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ORIGINAL ARTICLE—ALIMENTARY TRACT

Epitope peptides of *Helicobacter pylori* CagA antibodies from sera by whole-peptide mapping

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Abstract

Background *Helicobacter pylori* CagA has been found to be immuno-dominant protein and utilized for the diagnosis of the infection with *cagA*-positive strains. It is important to characterize the peptide epitopes capable of detecting serum anti-CagA antibodies to understand CagA immunogenicity.

Methods Sera from 171 Japanese patients were subjected for the epitope mapping study. Eighty seven peptides were designed from the CagA consensus sequence and were used for ELISA protocol to test the serum samples. The reacting anti-CagA IgG amounts to specific peptides were measured and compared.

Results The study revealed a strong reactivity of two peptides (c7-NNTEPIYAQVNKKKAGQAT and c8-AGQATSPEEPIYAQVAKKV) in *H. pylori*-infected group. Interestingly, these two peptides contained the well-known EPIYA-A and EPIYA-B region, respectively, which are two out of three CagA phosphorylation domains. Tyrosine-phosphorylation of these peptides reduced their reactivity in most sera. Moreover, additional peptides' mapping and chimeric-peptides' experiments indicated that the amino acids (QV and KK) accommodated in right-side flanking regions of both EPIYA-motifs were essential for their strong reactivity, whereas the third EPIYA-motif containing peptide (c12-GRSASPEPIYATIDFDEA) with differing flanking amino acids was not reactive in most cases.

Conclusions Our results suggest that the amino acid sequences constituted in the two reactive peptides are the important immunogenic regions of CagA which would be useful to develop next-generation peptide-based diagnostic assays.

Keyword *Helicobacter pylori* · CagA · IgG · ELISA · Peptide

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Background

Helicobacter pylori, the causative agent of various human gastroduodenal diseases such as chronic gastritis, peptic ulcers, and gastric cancer has been estimated to infect at least 50% of the world's population [1, 2]. The cure for *H. pylori* infection significantly decreases the risk of developing severe gastric complications [3]. However, for successful cure, it is important to choose the appropriate antibiotic regimens considering the regional resistance

rates [4]. Early and timely diagnosis is also important to initiate the treatment to reduce the development of severe disease progression [5].

Currently, several serological diagnostic methods are available and most of them are based on enzyme immuno assays (EIAs) using whole *H. pylori* bacterial cell lysate. As a type of EIA, many enzyme-linked immuno-sorbent assay (ELISA) kits for serology using whole *H. pylori* lysate are commercially available worldwide and the kits were evaluated comparatively and have been used clinically [6, 7]. However, ELISA using whole *H. pylori* lysate is limited in specificity and sensitivity, mainly due to the crude bacterial lysate preparations, therefore multiplex serology has been developed using recombinant proteins with a fusion protein-tag on fluorescent beads [8], or line immunoassays using multiple recombinant antigen proteins [9].

Cytotoxin-associated gene A protein (CagA) is the most studied virulence factor of *H. pylori* and has been identified as an antigenic protein [10–12]. Its immunogenic response has been reported for improved sero-diagnostic approaches in patients with gastroduodenal diseases [13–23]. Moreover, in several recent approaches utilizing multiple recombinant *H. pylori* proteins, high antibody reactivity was found against CagA [8, 24]. The prevalence of CagA among clinical isolates greatly varies according to the region and is reported to be nearly 100% in strains from East-Asia, whereas it remains as low as 50% in some Western countries [25]. Nearly all strains from the Japanese populations harbor East-Asian-type CagA (ABD-type), which is distinguished by the C-terminal repeated EPIYA and neighboring sequences from Western-type CagA (ABC-type) [26, 27]. Although the ABC-type CagA ELISA kit for serology has been already commercialized, a kit for the ABD-type has yet to be developed. We recently developed an ABD-type CagA-specific antibody-based diagnostic assay [28]; however, it must be improved in terms of the antibody cutoff value, which was relatively high, likely stemming from the large CagA (140 kDa) size, which increases the possibility of binding of non-specific antibodies to CagA fragments [28]. Therefore, to improve the sensitivity and reliability of next generation CagA-based diagnostic tests, it is important to characterize the epitope of CagA antibody. In this study, we performed the peptide-mapping study via ELISA to investigate the peptide epitopes capable of detecting anti-CagA antibodies from the sera of Japanese patients.

Methods

Serum samples and biopsy specimens from patients

A study protocol was reviewed and approved by Institutional Review Board (IRB) for research ethics committee of Oita University Faculty of Medicine. Written informed consent was obtained from all patients included in this study. Patients undergoing gastroscopy at Oita University Hospital between October 2015 and July 2016 were recruited. Exclusion criteria included a history of gastrectomy, allergic history to the medications used in this study, co-existence of serious concomitant illness, pregnancy, and treatment with antibiotics, bismuth-containing compounds, or proton pump inhibitors within 2 weeks of the study start date. Patients with confirmed *H. pylori* infection subsequently underwent *H. pylori* eradication therapy and were checked for successful eradication. The clinical presentations were recorded endoscopically, but suspected gastric cancer and MALT lymphoma were confirmed by histopathological examination. Blood samples were collected from all the patients and serum was separated and frozen at − 80 °C until analysis.

Processing of biopsy specimens for bacterial culture and histology

At the time of endoscopic examination, four biopsy specimens (two from both the greater curvature of the antrum and the middle of the body) for histological evaluation and two biopsy specimens (one from both the greater curvature of the antrum and the middle of the body) for *H. pylori* culture were obtained from each patient. Biopsy specimens for culture were processed as described previously [29]. The formalin-fixed biopsy specimens collected for histopathological examinations were embedded in paraffin and processed for hematoxylin and eosin (HE) and Giemsa staining as described previously [23].

H. pylori cagA genotyping

Small (1 μL) loopfull of *H. pylori* strains grown were suspended in 100 μL of Tris–EDTA (TE) buffer solution and DNA was extracted by same amount of phenol/chloroform/isoamyl alcohol (25:24:1) solution and then chloroform/isoamyl alcohol (24:1) solution. A 10 × diluted DNA solution in sterile distillated water was used for *cagA* genotyping using polymerase chain reaction (PCR). Analyses for *cagA*-positivity and EPIYA-repeat regions genotypes (East-Asian-type or Western type) were performed by PCR-based Sanger sequencing as described previously [27]. To confirm the *cagA*-negativity, PCR for *cag* empty site was performed using Luni1 and R5280 or Hp522R2 primers as described previously [27, 30], with additional primers to account for the genetic diversity within Japanese strains. The additional primers are as follows: eraF1 (ATAGGCAAACCAAACGCTGGAAAAAG) and eraF2 (CTTGCAAGTGATCGCTCAAAAATCATG). Luni1, eraF1, and eraF2 target the left outside sequence of *cag1*,

whereas R5280 and Hp522R2 target the right outside sequence of *cagA* in the *cag* pathogenicity island region.

Anti-*H. pylori* and anti-CagA antibody amounts

All the serum samples were subjected to whole *H. pylori* lysate E-plate ELISA with a cutoff value of 3.0 U/mL (Eiken Chemical, Tokyo, Japan) and automated latex-agglutination turbidimetric immunoassay test with a cutoff value of 10.0 U/mL (Denka Seiken Co., Ltd., Tokyo, Japan) to measure anti-*H.pylori* antibodies as recommended by the instructions. The serum samples were also subjected for anti-East Asian-type CagA ELISA according to our in-house method described previously [28], with a modified standard curve for antibody quantification. For this purpose, we constructed a new anti-East Asian-type CagA rabbit IgG antibody using m24 peptide (QKITDKVDNLNQAVSETKL) located in the middle region of East Asian-type CagA protein. The anti-m24 CagA rabbit IgG was constructed using 20 mer-m24N peptide [addition of asparagine (N) at N-terminal of m24 peptide] and then purified by m24N-peptide column affinity chromatography (Sigma, St. Louis, USA). For the standard curve of antibody detection in serum samples, serially diluted anti-m24 rabbit IgG was used as the primary antibody, simultaneously in six wells of recombinant-East-Asian-type CagA (rEA-CagA) protein (0.1 μg/well)-immobilized 96-well format ELISA [28]. Diluted anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (Jackson Immuno-Research Labs, West Grove, PA, USA) was then added as secondary antibody. The concentrations of anti-CagA antibodies (μg/mL) were calculated from the standard curve and then multiplied according to the dilution factor (1000 ×) of the original serum samples.

CagA-peptide series design

The CagA consensus sequence was obtained based on 114 East-Asian-type *cagA* sequences derived from our previous study in Japanese population [27]. From the consensus sequence, a series of 87 peptides with 19–20 amino acids, overlapping 5 amino acids of the adjacent peptide on each end were designed (Supplementary Appendix S1). The N-terminal peptides (n1–n33), middle-region peptides (m1–m24), and C-terminal peptides (c1–c27) constituted amino acids from 1–466, 462–801, and 797–1176 in consensus sequences, respectively. The peptide m24 was the same sequence as used for standard curve. Two or three peptides were prepared from the regions with highly variable amino acids (n26 and c20) substituting the second and the third most frequent amino acids (n26V, n26I, c20S, c20N, and c20D). Between the peptides c6 and c9 in C-terminal CagA, additional series of peptides and chimeric peptides were designed. To evaluate the anti-tyrosine-phosphorylated antibody in sera, three additional peptides (c7pY, c8pY, and c12pY) with tyrosine-phosphorylated of c7, c8, and c12 were also designed and synthesized commercially (Scrum Co. Ltd, Tokyo, Japan). The peptides were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, USA) as a 10 pmol/μL solution, then diluted 10 × with phosphate buffer saline (PBS) to be adjusted to 1 pmol/μL as stock solution, and stored at – 20 °C until use.

CagA epitope mapping by peptide ELISA method

Each peptide stock solution was diluted 100 × in 50 mM sodium carbonate buffer (pH 9.6) and 100 μL was immobilized to Nunc Immobilizer Amino 96-well plates (Thermo Fisher Scientific, Massachusetts, USA). The final amount of each peptide was 1 pmol/well. For a standard curve, the peptide m24 was also immobilized similar to the other peptides in six wells of first 8-well-column of the plate with bovine serum albumin (BSA) (negative control) and human IgG (positive control) in one well each (Supplementary Fig. S1). The peptide containing plates for immobilization were incubated at 4 °C for overnight in the dark.

Next day, the wells were washed three times with wash buffer (PBS with 0.1% Tween-20), masked with 200 μL of 50 mM ethanolamine/50 mM sodium carbonate buffer (pH 9.6) for 1 h, and washed three times. The wells were blocked with blocking solution (2% BSA in PBS) for 1 h, and washed three times. Patient serum samples were then diluted 1:200 with blocking solution and 100 μL/well were subjected to react with immobilized peptides for 2 h, and washed 5 times. Then, 100 μL/well of anti-human IgG conjugated with horseradish peroxidase (HRP) (Jackson Immuno-Research Labs, West Grove PA, USA) diluted 1:4000 was added as secondary antibody for 1 h, and washed 5 times. Finally, 100 μL/well of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (NACALAI TESQUE, Kyoto, Japan) was added and left for 6 min at room temperature to develop the color, then the reaction was stopped by adding 1 N H_2SO_4 (100 μL/well). The optical density (OD) was measured at 450 nm using a plate reader. The concentration of antibodies (ng/mL) reacting against each peptide was calculated from standard curve. Finally, to obtain the estimated concentration in patient serum, the reacting antibody concentrations were multiplied by the dilution factor (200×).

The ability of the phosphorylated peptides to detect the anti-tyrosine-phosphorylated antibody was evaluated using the mouse monoclonal p-Tyr (PY99) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and peroxidase-conjugated goat anti-mouse IgG (Jackson Immuno-

Research Labs, West Grove, PA, USA) as the primary and secondary antibodies, respectively.

Statistical analysis

The continuous variables of antibody amount measured against the peptides (ng/mL) were analyzed for their distribution. The analyses showed that the data were not normally distributed and were positively skewed. Therefore, the data of antibody amount measured against the peptides were expressed as a median (range) and the Mann–Whitney *U* test was applied to analyze the differences using R statistics software version 1.35. A *P* value of ≤ 0.01 (**) and ≤ 0.001 (***) was considered as statistically significant.

Results

Serum samples according to infection status

To evaluate the epitope characteristics of polyclonal CagA antibodies in sera of *H. pylori*-infected individuals, we initially recruited a total of 193 patients. Biopsy samples could not be collected from four patients undergoing anticoagulant therapy, and sera from two patients were missed. Therefore, 187 cases comprising of 115 before eradication therapy and 72 after eradication therapy were included in this study. As our gold standard, at least one of six biopsy specimens (2 cultures and 4 histology-staining) showing positive results was grouped as *H. pylori*-positive cases (62 cases including three cases of eradication failure). However, excluding seven cases revealed by histology only; overall, 55 patients were classified as *H. pylori*-positive. Fifty-six cases with all six biopsy specimens showing negative results for *H. pylori* infection before the eradication therapy were judged as *H. pylori*-negative. However, nine cases had endoscopic evidence of severe gastric atrophy, raising suspicion of natural eradication were excluded. Overall, 47 patients were classified as *H. pylori*-negative. In addition, 69 cases that were *H. pylori*-negative after successful eradication were categorized as eradicated cases (Fig. 1).

H. pylori cagA-genotype status and serum grouping

H. pylori isolates (55 strains) underwent *cagA* genotyping using DNA samples (Fig. 1). Two strains had no amplification of the *cagA* gene, but amplified the *cagA* empty site via PCR, were concluded to be *cagA*-negative. Of the remaining 53 *cagA*-positive strains, 49 (92.5%), 3 (5.7%), and one strain harbored ABD type (East-Asian), ABC type (Western) and AB-type of CagA, respectively (Table 1). Therefore, the majority of the patients were infected with *H. pylori* having East-Asian-type of CagA as expected.

We checked the characteristics of sera in these groups (Table 1). E-plate ELISA for anti-*H.pylori* IgG and the automated latex-agglutination turbidimetric-immunoassay (LATEX) for anti-*H.pylori* antibody showed similar trends of significantly higher levels of antibody in the *H. pylori*-positive group than in the *H. pylori*-negative and eradicated groups in both methods. We also measured the anti-CagA IgG using our in-house East-Asian-type CagA ELISA [28], and the median amount of antibodies in the *H. pylori*-positive group (10.5 μg/mL) was significantly higher than in the *H. pylori*-negative (7.9 μg/mL) and eradicated groups (8.8 μg/mL), although the *H. pylori*-negative group showed relatively high values, which is likely due to background detection.

The patients infected with *cagA*-negative strains (no. 2–18 and no. 2–44) showed low values of anti-East Asian CagA IgG (9.6 and 6.8 μg/mL, respectively), as expected. The high values of anti-*H.pylori* antibody (33.8 and 38.3 U/mL by Latex test, and 19.9 and 31.2 U/mL by E-plate for strains no. 2–18 and no. 2–44, respectively) were detected, as expected (Table 1). We therefore carefully evaluated the two *cagA*-negative cases separately which behaved like *H. pylori*-negative sera rather than like *H. pylori*-positive sera in their anti-CagA antibody response (8.2, 7.9, and 10.5 μg/mL, respectively) (Table 1). The reactivity of these two *cagA*-negative sera to all sets of CagA peptides could not distinguish to that of *H. pylori*-negative sera. So, we grouped these two sera together with *H. pylori*-negative cases during the first peptide-mapping step. Therefore, in further analyses, a total of 171 cases, comprising of 49 cases in *H. pylori*-negative or *cagA*-negative *H. pylori*-infected (N) group, 53 cases in *cagA*-positive *H. pylori*-infected (P) group, and 69 cases in eradicated (E) group, were recruited (Fig. 1).

Screening of CagA epitope peptides

To understand the antibodies' detecting abilities of the various CagA amino acid sequences, 28 representative gastritis sera comprising of 9 (including two sera form *H. pylori*-positive but *cagA*-negative strain possessing patients), 10, and 9 in each of three groups N, P, and E, respectively, were selected (Supplementary Fig. S2). CagA epitope mapping analysis was performed using each of the 87 peptides in a 96-well ELISA plate (Supplementary Fig. S1). The amount of serum antibody (ng/mL) reacting to each peptide was quantified using simultaneously detected m24-standard curve.

As a result, 20 peptides (n1, n19, n20, n21, n24, m4, m5, m24, m25/c1, c5, c7, c8, c9, c10, c11, c19, c20S, c21, c24, and c27) detected a significantly higher amount of

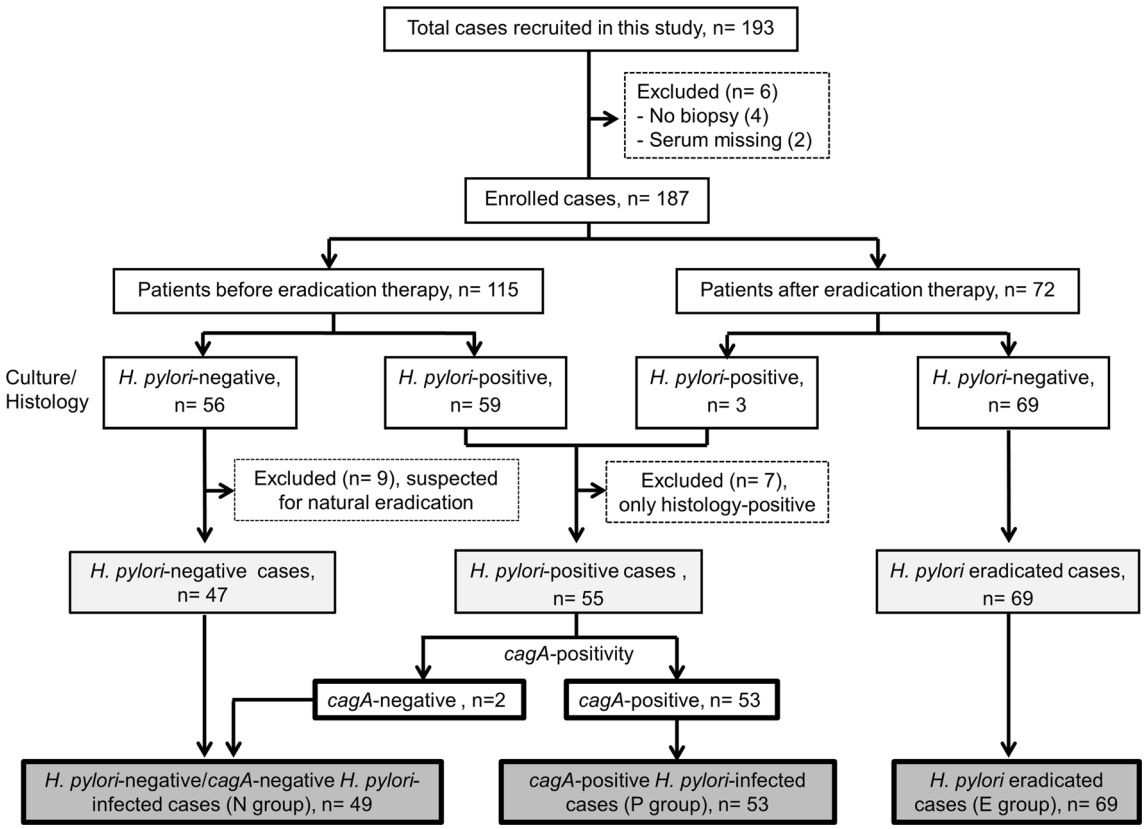

Fig. 1 Serum samples recruited in this study. Two cases infected with *cagA*-negative strains were included with *H. pylori*-negative group. Therefore, 49 cases in N group, 53 cases in P group and 69 cases in E group were enrolled in this study

antibodies ($P < 0.01$) in the representative P group sera than in the N group sera (Fig. 2). Similarly, 13 peptides (n1, n2, n9, n15, n17, n19, m4, c3, c4, c7, c8, c14, and c20S) detected from the representative P group sera than from the E group sera (Supplementary Fig. S3). Several peptides were reactive in N and E group sera, such as n6, n18, n31, and c12, indicating the regions that increase the background detection (Fig. 2 and Supplementary Fig. S3). The three peptides (c7, c8, and c21) showed high reactivity and were selected for further analysis.

The strong reactivity of peptides c7, c8, and c21

Next, we tested all 171 sera for the reactivity of peptides c7, c8, and c21 to detect the peptide-specific IgG (Fig. 3). The median IgG value to peptide c7 from P group (34.2 ng/mL) was significantly higher than values from N group (8.3 ng/mL) and E group (9.6 ng/mL) with $P < 0.001$ (Fig. 3a). Similarly, the median IgG values to peptide c8 from P group 35.3 ng/mL were significantly higher than values from N group (10.2 ng/mL), and E group (12.5 ng/mL) with $P < 0.001$ (Fig. 3b). The third candidate peptide c21 detected 3.7 ng/mL, 5.0 ng/mL, and 3.6 ng/mL from N, P, and E group sera, respectively. Differences of the N vs. P and P vs. E were statistically significant ($P = 0.0098$ and 0.0039, respectively) (Fig. 3c). Therefore, we concluded that c7 and c8 were the two best peptides that detected the significantly higher amounts of antibody from *H. pylori*-infected (P) group than from *H. pylori*-negative (N) or eradicated (E) group.

Serum reactivity of peptides m8 and m24

Previously we reported the strong reactivity of the peptides m8 and m24 among peptides from middle regions of CagA using Japanese child sera [22]. Here we re-evaluated the reactivity of these two peptides using Japanese adult sera in this peptide ELISA system. The median IgG amount of peptide m8 from N, P, and E group was 9.5 ng/mL, 10.3 ng/mL and 8.1 ng/mL, respectively, and only P vs. E was significant different ($P = 0.0019$) (Fig. 3d). The peptide m24 detected the median amount of 9.4 ng/mL, 11.5 ng/mL, and 10.0 ng/mL from N, P, and E group, respectively, and only N vs. P showed significant difference ($P = 0.0039$) (Fig. 3e). Therefore, the reactivity of m8 and m24 was not fully reproduced in this ELISA system using adult sera.

Table 1 Serum group characteristics and CagA status

Serum group	*H. pylori*-negative ($n = 47$)	*H. pylori*-positive					Eradicated ($n = 69$)
		cagA-negative ($n = 2$)	AB ($n = 1$)	ABC ($n = 3$)	ABD ($n = 49$)	*H. pylori*-positive (total)	
H. pylori antibody, latex test (U/mL)							
Median	2.5	36.1	9.3	33.0	43.8	$40.6^{\alpha\beta}$	5.7
(Min–max)	(0.1–75.5)	(33.8–38.3)		(11.8–111.4)	(3.7–124.2)	(3.7–124.2)	(0.2–49.3)
H. pylori-IgG, E-plate (U/mL)							
Median	0.40	25.6	19.0	17.0	18.5	$17.9^{\gamma\delta}$	1.8
(Min–max)	(0.02–20.7)	(19.9–31.2)		(9.2–17.7)	(3.1–97.0)	(3.1–97.0)	(0.04–13.8)
Anti-East Asian CagA-IgG (μg/mL)							
Median	7.9	8.2	14.8	7.8	10.7	$10.5^{\phi*}$	8.8
(Min–max)	(1.7–19.2)	(6.8–9.6)		(4.3–16.0)	(1.3–23.7)	(1.3–23.7)	(3.0–16.1)
Diseases							
Normal	6	0	0	0	0	0	0
Gastritis	33	2	1	3	42	48	59
Gastric ulcer	3	0	0	0	2	2	2
Duodenal ulcer	1	0	0	0	0	0	1
Gastric cancer	3	0	0	0	5	5	6
MALT lymphoma	1	0	0	0	0	0	1
Total	47	2	1	3	49	55	69

$^{\alpha\beta}$Significant level ($P < 0.0001$ each) between *H. pylori*-positive and *H. pylori*-negative group, and *H. pylori*-positive and eradicated group, respectively, for latex test

$^{\gamma\delta}$Significant level ($P < 0.0001$ each) between *H. pylori*-positive and *H. pylori*-negative group, and *H. pylori*-positive and eradicated group, respectively, for E-plate test

$^{\phi*}$Significant level ($P < 0.001$ and $P < 0.01$) between *H. pylori*-positive and *H. pylori*-negative group, and *H. pylori*-positive and eradicated group, respectively, for anti-East Asian CagA test (Mann–Whitney *U* test)

Peptides c7, c8, and c12 contain tyrosine-phosphorylated domains

We noted that both the c7 and c8 peptides share a common EPIYA-motif, containing a tyrosine phosphorylation domain of the CagA protein, EPIYA-A and EPIYA-B, respectively (Supplementary Appendix S1). We therefore tested the reactivity of the third EPIYA-motif (EPIYA-D)-containing peptide c12. As a result, the c12 peptide detected the median amount of 22.4 ng/mL, 26.7 ng/mL, and 19.3 ng/mL from the N, P, and E group sera, respectively, so the difference comparisons between both of N vs. P group ($P = 0.182$) or P vs. E group ($P = 0.0136$) were not significant according to our criteria at $P \leq 0.01$ (Fig. 3f). Therefore, the third EPIYA-D-containing c12 peptide was not reactive as were EPIYA-A- and EPIYA-B-containing peptides c7 and c8, respectively. Since the reactivity of c7 was correlated with the reactivity of c8, whereas not of c12 (Supplementary Fig. S4). Therefore, P group sera contained high amount of anti-CagA IgG recognized by both of EPIYA-A and B frequently, but not by EPIYA-D.

Reactivity of c7, c8, and c12 peptides together with their phosphorylated counterparts (c7pY, c8pY, and c12pY)

EPIYA-motifs A, B, and D in CagA are known as the domains that characteristically undergo post-translational tyrosine-phosphorylation inside host cells by tyrosine kinases after its injection into gastric epithelial cells [31]. Phosphorylated peptides have been found to alter the immune response [32]. Therefore, we compared the reactivity of the peptides c7, c8, and c12 to their tyrosine-phosphorylated (pY)-counterparts (c7pY, c8pY, and c12pY, respectively) in sera from all three groups.

For this analysis, we first confirmed the reactivity of the phosphorylated peptides (c7pY, c8pY, and c12pY) to the phosphorylated tyrosine-specific antibody (PY99). These phosphorylated peptides were strongly detected by the PY99 while non-phosphorylated peptides did not react at all which was consistent with previous studies using PY99 (Fig. 4a, b) [33, 34].

Interestingly, highly reactive sera to c7 and c8 from P group showed reduced reactivity to c7pY and c8pY

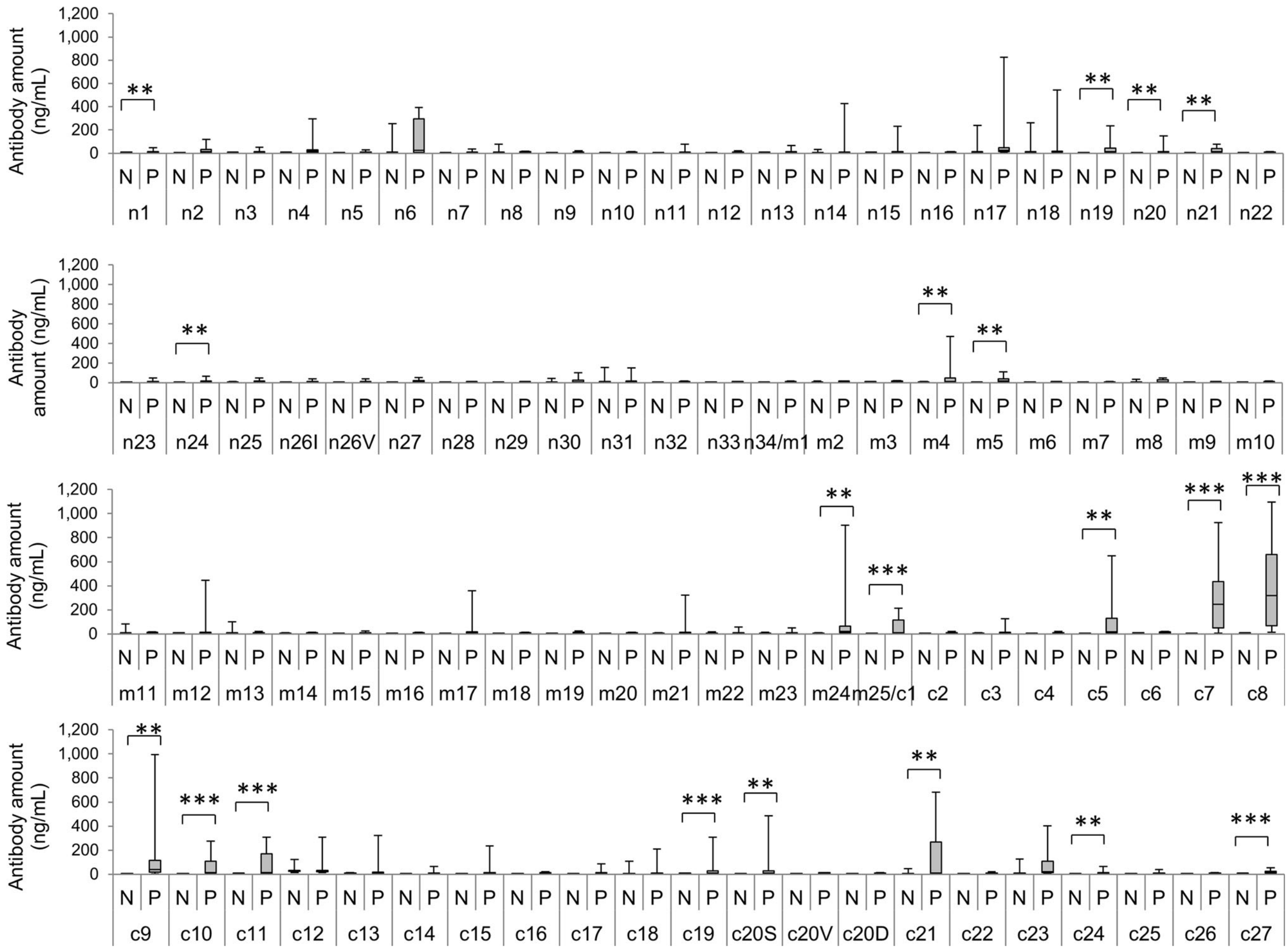

Fig. 2 Antibody amount measured against 87 peptides in representative sera from *H. pylori*-positive (P) and *H. pylori*-negative or *cagA*-negative *H. pylori*-positive (N) groups**.** Mann–Whitney *U* test (**$P \leq 0.01$ and ***$P \leq 0.001$)

(Supplementary Table S1). This fact indicates that Tyrosine (Y) in EPIYA is an epitope component in the c7 and c8 peptides. However, sometime higher reactivity to c12pY was seen in the N and E group sera, even being not reactive to c12 (Fig. 4c, e and Supplementary Table S1). The c12 and c12pY-reactive antibodies were largely unpredictable, which may suggest the capability of these peptides to detect cross-reacting antibodies of unknown origin.

The epitope region of c7 and c8 peptides

Next we confirmed the amino acid sequence variation of the c7 and c8 peptide regions in 53 *H. pylori* strains isolated from corresponding Japanese patient sera investigated in this study. It was clear that the peptide c7 and c8 regions were conserved (Fig. 5 and Supplementary Appendix S2) as expected from previous reports [35]. So, we aimed to narrow down our experimental scheme to identify the essential epitope region in peptides c7 and c8. Because both the c7 and c8 peptides contained the EPIYA-motif and a 5-amino acid overlapped sequence (AGQAT), it could be simultaneously capable of detecting antibodies. The additional peptide series, c6-6, c6-11, c7-6, c7-11, c8-6, and c8-11 were synthesized and these peptides together with c6, c7, c8, and c9 were reacted with 11 representative sera strongly reactive to the c7 and c8 peptides (Fig. 6a). The peptide c7-6, which covered the overlapped AGQAT together with incomplete EPIYA sequences, did not react to most of sera, suggesting the overlapped AGQAT sequence is not the epitope region of c7 and c8 peptides. However, the peptides c6-6 and c6-11, which contain the intact EPIYA-A together with QV, were able to detect antibodies. The peptide c6-11 which contained NGLK on the left side of the common sequence (NNTEPIYAQV) and NKKKA on the right side, showed the highest reactivity whereas the peptide c6-6 detected the lower amount of antibody in these c6-6, c6-11 and c7 peptides (Fig. 6b). Therefore, all or part of NKKKA might be responsible to increase the reactivity of EPIYAQV of c7. Similarly, the peptides c7-11 and c8-6 which contained the intact EPIYA of c8 together with QV were able to highly detect the antibodies. However, the peptide c7-11 which lacked right

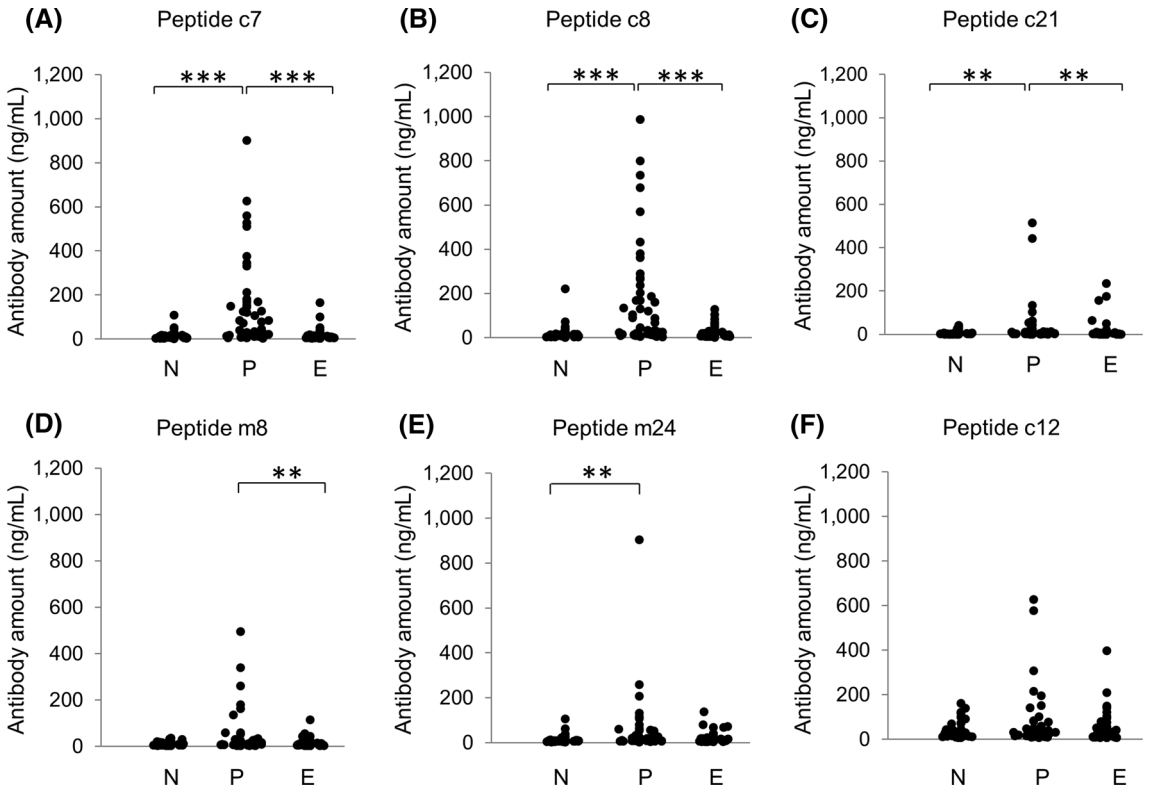

Fig. 3 Antibody amount detected against peptides c7, c8, c21, m8, m24, and c12. Antibody amount measured against c7, c8, c21, m8, m24, and c12 peptides from N group (*H. pylori*-negative or *cagA*-negative *H. pylori*-positive cases), P group (*cagA*-positive *H. pylori*-infected cases) and E group (*H. pylori*-eradicated) sera (**a**–**f**). The median amount of antibodies measured (ng/mL) was compared by Mann–Whitney *U* test. *P* values ≤ 0.01 (**) or ≤ 0.001 (***) were considered statistically significant

side flanking sequence (AKKV) to the common sequence (SPEEPIYAQV), showed lower reactivity than the peptide c8 (Fig. 6a, b). Therefore, similar to c7, all or part of AKKV might be responsible for increasing the reactivity of EPIYAQV of c8. Taking together, these results reveal that the EPIYA-motifs and QV of peptides c7 and c8, together with the common flanking amino acid sequences xKKx might be important for strong reactivity.

To understand the importance of each amino acid in the c8 peptide more extensively, chimeric peptides were generated (Fig. 6c). At first, the replacement of c8-specific sequence QVAKKV with c12-specific TIDFDE (negative switching) reduced its reactivity. Starting on this c8-c12 chimera, two more chimeric peptides c8-12a (replacing the QV of c8 with c12 specific TI) and c8-12b (replacing the AKKV of c8 with c12 specific DFDE) were tested by the same set of sera to understand the significance of QV and xKKx parts separately. Both the chimeric peptides recovered the reactivity depending on serum samples (Fig. 6d). From these results, we determined that AKKV or both QV and AKKV of c8 are important for the c8-peptide reactivity on 9 cases out of 11 serum samples. Moreover, the replacement of the c12-specific right side sequence TIDFDE with c8-specific sequence QVAKKV, c12-8 peptide (positive switching) showed almost fully recovered reactivity same as c8 peptide suggesting that the EPIYA-motif, together with the right side flanking sequence (QVAKKV) is important for the strong reactivity. Finally, the right side sequence of the c8 peptide (QVAKKV) was switched with the right side sequence of the c7 (QVNKKK) (the other positive switching) (Fig. 6d). This c8-c7 chimeric peptide was again very reactive, showing right ends of c7 and c8 are replaceable, supporting the correlated reactivity of c7 and c8 (Supplementary Fig. S4). Therefore, these chimeric peptide experiments support the idea of a common reactive amino acid sequence xxxE-PIYAQVxKKx (Fig. 6e) suggesting both QV and KK located to the right side of the EPIYA-A and EPIYA-B are essential amino acid residues for strong CagA-specific antibody binding.

Discussion

In this study, we reported several peptides strongly detected by specific antibodies from P group sera rather than N or E group sera. The peptides c7 and c8 were the two peptides located at the C-terminal regions containing the

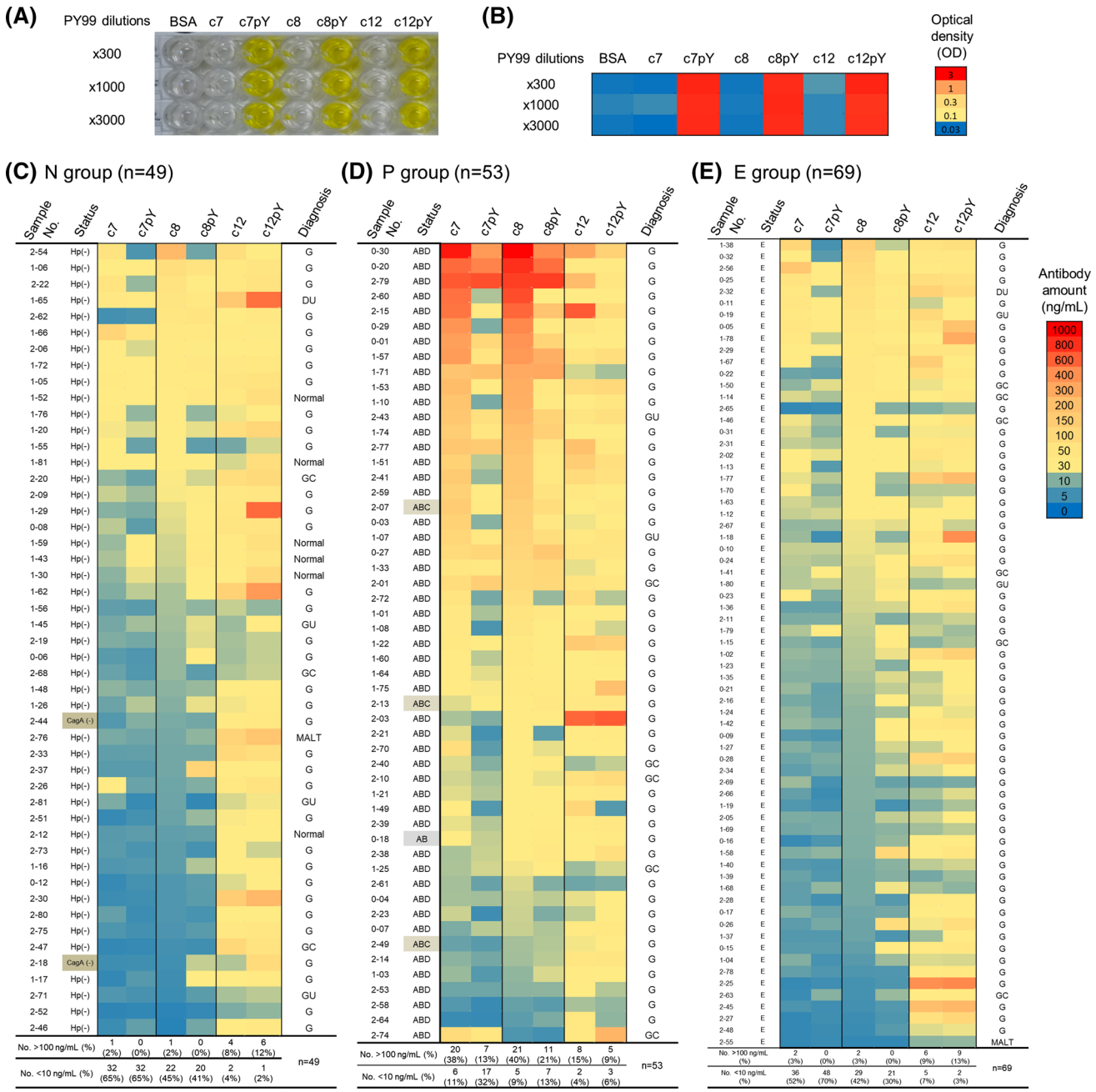

Fig. 4 Reactivity of phosphorylated peptides and amounts of IgG antibody detected by c7, c8, and c12 peptides as well as by their phosphorylated counterparts (c7pY, c8pY, and c12pY) in N, P, and E group sera. **a** The ELISA wells showing the reactivity of the BSA; bovine serum albumin (as negative control), peptides c7, c8, and c12 together with their phosphorylated counterparts (c7pY, c8pY and c12pY) as detected by the PY99 antibody in 3 dilutions (× 300, × 1000, and × 3000). **b** The reactivity represented by the color gradient as heat map with 3 dilutions of PY99 antibody. **c** Antibody amount detected from serum samples in N group. **d** In P group, and **e** E group. In the column of status in **c**, the two cases with tan color (no. 2–44 and no. 2–18) represent the *cagA*-negative *H. pylori*-infected cases. In the column of status in **d**, CagA-type shows the cases infected with *H. pylori* harboring CagA type (ABD/ABC/AB). The columns of diagnosis in **c**–**e** are G-gastritis; DU-duodenal ulcer; GU-gastric ulcer; GC-gastric cancer; MALT-MALT lymphoma; and Normal-histologically normal individual

well-known EPIYA-motifs, EPIYA-A and EPIYA-B, respectively, which could most efficiently detect antibodies in patient sera, identifying the region constituting in peptides c7 and c8 as one of the major immunogenic regions of CagA (Figs. 2, 3). So this is the first report that describes the EPIYA-motif regions as major immunogenic region.

In addition to the c7 and c8 peptides in this study using Japanese adult sera, we also replicated the m8 and m24

Fig. 5 Amino acid variation in the c7 and c8 peptide sequences identified from the strains with East Asian (ABD) type of CagA isolated from the corresponding patients of *H. pylori*-positive serum samples. The uppermost thick black horizontal lines represent the amino acid sequences constituted in peptide c7 and c8, respectively

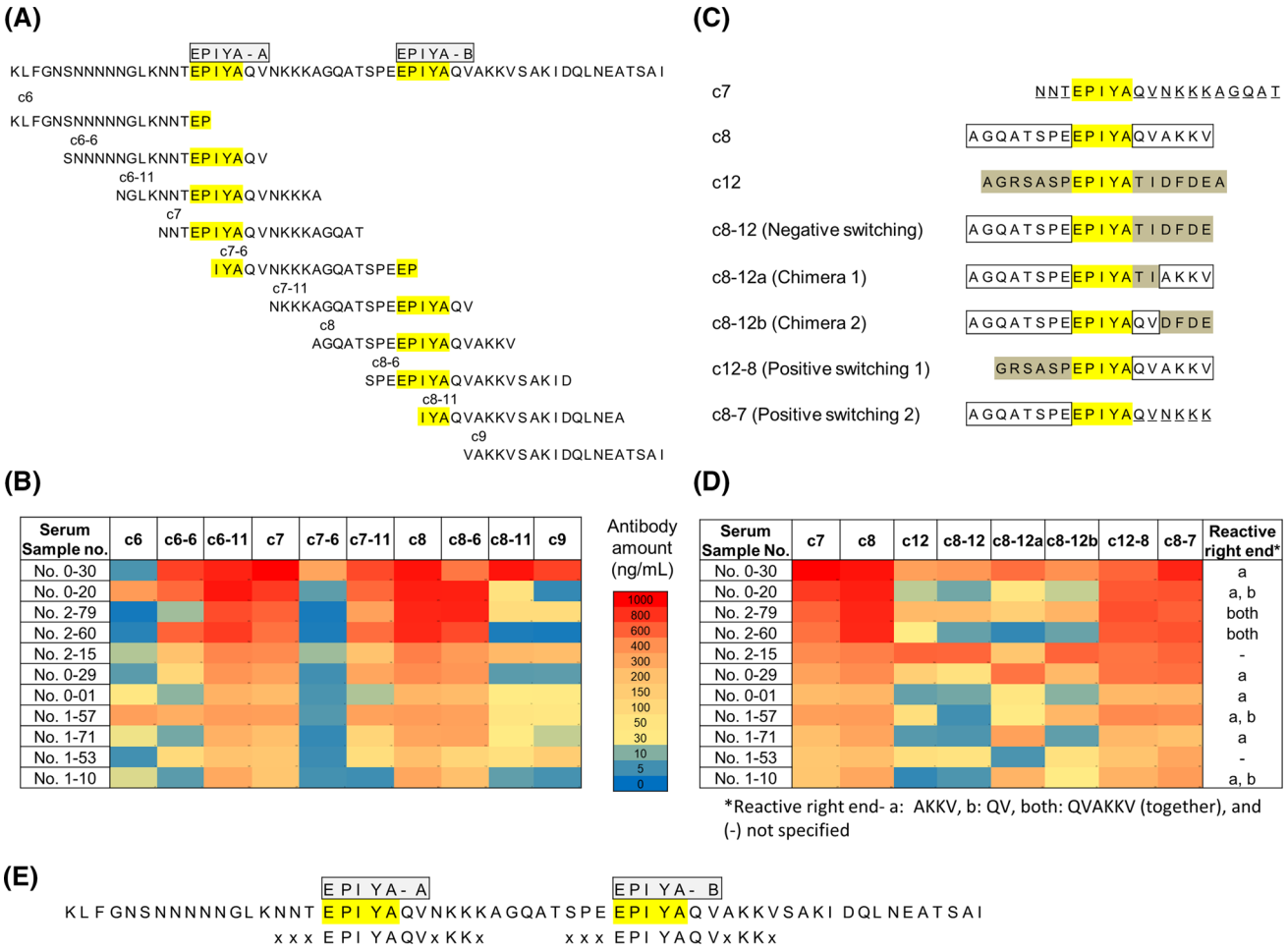

Fig. 6 Determination of immunogenic epitope region of CagA peptides c7 and c8. **a** The sequences of c7 and c8 neighbor peptides series and their reactivity in heat map. Four to five amino acids slipped peptides located between c6 and c9 of CagA consensus sequences were prepared. **b** Amount of antibody detected against different peptides in 11 representative sera presented as heat map below. **c** Chimeric peptides such as c8-12, c8-12a, and c8-12b replacing the QVAKKV, QV, and AKKV of c8 with c12 specific TIDFDE, TI, and DFDE, respectively. In peptide c12-8 the c12 specific TIDFDE is replaced with c8 specific QVAKKV and in peptide c8-7 the c8 specific QVAKKV is replaced with c7 specific QVNKKK to make positive switching 1 and 2, respectively. **d** The amount of antibody in same set of 11 representative sera presented as the color gradient of heat map. The reactive right end were judged if c8-12a or c8-12b was more reactive than c8-12, indicated as 'a' or 'b'; if both short chimeric peptides were less reactive than c8-12, both of QV and AKKV may be necessary, indicating "both". If c8-12 showed higher reaction than c8-12a and c8-12b the judgment was not specified indicating '–'. **e** CagA consensus sequences and the estimated epitope amino acid sequences of c7 and c8 peptides. The x is non-specific amino acid, but EPIYAQV and KK are common between c7 and c8 peptides

peptide reactivity; however, weakly but not strongly (Fig. 3). This result might be due to four reasons. (1) Used peptide mapping method. Previously peptides were covalently-immobilized via C-terminally added three cysteine residues on diamond like carbon (DLC)-chip. This time, we used a plate suitable for peptide ELISA (Nunc Immobilizer Amino plate) which was able to immobilize peptides covalently via N-terminal amino bases to the surface

of plate, so opposite direction of peptide immobilization may affect the reactivity of m8 and m24 peptides. (2) Used peptide sets. In the previous study, we used a set of peptides only from middle region of CagA, but in this study we used all set of CagA peptides. So, the previous work might emphasize middle-region reactive peptides by missing the most reactive C-terminus region peptides. (3) High amino acid sequence variation in m8 peptide among strains (Supplementary Appendix S3). When we checked the CagA amino acid sequence variation from 108 strains isolated in Oita University Hospital before we prepared all the peptides, although m24 (QKITDKVDNLNQAVSETKL) has very few variation along itself 19-mer peptide including East-Asian CagA specific E at the 16th position, m8 (RDLEDKLWAKGLSPQEANK) contains East-Asian CagA specific and also varied highly at the 8th position amino acid, W at most common, but with many substitutions. So, the variation of middle parts of peptide may reduce the serum reactivity to one type of immobilized m8 peptide. 4) The different age samples. Adult serum samples may be different in the reactivity to epitope peptides of CagA from child serum samples along the time of *H. pylori* chronic infection. However, more studies are needed to confirm these possibilities, especially about the 4th possibility.

The c7 and c8 reactive antibodies were the most highly detected anti-CagA antibodies in this epitope-mapping method; however, there should be other CagA antibodies not detected by this method, but detected by the CagA protein itself as high reactivity in CagA ELISA (Table 1). We can estimate that polyclonal human CagA antibodies may possess affinity towards many conformational epitopes, such as surface epitopes of CagA. The crystal structure of CagA from the reference strain 26695 has been previously reported and X-ray crystallography of a fragment of the CagA N-terminus revealed that the region has a structure comprised of three discrete domains [36]. The intrinsically disordered C-terminal region potentiates the pathogenic scaffold/hub function of CagA, mainly via EPIYA-repeat regions. The disordered nature of the C-terminal region CagA may fit to peptide mapping analysis to identify amino acid sequence-based epitopes more than structure-based epitopes on the surface of rigid parts of CagA. Moreover, the C-terminal region of CagA can be easily separated from structural N-terminal domains [22, 28, 37, 38]. Immunogenicity of different regions of CagA may differ based on its degree of presentation by antigen-presenting cells. The EPIYA-A- and –B-containing epitope peptides identified in this study may be involved in inflammation-related CagA pathogenicity during *H. pylori* infection.

Interestingly, the EPIYA-A and EPIYA-B areas were less antigenic in the tyrosine-phosphorylated state (EPIpYA) than its native state (EPIYA) in P group sera (Supplementary Table S1). The reduced antibody reactivity by tyrosine-phosphorylation in EPIYA indicates that the tyrosine is included in the epitopes of the c7- and c8-specific CagA antibody. Our narrow-down experiments revealed that the EPIYA and the surrounding sequences (xxxEPIYAQVxKKx) are important reactive epitopes, as they represent a common sequence of c7 and c8 peptides (Fig. 6e). The sequence was also conserved in c7 and c8 region of CagA protein in *H. pylori* strains isolated from the corresponding patients (Fig. 5). Since EPIYA-D containing peptide c12 was not as antigenic as c7 and c8 (Supplementary Table S1), in addition to the EPIYA, the surrounding sequences QVxKKx, must be regarded as a part of the c7 and c8 epitopes.

Post-translational modifications such as phosphorylation of peptide antigens have been found to alter the ability of T-cells to recognize the major histocompatibility complex (MHC) molecules [32], playing an important role in the immune response. In the current study, we measured the phosphorylated peptide (c7pY, c8pY, and c12pY)-specific antibodies, and found that c7pY and c8pY were less antigenic than c7 and c8 in P group sera. This observation indicates that after injection into gastric epithelial cells, the phosphorylated CagA may not be well recognized by the immune system. Until now, mouse experiments have demonstrated that the coccoid form of *H. pylori* is phagocytosed by dendritic cells (DCs) in Peyer's patches of the small intestine, resulting in Th1-type T-cell recruitment to the stomach and subsequent gastritis [39]. In this case, CagA antigen must be presented by DCs in its non-phosphorylated form together with other *H. pylori* antigens; therefore, our observation is supportive of this result. However, it is remarked that we occasionally observed higher levels of c7pY, c8pY, and c12pY-specific antibodies than those of the c7, c8, and c12-specific antibodies (Fig. 4c–e).

In addition to these characteristics, however, c12pY, the phosphorylated version of EPIYA-D containing peptide, was antigenic in approximately 12% of N group sera (Supplementary Table S1). Previously, the phosphorylated CagA-specific antibody titer (actually against EPIYA-D area) were analyzed and found a positive association with high gastritis scores in corpus in Japanese patients [40]. We did not analyze such association between anti-EPIpYA-D antibody and gastric diseases in this study. However, the sporadic reactivity of the c12pY to *H. pylori*-negative sera found in this study may suggest the presence of cross-reacting autoimmune antibodies in these sera.

In conclusion, the results depict the capabilities of the peptides c7 and c8 constituting EPIYA-A and EPIYA-B motifs, respectively, to detect the specific-IgG from Japanese patients sera. The EPIYA-motifs, together with the

amino acid structure of neighbor-motif (xxxE-PIYAQVxKKx), were essential for the immunogenicity of EPIYA-containing peptides c7 and c8. The peptides with small sequences could only detect the specific antibodies, whereas the combination of several reactive peptides could further improve the detection capabilities of ELISA-based diagnostic methods.

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Compliance with ethical standards

Conflict of interest The authors have declared that no conflict of interest exists.

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