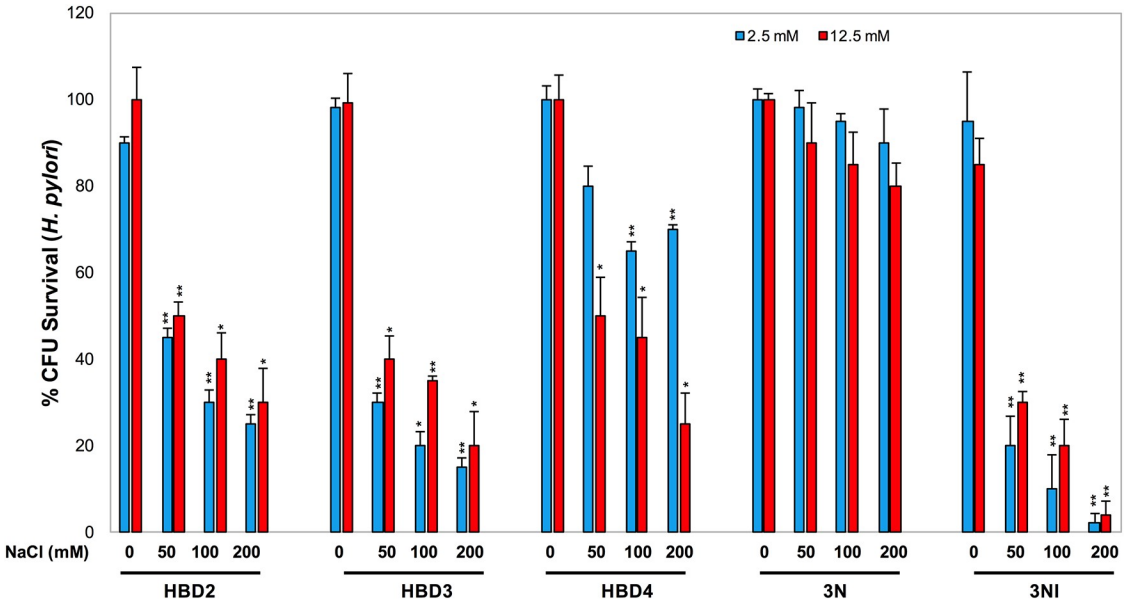

Fig 4. The 3NI analog has increased antibacterial activity against *H. pylori* in contrast to wild-type peptides. The antimicrobial activities of wild-type HBD2, HBD3, and HBD4 and the analogs 3N and 3NI were evaluated at two concentrations (2.5 and 12.5 μM) against *H. pylori* with 0, 50, 100, and 200 mM NaCl. Error bars show standard deviations (SDs) from 3 independent experiments. Statistical analysis was performed concerning salt point 0 at both two concentrations.

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Antibacterial activities of the 3NI analog and wild-type HBDs. Antimicrobial activity of HBD2-4 and the analogs 3N and 3NI was evaluated on *H. pylori*, cultured on HP agar for 4 d after a 1-h pre-incubation with or without chemically synthesized HBD-2-4 and 3N and 3NI analogs. The two peptide sequences are derived from HBD1 and HBD3. 3N analog is the HBD3 sequence without the N-terminal domain that HBD1 lacks. As for 3NI, this sequence is the most active and corresponds to 3N in which the inner HBD1 domain is responsible of the antimicrobial activity [36] substitutes the same domain of HBD3.

We evaluated two concentrations of every peptide (2.5 and 12.5 μM) and 4 NaCl concentrations (0, 50, 100, and 200 mM). Antibacterial activity of 3NI against *H. pylori* started at concentrations as low as 2.5 μM in the presence of at least 200 mM NaCl. The MIC values were between 12.5 and 25.0 μM. Each peptide exerted strong antibacterial activity at 12.5 μM against *H. pylori* except for the analog 3N, which showed little antibacterial effect at all four tested NaCl concentrations (Fig 4).

Discussion

The stomach protects against microbes using the low gastric pH and the secretion of antimicrobial peptides and mucins by epithelial cells. *H. pylori* gastritis correlates with up-regulation of various antimicrobials. For instance, the amounts of HBD2 but not HBD1 are increased in gastric mucosae of *H. pylori*-positive specimens [49,50]. Here, we compared the expression of HBD2-4 genes in the gastric mucosa with or without *H. pylori*, and we compared these changes to anti-*H. pylori* activity. Moreover, we analyzed the development of an epigenetic basis for cancer formation from HBD2 during *H. pylori*-associated gastric tumorigenesis. qPCR analysis of hBD2-4 gene expression revealed that expression powerfully increased HBD2 levels in *H. pylori*-infected gastric mucosa compared to uninfected, and this agreed with previously published data [32,39–42,46,47,51]. Interestingly there was no up-regulation of HBD3 and HBD4.

It has been suggested that HBD3 can be induced by *H*. *pylori*, but HBD3 was unaltered in the gastric antrum and imperceptible in biopsies of corpus in uninfected children [52–54]. Therefore, it is possible that previous studies reporting an increase of HBD3 gene expression in *H*. *pylori* gastritis are somewhat irrelevant in quantitative terms.

In addition, *H pylori* may induce the expression of HBD3 by activating NF-κB or other signaling pathways. Kawauchi et al., [41] found that anti-TLR-4 antibody partially repressed HBD3 expression in MKN45 cells after *H*. *pylori* induction. This suggests that different functions may be responsible for *H*. *pylori* infection-dependent HBD3 induction in gastric cells. Moreover, HBD3 mRNA expression levels did not relate with HBD2 in *H*. *pylori*-positive specimens. In addition, this also indicated that HBD3 and HBD4 mRNA expression in gastric epithelial cells is probably controlled via another mechanism.

In contrast to other studies [47,55] we observed no prominent HBD4 gene expression in the stomach. It is still unknown whether *H*. *pylori* itself or the inflammatory process-linked cytokines are responsible for HBD2 induction [47,56]. On the one hand, experiments in various gastric adenocarcinoma cell lines after *H*. *pylori* infection showed increased HBD2 and HBD3 expression [39,52,31,57]. On the other hand, induction with IL-1β also led to increased HBD2 expression [39].

Finally, we tested the *H*. *pylori tox*+ strain 60190 susceptibility toward the antimicrobial peptides of mucosal surfaces (HBD2-4 and analogs 3N and 3NI). In our experiments, the inducible defensins HBD2-HBD4 showed a robust antibacterial effect towards *H*. *pylori*.

DNA methylation analysis demonstrated that the methylation status of the HBD2 gene promoter was higher in *H*. *pylori*-negative chronic gastritis than *H*. *pylori*-positive chronic gastritis. Among the human beta-defensins (HBD2-4) analyzed, the grade of methylation was modulated only in HBD2. Surprisingly, we observed a correlation with HBD2 gene expression even if only 2 sites (-825 and -786) are demethylated at the HBD2 promoter. qRT-PCR analysis showed significant up-regulation of HBD-2 gene expression between *H*. *pylori*-negative chronic gastritis to *H*. *pylori*-positive chronic gastritis. It is well described that CpG methylation has an effect on native chromatin status, and this connection is modulated by methyl-binding proteins that preserve the capacity to engage chromatin repressor complexes on methylated DNA. In our previous work, we showed that the demethylation of a specific CpG site at the RET region enhancer promotes the shift of the methyl binding protein MeCP2, inducing transcriptional reactivation of the gene [58]. Moreover, it was observed that the demethylation of a single specific CpG site is required for *hIL2* (human interleukin 2 gene) transcription, and the epigenetic marker formed constitutes a memory of the regulatory event [59]. A similar result was obtained after single CpG demethylation of the L2RA locus gene, a subunit of the high affinity receptor for interleukin-2 (IL2). L2RA interprets a critical function in immune homeostasis. In fact, its single demethylation site corresponds to its expression in CD4+ T cells [60]. Finally, prominent demethylation of a single cytosine residue situated in the 5th exon of the PGLYRP3 gene is associated with an increment of mRNA level of PGLYRP3 [61]. This information supports our hypothesis that a single demethylation in one specific CpG dinucleotide could reactivate the gene.

We did not reveal a divergence in the DNA methylation and mRNA expression levels of HBD3 and HBD4 in chronic gastritis with and without *H*. *pylori* infection. The gap could be due to the failure of quantification molecules that have aberrant DNA methylation. An abnormal degree of methylation in non-cancerous tissues happens only in a percentage of cells, which is predicted to be highly variable. Qualitative analysis of methylation does not seem appropriate. Also, several CpG islands (CGI) and, even inside a CGI, diverse regions show different susceptibilities to aberrant DNA methylation [62], and analysis considering the different susceptibilities has not been done. More importantly, there are no studies on the relationship

between aberrant DNA methylation in the gastric mucosa and the risks of gastric cancer. It appears that modifications of DNA methylation could interact with *H. pylori* infection and other carcinogen agents and that these methylation alterations could affect cancer risk.

It has been suggested that this variance might occur because aberrant methylation develops in only a percentage of cells. Also, DNA methylation is intimately associated with metaplasia of the gastric mucosa. For example, hypermethylation of APC, THBD, and HAND1 was associated with gastric metaplasia [63]. Our findings may be interpreted as HBD2 epigenetic silencing occurring after GC generation rather than before the spread of cancer or during the formation of the epigenetic field. Another possible interpretation could be that an epigenetic action different from DNA methylation, like histone modification, is involved in increased mRNA expression [64].

Conclusion

Our study suggests that *H. pylori* can induce HBD2 expression and modulation of DNA methylation. It is possible that HBD2 induction minimizes competition by other more susceptible bacteria. Besides, *H. pylori* does not induce peptides like HBD3 and HBD4. The association of defensin elicitation and resistance to other bacteria may enable *H. pylori* to colonize the gastric mucosa where it can join epithelial cells and induce inflammatory and cancerous development.

Our study provides the first comprehensive analysis of transcript and DNA methylation levels of one of the most prominent human beta-defensins in patients infected with *H. pylori*.

A finer insight of the mechanisms regarding resistance and susceptibility of *H. pylori* against other antimicrobial peptides might favor the detection of possible targets for new eradication therapeutics.

Supporting information

S1 Fig. *DEFB1* gene expression analysis in gastric mucosal tissues. mRNA was extracted from 15 *H. pylori*-negative patients (N) and 10 -positive (G) patients. *DEFB1B* mRNA expression was measured using qPCR.
(TIFF)

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Author Contributions

Conceptualization: Raffaela Pero, Olga Scudiero.

Data curation: Tiziana Angrisano, Mariarita Brancaccio, Annarita Falanga, Lucia Lombardi.

Formal analysis: Annarita Falanga, Lucia Lombardi, Francesco Natale, Sonia Laneri, Barbara Lombardo.

Methodology: Mariarita Brancaccio, Francesco Natale.

Project administration: Raffaela Pero.

Supervision: Sonia Laneri, Stefania Galdiero, Olga Scudiero.

Visualization: Sonia Laneri, Barbara Lombardo.

Writing – original draft: Raffaela Pero, Olga Scudiero.

Writing – review & editing: Raffaela Pero, Stefania Galdiero, Olga Scudiero.

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