Helicobacter pylori vacA transcription is genetically-determined and stratifies the level of human gastric inflammation and atrophy

Charlotte G Sinnett,1 Darren P Letley,1 Geetha L Narayanan,1 Sapna R Patel,1 Nawfal R Hussein,1 Abed M Zaitoun,2 Karen Robinson,1 John C Atherton1,2

ABSTRACT

Aims Helicobacter pylori infection is the major cause of peptic ulceration and gastric cancer, and an important virulence determinant is its vaculating cytotoxin vacA. Previously, we have described allelic variation in vacA which determines toxin activity and disease risk. Here we aimed to quantify vacA mRNA expression in the human stomach, define its genetic determinants and assess how well it predicts gastric pathology.

Methods Gastric biopsies were donated by 39 patients with H. pylori infection attending for endoscopy at Queen’s Medical Centre, Nottingham, UK. Total RNA was extracted, and vacA mRNA quantified by reverse transcriptase quantitative PCR. Separate biopsies were historically scored for inflammation and atrophy using the updated Sydney system. H. pylori strains were isolated from further biopsies, and the nucleotide sequence upstream of vacA determined.

Results vacA mRNA levels in human stomachs varied by two orders of magnitude independently of vacA allelic type. Among vacA i1-type (toxic) strains, increased vacA expression was strongly associated with higher grade gastric inflammation (p<0.02), neutrophil infiltration (p<0.005) and the presence of atrophy (p<0.01). A polymorphism at nucleotide +28 near the base of a potential stem-loop structure within the S’ untranslated region was significantly associated with vacA transcript level and inflammation.

Conclusions Increased gastric vacA expression during H. pylori infection is associated with inflammation and premalignant pathology. The +28 nucleotide within the vacA S’ stem-loop stratifies disease risk among toxic vacA i1-type strains.

INTRODUCTION

Helicobacter pylori persistently colonises the stomach of approximately half the world’s population causing chronic gastritis, which usually remains asymptomatic. However, some individuals develop overt disease, such that H. pylori is the leading cause of peptic ulcer disease (PUD), mucosa-associated lymphoid tissue (MAL T) lymphoma and distal gastric adenocarcinoma (GC).1

Given the prevalence of infection, H. pylori-related gastric diseases are a major global health burden. In 2010, the estimated global deaths from PUD and GC were 0.25 million and 0.75 million, respectively.2 GC is estimated to be the third biggest cause of cancer-related death worldwide.3 Determinants of disease risk for a specific H. pylori-infected individual include: the virulence of the infecting strain, host factors such as gene polymorphisms and immune response, and environmental factors such as diet and smoking.1

Two important H. pylori virulence factors strongly associated with gastric and duodenal disease are the cytotoxin associated gene A (cagA) and the vaculat-

To cite: Sinnett CG, Letley DP, Narayanan GL, et al. J Clin Pathol 2016;69:968–973. doi:10.1136/jclinpath-2016-203641
particularly in East Asia, most _H. pylori_ strains are _vacA_ i1-type, making the intermediate region an unsuitable disease marker in such areas.\(^\text{14, 15}\)

_VacA_ transcription varies between _H. pylori_ strains grown in vitro.\(^\text{16, 17}\) We hypothesised that the amount of VacA produced during infection, as well as its activity, would have important implications for disease. We aimed to quantify _vacA_ transcript levels in gastric biopsies from patients with _H. pylori_ infection, correlate this with inflammation, atrophy and intestinal metaplasia, and identify genetic determinants of _vacA_ expression which may be additional markers of disease risk.

**MATERIALS AND METHODS**

**Bacterial strains**

_H. pylori_ strains were grown on blood agar base 2 plates supplemented with 5% horse blood (Oxoid, Basingstoke, UK) for 24–48 h at 37°C under humid, microaerobic conditions in a MACS-VA500 workstation (Don Whitley Scientific, Shipley, UK). Clinical _H. pylori_ isolates were genotyped for _vacA_ allelic type and cagA status by PCR as previously described.\(^\text{9}\)

**Patient samples**

Corporal gastric biopsies were donated by 39 patients with _H. pylori_ infection (median age 56 years, range 30–82 years; 51% male) attending the Queen’s Medical Centre, Nottingham, UK, for routine upper gastrointestinal endoscopy for dyspeptic symptoms. Informed written consent and approval from the Nottingham Research Ethics Committee 2 (08/H0408/193) was obtained. Patients taking proton pump inhibitors, non-steroidal anti-inflammatory drugs (NSAIDs), >150 mg/day of aspirin or antibiotics 2 weeks preceding endoscopy were excluded. Antibiotics 2 weeks preceding endoscopy were excluded. For histology, formalin-fixed corporal biopsies (two per patient to avoid patchy changes) were paraffin-embedded, sectioned and stained with H&E for assessment of inflammation and atrophy. Grading was performed using updated Sydney scoring\(^\text{18}\) (0=not present, 1=mild, 2=moderate and 3=substantial) by an experienced histopathologist (AMZ) blinded to other data. Histological scores were representative of both corporal biopsies examined for each patient. A single corporal biopsy for each patient was taken for RNA analysis and immediately preserved in RNAlater (Sigma-Aldrich, Poole, UK).

**Nucleotide sequencing of the vacA–cysS intergenic region**

Genomic DNA was extracted from clinical _H. pylori_ isolates as previously described.\(^\text{19}\) The _vacA_ sequence from nucleotides 520–1055 (GenBank U05676), including the intergenic region between _vacA_ and the upstream gene _cysS_ (cysteinyltRNA synthetase), was PCR-amplified using primers DL1\(^\text{20}\) and VA1-R,\(^\text{8}\) and the product sequenced by the Biopolymer Synthesis and Analysis Unit, University of Nottingham. Sequences were aligned using MegaAlign (DNAStar, Madison, Wisconsin, USA). Nucleotide positions where three or more strains differed to the consensus were defined as polymorphic.

**Reverse transcriptase quantitative PCR**

Total RNA was purified from gastric biopsies using rotor-stator homogenisation and an RNeasy Mini Kit (Qiagen, Manchester, UK). Residual genomic DNA was removed by DNA-free DNase treatment (Life Technologies, Paisley, UK). cDNA was reverse transcribed from 200 ng RNA using an Omniscript reverse transcriptase kit (Qiagen) and random hexamer primers (GE Healthcare Life Sciences, Little Chalfont, UK). For each sample, negative control reactions were prepared without reverse transcriptase to confirm the absence of genomic DNA. Triplicate reverse transcriptase quantitative PCRs (RT-qPCRs) were performed using a Quantitect SYBR Green PCR kit and a Rotor-Gene 3000 real-time PCR system (both Qiagen). _vacA_ mRNA levels were calculated relative to a comparator biopsy cDNA sample included in all experiments (arbitrarily assigned an expression level of 100) using the Pfaffl method,\(^\text{21}\) normalising to the _H. pylori_ reference gene 16S rRNA to correct for differences in _H. pylori_ density or sample amount. A 259 bp _vacA_ amplicon was amplified using primers VA1-F and VA1-R,\(^\text{8}\) and a 164 bp 16S rRNA amplicon amplified by primers 16S-rRNA-F (5′ CGATGAAAGTCTTAGGCTTG 3′) and 16S-rRNA-R (5′ ATAGGACATAAGCTGACTCG 3′). Thermocycling conditions were: 95°C for 15 min followed by 35 cycles of 95°C for 30 s, 56°C for 60 s and 72°C for 30 s. No template controls were included and melt curve analysis confirmed amplification specificity. Primer efficiencies, calculated from serial dilutions of the comparator sample, were 0.91 (R\(^2\)=0.98) and 0.57 (R\(^2\)=0.99) for _vacA_ and 16S rRNA, respectively.

**Statistical analysis**

Associations between gastric _vacA_ mRNA data and genotype, histological data, disease type and promoter region polymorphisms were evaluated by Mann–Whitney U tests or by Kruskal–Wallis analysis of variance (ANOVA) for multiple comparisons, with multiplicity adjusted pairwise p values calculated by Dunn’s test. Tests were performed using GraphPad Prism 6 software, and two-tailed p values less than 0.05 were taken as being statistically significant.

**RESULTS**

_H. pylori_ _vacA_ expression in the human stomach varies considerably between strains

While _vacA_ allelic type is a major determinant of gastric cancer risk among patients with _H. pylori_ infection, the association is imperfect, and we hypothesised that the amount of VacA produced by a strain may also be important. Reliable quantification of VacA production by _H. pylori_ in the human stomach is hampered by the lack of specific antibodies, so we aimed to assess levels of _vacA_ mRNA in gastric biopsies using RT-qPCR. We quantified _vacA_ mRNA in corporal gastric biopsies from 39 patients with _H. pylori_ infection. _VacA_ transcripts were detected in biopsies from 21 patients, with levels varying from 0.1 to 46.8 relative _vacA_ mRNA units. For the remaining 18 patients, _vacA_ expression was detected in vivo. By assaying serially diluted comparator sample cDNA, we determined our _vacA_ mRNA detection limit as being eightfold below the lowest positive value obtained from patient biopsy tissues. Thus strains with undetectable _vacA_ mRNA have expression below this level.

The level of _vacA_ expression in vivo does not correlate with _vacA_ type

We determined the _vacA_ allelic type of each strain by PCR\(^\text{8, 9}\) to assess whether this correlated with gastric _vacA_ mRNA level. Two patients were excluded because typing suggested the presence of multiple strains with different _vacA_ types, leaving 37 patients in our analysis. There was no significant difference in relative _vacA_ mRNA levels between toxic i1-type and non-toxic i2-type strains (medians 0.93 (n=27) and 0.96 (n=10), respectively, p=0.99, Mann–Whitney U test). There were only four patients infected with i2-type strains, too few to compare signal region types; however, one was a high- and two were low-level
transcribers. Moreover, one had undetectable vacA mRNA. We also assessed cagA status by PCR for all 39 infecting strains, and again there was no association with relative gastric vacA mRNA level (medians 0.93 for cagA+ strains (n=29) and 0.96 for cagA- strains (n=10), p=0.47).

**For toxic vacA i1-type strains, vacA mRNA levels in the human stomach are closely associated with gastric inflammation and precancerous change**

Next, we assessed whether H. pylori vacA expression in the stomach was associated with inflammation and precancerous damage by scoring histological parameters using the updated Sydney system.\(^8\) We previously showed that only i1-type strains are actively toxic in vitro.\(^9\) In agreement with this, 8 of the 10 patients infected with i2-type strains had mild (grade 1) inflammation and none had atrophy or intestinal metaplasia. Our main interest was why i1-type strains have different associations with inflammation and damage; so we limited subsequent analysis to 25 patients infected with i1-type strains for which histology samples could be located.

First we assessed the association between vacA expression and ‘chronic inflammation’ score, defined by the density of mononuclear inflammatory cells in the biopsy specimen. Median relative vacA mRNA level was significantly higher in patients with grade 3 inflammation than those with grade 1 inflammation (p<0.02; figure 1A). Patients with grade 2 inflammation had intermediate vacA mRNA levels. Seventeen patients had grade 1 or 2 neutrophil infiltration, referred to as ‘activity’ in the Sydney system, eight had no activity (grade 0) and none had grade 3 activity. Median relative vacA mRNA level for patients with grade 2 activity was significantly higher than for patients with no neutrophil infiltration (grade 0; p<0.005); patients with grade 1 activity were intermediate (figure 1C). Only six patients had gastric atrophy, all grade 1. Median relative vacA mRNA level was significantly higher in patients with atrophy than without (p<0.01; figure 1E). Only two patients had intestinal metaplasia, insufficient for statistical analysis; however, their median relative vacA level was 6.5 compared with 0.0 for the patients without intestinal metaplasia.

To avoid confounding effects from differences in vacA signal and mid-region type, and cagA status, we next stratified our data based on the 20 patients infected with vacA s1i1m1 cagA+ strains. We combined data for inflammation grades 2 and 3 to avoid small group sizes. Median relative vacA mRNA level was significantly higher for patients with grade 2 or 3 inflammation than grade 1 (p<0.05; figure 1B), and for patients with neutrophil infiltration than for those without (p<0.005; figure 1D). Only three patients infected with vacA s1i1m1 cagA+ strains had atrophy, too few for reliable statistical analysis (figure 1F).

**The level of vacA transcription in vivo does not correlate with PUD**

Of the 39 patients studied, 27 had past or present gastric or duodenal ulcers or erosions and were collectively defined as a PUD group. Only one patient had GC and was omitted from this analysis. The remaining 11 patients showed no pathology on endoscopy and were defined as a normal group. There was no significant difference in median relative vacA mRNA level between the PUD and normal groups (figure 2). However, our study was not powered to detect such a difference. There remained no significant difference in median relative vacA mRNA levels when comparing the 22 patients with active ulcers and the normal group (medians 0.76 and 0.0, p=0.50).

In vivo vacA mRNA levels are not associated with promoter polymorphisms, but are associated with a polymorphism in a potential 5’ stem-loop in the vacA transcript

Having shown that inflammation and premalignant disease were associated with increased vacA mRNA among toxigenic, i1-type strains, we considered it important to assess the genetic determinants of high level vacA expression in vivo. We compared the nucleotide sequence between vacA and the upstream gene cysS for 27 strains to identify polymorphisms that correlated with our gastric vacA mRNA data (see online supplementary figure S1). Interestingly, 31/110 nucleotides in the vacA promoter region (end of cysS to vacA transcriptional start site) were polymorphic compared with just 11/119 nucleotides in the 5’ untranslated region (5’ UTR) of the encoded transcript (p=0.0003, Fisher’s exact test). In agreement with previous studies,\(^17\)\(^23\) the vacA promoter had three polymorphisms: G/A (T(−32) within the −35 motif; and G/T(−14) and G/T(−7) within the −10 motif. Four polymorphisms were observed within a previously identified imperfect inverted repeat upstream of the −35 motif,\(^17\) with three strains having a perfect inverted repeat. None of these, or the remaining polymorphisms within the promoter region, correlated with gastric vacA mRNA level.

We recently described a second inverted repeat located between +4 and +30 in the vacA 5’ UTR, predicted to form a stem-loop structure at the 5’ end of the transcript (figure 3).\(^24\) We showed that this potential stem-loop is important for stabilising vacA mRNA: disrupting its formation led to reduced mRNA half-life and decreased steady-state vacA transcript levels.\(^24\) At the third pairing position from the base, the 5’ stem is consistently thymine (uracil in mRNA) but the 3’ pairing nucleotide (position +28) can be either guanine (G(+28)) or adenine (A(+28)), the latter potentially stabilising the stem-loop more efficiently. In our previous work, we showed that substituting adenine for guanine at position +28 significantly increased vacA mRNA steady-state levels in two different strain backgrounds.\(^24\) Here we report that strains possessing A(+28) expressed more vacA in gastric biopsies than those with G(+28) (p<0.01; figure 4A). Furthermore, i1-type strains with A(+28) were associated with more inflammation (p<0.05; figure 4B) and neutrophil infiltration (p<0.01; figure 4C) than those with G(+28).

**DISCUSSION**

The vacuolating cytotoxin is a major virulence determinant of H. pylori. Previously, we described vacA polymorphisms that affect its toxicity and devised simple PCR-based typing systems to identify these alleles among H. pylori strains.\(^9\)\(^22\)\(^24\)\(^31\)\(^32\) Subsequent vacA typing has shown that specific alleles, such as s1- and i1-types, are robust markers of increased disease risk in many Western populations.\(^6\) Here we show for the first time that in addition to differences in VacA toxicity between strains, vacA expression during H. pylori infection of the human stomach varies widely and is associated with the amount of inflammation and atrophy present. Previous studies have shown vacA expression varies widely between strains, but no correlation with promoter region polymorphisms was observed: sequence data were only reported for two strains studied by Forsyth et al,\(^4\) and there was insufficient variation at the +28 position in the 5’ UTR of the strains studied by Ayala et al\(^6\) to show the same association with vacA expression observed among our strains. An important difference in our study is that vacA mRNA levels were measured within gastric biopsies from

970 Sinnett CG, et al. J Clin Pathol 2016;69:968–973. doi:10.1136/jclinpath-2016-203641

Downloaded from http://jcp.bmj.com/ on 21 November 2018 by guest. Protected by copyright.
patients with the infection, rather than from in vitro cultures of isolated strains.

Despite many polymorphisms in the cysS–vacA intergenic region, we found only one that associated with gastric vacA mRNA expression, located at position +28 in the 5′ UTR. However, we cannot rule out that multiple polymorphisms affect vacA expression and have confounding effects on each other, masking any association with our expression data. Candidate polymorphisms are those located at −32, −14 and −7 within the promoter consensus sites. Indeed, replacing guanine with thymine at position −14 reduces vacA transcription twofold; so it is possible that the G/A polymorphism we observed at this position would affect vacA mRNA levels when assessed in an isogenic system.

It was interesting that the vacA 5′ UTR was significantly more conserved than the upstream nucleotide sequence containing the promoter sites. One explanation could be sequence constraints imposed by important secondary structure formation in...
this untranslated mRNA region. Indeed, we have recently identified a potential stem-loop structure from +4 to +30 in the vacA 5′ UTR which we showed was important for stabilising the vacA mRNA transcript. Interestingly, the only polymorphism significantly associated with gastric vacA mRNA level and inflammation, G/A(+28), was located within this stem-loop sequence. In our previous work, site-directed mutagenesis was used to replace guanine with adenine at +28 in the vacA 5′ UTR of strains 60190 and SS1, allowing more favourable pairing with uracil at position +6 and potentially increasing the stability of the stem-loop. We found that isogenic mutants with A(+28) had significantly higher steady-state levels of vacA mRNA than their wild-type controls with G(+28). This supports our current finding that clinical isolates with adenine at +28 are associated with increased vacA mRNA level in gastric biopsies from patients infected with H. pylori.

A recent study found vacA expression to be higher in patients with gastric cancer than in those with non-atrophic gastritis or duodenal ulcer. This agrees with our finding that higher vacA mRNA levels were associated with increased vacA expression in gastric tumors.
expression in the gastric mucosa is associated with increased inflammation and the presence of atrophy. Therefore, the disease risk of a patient with *H. pylori* infection is affected by both the VacA type of the infecting strain and the level of vacA expression during infection. This is important because the association between more active s1m1- and more recently i1-type strains and PUD or GC is not absolute. Furthermore, in countries where most *H. pylori* strains are s1i1-type, these markers of VacA toxicity do not allow differential determination of disease risk. Our finding that a natural polymorphism at nucleotide +28 of the vacA transcript affects vacA mRNA level shows that specific naturally occurring polymorphisms may affect vacA expression. This and other polymorphisms affecting vacA transcript levels may provide important additional markers for determining which patients are at greatest risk of developing severe gastric or duodenal disease, particularly among those infected with more toxic vacA i1-type *H. pylori* strains.

**Take home messages**

- Expression of the *Helicobacter pylori* vacuolating cytotoxin gene A (vacA) within the infected gastric mucosa varies considerably between strains.
- The level of vacA mRNA expressed in vivo correlates with gastric inflammation and premalignant pathology.
- A polymorphism at nucleotide +28 with the vacA 5′ untranslated region of the transcript is associated with gastric vacA expression level and inflammation, and may provide an additional marker of disease risk.

**Handling editor** Slade Jensen

**Acknowledgements** The authors thank the patients and staff at the Queen’s Medical Centre, Nottingham University Hospital.

**Contributors** JCA, DPL and CGS conceived and planned the study. CGS, GLN, JCA, DPL, KR and CGS wrote the manuscript.

**Funding** This work was supported by Cancer Research UK (C8968A/A11204); Nottingham University Hospitals Charities, and the National Institute for Health Research (NIHR), through the NIHR Biomedical Research Unit in Gastrointestinal and Liver Diseases at Nottingham University Hospitals NHS Trust and the University of Nottingham; and a Wellcome Trust VIP Award to DPL. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

**Competing interests** None declared.

**Ethics approval** Nottingham Research Ethics Committee 2 (08/H0408/195).

**Provenance and peer review** Not commissioned; externally peer reviewed.

**Open Access** This is an Open Access article distributed in accordance with the terms of the Creative Commons Attribution (CC BY 4.0) license, which permits others to distribute, remix, adapt and build upon this work, for commercial use, provided the original work is properly cited. See: http://creativecommons.org/licenses/by/4.0/