Kobe J. Med. Sci., Vol. 63, No. 2, pp. E58-E67, 2017

Associations between *cagA*, *vacA*, and the clinical outcomes of *Helicobacter pylori* infections in Okinawa, Japan

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Received 19July 2017 / Accepted 25 August 2017

Key words: Helicobacter pylori, CagA, VacA, Okinawa

***Helicobacter pylori*, which is involved in the pathogenesis of gastroduodenal disease, produces CagA and VacA as major virulence factors. CagA is classified into East Asian and Western types based on the number and sequences of its Glu-Pro-Ile-Tyr-Ala motifs. The *vacA* gene has three polymorphic regions: the signal (s), intermediate (i), and middle (m) regions. The lowest gastric cancer mortality rate is seen in Okinawa. On the Japanese mainland (Honshu), most *H. pylori* produce s1/m1-VacA, which exhibits strong toxicity, and East Asian-type CagA. However, the *H. pylori* detected in Okinawa produces s1/m2-VacA, which exhibits weak toxicity, or s2/m2-VacA, which is non-toxic, and Western-type CagA. Studies about the i-region of *vacA* have been performed around the world, but there have been few such studies in Japan. Therefore, the aim of this study was to assess the relationships between the clinical outcomes of *H. pylori* infections in Okinawa, *vacA* (especially the i-region genotype), and *cagA*. *H. pylori* strains that were collected from patients with gastric cancer or gastric ulcers in Okinawa only produced the i1-type VacA virulence factor. The vacuolating cytotoxin activity of i1-type VacA was stronger than that of i2-type VacA, suggesting that the i-region genotype of *vacA* is closely associated with vacuolating cytotoxin activity. These results indicate that the i-region genotype of *vacA* is a useful marker of both *H. pylori* virulence and the clinical outcomes of *H. pylori* infections in Okinawa, Japan.**

INTRODUCTION

Helicobacter pylori, which was discovered in 1983, is involved in the pathogenesis of various digestive diseases, such as gastritis, peptic ulcers, mucosa-associated lymphoid tissue lymphoma, and gastric cancer (GC). *H. pylori* produces multiple virulence factors, with the major ones being CagA and VacA. The *cagA* gene is one of the genes found in the cag pathogenesis island (cagPAI), and CagA is directly injected into epithelial cells via the bacterial type IV secretion system and then undergoes tyrosine phosphorylation. CagA contains Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs, which repeat several times in the C-terminal region (1,2), and the tyrosine phosphorylation of CagA occurs at the EPIYA motifs (3-5). CagA is classified into the East Asian (EA) and Western (W) types based on the number and sequence of its EPIYA motifs; EA-type CagA includes EPIYA-A, EPIYA-B, and EPIYA-D, while W-type CagA includes EPIYA-A, EPIYA-B, and one or more EPIYA-C motifs (1).

In 1988, it was reported that a vacuolating cytotoxin that was found in *H. pylori* culture solution denatured some cancer cell lines (6). Cover et al. identified the vacuolating cytotoxin as the VacA protein in 1992 (7), and other research groups reported the base sequence of the *vacA* gene (8-11). The *vacA* gene of *H. pylori* strain ATCC49503 encodes a 1,287-amino acid protoxin, which undergoes cleavage of its 33-amino-acid amino-terminal signal sequence and carboxy-terminal proteolytic processing to yield a mature secreted toxin of about 87-kDa, which consists of 821 amino acids (12). Mature VacA undergoes cleavage into a 37-kDa N-terminal fragment (p37) and a 58-kDa C-terminal (p58) fragment (12). p37 causes vacuole formation after entering cells (13, 14), and p58 is involved in the binding of the toxin to target cells and its subsequent invasion

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of the target cells (15). The *vacA* gene has three polymorphic regions: the signal (s), intermediate (i), and middle (m) regions (16, 17). The s-region, which is included in p37, encodes part of the VacA signal peptide. On the contrary, the m-region encodes part of p58 (16). The s-region of *vacA* is divided into s1 (s1a, s1b, and s1c) and s2 genotypes (16). s1-type VacA is secreted immediately and in greater quantities than s2-type VacA, and s1-type VacA has been suggested to be associated with peptic ulcers (16). In addition, the m-region, which is composed of about 300 amino acids, is classified into m1 (m1a and m1b) and m2 genotypes (16). m1-type VacA, but not m2-type VacA, induces vacuolation in HeLa cells. Therefore, the differences between the amino acid sequences of the m1 and m2 genotypes are related to the cell specificity of the toxin (17).

In Europe and America, it has been reported that *H. pylori* possessing s1/m1-type *vacA* were found in patients who had a history of peptic ulcers (16), but in Japan no relationship between s1/m1-type *vacA* and peptic ulcers has been detected (18). In 2007, the i-region was reported as a new polymorphic site in the *vacA* gene (19). The i-region is located between the s- and m-regions of *vacA* and is composed of different combinations of 3 clusters (A, B, and C) (19). The i-region is classified into the i1 and i2 genotypes according to the combination of clusters present. Strains with the i1 genotype are strongly associated with GC and vacuolating cytotoxin activity (19). Chung et al. detected the i3 genotype, in which cluster B or C is i1-like, and the other cluster in this pair is i2-like (20).

Most of the *H. pylori* strains collected in Japan produced s1/m1-type VacA, which exhibits strong toxicity, and EA-type CagA. However, most of the *H. pylori* strains collected in Okinawa, Japan, produce s1/m2-type VacA, which displays weak toxicity, or s2/m2-type VacA, which is non-toxic, and W-type CagA (18, 21, 22). Studies about the i-region of *vacA* have been performed worldwide, but few such studies have been conducted in Japan. Previously, it was reported that Okinawa has the lowest GC mortality rate in Japan (23, 24). In 2014, the GC mortality rate in Okinawa was 16.4 deaths/100,000 people, whereas the mean GC mortality rate in Japan was 37.7 deaths/100,000 people (25). Therefore, the aim of this study is to assess the relationships between the clinical outcomes of *H. pylori* infections, *vacA* (especially the i-region genotype), and *cagA* in Okinawa, Japan.

MATERIALS AND METHODS

H. pylori strains

One hundred and thirty-nine clinical *H. pylori* strains from Okinawa (Okinawa Chubu Hospital), Japan, were included in this study. The strains were isolated from patients with GC (N=34, 24.4%), gastric ulcers (GU; N=19, 13.7%), duodenal ulcers (DU; N=26, 18.7%), or chronic gastritis (CG; N=60, 43.2%). All patients gave written informed consent for use of their samples for the present study.

H. pylori culture conditions

The gastric biopsy specimens obtained from each patient were inoculated onto a Trypticase soy agar (TSA)-II/5% sheep blood plate and cultured for 3 days at 37ºC under microaerobic conditions (5% O_2, 5% CO_2, and 90% N_2). A single colony was collected from each primary culture plate, inoculated onto a fresh TSA-II plate, and then was cultured under the same conditions. *H. pylori* was harvested from each plate, transferred into Brucella broth liquid culture medium (BBL Microbiology Systems, Cockeysville, MD, USA) containing 10% fetal calf serum (FCS), and cultured for 24 hr with agitation under the same conditions. Some of the bacterial suspension was stored at -80ºC in phosphate-buffered saline (PBS) containing 20% glycerol. The DNA of each *H. pylori* strain was extracted from a pellet derived from the bacterial suspension using TE buffer consisting of 10 mM Tris-HCl (pH 8.0) and 1 mM ethylenediaminetetraacetic acid and was then stored at 4ºC, before being amplified via the polymerase chain reaction (PCR).

PCR amplification and typing of *vacA*

The primers used for the PCR amplification and typing of the s-, i-, and m-regions of *vacA* are shown in Table I. The amplification conditions were as follows: 94ºC for 1 min; 25 cycles of 94ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 5 min; followed by 72ºC for 10 min. The PCR products were separated by 1.2% agarose gel electrophoresis and examined under ultraviolet (UV) illumination. The PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The typing of the s and m regions of *vacA* were performed according to the method of Ito et al. (18), and the i-region was typed according to the method of Rhead et al. (19). Twelve Okinawa strains (OK107, OK139, OK144, OK158, OK160, OK180, OK181, OK185, OK187, OK204, OK205, and OK210), whose *vacA* sequences were reported previously, were also used in this study (GenBank accession numbers: AB190968, AB190974, AB190975, AB190977, AB190979, AB190981, AB190982, AB190983, AB190984, AB190986, AB190987, and AB190988, respectively).

Nucleotide sequence of the 3' region of *cagA*

The primers used for the PCR amplification and direct sequencing of the 3' region of *cagA* are shown in Table I. The amplification conditions were as follows: 94°C for 2 min; 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min; followed by 72°C for 10 min. The PCR products were separated by 1.5% agarose gel electrophoresis and examined under UV illumination. The PCR products were purified as described above. The direct DNA sequencing was performed using a BigDye terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems 3130 genetic analyzer (Applied Biosystems) according to the manufacturer's recommendations. The amino acid sequences of each gene were constructed and translocated from the nucleotide sequence, and then were aligned and analyzed using GENETYX version 7.0 (Software Development Co., Ltd., Tokyo, Japan).

Table I. Oligonucleotide primers used for the PCR analysis and DNA typing of *H. pylori cagA* and *vacA*.

Gene	Region	Primer	Sequence (5' to 3')	Reference
cagA				
		C2(+)	GAATTGTCTGATAAACTTGAAA	26
		C3(-)	GCGTATGTGGCTGTTAGTAGCG	
vacA				
		VAS-1F	AGCCGATAGCATCAGAGAAGAAC	18
		VAS-11R	TGTGGTGTATGCGTTGTAGGGGTT	
	s1a	vacA s1a-F	CTCTCGCTTTAGTAGGAGC	18
		VA1-R	CTGCTTGAATGCGCCAAAC	
	s1b	SS3-F	AGCGCCATACCGCAAGAG	18
		VA1-R	CTGCTTGAATGCGCCAAAC	
	s1c	vacA s1c-F	CTCTCGCTTTAGTGGGGYT	27
		VA1-R	CTGCTTGAATGCGCCAAAC	
	s2	SS2-F	GCTAACACGCCAAATGATCC	18
		VA1-R	CTGCTTGAATGCGCCAAAC	
	i1	VacF1	GTTGGGATTGGGGGAATGCCG	19
		VacA-C1R	TTAATTTAACGCTGTTTGAAG	
	i2	VacF1	GTTGGGATTGGGGGAATGCCG	19
		vacC2R	GATCAACGCTCTGATTTGA	
	m1a	VA3-F	GGTCAAAATGCGGTCATGG	18
		VA3-R	CCATTGGTACCTGTAGAAAC	
	m1b	VAm-F3	GGCCCCAATGCAGTCATGGAT	18
		VAm-R3	GCTGTTAGTGCCTAAAGAAGCAT	
	m2	VA4-F	GGAGCCCCAGGAAACATTG	18
		VA4-R	CATAACTAGCGCCTTGCAC	

Preparation of VacA

H. pylori strains were cultured at 37°C in Brucella broth liquid culture medium containing 10% FCS. The broth cultures were incubated in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2 on a gyratory shaker at 120 rpm for 72 hr. In order to count the number of *H. pylori*, the absorbance of the culture supernatants was measured at a wavelength of 560 nm (OD_{560}) using a NanoDrop ND-1000 spectrophotometer V3.2. (NanoDrop Technologies, Wilmington, DE, USA), and OD_{560} values ranging from 0.3 to 0.45 were recorded. The cultures were centrifuged at 6,000 × g for 15 min, and the supernatants were sterilized by passing them through a filter (pore size: 0.45 µm) (Minisart; Sartorius Hannover, Germany). The filtered culture supernatants were stored at -20°C. VacA from *H. pylori* ATCC49503 (GenBank accession number: HPU05676) was also prepared and used as a positive control for the Western blotting and neutral red dye uptake (NRU) measurements, which are described below.

Western blotting

Western blotting was used to confirm the presence of VacA in the samples used by NRU measurements. For electrophoresis, 20 µL of the VacA solution were loaded onto 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and then run at 80 mA. After gel electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P membranes; Millipore Bedford, MA, USA) at 100 V for 1 hr. The polyclonal anti-VacA antibody was kindly provided by Dr. T. Hirayama (Nagasaki University, Nagasaki, Japan). The Western blotting was performed as described previously (28), and enhanced chemiluminescent reagents were used to visualize the secondary antibody.

NRU

The human gastric adenocarcinoma cell line AZ-521 (Culture Collection of Human Science Research Resources Bank, Japan Health Science Foundation) was grown in Eagle's minimal essential medium (EMEM) containing 10% FCS and 1% antibiotic/antimycotic solution under a 5% CO_2 atmosphere at 37ºC. The AZ-521 cells were seeded into 96-well culture plates (2×10^4 cells in 100 µL/well; Corning Glass Works, Corning, NY, USA) and then were cultured as monolayers for 24 hr under a 5% CO_2 atmosphere at 37ºC. After transferring the culture supernatant to 80 µL of FCS-free EMEM, the cells were treated with 20 µL of VacA solution and incubated for 6 hr at 37ºC. To quantify vacuolating activity, the uptake of neutral red into the vacuoles of the VacA-treated cells was determined as described by Cover et al. (29). The cells were incubated with 50 µL of freshly prepared 0.05% neutral red solution in PBS containing 0.3% BSA for 10 min at room temperature and then were washed twice with 0.1 mL of PBS containing 0.3% BSA. After the addition of 0.2 mL of 70% ethanol in water containing 0.4% HCl, the absorbance of the cells at a wavelength of 540 nm (OD_{540}) was measured (30).

Statistical analysis

Excel 2013 (Statcell ver.4) was used to perform the statistical analyses. The chi-squared (χ^2) test or Fisher's exact probability test was used to analyze the associations between virulence factors and clinical outcomes. The differences in the OD_{540} values produced by NRU among the various *H. pylori* genotypes were analyzed using the Tukey-Kramer test. In all cases, p-values of <0.05 were considered to indicate a significant difference.

RESULTS

Genotypes of *vacA* and their associations with clinical outcomes

First, the genotypes of *vacA* were evaluated on the basis of three polymorphic regions: the s, i, and m regions. Among the 139 *H. pylori* strains that were collected in this study, 111 (79.9%), 6 (4.3%), 10 (7.2%), 2 (1.4%), 7 (5.0%), and 1 (0.7%) possessed the s1/i1/m1-type, s1/i1/m2-type, s1/i2/m2-type, s1/i3/m2-type, s2/i2/m2-type, and s2/i2/m2-m1b hybrid-type *vacA* gene, respectively. Two strains were *vacA*-negative (1.4%). Two strains possessed the i3-type *vacA* gene, and both of these strains had an i1-like cluster B and an i2-like cluster C (Table II).

Table II. Relationships between the s/i/m regions of *vacA* and the clinical outcomes of *H. pylori* infections in Okinawa.

Disease	N	s1/i1/m1	s1/i1/m2	s1/i2/m2	s1/i3/m2	s2/i2/m2	s2/i2/m2-m1b	Negative
GC	34	31	2	0	0	0	0	1
GU	19	19	0	0	0	0	0	0
DU	26	15	2	7	1	1	0	0
CG	60	46	2	3	1	6	1	1
Total	139	111	6	10	2	7	1	2

The numbers of *H. pylori* strains of each *vacA* genotype detected in various diseases are shown. GC: gastric cancer; GU: gastric ulcers; DU: duodenal ulcers; CG: chronic gastritis

All of the strains with s1/m1-type *vacA* genes carried i1-type *vacA* genes, and all of the strains with s2/m2-type *vacA* genes had i2-type *vacA* genes. Every type of i-region was detected among the strains that carried s1/m2-type *vacA* genes; however, none of the examined strains exhibited the s2/m1-type *vacA* genotype (Table II, Table III). Regarding the associations between the various *vacA* genotypes and clinical outcomes, all of the *H. pylori* strains that were collected from the patients with GC or GU had i1-type *vacA* genes. Among the *H. pylori* strains that were collected from the patients with DU, 17 (65.4%), 8 (30.8%), and 1 (3.8%) possessed i1-type, i2-type, and i3-type *vacA* genes, respectively. Regarding the *H. pylori* strains collected from the CG patients, 48 (81.4%), 10 (16.9%), and 1 (1.7%) had i1-type, i2-type, and i3-type *vacA* genes, respectively (Table IV). The s-region of *vacA* was not found to be related to the clinical outcomes of *H. pylori* infections ($p=0.063$), but the m- and i-regions were shown to be significantly related to clinical outcomes ($p<0.05$).

Genotypes of *cagA* and their associations with the i-region of *vacA*

Of the 139 *H. pylori* strains examined in this study, it was possible to type 133 for *cagA*, and the clinical outcomes of these 133 strains were as follows: GC: 34 (25.6%), GU: 16 (12.0%), DU: 26 (19.5%), and CG: 57 (42.9%) (Table V). In total, 105 (79.0%) *H. pylori* strains possessed EA-type *cagA* genes, and 20 (15.0%) had W-type *cagA* genes. There were 8 (6.0%) *cagA*-negative strains (Table III). Two *vacA*-negative *H. pylori* strains possessed EA-type *cagA* genes. Among the 133 *H. pylori* strains in which it was possible to type *cagA*, the i-region of the *vacA* gene was classified as i1, i2, i3, and *vacA*-negative in 102 (97.1%), 1 (1.0%), 0 (0.0%), and

2 (1.9%) of the *H. pylori* strains with EA-type *cagA* genes, respectively; 7 (35.0%), 11 (55.0%), 2 (10.0%), and 0 (0.0%) of the *H. pylori* strains with W-type *cagA* genes, respectively; and 2 (25.0%), 6 (75.0%), 0 (0.0%), and 0 (0.0%) of the *cagA*-negative *H. pylori* strains, respectively (Table III).

Table III. Genotypes of the i-region of *vacA* and *cagA* in *H. pylori* strains isolated in Okinawa.

		vacA i-region genotype (no. of strains)			Total
	Gene	i1	i2	i3	
vacA	s1/m1	111	0	0	111
	s1/m2	6	10	2	18
	s2/m2	0	8	0	8
	Total	117	18	2	137

		vacA i-region genotype (no. of strains)			*vacA*-negative	Total
	Gene	i1	i2	i3		
cagA	East Asian	102	1	0	2	105
	Western	7	11	2	0	20
	Negative	2	6	0	0	8
	Total	111	18	2	2	133

Among the 139 *H. pylori* strains, 137 were *vacA*-positive, and the i-region genotypes of the *vacA* genes detected in the 137 *vacA*-positive *H. pylori* strains are shown in the upper table. In the lower table, the results for 133 *H. pylori* strains in which it was possible to type *cagA* are shown. s2/i2/m2-m1b *vacA* is included in s2/i2/m2 *vacA*.

Table IV. Relationships between the *vacA* s-, m-, i-region genotypes of *H. pylori* strains isolated in Okinawa and the clinical outcomes of *H. pylori* infections.

Clinical outcome	*vacA* s-region genotype (no. of strains)		Total
	s1	s2	
GC	33	0	33
GU	19	0	19
DU	25	1	26
CG	52	7	59
Total	129	8	137

Clinical outcome	*vacA* m-region genotype (no. of strains)		Total
	m1	m2	
GC	31	2	33
GU	19	0	19
DU	15	11	26
CG	46	13	59
Total	111	26	137

Clinical outcome	*vacA* i-region genotype (no. of strains)			Total
	i1	i2	i3	
GC	33	0	0	33
GU	19	0	0	19
DU	17	8	1	26
CG	48	10	1	59
Total	117	18	2	137

For the 137 *vacA*-positive *H. pylori* strains, the numbers of *H. pylori* strains that exhibited each *vacA* genotype in various diseases are shown. GC: gastric cancer; GU: gastric ulcers; DU: duodenal ulcers; CG: chronic gastritis

Table V. Associations among clinical outcomes, *cagA*, and the i-region genotype of *vacA*.

(A) GC

		vacA							Total
		s1/i1/m1	s1/i1/m2	s1/i2/m2	s1/i3/m2	s2/i2/m2	s2/i2/m2-m1b	Negative	
cagA	East Asian	29	2	0	0	0	0	1	32
	Western	2	0	0	0	0	0	0	2
	Negative	0	0	0	0	0	0	0	0
	Total	31	2	0	0	0	0	1	34

(B) GU

		vacA							Total
		s1/i1/m1	s1/i1/m2	s1/i2/m2	s1/i3/m2	s2/i2/m2	s2/i2/m2-m1b	Negative	
cagA	East Asian	16	0	0	0	0	0	0	16
	Western	0	0	0	0	0	0	0	0
	Negative	0	0	0	0	0	0	0	0
	Total	16	0	0	0	0	0	0	16

(C) DU

		vacA							Total
		s1/i1/m1	s1/i1/m2	s1/i2/m2	s1/i3/m2	s2/i2/m2	s2/i2/m2-m1b	Negative	
cagA	East Asian	13	1	1	0	0	0	0	15
	Western	2	1	6	1	0	0	0	10
	Negative	0	0	0	0	1	0	0	1
	Total	15	2	7	1	1	0	0	26

(D) CG

		vacA							Total
		s1/i1/m1	s1/i1/m2	s1/i2/m2	s1/i3/m2	s2/i2/m2	s2/i2/m2-m1b	Negative	
cagA	East Asian	39	2	0	0	0	0	1	42
	Western	2	0	3	1	2	0	0	8
	Negative	2	0	0	0	4	1	0	7
	Total	43	2	3	1	6	1	1	57

For the 133 *H. pylori* strains in which it was possible to type *cagA*, the numbers of *H. pylori* strains that exhibited each *vacA* genotype in various diseases are shown. GC: gastric cancer; GU: gastric ulcers; DU: duodenal ulcers; CG: chronic gastritis

Associations between clinical outcomes, *cagA*, and the i-region of *vacA*

All of the *H. pylori* strains that were collected from the patients with GC exhibited i1-type *vacA* genes, and 31 (91.2%), 2 (6.1%), and 1 (2.9%) were EA-type *cagA*, W-type *cagA*, and *cagA*-negative strains, respectively. Regarding the *H. pylori* strains collected from the patients with GU, only i1-type *vacA* and EA-type *cagA* genes were detected in these strains. Of the *H. pylori* strains that were collected from the patients with DU, 15 (57.7%) possessed EA-type *cagA* genes, 10 (38.5%) had W-type *cagA* genes, and 1 (3.8%) was *cagA*-negative. The i-region of *vacA* was classified as i1, i2, and i3 in 14 (93.3%), 1 (6.7%), and 0 (0%) of the *H. pylori* that possessed EA-type *cagA* genes, respectively; 3 (30.0%), 6 (60.0%), and 1 (10.0%) of the *H. pylori* strains with W-type *cagA* genes, respectively; and 0 (0%), 1 (100.0%), and 0 (0%) of the *cagA*-negative *H. pylori* strains. Of the *H. pylori* strains collected from the patients with CG, 42 (73.7%) possessed EA-type *cagA* genes, 8 (14.0%) had W-type *cagA genes*, and 7 (12.3%) were *cagA*-negative. In addition, the i-region of *vacA* was classified as i1, i2, i3, and *vacA*-negative in 41 (97.6%), 0 (0.0%), 0 (0.0%), and 1 (2.4%) of the *H. pylori* strains that possessed EA-type *cagA* genes, respectively; 2 (25.0%), 5 (62.5%), 1 (12.5%), and 0 (0.0%) of the *H. pylori* strains with W-type *cagA* genes, respectively; and 2 (28.6%), 5 (71.4%), 0 (0%), and 0 (0.0%) of the *cagA*-negative *H. pylori* strains, respectively (Table V). Taken together, the Okinawa *H. pylori* strains collected from GC and GU were

only i1-type *vacA* genes, although statistical analyses could not be performed due to the biased sample data. In the *H. pylori* strains collected from DU and CG, the significant relation between the genotypes of *cagA* and *vacA* ($p<0.05$) were observed, so it means that the rate of Okinawa *H. pylori* strains with W-type *cagA* genes and m2-type or i2-type *vacA* genes tended to be high in DU and CG.

Evaluation of NRU

Vacuoles induced by VacA form both late endosomes and lysosomes (24), and neutral red is taken up by and accumulates in lysosomes in viable cells. Therefore, NRU is widely used to measure vacuolating cytotoxin activity (20, 24). To compare the vacuolating cytotoxin activity associated with the various types of *vacA* i-regions, we examined NRU in 19 *H. pylori* strains, which consisted of 6 strains with s1/i1/m2-type *vacA* genes; 8 strains with s1/i2/m2-type *vacA* genes; 2 strains with s1/i3/m2-type *vacA* genes; 2 strains with s1/i1/m1-type *vacA* genes; and *H. pylori* ATCC49503, which possesses s1/i1/m1-type *vacA* genes, as a positive control (Figure 1). The presence of VacA in the samples used by this analysis was confirmed by Western blotting. As a result, OD_{540} values of 0.427 ± 0.179, 0.159 ± 0.032, 0.313 ± 0.258, and 0.49 ± 0.164 were obtained for s1/i1/m2-type VacA, s1/i2/m2-type VacA, s1/i3/m2-type VacA, and s1/i1/m1-type VacA, respectively (mean ± SE). The OD_{540} value obtained for *H. pylori* ATCC49503 was 0.67, and s1/i1/m1-type VacA exhibited the highest OD_{540} value, as we expected. There were significant differences between the vacuolating cytotoxin activity of s1/i1/m2-type VacA and s1/i2/m2-type VacA and between that of s1/i1/m1-type VacA and s1/i2/m2-type VacA, but we not detect any such differences between s1/i2/m2-type VacA and s1/i3/m2-type VacA, between s1/i1/m1-type VacA and s1/i1/m2-type VacA, or between s1/i1/m2-type VacA and s1/i3/m2-type VacA.

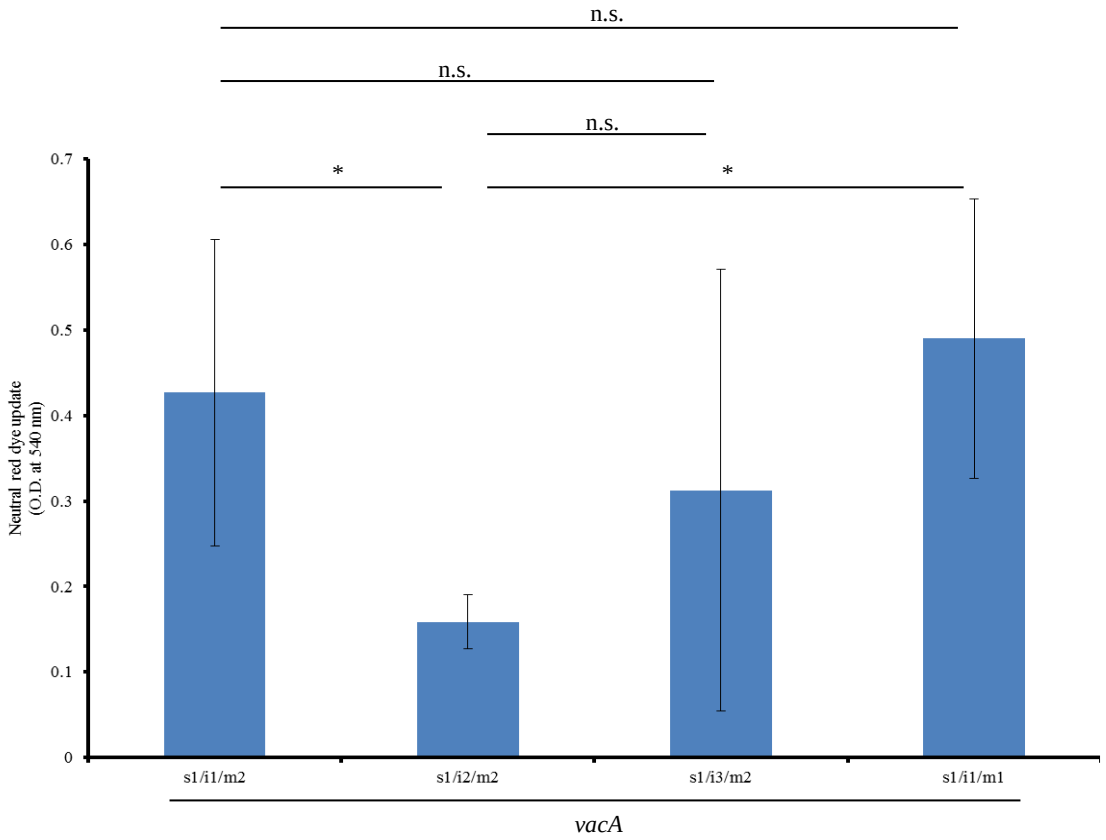

Figure 1. NRU induced by VacA OD_{540} values of 0.427 ± 0.179, 0.159 ± 0.032, 0.313 ± 0.258, and 0.49 ± 0.164 were obtained for s1/i1/m2-VacA, s1/i2/m2-type VacA, s1/i3/m2-type VacA, and s1/i1/m1-type VacA, respectively (mean ± SE). The OD_{540} value of *H. pylori* ATCC49503 (the positive control) was 0.67. The asterisks indicate significant differences ($p<0.05$) according to the Tukey-Kramer test. n.s.: not significant

DISCUSSION

Previously, Yamaoka et al. reported that the s- and m-genotypes of *vacA* are not useful as disease markers in East or Southeast Asia (27, 31-34). Similarly, it was also shown that the i-region of *vacA* is not useful as a disease determinant in these areas (35). In this study, we investigated the relationship between the clinical outcomes of *H. pylori* infections, *vacA* (especially the i-region genotype), and *cagA* in Okinawa, Japan, because Okinawa has the lowest GC mortality rate in Japan (23, 24). In Okinawa, the m- and i-regions, but not the s-region, of *vacA*, were found to be related to the clinical outcomes of *H. pylori* infections. In our previous study (22), it was demonstrated that all of the *H. pylori* strains collected from patients in Fukui, which is a typical prefecture located on the Japanese mainland (Honshu), possessed s1/i1/m1-type *vacA* genes. The *H. pylori* strains collected from Fukui also had EA-type *cagA* genes (22). In this study, the *H. pylori* strains of s1/i1/m1-*vacA* genes and EA-type *cagA* genes in Okinawa were related to GC and GU, and this tendency was also observed in the *H. pylori* strains collected from Fukui. On the other hand, the results from the *H. pylori* strains except GC and GU in Okinawa were different from those of Fukui. The reasons for this might include differences in the geographic characteristics and culture of Okinawa, which is an island located in the southwest of Japan and has a different culinary culture and history from the other areas of Japan. Taking these findings together, the typing of *vacA* and *cagA* might only be useful in Okinawa.

In a study carried out in Iran by Rhead et al., it was reported that most of the *H. pylori* strains with s1/m1-type *vacA* genes had i1-type *vacA* genes, whereas all of the strains with s2/m2-type *vacA* genes had i2-type *vacA* genes, and infection with an i1-type *H. pylori* strain was found to be associated with GC (19). Similarly, in Okinawa all of the *H. pylori* strains that possessed s1/m1-type *vacA* genes had i1-type *vacA* genes, and i1-type *H. pylori* strains were associated with GC. In addition, the i1-type *H. pylori* strains were associated with GU in Okinawa, and infection with an i2-type *H. pylori* strain was very rare among the patients with GC or GU. Therefore, the i1-type of *vacA* might be a risk factor for GC and GU. Most of the *H. pylori* strains with EA-type *cagA* genes had the i1-type of *vacA*, whereas the i1-, i2-, and i3-types of *vacA* were detected in the *H. pylori* strains with W-type *cagA* genes. These results suggest that the EA-type *cagA* genotype is a risk factor for GC. On the contrary, patients that are infected with *H. pylori* strains possessing i2-type *vacA* genes might be at low risk of developing of GC or GU. Regarding vacuolating cytotoxin activity, which was measured via NRU in this study, i1-type VacA exhibited stronger vacuolating cytotoxin activity than i2-type VacA, while there was no significant difference between the vacuolating cytotoxin activity levels of the m1- and m2- types of VacA. These results indicate that the i-region genotype of *vacA* might be closely associated with the degree of vacuolating cytotoxin activity.

In conclusion, the i-region genotype of *vacA* is a useful marker of both *H. pylori* virulence and the clinical outcomes of *H. pylori* infections in Okinawa, Japan, and the typing of the i-region of *vacA* might be helpful for evaluating the toxicity of *H. pylori* and predicting *H. pylori*-related diseases.

ACKNOWLEDGEMENTS

We are very grateful to Yoshihide Keida (Division of Internal Medicine, Okinawa Chubu Hospital, Uruma, Japan) and Toshiya Hirayama (Department of Bacteriology, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan) for the research assistance they provided. None of the authors has any conflicts of interest or any financial ties to disclose.

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