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RESEARCH ARTICLE

Nepalese *Helicobacter pylori* Genotypes Reflects a Geographical Diversity than a True Virulence Factor

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Abstract

Background: The data about the association between *Helicobacter pylori* putative virulence factors; *iceA* and *jhp0562/β-(1,3)galT* with clinical outcomes are still controversial. We identified and analyzed two putative *H. pylori* virulence factors in Nepalese strains. **Methods:** The *iceA* and *jhp0562/β-(1,3)galT* allelic types were determined by polymerase chain reaction amplification. Histological analysis were classified according to the updated Sydney system and the Operative Link on Gastritis Assessment (OLGA) system. **Results:** Among 49 strains, *iceA1* negative/*iceA2* positive (*iceA2*-positive) was predominant type (57.1%, 28/49) and 20 (40.8%) were *iceA1* positive/*iceA2* negative. The remaining one (2.0%) was positive for both *iceA1* and *iceA2* (*iceA1/iceA2*-mixed). Patients infected with *iceA1*-positive strains tended to be higher OLGA score than *iceA2*-positive strains [1.45 [1] vs. 0.07 [0.5], P = 0.09, respectively). The *jhp0562* negative/*β-(1,3)galT* positive was predominant type (25/51, 49.0%), followed by double positive for *jhp0562/β-(1,3)galT* (15/51, 29.4%) and *jhp0562* positive/*β-(1,3)galT* negative (11/51, 21.6%). Activity in the corpus was significantly higher in *jhp0562* negative/*β-(1,3)galT* positive than double positive of *jhp0562/β-(1,3)galT* positive [mean (median); 1.24 (1) vs. 0.73 (1), P = 0.03]. There was association between *iceA* and subtype of *vacA* signal region (e.g., s1a, s1b or s1c) and combination subtypes of signal and middle regions (e.g., s1a-m1c) (P = 0.02, r = 0.29; and P = 0.002, r = 0.42, respectively). In addition, *jhp0562/β-(1,3)galT* genotypes associated with *cagA* pre-*EPIYA* type (e.g., 6 bp-, 18 bp-, or no deletion-type) (P = 0.047, r = 0.15). **Conclusion:** The inconsistency results of the association between *iceA*, *jhp0562/β-(1,3)galT* and histological scores suggesting that these genes may associate with genetic heterogeneity rather than as a true virulence factor.

Keywords: *Helicobacter pylori*- *iceA*- *jhp0562/β-(1,3)galT*- Nepal

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Introduction

Helicobacter pylori was recognized as a type I carcinogen and accepted as the primary cause of chronic gastritis, peptic ulcers and gastric cancer (Suerbaum and Michetti, 2002). However, epidemiological data showed that a small proportion of infected patients develop more severe diseases such as peptic ulcers and gastric cancer suggesting the bacteria contain some factors express harmfulness (virulence) properties that might be available in a few strains however not in others (Yamaoka, 2010). A number of important *H. pylori* virulence factors have been identified including cytotoxin-associated gene A (CagA), vacuolating cytotoxin (VacA) and outer membrane proteins such as outer inflammatory protein (OipA), blood group antigen binding adhesin (BabA), sialic acid-binding adhesin (SabA) and helicobacter outer membrane protein Q (HopQ) (Atherton et al., 1995; Belogolova et al., 2013; Covacci et al., 1993; Mahdavi et al., 2002; Sheu et al., 2003; Yamaoka et al., 2000).

The two initial studies proposed *IceA* (induced by contact with epithelium) as a putative virulence factor based on two main allelic variants, *iceA1* and *iceA2* (Peek et al., 1998; van Doorn et al., 1998). The presence of *iceA1* was associated with peptic ulcers, independent with the *cagA* and *vacA* status (van Doorn et al., 1998), and *IceA* induced mucosal interleukin-8 expression and acute antral inflammation (Peek et al., 1998; Xu et al., 2002). In contrast, subsequent studies failed to reproduce the significant role of this gene when was tested in other populations (Ito et al., 2000; Nishiya et al., 2000; Yamaoka et al., 1999). Although a meta-analysis revealed

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the importance of the presence of *iceA* for peptic ulcers, the significancy was not strong (Shiota et al., 2012). In addition, the *iceA1* mechanism to contribute on the development of peptic ulcers remains unclear as similar as the function of the *iceA2* product (Shiota et al., 2012).

On the other hand, gene encode a glycosyltransferase, *jhp0562*, which involved in the synthesis of lipopolysaccharide (LPS) and *β-(1,3)galT (jhp0563)*, which involved in the Lewis (Le) antigen expression of LPS were reported to be associated with peptic ulcers diseases but only in children (Oleastro et al., 2010). Our previous study indicated that the absence of *β-(1,3)galT* was an independent factor discriminating peptic ulcers from gastritis, however only in the U.S, but not in the Japanese population (Matsuda et al., 2011). In Indonesian strains, inflammation in the corpus was significantly higher only in double positive of *jhp0562/β-(1,3)galT* than *jhp0562* negative/β-(1,3)galT positive [β-(1,3)galT positive] (Miftahussurur et al., 2015a). As similar as the *iceA*, the association between *iceA*, *jhp0562/β-(1,3)galT* and clinical outcomes is still unclear. It is still needed further evidence about the consistency of these virulence factors across populations and regions.

Nepal is a small landlocked country in South Asia with Kathmandu as the capital. The age-standardized incidence rate (ASR) of gastric cancer in Nepal is reported to be 5.3 cases per 100,000 population per year (available from the International Agency for Research on Cancer, GLOBOCAN 2012; http://globocan.iarc.fr). Our previous study revealed that the prevalence of *H. pylori* infection on dyspeptic patients assessed using a combination of three diagnostic tests, in Kathmandu, Nepal was 38.4% (Miftahussurur et al., 2015b). We found that the mountainous population have high-risk gastric mucosal status and considered a high-risk population in Nepal (Miftahussurur et al., 2015a). The Nepalese predominant genotypes were of the characteristic South Asian genotypes, Western-type-*cagA* with no deletion, s1a, m1c, and a high proportion of m2 (Miftahussurur et al., 2015b). Although we found a significantly higher antral histological scores in patients infected with *vacA* s1 than in those infected with s2 genotypes, opposite results were found in between subtype of *cagA* which associated with *H. pylori* recombination (Miftahussurur et al., 2015b). In total, both of the two most extensively studied *H. pylori* virulence factors, *cagA* and *vacA*, could not explain the low incidence of gastric cancer in Nepal. Therefore, in this study we identified and analyzed two putative *H. pylori* virulence factors in Nepalese strains.

Materials and Methods

We used 51 *H. pylori* DNA from previous publications (Miftahussurur et al., 2015b). They were extracted from the *H. pylori* colonies using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) which were cultured from antral gastric biopsy specimen of patients who received endoscopy services section at the Gastroenterology Department, Tribhuvan University Teaching Hospital (TUTH), Kathmandu, Nepal (Miftahussurur et al., 2015b). The strains isolated from 41 gastritis patients, 7 from peptic ulcers and 3 from gastric cancer. The *iceA* and *jhp0562/β-(1,3)galT* allelic types were determined by PCR amplification as described previously (Matsuda et al., 2011; van Doorn et al., 1998). Primers *iceA1F* and *iceA1R* yielded a fragment of 247 bp for the *iceA1* allele, and primers *iceA2F* and *iceA2R* yielded a fragment of 229 or 334 bp (van Doorn et al., 1998). The primers generated two PCR products with 404 and 704 bp for *jhp0562* and *β-(1,3)galT*, respectively (Matsuda et al., 2011). Written informed consent was obtained from all participants, and the study protocol was approved by the Ethics Committee of TUTH and Oita University Faculty of Medicine, Japan.

Experienced endoscopists also collected one sample each from the lesser curvature of the antrum and from the greater curvature of the corpus for histological testing. They were fixed in 10% buffered formalin and were embedded in paraffin. Serial sections were stained with hematoxylin and eosin as well as May–Giemsa stains. The degree of inflammation, neutrophil activity, atrophy, intestinal metaplasia, and bacterial density were classified into four grades according to the updated Sydney system: 0, normal; 1, mild; 2, moderate; and 3, marked (Dixon et al., 1996). Samples with grade 1 or more atrophy were considered atrophy-positive (Bornschein et al., 2012). In addition, the gastritis stage was assessed based on topographic locations (antrum and corpus), according to the Operative Link on Gastritis Assessment (OLGA) system (Rugge et al., 2007).

Data analysis

Discrete variables were tested using the chi-square test; continuous variables were tested using the Mann-Whitney U and t-tests. A P value of < 0.05 was accepted as statistically significant. The SPSS statistical software package version 18.0 (SPSS, Inc., Chicago, IL) was used for all statistical analyses. The Somers'd rank coefficients (r) were determined to evaluate the association between the *iceA*, *jhp0562/β-(1,3)galT* genotype with other virulence factors.

Results

Genotypes and histology

Two isolates did not yield any PCR product for *iceA*, including one patient with peptic ulcers. In total, *iceA1* positive/*iceA2* negative (*iceA1*-positive) was detected in 20 (40.8%) of 49 strains examined, and i*ceA1* negative/*iceA2* positive (*iceA2*-positive) was predominant type (57.1%, 28/49). The remaining one (2.0%) was positive for both *iceA1* and *iceA2* (*iceA1*/*iceA2*-mixed). Among strains from peptic ulcers patients (n = 7), four were *iceA2*-positive type, one was *iceA1*-positive type, and one was *iceA1*/i*ceA2* mixed. In contrast, 2 of 3 gastric cancer patients were infected with *iceA1*-positive type. Although there were no significant for all histological scores, patients infected with *iceA1*-positive strains tended to be higher *OLGA* score than *iceA2*-positive strains (1.45 [1] vs. 0.07 [0.5], P = 0.09, respectively), Table 1).

β-(1,3)galT positive was predominant type (25/51, 49.0%), followed by double positive for *jhp0562/β-(1,3) galT* (15/51, 29.4%) and *jhp0562* positive/β-(1,3)galT

Table 1. Histological Scores According to *iceA* Status

	iceA1 (+)/ iceA2 (-)	*iceA1 (-)/ iceA2 (+)*	*P*
n	20	28	
Antrum			
Activity	1.45 (1)	1.39 (1)	0.76
Inflammation	1.85 (2)	1.64 (1)	0.23
Atrophy	1.35 (1)	1.07 (1)	0.11
Intestinal metaplasia	0.10 (0)	0.07 (0)	0.73
Corpus			
Activity	0.95 (1)	1.11 (1)	0.49
Inflammation	0.85 (1)	0.89 (1)	0.73
Atrophy	0.15 (0)	0.11 (0)	0.98
Intestinal metaplasia	0.15 (0)	0.00 (0)	0.24
OLGA score	1.45 (1)	0.07 (0.5)	0.09

Histology data are presented as mean (median)

Table 2. Histological Scores According to *jhp0562/β-(1,3) galT* Status

	jhp0562 (+)/β-(1,3)galT (-)	*jhp0562 (-)/β-(1,3)galT (+)*	*jhp0562 (+)/β-(1,3)galT (+)*
n	11	25	15
Antrum			
Activity	1.45 (2)	1.44 (1)	1.27 (1)
Inflammation	1.55 (2)	1.84 (2)	1.60 (2)
Atrophy	1.36 (1)	1.20 (1)	1.07 (1)
Intestinal metaplasia	0.09 (0)	0.08 (0)	0.07 (0)
Corpus			
Activity	1.00 (1)	1.24 (1)	0.73 (1)*
Inflammation	0.91 (1)	0.92 (1)	0.80 (1)
Atrophy	0.18 (0)	0.04 (0)	0.27 (0)
Intestinal metaplasia	0.00 (0)	0.00 (0)	0.20 (0)
OLGA score	1.36 (1)	1.28 (1)	1.13 (1)

Histology data are presented as mean (median); *P, 0.03 compared to *jhp0562 (-)/β-(1,3)galT (+)*

negative (*jhp0562* positive) (11/51, 21.6%). There was no association between *jhp0562/β-(1,3)galT* genotype with clinical outcomes. Histological analysis showed that only activity in the corpus was significantly higher in β-(1,3) galT positive than double positive of *jhp0562/β-(1,3)galT* positive [mean (median); 1.24 (1) vs. 0.73 (1), P = 0.03], but not lower than *jhp0562* positive (P = 0.49, Table 2).

Association between iceA, jhp0562/β-(1,3)galT and other virulence factors

We collected data of other virulence factors including *cagA* type, *cagA* pre-*EPIYA* type, *vacA* signal and middle region subtypes from previous publication (Miftahussurur et al., 2015b), and determined the association with *iceA* and *jhp0562/β-(1,3)galT* genotypes. There was association between *iceA* with subtype of *vacA* signal region (e.g., s1a, s1b or s1c), and *iceA* with combination subtypes of signal and middle regions (e.g., s1a-m1c) (P = 0.02, r = 0.29; and P = 0.002, r = 0.42, respectively). In addition, *jhp0562/β-(1,3)galT* genotypes associated with *cagA* pre-*EPIYA* type (e.g., 6 bp-, 18 bp-, or no deletion-type) (P = 0.047, r = 0.15).

Discussion

We revealed that the Nepalese strains considered to be *iceA2*-positive predominant but failed to show a positive association between the *iceA* status and clinical outcomes or with histological scores. Although *iceA1*-positive were predominant in gastric cancer patients and tended to more severe histological scores than *iceA2*-positive, it is not statistically significant. In fact, a meta-analysis revealed that only in 6 of 41 studies found a significantly lower prevalence of *iceA2* in peptic ulcers patients compared with gastritis (Shiota et al., 2012). In contrast, *iceA2*-positive predominant in Nepalese strains confirmed a reflection a geographical diversity than associated with clinical outcomes. Subjects who living in the area infected with major strains of Western-type-*cagA*, are also contained *iceA2*-positive genotype. For examples, Americans (78.6%) (Graham and Yamaoka, 2000) and Brazilian (90.1%) were infected with *iceA2* predominant strains (Ashour et al., 2001). In contrast, in countries with East-Asian type *cagA* strains as a major genotype also possess *iceA1*-positive, e.g., Japan (61.6%) and Korea (69.6%) (Yamaoka et al., 1999). Interestingly, Mexican carried both genes *iceA1* and *iceA2* (Gonzalez-Vazquez et al., 2012). Overall, the prevalence of *iceA1* was significantly higher in Asian countries (64.6%, 1,791/2,771) than in Western countries (42.1%, 935/2,218) (Shiota et al., 2012). The hypothesis is also supported the result of this study that *iceA* associated with subtype of *vacA* signal region or with combination subtypes of signal and middle regions. Previous studies suggested that *vacA* subtype enabled to assume the migration of human populations (Mukhopadhyay et al., 2000; Yamaoka et al., 2002). The *vacA* s1c a-m1b types are common in East Asia; s1a-m1c types are common in South Asia, and the s1b-m1a type is common in Africans and Europeans (Suzuki et al., 2012). *H. pylori* genetic diversity is about 50-fold greater than that of the human population and is greater than within most other bacteria which provide additional information for population genetic analysis (Achtman et al., 1999; Li and Sadler, 1991).

We found that *β-(1,3)galT*-positive was predominant type in Nepalese strains. Previous studies reported that *jhp0562* positive (81%) genotype are common in the Japanese population, while double positive of *jhp0562/β-(1,3)galT* is a major genotype in the US and Indonesian population (Matsuda et al., 2011; Miftahussurur et al., 2015c). However, we found an inconsistent results that β-(1,3)galT positive had severe histological score in corpus than double positive of *jhp0562/β-(1,3)galT* obscures the role of *jhp0562* as an independent discriminating factor for distinguishing peptic ulcers from gastritis. For example, the two previous studies reported a strong positive association of *jhp0562* with the cag pathogenicity island (PAI) (Oleastro et al., 2010) or *cagA* (Matsuda et al., 2011). In addition, all *cagA*-positive strains were also positive for *jhp0562* in Japanese

population (Matsuda et al., 2011). It is suggesting that strains possess *jhp0562*-positive are more virulent and the two homolog genes were inversely correlated, the products of the two genes may have the same cell function, thus producing functional redundancy. In the fact, this gene was also failed as a discriminant factor when was analyzed in Japanese population (Matsuda et al., 2011). In addition, our previous study showed the opposite results that inflammation in the corpus was significantly higher in double positive of *jhp0562/β-(1,3)galT* than β-(1,3)galT positive which might be related with the less virulent *H. pylori* strains (Miftahussurur et al., 2015c). Importantly, although the Le antigenic structures were reported to be important for bacterial colonization, adhesion, and evasion of host immune response (Appelmelk and Vandenbroucke-Grauls, 2000; Heneghan et al., 2000), the role of these in *H. pylori* infection has not been elucidated. Both of these factors are not fulfilled criteria by Lu et al. that proposed several requirements to determine a virulence factor (Lu et al., 2005). It is including (1) There should have a disease or other in-vivo correlation; (2) There should be epidemiologic consistence across populations and regions; (3) There should be biologic plausibility and biologic activity should be reduced or eliminated by gene deletion; (4) Biologic activity should be restored by complementation. Although it is indisputable that bacterial factors are often key factor determining the outcome, several discrepancies in the epidemiological data are yet unexplained. Some putative virulence factors maybe are not a true virulence factors and just co-express of other establish factors (e.g., *CagA*). Importantly, the linkages of the virulence factors may have a certain biological significance, and may somehow interact with each other; *cagA*-positive strains also possess a *vacA* s1/m1 region type and they are further closely linked to the presence of the *babA* and *oipA* "on" status (Yamaoka, 2010).

In conclusions, we found an inconsistency of the association between *iceA*, *jhp0562/β-(1,3)galT* and clinical outcomes results which stimulate a hypothesis that these genes may associate with genetic heterogeneity or geographical differences than as a true virulence factors.

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