PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Helicobacter pylori cagA, *iceA* and *vacA* genotypes in patients with gastric cancer in Taiwan

Hwai-Jeng Lin, Chin-Lin Perng, Wen-Ching Lo, Chew-Wun Wu, Guan-Ying Tseng, Anna Fen-Yau Li, I-Chen Sun, Yueh-Hsing Ou

Hwai-Jeng Lin, I-Chen Sun, Division of Gastroenterology, Department of Medicine, VGH-TAIPEI, Taiwan, China
Chin-Lin Perng, I-Lan Hospital, Department of health, Taiwan, China
Wen-Ching Lo, Zhongxiao Municipal Hospital, Taipei, Taiwan, China
Chew-Wun Wu, Division of General Surgery, Department of Surgery, VGH-TAIPEI, Taiwan, China
Guan-Ying Tseng, Ton-Yen General Hospital, Hsin-Chu, Taiwan, China
Anna Fen-Yau Li, Department of Pathology, VGH-TAIPEI, Taiwan, China
Yueh-Hsing Ou, Institute of Biotechnology in Medicine, School of Medical Technology and Engineering, and School of Medicine, National Yang-Ming University, Taiwan, China
Supported by VGH 91-274, NSC 91-2314-B075-127
Correspondence to: Professor Hwai-Jeng Lin, Division of Gastroenterology, Department of Medicine, VGH-TAIPEI, Shih-Pai Rd, Sec 2, Taipei, 11217, Taiwan, China. hjlin@vghtpe.gov.tw
Telephone: +886-2-28712121 Ext. 2015 **Fax:** +886-2-28739318
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Abstract

AIM: *Helicobacter pylori* (*H pylori*) has been linked to chronic gastritis, peptic ulcer, gastric cancer and MALT-lymphoma. The link of genotypes of *H pylori* to gastric cancer remains controversial. The aim of this study was to investigate the *H pylori vacA* alleles, *cagA* and *iceA* in patients with gastric cancer in Taiwan.

METHODS: Patients with gastric cancer, peptic ulcer and chronic gastritis were enrolled in this study. We obtained biopsy specimens from the stomach at least 2 cm away from the tumor margin in patients with gastric cancer, and from the antrum of stomach in patients with peptic ulcer or chronic gastritis. DNA extraction and polymerase chain reaction were used to detect the presence or absence of *cagA* and to assess the polymorphism of *vacA* and *iceA*.

RESULTS: A total of 168 patients (gastric ulcer: 77, duodenal ulcer: 66, and chronic gastritis: 25) were found to have positive PCR results of the biopsy specimens from patients with peptic ulcer and chronic gastritis. We found positive *cagA* (139/168, 83%), m2 (84/168, 50%) and *iceA1* (125/168, 74%) strains in the majority of patients. In patients with gastric cancer, the *vacA* s1a and s1c subtypes were less commonly found than those in non-cancer patients (35/66 *vs* 127/168, $P = 0.0001$ for s1a and 13/66 *vs* 93/168, $P < 0.0001$ for s1c). In the middle region, the m1T strain in patients with gastric cancer was more than that of non-cancer patients (23/66 *vs* 33/168, $P = 0.02$).

CONCLUSION: In Taiwan, *H pylori* with positive *vacA* s1a, *cagA* and *iceA1* strains are found in the majority of patients with gastric cancer or non-cancer patients. In patients with gastric cancer, the *vacA* s1a and s1c subtypes are less and m1T is more than in patients with peptic ulcer and chronic gastritis.

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INTRODUCTION

Helicobacter pylori (*H pylori*) is one of the most common bacterial infections of humans[1]. It has been closely linked to chronic gastritis, peptic ulcer, gastric cancer and MALT-lymphoma[2,3]. It has heterogeneous genotypes and phenotypes[4,5].

The International Agency for Research on Cancer of the World Health Organization recommends that *H pylori* be classified as a group I carcinogen [6]. In a long-term follow-up study, gastric cancers developed in 36 (2.9%) of the 1 246 infected and none of the 280 uninfected patients ($P<0.001$)[7]. Enomoto *et al.* and Uemura *et al.* also confirmed that very few patients who had gastric cancer were not infected with *H pylori*[7,8].

The clinical outcome of *H pylori* infection was supposed to be linked to certain strains, *e.g.* vacuolating cytotoxin (*vacA*) and the cytotoxin-associated gene (*cagA*)[9,10]. However, controversy exists concerning the relationship between *H pylori* and gastric cancer. It is now evident that approximately 25-50% of the world's population is infected by *H pylori*. Why, then, did only a minority of them develop gastric cancer? A report of an epidemiological study in Africa suggested that *H pylori* infection did not always directly correlate with the risk for gastric cancer[11]. The same phenomenon occurred in southern Asia[12]. The prevalence of *H pylori* is high in India and Bangladesh, but low gastric cancer rates have been reported. In addition, Louw et al did not find difference in the prevalence of *H pylori* infection when comparing gastric cancer cases with matched controls[13].

How could the above phenomenon be explained? Although *H pylori* infection is very common, there is geographic distribution of different subtypes[14]. Is it possible that different subtypes of *H pylori* cause different outcomes? In Taiwan, specific genotypes of *H pylori* have been found in patients with peptic ulcer or non-ulcer dyspepsia[15,16]. There is no report concerning the genotype of gastric cancer in Taiwan so far. The aim of this study was to determine the *vacA*, *cagA*, and *iceA* genotypes of *H pylori* in patients with gastric cancer as compared with those in patients with peptic ulcer or chronic gastritis in Taiwan.

MATERIALS AND METHODS

Patients with gastric cancer, peptic ulcers (gastric ulcer or duodenal ulcer, at least 5 mm in diameter) or chronic gastritis were invited to enter the study. Patients with pregnancy, bleeding tendency (platelet count less than 50 000/mm^3, prothrombin time less than 30%, or taking anti-coagulants), age under 10, or over 90 years, and inability to cooperate were excluded from the study. The study was approved by the Clinical Research Committee of the Veterans General Hospital, Taipei.

Endoscopic examination and biopsy were performed after informed consent was obtained. In patients with peptic ulcer or chronic gastritis, we took one specimen from the antrum of

each patient for rapid urease test, one specimen from the gastric antrum for DNA extraction and PCR assay. In patients with gastric cancer, we took two specimens from the stomach at least 2 cm from the cancer part for rapid urease test and DNA extraction and PCR assay. Lysates of gastric mucosa biopsy specimens were used for PCR. DNA of gastric biopsy specimens was extracted according to the method described by Boom[17]. Briefly, biopsy specimens were homogenized in guanidinium isothiocyanate, using a sterile micropestle. DNA was extracted, washed and eluted in 100 μL of 10 mnol/L Tris-HCL (pH 8.3). Two microliters of the eluted DNA were used for each PCR reaction.

All pathological samples from patients with gastric cancer were evaluated by a single experienced pathologist, and classified in accordance with the Lauren classification as diffuse, intestinal or mixed types[18]. The description of advanced gastric cancer was based on Borrmann's classification[19]. The morphological subtypes of early gastric cancer were classified according to the guidelines of Japanese Endoscopy Society[20].

Oligonucleotide primers for PCR amplification of specific segments are shown in Table 1[15,21,22]. For *vacA* evaluation, the PCR program comprised 35 cycles of denaturation (at 94 °C for 1 min), annealing (at 56 °C for 2 min, extension at 72 °C for 1 min), and one final extension (at 72 °C for 10 min). For *cagA*, amplification was performed with 35 cycles of denaturation (at 94 °C for 1 min, annealing at 56 °C for 2 min, extension at 72 °C for 1 min), and one final extension (at 72 °C for 5 min). For *iceA* amplification, amplifications were performed with 40 cycles of denaturation (at 95 °C for 30 s), annealing (at 50 °C for 45 s), extension (at 72 °C for 45 s) and one final extension (at 72 °C for 10 min).

The association between *H pylori* genotypes and clinical diseases was determined using the chi-square test with Yates' correction or Fisher's exact test when appropriate. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Patients with peptic ulcer and chronic gastritis

Between January 2002 and Feburary 2003, a total of 278 patients with peptic ulcer or chronic gastritis were included in this study. There were 200 males and 78 females with a mean age of 62.1 years, 95% C.I.: 61.1-64.3 years. One hundred and forty patients had gastric ulcers, 101 patients had duodenal ulcers, 37 patients had chronic gastritis. Among these patients, 168 patients (65.9%) (comprising 25 patients with chronic gastritis, 77 patients with gastric ulcer, and 66 patients with duodenal ulcer) were found to have positive PCR (Table 2). Of them, the urease test was found to be positive in 152 patients (90.5%). There was no significant difference in age of the patients with chronic gastritis (mean: 51 years, 95% CI : 42.8-59.2 years), gastric ulcer (65.3 years, 62.3-68.9), duodenal ulcer (58.9 years, 54.7-63.1) and gastric cancer (69 years, 67-91).

In the s-region, *vacA* s1a was most frequently found (76%, 127/168, $P<0.001$ *vs* s1b, s1c and s2) followed by s1c (93/168, 55%), s1b (9/168, 5%), and s2 (2/168, 1%) (Table 3). In the m-region, m2 was most frequently found (84/168, 50%) ($P<0.0001$ *vs* m1T and m1), followed by m1T (33/168, 20%) and m1 (2/168, 2%). *CagA* was found in 139 patients (83%). *IceA1* was found most commonly in comparison with *ice A2* (125 *vs* 29, $P<0.0001$).

Patients with gastric cancer

A total of 167 patients with gastric cancers were enrolled in this study (mean age: 69 y/o, 95% CI: 67.0-91.0, sex M/F: 130/37). We obtained specimens through endoscopic biopsy from 66 patients and surgery from 101 patients. After PCR assessment of gastric specimens, a total of 66 patients (39.5%) were found to be positive (24 patients from endoscopic biopsy and 42 patients from surgical specimens) (Table 3). We found early gastric cancer in seven patients (type IIc: 2, IIc+III: 5) and advanced gastric cancer in 59 patients (Borrmann type I: 7, II: 28, III: 14, IV: 10) (Table 4).

Among these patients, 35 (53%) were found to have *vacA* s1a ($P<0.001$ *vs* s1c, s1b, and s2), followed by s1c (13 patients, 20%) ($P<0.001$ *vs* s1b and s2) and s1b (1 patient, 2%), s2 (1 patient, 2%) (Table 3). In the m-region, m2 was most commonly found (32 patients, 48%) ($P<0.0001$ *vs* m1) followed by m1T (23 patients, 35%) and m1 (1 patient, 2%). In the *iceA* subtypes, *iceA1* was most commonly found (39 patients, 59%) followed by *ice A2* (15 patients, 23%) ($P<0.0001$). *CagA* was found in 76% (50/66) of the patients.

The genotypes between early gastric cancer and advanced gastric cancer were similar (Table 4). There was no difference of genotypes according to Borrmann's classification (Table 5). Regarding the histological classification, there was no difference of genotypes among the diffuse type, intestinal type and mixed types (Table 6).

Table 1 Oligonucleotide primers used for *cagA*, *vacA* and *iceA* genotyping

Region detected	Primer designation	Primer sequence	Size of PCR product (bp)	References
s1 and s2	VA1-F	5'ATGGAAATACAACAAACACACC3'	259/286	14
	VA1-R	5'CTGCTTGAATGCGCCAAACTTTATC3'		
s1a	SS1-F	5'GTCAGCATCACACCGCAAC3'	190	20
s1b	SS3-F	5'AGCGCCATACCGCAAGAG3'	187	20
s1c	S1C-F	5'CTYGCTTTAGTRGGGYTA3'	213	26
M1	VA3-F	5'GGTCAAAATGCGGTCATGG3'	290	20
	VA3-R	5'CCATTGGTACCTGTAGAAAC3'		
M1T	m1T-F	5'GGTCAAAATGCGGTCATGG3'	290	14
	m1T-R	5'CTCTTAGTGCCTAAAGAAACA3'		
M2	VA4-F	5'GGAGCCCCAGGAAACATTG3'	352	20
	VA4-R	5'CATAACTAGCGCCTTGCAC3'		
iceA1	iceA1F	5'GTGTTTTTAACCAAAGTATC3'	247	21
	iceA1R	5'CTATAGCCASTYTCTTTGCA3'		
iceA2	iceA2F	5'GTTGGGTATATCACAATTTAT3'	229	21
	iceA2R	5'TTRCCCTATTTTCTAGTAGGT3'		
lcagA	lcagAD008	5'ATAATGCTAAATTAGACAACTTGAGCGA3'	297	8
	lcagAR008	5'TTAGAATAATCAACAAACATCACGCCAT3'		

Table 2 Genotypes of *H pylori* in patients with non-gastric cancer (chronic gastritis, gastric ulcer and duodenal ulcer) (%)

Diagnosis	No of Patients	s1a	s1b	s1c	s2	s1a +s1c	s1as +s1b	s1b +s2	s1a+ s1b+s1c	m1	m1T	m2	m1T +m2	cagA	iceA1	iceA2	iceA1 +iceA2
Chronic gastritis	25	21 (84)	0 (0)	14 (56)	0 (0)	11 (44)	0 (0)	0 (0)	0 (0)	0 (0)	8 (32)	12 (48)	2 (8)	22 (88)	22 (88)	2 (8)	0 (0)
Gastritic ulcer	77	59 (77)	1 (1)	42 (55)	0 (0)	36 (47)	0 (0)	0 (0)	0 (0)	0 (0)	11 (14)	45 (58)	1 (1)	63 (82)	54 (70)	17 (22)	3 (4)
Duodenal ulcer	66	47 (71)	8 (12)	37 (56)	2 (3)	30 (45)	7 (11)	2 (3)	4 (6)	2 (3)	14 (21)	27 (41)	7 (11)	54 (82)	49 (74)	10 (15)	3 (5)

P>0.05 for all genotypes among three groups.

Table 3 Genotypes of *H pylori* in patients with gastric cancer (GC) and non-gastric cancer (non-GC) (%)

Diagnosis	No of Patients	s1a	s1b	s1c	s2	s1a+s1c	s1a+ s1b+s2	s1a+ s1b+s1c	m1	m1T	m2	m1T +m2	cagA	iceA1	iceA2	iceA1 +iceA2
GC	66	35[b] (53)	1 (2)	13[l] (20)	1 (2)	7 (11)	1 (2)	0 (0)	1 (2)	23[a] (35)	32[h] (48)	4 (6)	50 (76)	39[f] (59)	15 (23)	2 (3)
Non-GC	168	127[d] (76)	9 (5)	93[l] (55)	2 (1)	77 (46)	2 (2)	4 (2)	2 (2)	33[a] (20)	84[j] (50)	10 (6)	139 (83)	125[f] (74)	29 (17)	6 (4)

[b]P<0.001 *vs* s1b, s1c and s2 of non-GC, [d]P<0.0001 *vs* m1T, m1 of non-GC, [f]P<0.0001 *vs* ice A2 of non-GC, [h]P<0.001 *vs* s1c, s1b and s2 of GC, [j]P<0.0001 *vs* m1 of GC, [l]P<0.0001, [a]P = 0.02.

Table 4 Genotypes of *H pylori* in patients with early or advanced gastric cancer (%)

Diagnosis	No of Patients	s1a	s1b	s1c	s2	s1a+s1c	s1a+ s1b+s2	m1	m1T	m2	m1T+m2	cagA	iceA1	iceA2	iceA1 +iceA2
EGC	7	4 (57)	0 (0)	2 (29)	0 (0)	1 (14)	0 (0)	0 (0)	1 (14)	3 (43)	0 (0)	6 (86)	4 (57)	1 (4)	0 (0)
AGC	59	31 (53)	1 (2)	11 (19)	1 (2)	6 (10)	1 (2)	1 (2)	22 (37)	29 (49)	4 (7)	44 (75)	35 (59)	14 (24)	2 (3)

Table 5 Genotypes of *H pylori* in patients with advanced gastric cancer according to Borrmann's classification (%)

Diagnosis	No of Patients	s1a	s1b	s1c	s2	s1a+s1c	s1a+ s1b+s2	m1	m1T	m2	m1T+m2	cagA	iceA1	iceA2	iceA1 +iceA2
I	7	4 (57)	0 (0)	2 (29)	0 (0)	1 (14)	0 (0)	0 (0)	5 (71)	1 (14)	0 (0)	5 (71)	5 (71)	1 (14)	0 (0)
II	28	15 (54)	1 (4)	3 (11)	1 (4)	3 (11)	1 (4)	1 (4)	8 (29)	13 (46)	1 (4)	20 (71)	17 (61)	6 (21)	1 (4)
III	13	6 (46)	0 (0)	5 (38)	0 (0)	2 (15)	0 (0)	0 (0)	3 (23)	8 (62)	1 (8)	11 (85)	8 (62)	3 (23)	0 (0)
IV	10	5 (50)	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	5 (50)	7 (70)	2 (20)	8 (80)	5 (50)	4 (40)	1 (10)

Table 6 Genotypes of *H pylori* in patients with gastric cancer according to histological classification. (%)

Diagnosis	No of Patients	s1a	s1b	s1c	s2	s1a+s1c	s1a+ s1b+s2	m1	m1T	m2	m1T+m2	cagA	iceA1	iceA2	iceA1 +iceA2
Intestinal	28	15 (54)	0 (0)	5 (18)	0 (0)	3 (5)	0 (0)	0 (0)	7 (25)	16 (57)	2 (7)	21 (75)	16 (57)	8 (29)	1 (4)
Diffuse	24	12 (50)	1 (4)	4 (17)	1 (4)	2 (8)	1 (4)	0 (0)	9 (38)	10 (42)	1 (4)	18 (75)	15 (63)	4 (17)	1 (4)
mixed	11	5 (45)	0 (0)	3 (27)	0 (0)	1 (9)	0 (0)	1 (9)	5 (45)	5 (45)	1 (9)	9 (82)	6 (55)	3 (27)	0 (0)

Comparison of gastric cancer patient with non-cancer (peptic ulcer and chronic gastritis) patients

In patients with gastric cancer, the *vacA* s1a and s1c subtypes were less commonly found than those in non-cancer patients (35/66 *vs* 127/168, P<0.001 for s1a and 13/66 *vs* 93/168, P<0.0001 for s1c) (Table 3). In the middle region, the m1T in patients with gastric cancer was more than that in non-cancer patients (23/66 *vs* 33/168, P = 0.02). There was no difference in *iceA* and *cagA* between patients with gastric cancer and non-cancer status.

DISCUSSION

This is the first sudy to investigate the allelic variations of *H pylori vacA*, *cagA* and *iceA1* in gastric cancer patients in Taiwan. The results showed *vacA* s1a, m2, and *iceA1* predominated in patients with gastric cancer and those without.

H pylori has become a world-wide infective agent ranging from 25% in developed countries to more than 80% in the developing world[23]. Not all individuals infected with *H pylori* developed gastric illness and this might be related to various factors such as environmental factors, host genetic factors, and bacterial virulent ability[24]. Certain genotypes (*e.g*. *cagA*, *vacA* s1a) have been closely related to severe clinical outcome and response to anti- *H pylori* therapy[25,26]. However, these findings were not supported by other studies[27].

Different genotypes of *H pylori* have been confirmed in patients with peptic ulcer or non-ulcer dyspepsia from diverse geographic areas[14,23]. For example, in Northern and Eastern Europe, 89% strains were *vacA* s1a. *VacA* s1a and s1b were equally present in France and Italy. In Spain and Portugal, 89% of the strains were *vacA* s1b. While in north America, s1a and s1b were equally prevalent. *VacA* s1c was only found in East Asia. In Taiwan, *H pylori* with *vacA* s1a was the major strain[15,16]. Because of this diversity, it is interesting to analyze the genotypes in different areas.

In this study, predominance of *vacA* s1a was found in patients with gastric cancer (53%) and non-cancer status (76%). Our findings were similar to those reported by other authors in patients with peptic ulcer or non-ulcer dyspepsia[15,16]. In Hong Kong and Korea, a low incidence of *vacA* s1a subtype was found[28,29]. The previous Taiwan reports gave no data concerning *vacA* s1c[6,15]. *VacA* s1c was frequently found (20% in gastric cancer and 55% in non-cancer) in this study. In contrast, *vacA* s1b and s2 were rare. Our findings were compatible with those

in mainland China[30]. A high incidence of *vacA* s1c in this study was similar to the reports of Hong Kong[28], Korea[29], and Japan[31], but different from those of the Western world[14].

Concerning the m-region of *vacA*, m1 strains predominated in most Western reports[14,23]. However, there were few m1 subtypes (2% in cancer and 2% in non-cancer) in this study. We used a modified primer (m1T)[15] and found that some patients (35% in gastric cancer, 20% in non-cancer) with *H pylori* infection contained this genotype. M2 strains predominated (48% in gastric cancer and 50% in non-cancer) in this study. Our findings were consistent with reports from our previous experience[32], other studies in Taiwan[15,16], Hong Kong[28], and mainland China[30]. In contrast, Japan and Korea had a much lower incidence of m2 strain[27,29]. We could not detect the m-region in some patients (15% in gastric cancer and 28% in non-cancer). This indicates a great variation in the *vacA* region in Taiwan, particularly in the mid-region locus. *H pylori* may have a different geographic evolution in Taiwan even compared with other East Asian countries.

IceA1 has been suggested to be related to peptic ulcer disease[22,33]. But, like other authors, we doubted this finding[27-29,32]. It has been found that *IceA1* is the predominant subtype of *ice* in the East Asia, while *iceA2* is the predominant subtype in the USA and Columbia[27]. In this study, we found *iceA1* was the predominant subtype and showed no difference in patients with gastric cancer and non-cancer status.

The clinical relevance of putative virulence-associated genes of *H pylori* in patients with gastric cancer is a matter of controversy. Enomoto *et al*. found that 98% of patients with gastric cancer were *H pylori*-positive[12]. Many studies suggested the strong association of certain genotypes of *H pylori* with gastric cancer[34-38]. A significant association (o.r. 2.94) between *cagA* and gastric cancer was found in young Italian patients[34]. Miehlke *et al*. suggested a significant association between the *H pylori vacA* s1, m1, *cagA* and gastric cancer[35]. Kidd *et al*. confirmed that the *vacA* s1b, m1 and *iceA1* were closely linked to gastric cancer in South Africa[36]. van Doorn *et al.* found a significant association between the presence of ulcers or gastric cancer and the presence of *vacA* s1 and *cagA*[37]. Basso *et al* and Qiao *et al*. also concluded that *H pylori* infection caused by *cagA* positive/*vacA* s1 was a frequent finding in patients with gastric cancer[38,39].

However, some authors have presented different observations. Mitchell *et al*. compared serum antibody to *cagA* antigen in patients with gastric cancer and normal subjects[40]. They found no association between *cagA* and gastric cancer in Chinese subjects. Other authors also confirmed no relationship between *cagA* status and the risk of gastric cancer[41]. Some Japanese studies did not support the link of *vacA* and *cagA* with gastric cancer[42,43]. In these Japanese studies, the majority of the controls had positive *vacA* and *cagA*. Therefore, they obtained a different result as compared with those of the Western studies. In addition, the case number was small in their series. Increased number is needed to avoid bias. In this study, we found no difference in *cagA* between gastric cancer and non-cancer status. But, we found less *vacA* s1a, s1c and more m1T in patients with gastric cancer.

There is a paucity of *iceA* allele data in isolates from patients with gastric cancer. Gastric cancer isolates from Japan and Korea were distinguished by the prevalence of *iceA1* (67%) while 75% of isolates from the USA were *iceA2*[27]. In this study, we found that iceA1 predominated (59%) in patients with gastric cancer.

Patients with histologic findings of severe gastric atrophy, corpus-predominant gastritis or intestinal metaplasia are at an increased risk for gastric cancer. *H pylori* carrying the *cagA* gene might have promoted the atrophic metaplastic mucosal lesions that represent the pathway in multistep intestinal type gastric oncogenesis[25,44]. Correa *et al*. and Uremura *et al*. found that severe atrophic gastritis accompanying intestinal metaplasia caused by persistent *H pylori* infection was closely related to the development of intestinal type gastric cancer[9,45]. But, some authors present different results. No significant relationship was found between *H pylori* and diffuse type gastric cancer because atrophic change was not evident in these patients[46,47]. In addition, other authors did not support this finding due to epidemiological and pathological evidence[48,49]. In this study, we found no difference in genotypes among diffuse, intestinal and mixed types of gastric cancer. In addition, there was no difference of genotypes in patients with early and advanced gastric cancer. However, the case number should be increased to avoid type II error.

In conclusion, *vacA* s1a, m2, and *iceA1* predominate in patients with gastric cancer. As compared with those of non-cancer patients, patients with gastric cancer have less *vacA* s1a, s1c and more m1T subtypes. Genotypes are similar according to morphological and pathological classification.

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