Simple Method for Determination of the Number of *Helicobacter pylori* CagA Variable-Region EPIYA Tyrosine Phosphorylation Motifs by PCR

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Received 30 June 2004/Returned for modification 17 August 2004/Accepted 8 October 2004

Helicobacter pylori strains possessing the *cag* pathogenicity island are associated with the development of gastric cancer. The CagA protein is translocated into epithelial cells and becomes phosphorylated on tyrosine residues within EPIYA motifs, which may be repeated within the variable region of the protein. Strains possessing CagA with greater numbers of these repeats have been more closely associated with gastric carcinogenesis. Phosphorylated CagA leads to epithelial cell elongation, which is dependent on the number of variable-region EPIYA motifs. Thus, determination of the degree of CagA phosphorylation and the number of EPIYA motifs appears to be more important than detection of *cagA* alone. Determination of the number of EPIYA motifs by nucleotide sequencing, however, is a laborious and expensive process. We describe here a novel and rapid PCR method for determination of the pattern of repeats containing the EPIYA motif. This will aid in the identification of those strains that may be more likely to cause disease.

Helicobacter pylori is estimated to infect the stomachs of half the world's population and is associated with the development of gastroduodenal diseases, including peptic ulceration and gastric cancer. Strains possessing the *cag* pathogenicity island (PaI) are more likely to cause disease than those lacking this locus (17–19, 21). The *cag* PaI comprises 27 to 31 genes and encodes a type IV secretory system (11) that forms a syringe-like structure that penetrates epithelial cells, facilitating the translocation of CagA (1, 11) but also inducing the secretion of proinflammatory cytokines and chemokines such as interleukin-8 (31). Within the cytosol, CagA may become phosphorylated (4, 10, 22, 25, 28) by Src family kinases (4, 27, 29) and can then interact with the phosphatase SHP-2 (15, 16, 33), leading to epithelial cell elongation and formation of the “hummingbird” phenotype (9, 25, 26).

Phosphorylation of CagA occurs within tyrosine phosphorylation motifs (TPMs) containing the EPIYA sequence (5, 15, 20, 28, 30). CagA proteins show size variation due to the presence of repeat sequences containing the EPIYA motif within the C-terminal variable region (7, 12, 29, 32). We and others have shown that CagA proteins possessing greater numbers of EPIYA repeats increase phosphorylation of the protein (3, 15, 29), increase the extent of hummingbird phenotype formation (3, 15), and are more likely to be associated with the development of gastric cancer (7, 32). Determination of the number of TPMs within the CagA variable region may therefore be more important than determination of the presence of *cagA* alone.

Nucleotide sequencing of the *cagA* variable region is a time-consuming and expensive procedure. Owen et al. recently de-

veloped a restriction fragment length polymorphism-PCR approach to determine the potential number of *cagA* TPMs (23), based on the TPM motif (R/K)X_{2–3}(D/E)X_{2–3}Y, which was considered to be the motif phosphorylated within CagA (9, 22). However, using mutagenesis and mass spectrometry, it has recently been shown that all EPIYA motifs may undergo phosphorylation (5, 15, 20, 29, 30). We have therefore developed a simple and rapid PCR-based method to determine the number and type of EPIYA motifs present within the *cagA* 3' variable region.

MATERIALS AND METHODS

H. pylori strains (Table 1) were grown on blood-agar plates (Oxoid, Basingstoke, United Kingdom) in a microaerobic environment within a MACS-500

TABLE 1. *H. pylori* strains used in this study

Strain	Origin	Disease status	TPM pattern
AB3	Scotland ^a	Gastritis	P1P2P3 ^c
AB5	Scotland ^a	Atrophy	P1P2P3P3 ^c
AB22	Scotland ^a	Atrophy	P1P2 ^c
AB29	Scotland ^a	Peptic ulcer	P1P2P3P3P3 ^c
AB36	Scotland ^a	Atrophy	None ^c
AB55	Scotland ^a	Atrophy	P1P2 ^c
GC77	South Africa ^b	Gastric cancer	P1P2P3P3P3 ^c
GC78	South Africa ^b	Gastric cancer	P1P2P3P1P2P3 ^c
GC102	South Africa ^b	Gastric cancer	P1P2P3P3 ^c
HP508	South Africa ^b	Gastritis	P1P2P3 ^c
Z7	China ^c	Gastritis	P1P2P3 ^f
Z11	China ^c	Peptic ulcer	P1P2 ^f
Z28	China ^c	Gastritis	P1P2P3 ^f
J99	United States ^d	Peptic ulcer	P2P3 ^d

^a El-Omar et al. (14).

^b Kidd et al. (18).

^c This study.

^d Alm et al. (2).

^e Argent et al. (3).

^f Accession numbers AJ832141, AJ832142, and AJ832145.

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TABLE 2. Primers used for the amplification of *cagA* TPMs

Primer	Sequence (5'-3')	Motif amplified
cag2 ^a	GGAACCTAGTCGGTAATG	
cag4 ^a	ATCTTTGAGCTTGTCTATCG	
cagA28F	TTCTCAAAGGAGCAATTGGC	
cagA-P1C	GTCCTGCTTTCTTTTATTAACCTKAGC	P1
cagA-P2CG	TTTAGCAACTTGAGCGTAAATGGG	P2
cagA-P2TA	TTTAGCAACTTGAGTATAAATGGG	P2
cagA-P3E	ATCAATTGTAGCGTAAATGGG	P3

^a Data from Rudi et al. (24).

workstation (Don Whitley Scientific, Shipley, United Kingdom) for three passages before extraction of genomic DNA as previously described (6). PCR amplification of the *cagA* 3' variable region was performed as previously described by using the primers cag2 and cag4 (24), and sequencing of the 550- to 800-bp products was performed by the Biopolymer Synthesis and Analysis Unit (Queen's Medical Centre, University of Nottingham, Nottingham, United Kingdom). Determination of the number of nucleotide sequences encoding the EPIYA motifs, and the types of motifs encoded, was carried out by using the forward primer cag2 (or cagA28F) and the reverse primers cagA-P1C, cagA-P2CG, cagA-P2TA, and cagA-P3E (Table 2). A reaction mixture containing 0.2 mM concentrations of each deoxynucleoside triphosphate, a 0.4 nM concentration of the forward primer, a 0.08 nM concentration of the reverse primer, 0.05 U of *Taq* DNA polymerase (Roche Diagnostics, Penzberg, Germany)/ μ l, and 1 μ l of genomic DNA in buffer (10 mM Tris-HCl [pH 8.3], 1.5 mM magnesium chloride, 50 mM potassium chloride) was incubated at 95°C for 90 s, followed by 35 cycles at 95°C for 30 s, 57°C for 60 s, and 72°C for 30 s and a final extension at 72°C for 5 min. PCR products were separated on a 1.5% (wt/vol) agarose gel.

RESULTS AND DISCUSSION

CagA proteins differ in size due to the presence of repeat regions containing EPIYA motifs, and we have previously shown that *H. pylori* strains isolated from South Africa possess three to six TPMs within this variable region (3). PCR amplification of the entire *cagA* variable region by using primers cag2 and cag4 (24) can give a size-based estimation of the likely number of TPMs. However, some *H. pylori* strains possess *cagA* with insertions or deletions between TPM repeats, thereby generating size variations without affecting the number of EPIYA motifs. Determination of the exact number of EPIYA motifs has therefore previously involved sequencing the entire *cagA* variable region, a laborious and generally expensive process.

To simplify determination of the number and type of EPIYA motifs present, we developed a PCR-based approach to identify individual EPIYA motifs, using a single forward primer and multiple reverse primers. Analysis of the sequences of *cagA* variable regions showed that there are three types of repeat region containing the EPIYA motif present: the first (P1) position has the consensus sequence EPIYA(Q/K)VNKKK(T/A)GQ, the second (P2) has the consensus sequence EPIY(A/T)QVAKKV, and the third (P3) has the consensus sequence EPIYATIDDLGGPFPL. The third (P3) position, however, is different in *H. pylori* strains isolated from East Asia, having the sequence EPIYATIDFDEANQAG. In

FIG. 1. PCR amplification of *cagA* variable-region TPMs from South African *H. pylori* strains. (A and C) Genomic DNA from *H. pylori* strains HP508, GC102, and GC78 (A) and GC77 (C) were used to PCR amplify the *cagA* variable-region EPIYA motifs, using the forward primer cagA28F and the reverse primers cagA-P1C (P1), cagA-P2CG and cagA-P2TA (equimolar mixture; P2), or cagA-P3E (P3). M, size markers (in base pairs). (B) Schematic representation of the *cagA* variable region from *H. pylori* strain GC77 showing the EPIYA motifs (gray), the annealing positions of the cagA28F (A28F) and cag2 (2) forward primers and the cagA-P1C (P1C), cagA-P2TA (P2TA), cagA-P2CG (P2CG), and cagA-P3E (P3E) reverse primers, and the expected sizes of the amplified products.

FIG. 2. PCR amplification of *cagA* variable-region TPMs from *H. pylori* strains. Genomic DNA from *H. pylori* strains J99, AB36, AB5, AB29, Z11, and Z28 were used to amplify the *cagA* variable-region EPIYA motifs, using the forward primers *cagA*28F (J99, AB36, AB5, and AB29) or *cagA*2 (Z11 and Z28) and the reverse primers *cagA*-P1C (P1), *cagA*-P2CG and *cagA*-P2TA (equimolar mixture; P2), or *cagA*-P3E (P3). *H. pylori* strain J99 lacks the P1 motif, and strain AB36 does not possess *cagA*. M, size markers (in base pairs).

order to amplify the three types of TPM motif, we designed primers complementary to the nucleotide sequences encoding these repeats (Table 2). The original P1 reverse primer (*cagA*-P1) was found to preferentially amplify the P2 repeat, due to high identity between the primers, and so was redesigned to amplify the A(Q/K)VNKKK(T/A)G repeat instead (*cagA*-P1C). The P2 primers (*cagA*-P2CG and *cagA*-P2TA) were designed to amplify the PIY(A/T)QVAK repeat, and the P3 primer (*cagA*-P3E) was designed to amplify the PIYATID repeat (Table 2).

Figure 1 shows the amplification of TPM repeats within the *cagA* variable regions of South African *H. pylori* strains known to possess three to six EPIYA motifs by nucleotide sequencing (3). From the sequences we expected that the strains would have the EPIYA motif patterns P1P2P3 (HP508), P1P2P3P3 (GC102), P1P2P3P3P3 (GC77), and P1P2P3P1P2P3 (GC78), which are the exact patterns obtained by PCR (Fig. 1). We predicted that, with the forward primer *cagA*28F, which anneals to nucleotide positions 546053 to 546072 in the *H. pylori* strain J99 chromosome, the amplified fragment sizes would be 264 to 291 bp for the first P1 site, 309 to 336 bp for the first P2 site, and 465 to 498 bp for the first P3 site. For *cagA* from strain GC77, which has three copies of the P3 motif, we predicted that amplification of the P1 and P2 sites would generate fragments of 264 and 309 bp, respectively, and that amplification of the P3 sites would produce products of 468, 570, and 672 bp (Fig. 1B), which are the approximate sizes generated in the PCR amplifications (Fig. 1C). PCR with the forward primer *cagA*2 in place of *cagA*28F generates the same products except that, as predicted, they are ca. 99 bp shorter in length.

We next looked at *cagA* variable regions from *H. pylori* strains from other populations, along with a control from genome sequence strain J99 (ATCC 700824) and from a strain lacking *cagA* (Fig. 2). The *cagA* from strain J99 has the EPIYA motif pattern P2P3 and therefore lacks a P1 motif, and this can be seen in the PCR data. There are no products generated for the *cagA*-negative sample (AB36), whereas *cagA* from strains AB3, AB5, AB22, AB29, and AB55 predict the EPIYA motif patterns P1P2P3, P1P2P3P3, P1P2, P1P2P3P3P3, and P1P2, respectively (Fig. 2). To confirm that our PCR-based prediction of TPMs was correct, we performed nucleotide sequencing of the entire *cagA* 3' variable region from strains AB3, AB5, AB22, AB29, and AB55 after amplification of this region with primers *cagA*2 and *cagA*4 (Table 3). This showed that the *cagA* variable region from these strains possessed the exact patterns of EPIYA motifs expected from the PCR data.

Although the PCR prediction of EPIYA motifs proved reliable with "Western" isolates of *H. pylori*, we also wanted to determine whether this method could be used to type the *cagA* variable regions of *H. pylori* strains from East Asia that are known to possess differences in sequence. To do this, we PCR amplified the *cagA* EPIYA motifs from *H. pylori* strains isolated from China. Strain Z11 has a nucleotide sequence that encodes a Western type of *CagA*, with a P1P2 motif pattern, whereas strains Z7 and Z28 have an "East Asian" type of *cagA*, with a P1P2P3 pattern. The reverse primer *cagA*-P3E was designed to amplify the P3 motif within all types of *cagA* by annealing to the nucleotide sequence encoding the EPIYATID motif (Table 2). PCR amplification of *cagA* from Chinese *H. pylori* strains, carried out with the forward primer

TABLE 3. Nucleotide sequence analysis of *H. pylori* strains^a

Strain	Motifs P1 and P2	Motif P3
AB3	EPIYAKVNKKKTGQVASPEEPIYQVAKKVN	SVSPEPIYATIDDL
AB5 ^b	EPIYAQVNKKKAGQVASPEEPIYQVAKKVK	SANHEPIYATIDDL SASPEPIYATIDDL
AB22	EPIYAKVNKKKAGQVASPEEPIYAQVAKKVN	
AB29 ^c	EPIYAKVNKKKAGQVASPEEPIYAQVAKKVN	SVSPEPIYATIDDL
AB55	EPIYAKVNKKKAGQVASPEEPIYAQVAKKVN	

^a P1, P2, and P3 EPIY(A/T) motifs are shown in boldface. The annealing regions for the reverse primers are shown in italics.

^b Strain AB5 has two P3 motifs, as shown in the final column.

^c Strain AB29 has three identical P3 motifs, as shown in the final column.

cag2, again revealed the predicted patterns (Fig. 2). Strain Z11 has the EPIYA motif pattern P1P2, whereas strains Z7 and Z28 have the pattern P1P2P3. Amplification with the forward primer cagA28F gave the same patterns, although we found that, with East Asian types of *cagA*, PCRs with the cag2 primer gave clearer results.

We have therefore shown that PCR can be used to determine the number of *cagA* variable-region EPIYA motifs in strains from Africa, Britain, and China. Although we have not tested *cagA* variable regions from other populations, we expect that this method would be compatible for use with almost all *H. pylori* strains. The methodology we describe is simple and inexpensive; after genomic DNA extraction, PCRs and analysis can be carried out within a few hours, and this method allows the rapid screening of multiple strains. Although most strains of *H. pylori* possess the three types of TPM analyzed here, or minor variations in these motifs, we cannot exclude the possibility that some strains may rarely possess alternative sequences that would not be recognized by our primers.

Our new method will help in further work to elucidate the biological and clinical importance of multiple tyrosine phosphorylation sites. In particular, it is important that we separately identify P1, P2, and P3 sites since the relative importance of each type of TPM containing the EPIYA repeat remains unclear. Although Higashi et al. (15) showed that the P3 motif of CagA from strain NCTC 11637 (which has a P1P2P3P3P3, or ABCCC, TPM pattern) became more phosphorylated within epithelial cells than the other motifs and induced the hummingbird phenotype in more cells, we (3) showed that CagA from *H. pylori* strain GC78 (which has a P1P2P3P1P2P3 pattern; Table 1) became more phosphorylated and induced the hummingbird phenotype more cell than CagA from strain GC77 (which has a P1P2P3P3P3 pattern; Table 1). Finally, we point out that aspects of CagA other than its level of tyrosine phosphorylation may impact its cellular effects. CagA from East Asian strains interacts with SHP-2 phosphatase via the binding motif pY-(S/T/A/V/I)-X-(V/I/L)-X-(W/F) (13), which is present in CagA from East Asian *H. pylori* strains but not in Western strains and leads to greater activation of the phosphatase (8, 15). However, despite the absence of this consensus sequence in non-East Asian CagA proteins, it is clear that these strains can still induce formation of the hummingbird phenotype (3, 8). Whether strains with an East Asian SHP-2 binding motif are associated with more severe disease is a further question worth addressing.

ACKNOWLEDGMENTS

This study was funded by a grant from Cancer Research UK. Y.Z. was funded by a scholarship from the Jiangsu Provincial Department of Education of the People's Republic of China to conduct research in the United Kingdom. J.C.A. is funded by a Senior Clinical Fellowship from the Medical Research Council (United Kingdom).

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