Analysis of the relationship between invasive capability of Helicobacter pylori and gastroduodenal diseases

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Helicobacter pylori (H. pylori) may enter into host cells, maybe as a facultative intracellular pathogen. This study aims to reveal the roles of internalized H. pylori in the bacterial pathogenesis. Transmission electron microscopy was used to observe the invasion of H. pylori. Invasion rates of H. pylori (two standard strains and 43 clinical strains) were examined by gentamicin invasion assay. The cagA, cagE and vacA genes of H. pylori were detected by PCR. The cagA 3’region (cagA-EPIYA) of each strain was sequenced. The secretion of IL-8 from AGS cells and activity of NF-κB induced by intracellular H. pylori were tested by ELISA and the dual-luciferase reporter assay system, respectively. It was found that H. pylori could adhere to and invade AGS cells, then continue to survive and multiply in the cytoplasm. The average invasion rate of H. pylori gastric cancer plants and that of ulcer plants were both higher than that of gastritis plants ($P<0.0001$). In the clinical strains, cagA, vacA and cagE were all positive; cagA-EPIYA genotypes included ABD 90.7% (39/43) and ABBD 9.3% (4/43), all without comparability. Notably, the average invasion rate of H. pylori vacA s1c-i1-m1b plants was higher than that of vacA s1c-i1-m2 plants ($P=0.0445$). In addition, the intracellular H. pylori all could induce IL-8 secretion, which was decreased after cells were pretreated with anti-β1-integrin antibody or SN-50 (an NF-κB inhibitor). The intracellular H. pylori all activated NF-κB, which would be inhibited after cells were pretreated with anti-β1-integrin antibody. These results demonstrate that H. pylori invasive ability and disease severity have a positive correlation, and this intension of invasive ability is associated with the vacA mid-region, not with cagA, cagA-EPIYA or cagE. It is possible that cagA and cagE are essential for the bacterial invasion. Internalized H. pylori can activate NF-κB signal pathway and induce IL-8 secretion, which suggests that H. pylori invasion may be an important strategy to play a role in the development of H. pylori associated diseases.

INTRODUCTION

Helicobacter pylori (H. pylori), a microaerophilic organism, infects about one-half of the population on the earth; however, only 15–20% of H. pylori positive people experience gastroduodenal diseases, including cancer, ulcers and chronic gastritis (Peek & Blaser, 2002). Both the conflict between high infection rate and low morbidity, and the difference in clinical outcomes may be related to bacterial pathogenicity, host susceptibility and environment (Kalali et al., 2014). In particular, bacteria virulence factors have attracted significant attention, but have still not been completely interpreted.

For a long time, H. pylori has been counted as a noninvasive organism, adhering to gastroduodenal epithelial cells. As bacterial research continues, a number of scientists have observed that H. pylori is able to invade into cells, even reside and multiply within cells, and may be a facultative intracellular pathogen (Chu et al., 2010; Dubois & Borén, 2007; Ito et al., 2008).

H. pylori invasion involves the host and itself. On the one hand, the invasion may depend on bacterial virulence factors, such as cag pathogenicity island (PAI), VacA, OipA or BabA, which however is still controversial (Chu et al., 2010; Ito et al., 2008). On the other hand, this invasion may also need the host’s β1-integrin as a receptor, but the β1-integrin antibody could not completely interdict this invasion, and the mechanism has not been clarified (Ito et al., 2008; Su et al., 1999). It has been observed that internalized H. pylori could express cagA, vacA and babA2...
within the host cells (Necchi et al., 2007; Semino-Mora et al., 2003), but it has not been reported whether these virulence factors in the host cells still could play a role as usual, and whether they may aid the invasion, by being involved in this bacterial pathogenicity.

In summary, many problems and controversies about \textit{H. pylori} invasion still have not been perfectly resolved. Here, we report our research regarding the relationship between invasion and disease, invasion and virulence factors, as well as invasive roles in disease development.

**METHODS**

**Bacterial strains and culture.** The 43 \textit{H. pylori} clinical isolates were conserved by our laboratory, and had been isolated from 43 gastroduodenal biopsy samples collected from the Department of Gastroenterology, Affiliated Union Hospital of Fujian Medical University, Fujian Provincial Hospital gastrointestinal endoscopy room and Fujian Provincial Tumour Hospital endoscopy room. All isolates were tested by Gram stain and rapid urease identification. \textit{H. pylori} standard strains (NCTC11637, 26695 and SS1) were obtained from the Chinese Center for Disease Control and Prevention. \textit{H. pylori} was cultured on columbia agar plates containing 7% fresh \textit{H. pylori} standard strains. \textit{H. pylori} selective supplement (Oxoid) under a microaerobic atmosphere (10% CO\textsubscript{2}, 5% O\textsubscript{2} and 85% N\textsubscript{2}) in 85% humidity in a Culture Safe CO\textsubscript{2} Incubator (LEE, Touch 190S) at 37°C. Fresh plates were started from glycerol stocks and subcultured every 3–5 days.

**Cell culture.** The human gastric adenocarcinoma epithelial cell line (AGS) was obtained from the Institute of Cell Biology, Chinese Academy of Science (Shanghai, China) and was maintained at 37°C in 5% CO\textsubscript{2} humidified atmosphere in HAM’S-F12 medium supplemented with 10% fetal bovine serum (FBS; HyClone).

**Gentamicin invasion assay.**

**Transmission electron microscopic examination.** AGS cells were infected with \textit{H. pylori} NCTC 11637 at an m.o.i. of 100 for 2.5 h, 4 h, 6 h, 12 h and 24 h at 37°C. Cells were harvested by trypsinization, fixed with 3% glutaraldehyde and postfixed in 1% osmic acid. After embedding, sections were observed by transmission electron microscopy (TEM; Philips EM208).

**Gentamicin protection assay** (Chu et al., 2010; Ito et al., 2008). About 7×10\textsuperscript{5} AGS cells were seeded per well in 12-well tissue culture plates and incubated for 15 h. \textit{H. pylori} standard strains (NCTC11637 and SS1) and clinical strains (43 plants, including 15 plants from gastritis, 15 plants from peptic ulcer and 13 gastric cancer plants) were respectively added to each well at an m.o.i. of 100, at 37°C for 2 h. The infected cells were washed six times and treated with gentamicin-containing (100 μg ml\textsuperscript{-1}) culture medium for 2 h to kill extracellular bacteria. After this treatment, no bacteria were cultured on columbia agar plates and incubated for 15 h, 4 h, 6 h, 12 h and 24 h at 37°C. The number of colony forming units (c.f.u.) was counted. The c.f.u. at 4 h p.i. gives the number of invasive bacteria. The concentration of gentamicin in our experiments was therefore much greater than the MIC and theoretically sufficient to eliminate all external \textit{H. pylori}.

**H. pylori main virulence genes analysis.** Bacteria were extracted and used for genomic DNA preparation with the High Pure PCR Template Preparation kit (Roche) according to the manufacturer’s instructions, including \textit{H. pylori} clinical strains (43 plants), standard strains (NCTC11637, 26695 and SS1) and \textit{Escherichia coli} (DH5\textalpha, as negative control). The isolated DNA was eluted and diluted to 50 ng μl\textsuperscript{-1} and stored at −20°C until use. PCR was performed in a volume of 50 μl containing 0.2 μM of each primer, 25 ng of extracted genomic DNA and 2× Taq PCR MasterMix (TIANGEN). Amplifications were carried out as follows: 94°C for 5 min; 94°C for 30 s, annealing for 30 s, 72°C for 30 s (except \textit{cagA} 3’ region, 72°C for 1 min) × 30 cycles; 72°C for 7 min. The primer sequences, annealing temperatures and the expected size of PCR products are summarized in Table 1. All reactions included one negative control (DH5\textalpha) and one positive control (26695). PCR amplification was performed in duplicate for each DNA sample. The PCR products were examined by 1.5% agarose gel electrophoresis, and then analysed by the image analysis system.

The PCR products of the \textit{cagA} 3’ region were sequenced by the fluorescent deoxy terminator method (Sangon Biotech). The sequenced results were matched against nucleotide sequences presented in GenBank using the BLASTN program, and then \textit{cagA}-EPYA analysed with BioEdit software.

**The cell function of AGS cells influenced by \textit{H. pylori} invasion.**

**H. pylori invasion.** \textit{H. pylori} (high invasiveness plant NCTC11637 and T494, low invasiveness plant SS1 and T049) invaded into AGS cells at an m.o.i. of 100 for 2 h. In the anti-β1-integrin antibody invasion assays, pretreatment with culture medium containing 5 μg ml\textsuperscript{-1} of anti-β1-integrin antibody (Santa Cruz) was performed before the addition of the bacterial suspension, followed by 40 min incubation. In the SN-50 (an NF-κB inhibitor) invasion assay, pretreatment with culture medium containing 20 μM of SN-50 (Calbiochem) was performed before the addition of the bacterial suspension, followed by 2 h incubation. The infected cells were washed six times with gentamicin-containing (100 μg ml\textsuperscript{-1}) culture medium, and then cultured with the same gentamicin-containing medium before the samples were harvested. All tests were under administration (the simplex \textit{H. pylori} invasion, the anti-β1-integrin antibody invasion and the SN-50 invasion) and control (the according invasion without \textit{H. pylori}).

**IL-8 secretion assay.** AGS cells were seeded into 96-well plates with 5×10\textsuperscript{4} cells per well and then manipulated as described above, but after the bacterial suspension was added, the culture medium should not contain FBS. At 48 h after \textit{H. pylori} invasion, the supernatants were collected and stored at −80°C before analysis. The level of IL-8 in the supernatants was determined by ELISA using a Human IL-8 ELISA kit (NeoBioscience Technology) according to the manufacturer’s instructions. Results were representative of three individual experiments in triplicate.

**NF-κB activation assay.** AGS cells (2.5×10\textsuperscript{5}) were cotransfected with pNF-κB-luc (Clontech) and pRL-TK, an internal control renilla luciferase expression vector (Promega), using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. At 24 h after transfection, AGS cells were invaded by \textit{H. pylori} as described above. Cells were harvested 12 h post-invasion, and then, renilla-normalized firefly luciferase activities were measured by the dual-luciferase reporter assay system (Promega). Results were representative of three individual experiments in triplicate.
Table 1. Details of the PCR primers used in the study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Annealing temperature (°C)</th>
<th>Product length (bp)</th>
<th>References</th>
</tr>
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<tr>
<td>cagA</td>
<td>cagA F AACAGGACAAATGCGTACGCC</td>
<td>52</td>
<td>701</td>
<td>Kauser et al.(2005)</td>
</tr>
<tr>
<td></td>
<td>cagA R TATTAATGGGTTCTTCTGTGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cagA 3’ region</td>
<td>cagA 3’F ACCCTATGGGTTAAATTGGTTA</td>
<td>50</td>
<td>600–800</td>
<td>Yamaoka et al.(1998)</td>
</tr>
<tr>
<td></td>
<td>cagA 3’R GTAATTGTCTGTTTGGCG</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>cagE</td>
<td>cagE F GCGATTGTAAATTGGCTTGTAG</td>
<td>52</td>
<td>329</td>
<td>Kauser et al.(2005)</td>
</tr>
<tr>
<td></td>
<td>cagE R GAATGGTTATAAAAAATAACGTGCCC</td>
<td></td>
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<tr>
<td>vacA s1a</td>
<td>S1a-F CTCTCAGGTATTAGGAGG</td>
<td>55</td>
<td>213</td>
<td>Yamazaki et al. (2005)</td>
</tr>
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<td></td>
<td>VAI-R CTGCTTGAATGGCGCAACAC</td>
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<tr>
<td>vacA s1b</td>
<td>SS3-F AGCCGCATACCCGCAAGAG</td>
<td>55</td>
<td>187</td>
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<td></td>
<td>VAI-R CTGCTTGAATGGCGCAACAC</td>
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<tr>
<td>vacA s1c</td>
<td>S1c-F CTCTCAGGTATTAGGAGG</td>
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<td></td>
<td>VAI-R CTGCTTGAATGGCGCAACAC</td>
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<tr>
<td>vacA s2</td>
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<td>55</td>
<td>199</td>
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<td>VAI-R CTGCTTGAATGGCGCAACAC</td>
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<td>vacA i1</td>
<td>VacF1 GGTGGATTGGGGGAATGCCG</td>
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<td>500</td>
<td>Rhead et al. (2007)</td>
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<td></td>
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<tr>
<td>vacA i2</td>
<td>VacF1 GGTGGATTGGGGGAATGCCG</td>
<td>53</td>
<td>500</td>
<td>Rhead et al. (2007)</td>
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<td>C2R GTAATTGTCTAGTTTCGC</td>
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<td>vacA m1b</td>
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<td>VAm-F GGCACAATGCGATGCATTGATG</td>
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<td>vacA m2</td>
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<td>54</td>
<td>400</td>
<td>Kauser et al. (2005)</td>
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<tr>
<td></td>
<td>VA4-R CATTACAGCGCGCTTGAGCAC</td>
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</table>

Statistical analyses. All results were analysed by SPSS software (SPSS 18.0; SPSS). Data were expressed as the mean ± standard deviation (s.d.). The P-value set at <0.05 was regarded as being statistically significant. For gentamicin invasion assays, the Kruskal–Wallis test was used to compare average invasion rates among H. pylori clinical strains obtained, as shown in Fig. 2. The average invasion rate of H. pylori gastritis plants [2.7±1.7(×10⁻²)] and that of ulcer plants [2.9±1.9(×10⁻²)] were both higher than that of gastritis plants [1.2±0.6(×10⁻²)] (x²=17.64, P<0.0001), but there was no significant difference between gastric cancer plants and ulcer plants (Fig. 2). The results suggest that the invasive ability of H. pylori may be related to the severity of the disease.

RESULTS

H. pylori invasive visualization analysis

When TEM was used to observe the invasion of H. pylori NCTC11637, the bacteria were found to adhere and enter into AGS cells (Fig. 1). After infecting for 2.5 h, H. pylori mainly attached closely to cell membrane, the bacteria tightly combined with cellular microvillus. After invading, H. pylori was enveloped by double-layer membrane vesicles (4 h). The elongating and dividing H. pylori were found in the cytoplasm (6 h and 12 h). After infecting for 6 h, the invasion appeared to reach the peak and many bacteria were observed in cytoplasm. After infecting for 12 h, adhering, invading, dividing and lysing of the bacteria all could be observed. After infecting for 24 h, most of intracellular H. pylori lysed. The results demonstrate that H. pylori can adhere and invade into cells, then continue to survive and multiply in the cytoplasm, which suggests that invasion of H. pylori may take part in damage to host cells.

The disease types analysis

By gentamicin invasion assays, average invasion rates of H. pylori clinical strains were obtained, as shown in Fig. 2. The average invasion rate of H. pylori gastric cancer plants [2.7±1.7(×10⁻²)] and that of ulcer plants [2.9±1.9(×10⁻²)] were both higher than that of gastritis plants [1.2±0.6(×10⁻²)] (x²=17.64, P<0.0001), but there was no significant difference between gastric cancer plants and ulcer plants (Fig. 2). The results suggest that the invasive ability of H. pylori may be related to the severity of the disease.

H. pylori main virulence genes analysis

By PCR and sequencing, the main virulence genotypes of H. pylori clinical strains were tested, as shown in Table 2. CagA, vacA and cagE were all positive in the 43 clinical strains, but all negative in DH5α (as a negative control). The cagA-EPIYA genotypes detected in the clinical strains included ABD, 90.7 % (39/43) and ABBBD, 9.3 % (4/43), which suggests that there is no significant relationship between cagA-EPIYA genotypes and the bacterial invasive ability. The vacA genotypes detected in clinical strains included s1c-i1-m2, 46.5 % (20/43) and s1c-i1-m1b, 53.5 %
(23/43). The average invasion rate of \emph{H. pylori} vacA s1c-i1-m1b plants [2.7 ± 2.0 (× 10^{-2})\%] was higher than that of vacA s1c-i1-m2 plants [1.7 ± 0.8 (× 10^{-2})\%] (Z=2.0091, P=0.0445) (Fig. 3), which suggests that the invasive ability of \emph{H. pylori} may be related to vacA genotypes.

As frequently used standard strains, NCTC11637 and SS1 were tested in our study to possess \textit{cagA}, \textit{vacA} and \textit{cagE}. And their \textit{cagA}-EPIYA/ vacA genotypes were AB/s1a-i1-m1a (NCTC11637) and ABC/s2-i2-m2 (SS1). The invasion rate of NCTC11637 was 4.1 ± 0.12 (× 10^{-2})\% and that of SS1 was 1.1 ± 0.19 (× 10^{-2})\%.

With comparative analysis on the invasion rates of the 43 clinical strains and two standard strains, NCTC11637 and T494 [7.7 ± 1.12 (× 10^{-2})\%] were chosen to stand for high
invasiveness plants, and SS1 and T049 [0.6 ± 0.01(×10^{-2}%) ] were chosen to stand for low invasiveness plants, which would be used in follow-up functional studies.

**The cell function of AGS cells influenced by *H. pylori* invasion**

*H. pylori* high invasiveness plants NCTC11637 and T494, and low invasiveness plants SS1 and T049 were analysed with respect to the effect on the cell function of AGS cells.

**IL-8 secretion analysis.** By ELISA, the secretion of IL-8 from AGS induced by intracellular NCTC11637, T494, SS1 or T049 was tested, as shown in Fig. 4. All four strains could induce IL-8 secretion, compared with no *H. pylori* invasion. In the each bacterial group, the concentrations of IL-8 secretion from AGS cells induced by *H. pylori* only were higher than those additionally pretreated with anti-β1-integrin antibody and additionally pretreated with SN-50 (*P*<0.05) (Fig. 4). The concentrations of IL-8 secretion from a large variety of strains convincingly demonstrate that intracellular *H. pylori* can induce IL-8 secretion from AGS cells, and that this ability may depend on amount of the intracellular bacteria.

**DISCUSSION**

In the traditional view, *H. pylori* is considered as an extracellular bacteria, but increasing studies in *vitro* and *in vivo* have shown that *H. pylori* can invade into cells and it is also an intracellular organism. Using TEM and immunohistochemistry (IHC) technique, some researchers found that *H. pylori*, in specimens including gastritis, peptic ulcer, precancerosis and gastric cancer, can invade into epithelial cells and even lamina propria in gastric mucosa (Dubois & Borén, 2007; Ozbek et al., 2010). By immunofluorescence (IF) technique, internalized *H. pylori* was observed in AGS cells (Amieva et al., 2002; Chu et al., 2010; Coray et al., 2012; Kwok et al., 2002). Other studies verified that *H. pylori* can enter into AGS, MKN45, Huh7, HEp-2, HeLa and so on, and noted that invasive capability was not similar according to different host cells (Dubois & Borén, 2007; Ito et al., 2008). In our study, using TEM, we also observed that *H. pylori* entered into AGS cells where it could continue to survive and multiply. In our gentamicin invasion assays, the key to this finding is that a large number of *H. pylori* strains, not only including two standard strains, but also including 43 different clinical isolates, all could be reisolated and cultured from corresponding cell lysates. These data from a large variety of strains convincingly demonstrated that *H. pylori* was a facultative intracellular organism. Moreover, in our research, the invasion rates were approximately 0.46 (×10^{-2}%) to 9.08 (×10^{-2}%) for the clinical strains. In general, those of gastric cancer plants and ulcer plants were high, and that of gastritis plants was low. We can conclude that the severity of the disease may depend

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**Table 2. *H. pylori* main virulence genes**

<table>
<thead>
<tr>
<th>Disease types</th>
<th>cagA</th>
<th>cagA-EPIYA</th>
<th>vacA</th>
<th>slc-ii-mlb</th>
<th>slc-ii-m2</th>
<th>cagE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptic ulcer (n=15)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>Gastric cancer (n=13)</td>
<td>13</td>
<td>0</td>
<td>1</td>
<td>8</td>
<td>5</td>
<td>13</td>
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<tr>
<td>Gastritis (n=15)</td>
<td>15</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>9</td>
<td>15</td>
</tr>
</tbody>
</table>

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**NF-κB activation analysis.** The relative luciferase activity of NF-κB following invasion by the four strains is shown in Fig. 5. Compared with the control (*P*<0.01) (Fig. 5), the four strains all activated NF-κB in AGS cells. After AGS cells were pretreated with anti-β1-integrin antibody, the activity of NF-κB declined (*P*<0.01) (Fig. 5). Activation by high invasiveness plants was stronger than activation by low invasiveness plants (*P*<0.0001) (Fig. 5). The results suggest that intracellular *H. pylori* can activate NF-κB, and this ability may depend on amount of the intracellular bacteria.
on the invasive ability of *H. pylori*. The more powerful the invasive ability is, the more serious the disease is. Up to now, the similar opinion reported is only that invasion of *H. pylori* was related to *H. pylori* associated disease, but the relationship was not explained and speculated from biopsy specimens (Dubois & Boren, 2007). In our study, *H. pylori* from clinical patients was tested in AGS cells, so this relationship between invasion and disease was completely verified under *in vitro* conditions. Our group is researching invasion in C57BL/6 mice, and will search for more evidence *in vivo* to verify this character of *H. pylori*. Wilkinson and el-Shoura have demonstrated that internalization of a large number of *H. pylori* was related to gastric epithelial cell damage and disintegration (el-Shoura, 1995; Wilkinson et al., 1998), which supports our viewpoint. We speculate that *H. pylori* invasiveness may play a role in its pathogenicity, and that the virulence of *H. pylori* may depend on its invasive ability.

So far, the role of *H. pylori* virulence factors in its invasiveness is controversial. The associated virulence factors reported include mainly cagPAI, cagA, vacA and so on (Dubois & Boren, 2007; Ito et al., 2008).

As an acknowledged virulence factor, CagA is injected into host cells by the Cag-type IV secretion system, primarily regulates cell morphology and polarity, and activates or inactivates multiple signal molecules in a phosphorylation-dependent or phosphorylation-independent way (Backert et al., 2010; Posselt et al., 2013). Even CagA possesses oncogenic potential to induce the development of gastrointestinal cancer, depending on intact Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs (Ohnishi et al., 2008). CagE resides within cagPAI, as an ATPase, supplies energy to participate in CagA translocation and may be important to cause *H. pylori* associated neoplasma (Fischer, 2011; Rizzato et al., 2012). The isogenic cagE-knockout mutant strain (∆cagE mutant) has even replaced the functional cagPAI-knockout strain in some research (Matsumoto et al., 2007). In previous studies, when expression of CagA was abolished, *H. pylori* would reduce internalization activity, so CagA had been believed to act in bacterial invasion (Lai et al., 2008; Petersen et al., 2001). On the contrary, Amieva and co-workers observed that the ∆cagA mutant was similar in entry into cells compared to the wild-type strain, suggesting that CagA was not required for *H. pylori* internalization.

**Fig. 3.** The relationship between invasive ability and vacA genotypes assays. The ability of invasion of 43 clinical strains was assessed by gentamicin invasion assays. Corresponding vacA genotypes were analysed by PCR amplification (*P*<0.05).

**Fig. 4.** IL-8 secretion from AGS cells induced by intracellular *H. pylori*. The ability of inducing IL-8 secretion of NCTC11637, T494, SS1 and T049 was assessed by IL-8 ELISAs. AGS cells were infected with the four strains as indicated. At 48 h after invasion, IL-8 secretion was observed. The group with simplex *H. pylori* invasion was compared with the group without invasion (#*P*<0.01). For *H. pylori* invasion, the group pretreated with anti-integrin β1 and the group pretreated with SN-50, respectively, were compared with the group not pretreated (*P*<0.05). With simplex *H. pylori* invasion, the high invasiveness groups were compared with the low invasiveness groups (**)P*<0.0001).
(Amieva et al., 2002). In our study, the 43 strains of bacteria with different invasiveness, and isolated from various mild or serious diseases, were all cagA positive, mostly with the EPIYA-ABD genotype. Our findings could not support the relationship between cagA or its EPIYA motifs and gastric carcinoma. Meanwhile, these observations hint that cagA and its EPIYA motifs may fail to influence bacterial internalization ability. There are possibly some other genes that have more important roles than cagA in affecting the bacterial invasiveness or pathogenicity. Regrettfully, cagE is also not one, because all were cagE positive in the 43 strains. Our findings (including standard strains NCTC11637 and SS1) could not exclude that cagA or cagE may be necessary for H. pylori to enter into host cells, which will need to be tested by according mutant strains in future.

VacA, another crucial virulence factor, can induce vacuolization, apoptosis and the inhibition of T-cell functions. (Posselt et al., 2013). In its gene sequence, polymorphism of the signal sequence (allele types s1a, s1b, s1c or s2), intermediate region (allele types i1 or i2) and mid-region (allele types m1a, m1b or m2) have been observed (Kauser et al., 2005; Rhead et al., 2007; Yamazaki et al., 2005). The role of VacA in invasiveness has been reported. On the one hand, some previous studies showed that vacA did not contribute to the bacterial invasion and even suppress it (Amieva et al., 2002; Chu et al., 2010; Terebiznik et al., 2006). On the other hand, other researchers reported that levels of ΔvacA mutant internalization activity were reduced (Lai et al., 2008; Petersen et al., 2001). In our study, the signal sequences and intermediate regions of vacA from the 43 clinical strains tested were all identical, respectively, and there were only differences in mid-regions; some were m1b, others were m2. The average invasion rate of m1b plants was higher compared to m2 plants, which prompted that the vacA mid-region was associated with H. pylori internalization. Rhead and colleagues had concluded that vacA intermediate region was an important determinant of H. pylori toxicity (Rhead et al., 2007). However, in our study, the intermediate regions from 43 clinic strains tested all were the same, which suggested that there was no relationship between the diversity of the intermediate region and clinical disease. In addition, according to our above opinion, virulence of H. pylori depends on its invasive ability, so we can conclude that the toxicity of H. pylori with vacA m1b genotype is stronger than that with m2 genotype. This deduction is in line with the findings of Atherton et al., who showed that infection with vacA m1 bacteria was associated with much greater cell damage than with m2 bacteria (Atherton et al., 1997).

Lots of evidence from previous research has suggested that H. pylori could induce IL-8 production from gastric epithelial cells, in vivo and vitro (Allison et al., 2009; Crabtree et al., 1994; Crowe et al., 1995; Fischer et al., 2001). By the whole genome analyses, Eftang and colleagues found that IL-8 was the most significantly upregulated gene in epithelial cells in reaction to H. pylori infection (Eftang et al., 2012). IL-8 may play a crucial role in H. pylori infection and pathopoiesis. In these previous studies, it was all extracellular H. pylori that cells met with.

**Fig. 5.** NF-κB activation by intracellular H. pylori in AGS cells. The ability to active NF-κB by intracellular H. pylori in AGS cells was assessed by NF-κB activation assays. The plasmids pNFκB-luc and pRL-TK were co-transfected into AGS cells. After 24 h, AGS cells were infected with the four strains as indicated. At 12 h after invasion, luciferase activity was measured. The ability of activation was assessed by the relative luciferase activity of NF-κB. The group with simplex H. pylori invasion was compared with the group without invasion (##P<0.01). For H. pylori invasion, the group pretreated with anti-integrin β1 was compared with the group not pretreated (*P<0.01). With simplex H. pylori invasion, the high invasiveness groups were compared with the low invasiveness groups (**P<0.0001).
To date, no one knows whether intracellular H. pylori also contributes to IL-8 secretion. We made H. pylori with different invasiveness enter into AGS cells, and detected the concentration of IL-8 in supernatant. Meanwhile we demonstrated that intracellular H. pylori could induce IL-8 secretion and this induction could be inhibited when the bacterial entrance was inhibited. IL-8 is a pro-inflammatory cytokine, and its release in extracellular H. pylori exposure often involves in the NF-kB signal pathway. In our studies, we noted that the intracellular organisms could activate NF-kB, and SN-50 as an NF-kB inhibitor, could mostly suppress the induction of IL-8. This suggests that induction of IL-8 by intracellular H. pylori may principally involve the NF-kB signal pathway, which is similar to extracellular H. pylori; the latter owns NF-kB and other pathways to induce IL-8 secretion (Backert & Naumann, 2010; Lee et al., 2013).

NF-kB, as a transcription factor, primarily regulates lots of intracellular proteins, influencing immunity, inflammation, transformation, proliferation, metastasis and so on. It is closely related with neoplasm (Lamb & Chen, 2013). It is clear that extracellular H. pylori infection can activate NF-kB, but it is inconclusive about the intracellular bacteria. Our group noted that when the pathogens entered into epithelial cells, NF-kB also could be activated, which could be reduced following the inhibition of entrance. The results hinted that intracellular H. pylori might induce host cell injury via activation the NF-kB signal pathway. In our research, internalized H. pylori was capable of activating NF-kB and inducing IL-8 release, and this capacity depends on bacterial invasive ability, suggesting that H. pylori may be similar to other invasive pathogens such as Shigella and Salmonella, whose invasiveness plays a certain role in pathogenesis. Again, this deduction confirms our above initial opinion; virulence of H. pylori depends on its invasive ability.

In conclusion, we show that H. pylori invasive ability and disease severity have a positive correlation. This invasion of invasive ability is associated with vacA mid-region, not with cagA, cagA-EPIYA or cagE. However, the results do not exclude the possibility that cagA and cagE are essential for the bacterial invasion. Internalized H. pylori can activate the NF-kB signal pathway and induce IL-8 secretion, which suggests that H. pylori invasion may be an important strategy to play a role in the development of diseases. Many puzzles and problems regarding H. pylori invasion, involving virulence factors, IL-8, NF-kB and so on, still need to be explored in depth.

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