**Helicobacter pylori** participates in the pathogenesis of IgA nephropathy

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**ABSTRACT**

Increasing evidences have shown that *Helicobacter pylori* (*Hp*) is a pathogen closely related to extra-gastric disorders. Our previous *in vitro* studies had demonstrated that *Hp* infection, at least via cytotoxin-associated gene A protein (CagA), might play an important role in the pathogenesis of IgA nephropathy (IgAN) by stimulating proliferation and ectopic synthesis of aberrantly glycylated IgA1 of B cells. However, the relevant clinical evidence of IgAN resulted from *Hp* infection remain to be elucidated. This study aimed to investigate the risk incidence of IgAN caused by *Hp* infection. 22 primary IgAN, 20 non-IgA nephropathy (n-IgAN), and 30 healthy controls were included in this study. We found that the rate of IgG anti-*Hp* seropositivity was significantly improved in IgAN, but the current *Hp* infection was similar in all groups. The production and underglycosylation of IgA1 tended to increase in IgAN patients with IgG anti-*Hp* seropositivity. A tendency toward increased the risk of clinical prognosis was seen in IgAN with *Hp* infection. *Hp* antigen and CagA were only deposited in renal tubules, and enhanced antigen deposition in response to *Hp* was observed in IgAN. Our study suggested that *Hp* infection might have a pathogenic role in IgAN through giving rise to strongly mucosal immune response, and based on damage of renal tubular.

**Introduction**

IgA nephropathy (IgAN) is one of the most common causes of glomerulonephritis in the world. IgAN is characterized by mesangial deposition of immune complex, primarily containing IgA1, accounting for more than 50% biopsy-proved primary glomerulonephritis in Asia. Approximately 30–50% of patients will progress to end-stage renal disease (ESRD) within 20 years since the initial diagnosis. While IgAN patients that reach ESRD can choose kidney transplant as the treatment, the recurrence rates of this disease is up to 50% in patients with renal allograft, which suggests that extra-renal factors take the primary responsibility of the pathogenesis of IgAN.

In general, after an upper respiratory infection or a gastrointestinal infection, IgAN may relapse or aggravate. It is found that tonsillectomy may relieve renal dysfunction in patients with IgAN. IgA1 often deposits in kidney in the form of polymeric IgA1 which is mainly produced by the mucosa-localized plasma cells. All above implicate that mucosal infection may be the pathogenic risk factor in IgAN and antigens of microbial origin may participate in the pathogenesis of IgAN.

*Helicobacter pylori* (*Hp*), a spiral-shaped gram-negative bacterium habitated in the mucus layer of the human stomach, is considered to be the main cause of a number of gastrointestinal diseases such as chronic gastritis and peptic ulcers. Without medical eradication, the infection generally persists for decades, resulting in a persistent low-graded inflammation and immune response in local and even remote tissue. Increasing evidences have shown that *Hp* is a pathogen closely related to extra-gastric disorders.

For the first time, Barratt et al. reported the systemic antibody response to mucosal *Hp* infection in IgAN. It is reported that IgAN was associated with both a greater rate of IgA anti-*Hp* seropositivity and a more pronounced IgA anti-*Hp* antibody response, which conclude that *Hp* might be a potential IgAN-pathogenic antigen. In two recent studies, it was demonstrated that coccoid *Hp* was present in palatine tonsil. Moreover, the prevalence of *Hp* was 100% in the IgAN group, which may indicate that *Hp* may have a...
pathogenic role in the development of IgAN. While our previous in vitro studies had demonstrated that Hp infection, at least via CagA, might participate in the pathogenesis of IgAN by stimulating B-cell proliferation, influencing the production and glycosylation of IgA1, the clinical evidence by which Hp infection participates in the pathogenesis of IgAN have not been fully elucidated.

The aim of present study is to investigate the risk incidence of IgAN result from Hp infection, the effect of Hp infection on the production and underglycosylation of IgA1, and clinical prognosis in patients with IgAN.

**Materials and methods**

**Patients and controls**

This study included 22 patients with primary IgAN and 20 patients with non-IgA nephropathy (n-IgAN) primary glomerulonephritis diagnosed by renal biopsy. Two cases of focal segmental glomerulosclerosis (FSGS), 4 cases of membranous nephropathy (MN), 4 cases of mesangial proliferative glomerulonephritis (MsPGN), and 10 cases of minimal change disease (MCD) were composed of the n-IgAN patients. Patients showing symptom of other secondary glomerulonephritis, such as systemic lupus erythematosus, vasculitis, or Henoch–Schönlein purpura, were excluded for analysis. The primary IgAN group consisted of 9 males and 13 females and the mean age was 32.27 ± 11.57 years. The n-IgAN primary glomerulonephritis group consisted of 11 males and 9 females and the mean age was 36.60 ± 11.57 years.

The healthy controls group consisted of 30 residents of the same geographic area matched by age and gender with the patients. The healthy controls consisted of 18 males and 12 females. The mean age was 38.96 ± 9.22 years. All individuals were at least 25 years old (range 25–61 years).

Complete clinical data, including age, gender, course of disease, serum creatinine, total serum IgA, complement C3 and estimated glomerular filtration rate (eGFR) were collected at the time of renal biopsy.

**Detection of Hp by[^14]C-UBT and ELISA**

The carbon 14-urea breath test (^[^14]C-UBT) for the diagnosis of Hp status in patients and controls and enzyme-linked immunosorbent assay (ELISA) for the detection of Hp-IgG antibodies in the serum were performed in patients and controls.

The current infection of Hp was determined by a positive reaction in ^[^14]C-UBT. Urea[^14]C capsules were taken on an empty stomach or at least 3 h after a meal. Fifteen minutes later, collect breath samples by breath card and analyzed for the content of carbon 14-labeled CO₂ using a Hp analyzer (HUBT-20A, Guangdong, China). The results were judged according to disintegrations per minute (DPM) values. A DPM value of more than 99.0 was considered to be positive for Hp infection (current infection) and a value of equal or less than 99.0 was considered to be negative.

IgG antibodies to Hp was measured by Hp-IgG ELISA kit (Sigma-Aldrich, USA) according to the manufacturer’s protocol. Brought all specimens and kit reagents to room temperature (18–26°C) and gently mixed. Dispensed diluted serum, calibrator, and control into the appropriate wells and incubated for 20 min at RT. After washing, enzyme conjugate was added and then incubated for 20 min at RT. Plates were treated with tetramethyl benzidine (TMB) for 10 min and read OD at 450 nm using ELISA reader within 15 min.

**Detection of serum IgA1 and underglycosylation IgA1 by ELISA**

Serum samples from patients were collected before renal biopsy, and stored at −20°C until assaying. Serum IgA1 and underglycosylation IgA1 were respectively quantified by ELISA and helix aspersa agglutinin (HAA) lectin binding assay, according to a protocol established previously.

As described earlier, serum IgA were measured by ELISA, in which 96-well plates were coated with goat anti-human IgA1 antibody (Southern Biotechnology Associates, Birmingham, AL) overnight at 4°C. After blocking with 5% BSA, serum samples were added to each well in duplicate and incubated for 1 h at 37°C. After washing, horseradish peroxidase-conjugated goat anti-human IgA antibody (Southern Biotechnology Associates, Birmingham, AL) were added and then incubated for 1 h at 37°C. The signal was developed with TMB and read at 450 nm using Bio-Rad 550.

The underglycosylation of IgA1 were determined by HAA lectin binding assay. As described earlier, 96-well plates were coated with goat anti-human IgA antibody and blocked. Serum samples were added in duplicate and incubated for 3 h at 37°C. The captured IgA1 was subsequently desialylated by the treatment with neuraminidase from *Vibrio cholerae* (Roche, Penzberg, Germany) and incubated for 3 h at 37°C. After washing, plates were then treated for 3 h at 37°C with biotinylated HAA lectin. The lectin binding was measured with avidin-horseradish peroxidase conjugate (Sigma-Aldrich). The color development was performed as above.
Immunohistochemistry

Immunohistochemistry was performed on paraffin sections to detect *Hp* antigen and cytotoxin-associated gene A protein (CagA) in renal tissue. Renal tissues were only collected 17 specimens from IgAN group and 19 specimens from n-IgAN group.

Consecutive section was prepared at a thickness of 5 μm for immunohistochemistry. The sections were dewaxed in xylene, rehydrated in a graded ethanol series. Endogenous peroxidase was inactivated by incubating the sections with 3% hydrogen peroxide for 10 min. After blocking with 10% goat serum in PBS, the sections were incubated with the primary antibody at 4°C overnight and then were incubated for 30 min with the secondary antibodies. The primary antibody used was mouse anti-*Hp* monoclonal antibody (Spring Bioscience, USA) and mouse-raised monoclonal anti-*Hp* CagA antibody (Santa Cruz Biotechnology, USA), respectively. Slides were then developed with 3, 3'-diaminobenzidine (DAB) for 5 min and counterstained with hematoxylin.

Statistical analyses

All data were analyzed using SPSS software (version 17.0; SPSS, Chicago, IL). The chi-square test and independent Student’s t-test were used for comparison between groups, where *p* < .05 was considered to be significant.

Results

Hp current infection

Of the 22 patients with IgAN screened, 6 patients (27.27%) had a positive 14C-UBT that meant a current infection of *Hp*, which was similar to the frequency of positivity in n-IgAN and healthy controls in which 6 of 20 subjects (30%) and 10 of 30 subjects (33.33%) were infected with *Hp*. There was no significant difference among the three groups (Table 1).

IgG antibodies to *Hp*

A high rate of IgG anti-*Hp* seropositivity was surprisingly found in IgAN, which was 54.55% (12/22). However, the rate of IgG anti-*Hp* seropositivity were 20% (4/20) and 10% (3/30) in n-IgAN and healthy controls, respectively (Table 1). There had an obviously statistical difference.

Effect of *Hp* infection on IgA1 production and glycosylation of IgA1 in IgAN

To investigate the serum IgA1 content, an IgA1-specific ELSIA assay was performed. The results showed that the production of IgA1 was lightly increased in IgAN patients with IgG anti-*Hp* seropositivity. When compared with patients with seronegativity, however, there was no statistically significance (Figure 1(A)).

IgA1 in IgAN patients with IgG anti-*Hp* seropositivity presented increased underglycosylation level when compared with that from patients who were seronegative for *Hp*-IgG (Figure 1(B)).

Effect of *Hp* infection on clinical prognosis of IgAN

Compared with IgAN with *Hp*-IgG seronegativity, IgAN with *Hp*-IgG seropositivity showed increased total serum IgA and IgA/C3 ratio, however, decreased eGFR. Considering the limited sample size, the above data demonstrated no statistical difference, but it still deserved our attention. Other variables, including age and course of disease were not significantly different between the two groups of *Hp*-IgG seropositive IgAN and *Hp*-IgG seronegative IgAN (Table 2).

For the patients that seropositive for *Hp*-IgG, total serum IgA and IgA/C3 in IgAN patients were greater than those in n-IgAN patients (Table 2).

Localization of *Hp* antigen in kidney tubules determined by immunohistochemistry

Some specimens revealed granular deposits giving a positive reaction with monoclonal anti-*Hp* antibody. However, the most interesting thing is the positive labeling only exists in renal tubules. No positive staining was observed in glomerulus. Immunostaining of different *Hp* antigen in additional consecutive section, revealed that the CagA, the key virulence factor of *Hp*, was also co-localized with *Hp* antigen in the same renal tubules (Figure 2(A)).

The deposition of *Hp* antigen and CagA in IgAN renal tissue was more serious than those in n-IgAN. Mean IOD analysis showed that the mean IOD of *Hp* antigen and CagA in IgAN were significantly greater than those of n-IgAN (Figure 2(B,C)). The degree of *Hp* antigen and CagA deposition were divided into mild and severe according to the limited mean IOD of 0.005 and 0.0025.

| Group      | Current infection of *Hp* (%) | Serum IgG anti-*Hp* (%) | Total |
|------------|-------------------------------|------------------------|-------|
| IgAN       | 6 (27.27%)                    | 12 (54.55%)*           | 22    |
| n-IgAN     | 6 (30%)                       | 4 (20%)                | 20    |
| Healthy control | 10 (33.33%)               | 3 (10%)                | 30    |

*p < .05, compared with n-IgAN or healthy control group.

Table 1. The positivity rate of *Hp* infection.
respectively. A mean IOD of more than 0.005 or 0.0025 was considered to be severe for Hp antigen or CagA deposition, respectively. A mean IOD of equal or less than 0.005 or 0.0025 was considered to be mild, respectively. Accordingly, the rate of severe deposition was significantly higher in IgAN, which was 80% (8/10). However, all of deposition in n-IgAN were mild (Table 3).

In IgAN with IgG anti-Hp seropositivity, 8 of 9 (88.9%) specimens had kidney tubular deposits that produced from the positive reaction with anti-Hp antibody and anti-CagA antibody. However, the rate of deposition in IgAN with IgG anti-Hp seronegativity was 2 of 8 (25%). In n-IgAN, the rate of depositional positivity was not different between anti-Hp IgG seropositive and seronegative patients (Table 3).

**Discussion**

*Hp* infection is a common chronic infection, and has increasingly become a focus of attention owing to its close relationship with extra-gastric disorders. It had been reported that, compared with *Hp*-infected controls without renal disease, *Hp*-infected patients with IgAN had a similar rate of IgG anti-*Hp* seropositivity. But IgAN patients with infections caused by *Hp* produced a much greater IgG anti-*Hp* response. In the present study, we found that the incidence of current *Hp* infection in IgAN was similar with n-IgAN and controls. However, the rate of IgG anti-*Hp* seropositivity was striking greater in IgAN. Positive 14C-UBT just represented current infection of *Hp*, while IgG anti-*Hp* seropositivity indicated people had been exposed to *Hp* in the past due to demonstrable antibody levels for years following the initial exposure and *Hp* requires a longer time than do other bacteria to cause infection. This means that patients with 14C-UBT-negative maybe have a seropositivity of IgG anti-*Hp*. One possible explanation for this enhanced rate of IgG anti-*Hp* seropositivity was that in IgAN the mucosa-driving, immune response was abnormal. When exposed to *Hp*, IgAN was more easily to produce strong mucosal immune response.

The IgA1 from IgAN patients with mucosal *Hp* infection had a higher level of lectin binding, which indicated that mucosal *Hp* infection induced underglycosylation of IgA1, and the increasing of underglycosylation IgA1 in the circulation maybe caused by the abnormal reaction of system to mucosal antigen. It was reported that there was increased mucosal-type IgA1 against * Hp* in the serum of IgAN patients with mucosal *Hp* infection. Our prior study demonstrated that CagA, a major
virulence factor of \( Hp \), could lead to underglycosylation of \( IgA1 \) and promote the production of \( IgA1 \) in the B cell line, DAKIKI cells.\(^{13}\) In the present study, we found that \( Hp \) infection have effect on the production and underglycosylation of \( IgA1 \) in IgAN. Although this difference did not demonstrate statistical significance for the limited sample size in this study. A larger-sized sample should be required for further clinical study.

**Table 3.** The deposition of \( Hp \) antigen and CagA in renal tissue.

| Group   | Serum Hp-IgG | Total | The deposition of \( Hp \) antigen and CagA, \( n \) (%) | Serve (mean IOD >0.005/0.0025) | Mild (mean IOD <0.005/0.0025) |
|---------|--------------|-------|------------------------------------------------------|-------------------------------|-------------------------------|
| IgAN    | +            | 9     | 8 (88.9%)\(^{a}\)                                     | 8 (80%)\(^{b}\)               | 2 (20%)                       |
|         | −            | 8     | 2 (25%)                                              |                              |                               |
| n-IgAN  | +            | 4     | 3 (75%)                                              | 0                             | 16 (100%)                     |
|         | −            | 15    | 13 (86.7%)                                           |                               |                               |

\(^{a}\)p < .05, compared with the Hp-IgG seronegative IgAN;
\(^{b}\)p < .05, compared with n-IgAN.

**Figure 2.** Localization of \( Hp \) antigen and CagA in kidney tubules. (A) The images represented the deposition of \( Hp \) antigen and CagA in IgAN and n-IgAN renal tissue, respectively (Original magnification 400 ×). \( Hp \) antigen and CagA were stained in brown color by immunohistochemistry using monoclonal antibody against \( Hp \) and CagA. IgAN and n-IgAN specimens revealed granular deposits giving a positive reaction with monoclonal anti-\( Hp \) antibody and anti-CagA antibody only in renal tubule, and the deposition in IgAN was more obviously than that in n-IgAN. No positive staining was observed in glomerulus. (B) The IOD analysis of \( Hp \) antigen deposition in renal tissue. The deposition of \( Hp \) antigen in IgAN was significantly increased than that in n-IgAN renal tissue. \(^{*}\)p < .05, compared with the n-IgAN group. (C) The IOD analysis of CagA deposition in renal tissue. The deposition of CagA in IgAN was significantly increased than that in n-IgAN renal tissue. \(^{*}\)p < .05, compared with the n-IgAN group.
Local inflammation resulted from Hp has systemic effects and persistent Hp infection was not only able to induce lesions locally but also to induce lesions of other remote sites. These facts indicated that Hp might be involved in the pathogenesis of extra-gastric disorders. The autoimmune reaction induced by Hp infection might not only play a role in chronic gastritis but also be associated with the pathogenesis of other diseases in other organ, such as renal tubular cells. It was reported that of 71 antibodies that reacted with Hp in gastric mucosa, 11 antibodies reacted with renal tubular cells in other organ, such as renal tubular cells. In the present study, we found that monoclonal anti-Hp antibody and anti-CagA antibody only reacted with renal tubules. In addition, the degree of Hp antigen and CagA deposition were obviously severe in IgAN and an enhanced rate of deposition was observed in IgAN patients with Hp-IgG seropositivity. It once again indicated that the immune system was abnormal in IgAN and the immune response to Hp infection which more easily produced in IgAN patients would be stronger.

A rapidly progressive irreversible kidney failure ranging from a benign condition resulted in a highly variable clinical course of IgAN. Early detection and intervention of risk factors was important to delay the progression of IgAN nephropathy and prevent the emergence of adverse outcomes. Numerous studies showed that proteinuria and low GFR increased the risk of ESRD in IgAN patients. In the present study, we found that Hp infection could increase the risk of clinical prognosis of IgAN when compared with n-IgAN. In addition, Hp infection had a tendency to aggravate renal function in IgAN patients, although we had not obtained the data with statistical significance due to the limitation of the sample size.

In conclusion, our results showed that long time infection of Hp infection increased the antibodies against Hp, promoted the production of IgA1 and its underglycosylation, aggravated renal function and made more severe degree of antigen deposition in IgAN. This study suggested that Hp might participate in pathogenesis of IgAN through giving rise to strongly mucosal immune response.

Disclosure statement
The authors report no conflicts of interest.

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