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Original Article

Correlation between persistent chlamydia pneumoniae infection and primary IgA nephropathy

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Abstract: To investigate the correlation between chlamydia pneumoniae (CP) infection and IgG nephropathy (IgAN). Seventy cases with primary IgAN were chosen along with 70 healthy blood donors and 12 cases which died of accidents, from which serum samples and kidney tissues obtained at autopsies were collected, respectively. CP IgG and CP IgA antibody titers in the serum were detected by using indirect immunofluorescence assay (IIF), and CP DNA in kidney tissues was detected using quantitative fluorescence PCR. The correlations of CP infection to positive CP DNA in kidney tissues, clinical manifestations of IgAN and pathological changes in kidney tissues were investigated. IgAN group had a significantly higher incidence of persistent CP infection than healthy blood donor group ($P<0.01$). No significant difference was found in the proportion of acute CP infection, previous infection and non-infection between different clinical subtypes of IgAN ($P>0.05$). The incidence of persistent CP infection in cases with severe proteinuria and persistent renal insufficiency was higher than in cases without severe proteinuria ($P<0.05$). Glomerular pathological injury scores and tubulointerstitial scores in cases with persistent CP infection were higher than those in cases without persistent CP infection ($P<0.05$). Renal pathological changes of cases with persistent CP infection were more severe than those of cases without persistent CP infection. Positive rate of CP DNA was higher among cases with severe proteinuria and persistent renal insufficiency than in cases without severe proteinuria ($P=0.012$). Glomerular pathological injury scores ($P<0.05$) and tubulointerstitial scores ($P<0.01$) in cases positive for CP DNA in kidney tissues were higher compared to negative cases; cases positive for CP DNA presented with more severe pathological changes than negative cases. Persistent CP infection was correlated with positive CP DNA in kidney tissues ($P<0.01$). The onset of IgAN was associated with persistent CP infection, but not significantly correlated with previous CP infection and acute CP infection.

Keywords: IgA, glomeruli, chlamydia pneumoniae (CP), infection, proteinuria

Introduction

Chlamydia pneumoniae (CP) is a common pathogen of respiratory tract infection. It is found that many chronic diseases including atherosclerosis are related to CP infection [1-3]. As to the pathogenesis of IgA nephropathy (IgAN), most studies focus on the relationship between viral and bacterial infection and the onset and development of IgAN [4]. However, the true pathogens leading to IgAN are still unidentified. IgA nephropathy progressive glomerular sclerosis and atherosclerosis share many similarities in pathogenesis and mechanism of deterioration where immune-mediated inflammatory reactions play crucial roles. Theoretically, CP may infect kidney tissues and is associated with IgAN. However, the correlations between CP infection and IgAN are not fully corroborated by histological evidences. The present study was a prospective trial on the correlation between CP infection and IgAN in 70 cases with primary IgAN.

Materials and methods

Subjects

Seventy cases clinically and pathologically with primary IgAN and treated at the First Affiliated Hospital of Xinxiang Medical College between March 2008 and March 2011 were collected.

The cases included 38 males and 32 females who were aged 19.1±7.3 years (9-40 years) and renal biopsies were carried out 7 days to 6 years after onset. Clinical manifestations were as follows: recurrent gross hematuria in 15 cases (21.43%), asymptomatic abnormal urinalysis in 24 cases (34.92%), nephritic syndrome in 5 cases (7.14%), non-renal severe proteinuria in 11 cases (15.71%), acute nephritis in 8 cases (11.43%) and persistent renal insufficiency in 7 cases (10%). Besides, 80 healthy blood donors at Xinxiang Central Blood Station were recruited, including 44 males and 26 females aged 18-40 years (average, 25.7±5.6 years). For control group, 12 cases who died of accidents were selected and autopsies revealed no obvious lesions of kidney tissues by Xinxiang Forensic Medicine Center. Of 12 accidental deaths, 9 were males and 3 were females who were aged 12-43 years (average, 31±16.4 years). Informed consent was obtained from all subjects, and the protocol was approved by Ethics Committee in the hospital.

Reagents and equipments: CP antibody detection kit (Euroimmun, Germany); CP quantitative fluorescence PCR kit (Da An Gene Co., Ltd. of Sun Yat-sen University); 6-carboxy-fluorescein (FAM) and 6-carboxy-tetramethylrhodamine (TAMRA) (Perkin Elmer, USA).

Methods

Detection of serum CP antibodies: On the second day after hospitalization and before blood donation, 2-3 mL of venous blood was sampled, respectively. The serum was isolated and stored at -80°C. Venous blood sampling was performed again 4 weeks after hospitalization for some IgAN patients. CP antibodies were detected using IIF according to the instruction in the manual. TITERPLANETM technique was used to detect CP antibodies and serum was diluted in specific proportion using PBS-Tween buffer. Into each reaction zone of the plate, 25 μL of the diluted serum was added, and 25 μL of diluted positive and negative serum containing CP antibodies was also added, respectively. After incubation at room temperature for 30 min, the plate was washed with PBS-Tween buffer for 1 min, and goat anti-human IgG and IgA antibodies labeled with Evans blue were added dropwise and incubated at room temperature for 30 min. The cells were washed by PBS-Tween buffer and the slide was sealed. Appearance of evenly scattered particles emitting bright green fluorescence with the size of millet on dark background image under the fluorescence microscope was defined as positive; otherwise the result was negative. For positive cells, dilution was performed continually and comparison was made with negative serum under the same dilution. Endpoint of titration was defined as the appearance of evenly scattered particles emitting yellow-green fluorescence with the size of needle point on dark background image under the fluorescence microscope. The highest dilution was considered the antibody titer. For each experiment, positive control, negative control and blank control were set up, respectively. Criteria for serological diagnosis of CP infection: acute CP infection: an increase of IgG or IgM in double serum samples by 4 times or above; IgM ≥1:16; IgG ≥1:512. Diagnosis was made if any of the above three criteria was met; previous CP infection: 1:16≤ IgG <1:512; chronic CP infection: IgA ≥1:100 with the possibility of acute infection precluded [5].

Detection of CP DNA in kidney tissues: CP DNA in kidney tissues was detected using quantitative fluorescence PCR in accordance with the instruction enclosed in the kit. The target gene fragment was CP-specific 16S rRNA gene with the length of 278 bp. The primer sequence HM-1 was 5' TGA CAA CTG TAG AAA TAC AGC 3' and the complementary strand HR-1 was 5' ATT TAT AGG AGA GAG GCG 3'. Probes: 5'-end labeled fluorescent reporter group FAM, 3'-end labeled fluorescent quencher group TAMRA. The kidney cortex was crushed and added with 50 μL of DNA extraction liquid with proper mixing. After reaction for 10 min at 100°C and standing at 4°C for 6-8 h, centrifugation was performed at 10000 r/min for 5 min and 5 μL of supernatant was taken to measure $A_{260/280}$ ratio using ultraviolet spectrophotomter. DNA content was calculated, and the remaining extract was preserved at -20°C. For each batch of samples, negative control, positive control and blank control were set up, respectively; sterile deionized water was used as negative control. The quality control standard as positive control template of 10^7 copies/mL was diluted to 10^6, 10^5, 10^4, 10^3 and 10^2 copies/ml, respectively. Centrifugation was performed at 6 000 r/min for several seconds using 2 μL of the sample, quality control standard or sterile deionized water, respectively, which was followed by PCR

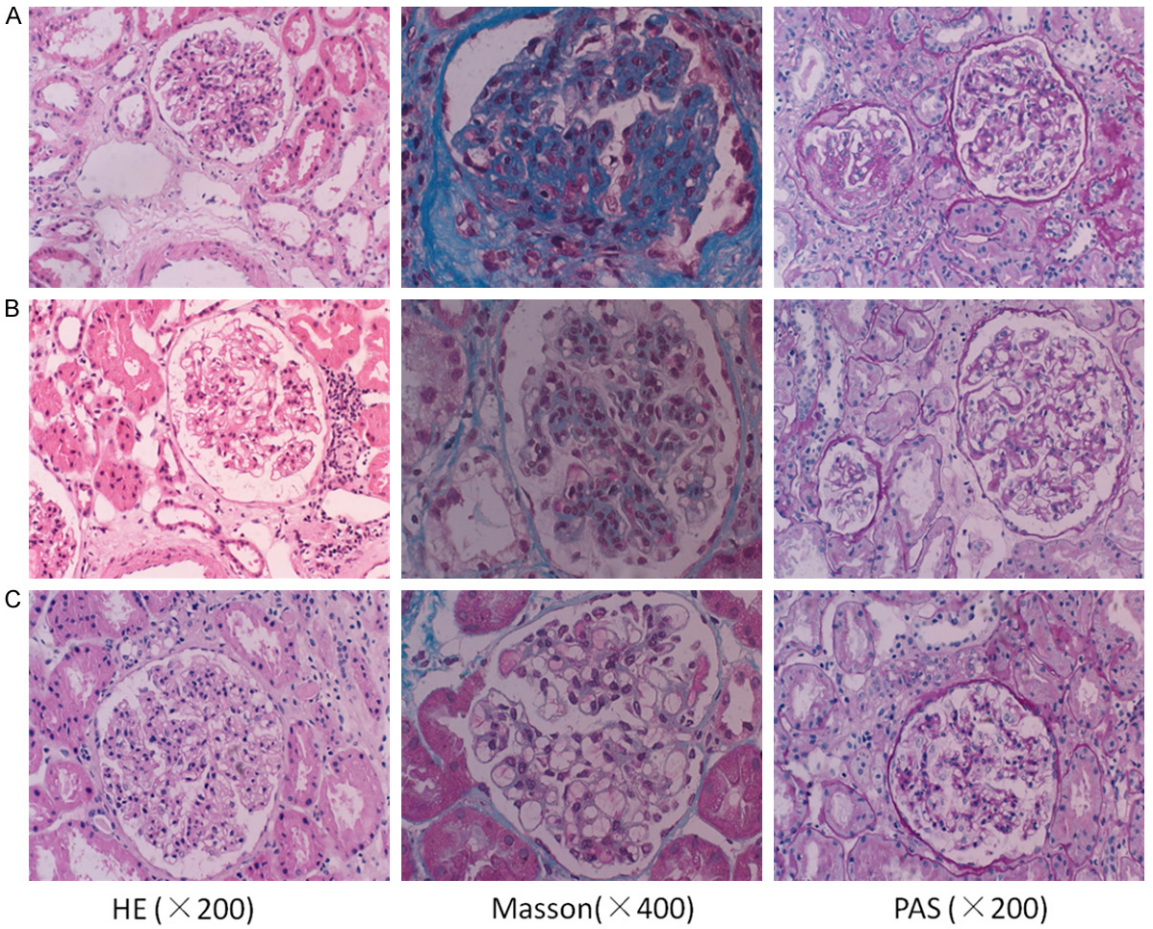

Figure 1. The morphological photos of each group. (A. Persistent renal insufficiency; B. Severe proteinuria; C. Non-severe proteinuria).

Table 1. Comparison of percentages of acute CP infection, previous infection and non-infection among different types of IgAN

Clinical classification	*n*	Non-infection	Previous infection	Acute infection
Non-severe proteinuria	47	7	29	11
Severe proteinuria	16	3	9	4
Persistent renal insufficiency	7	3	4	0
Total	70	13	42	15

Note: Severe proteinuria included nephritic syndrome and non-renal severe proteinuria; non-severe proteinuria included recurrent gross hematuria, asymptomatic abnormal urinalysis, and acute nephritic syndrome.

amplification. The 25 μL PCR reaction system consisted of 10× PCR buffer 2.5 μL, primer 16 pmol/L each, fluorescent probe 10 pmol/L, dNTPs 0.5 μL (10 mmol/L), Taq DNA polymerase 1.5 U and sample 2 μL. PCR conditions: predenaturation at 93°C for 2 min, 93°C for 45 s, 55°C for 50 s, 72°C for 50 s, 40 cycles. GeneAmp 5700 SDS was used for data analysis. Copy number of CP 16S rRNA in every microgram of samples was obtained by conversion. Amplified product of 8 μL was mixed with 2 μL of loading buffer and electrophoresis was carried out using 15 g/L agarose gel (containing 0.5 μg/mL ethidium bromide) at 5 V/cm for 60 min. The results were observed under the ultraviolet light and photos were taken.

Evaluation of pathological lesions of kidney tissues: Memphis scoring system was used to evaluate glomerular and tubulointerstitial injury combining with relevant literature [6, 7]. Twenty non-overlapping fields (×200) were selected under the microscope, and the scope of glomerular and tubulointerstitial lesions was esti-

Table 2. Comparison of incidence of persistent CP infection among different clinical subtypes

Clinical subtype	*n*	Non-persistent infection	Persistent infection	Incidence of persistent infection (%)
Non-severe proteinuria	47	42	5	11.90
Severe proteinuria	16	11	5	31.25[a]
Persistent renal insufficiency	7	4	3	42.86[a]
Total	70	57	13	18.57

[a]*P*<0.05, compared to non-severe proteinuria.

mated. Criteria for evaluating glomerular lesions: Normal mesangial matrix with broadening of matrix not exceeding the capillary diameter and showing a segmental distribution (0 point); open capillary lumen with broadening of matrix slightly exceeding the capillary diameter and showing a diffuse distribution (1 point); compression and damage by the hyperplasia tissues to glomerular capillary loops, with broadening of matrix and aggravated diffuse distribution (2 points); severe compression and damage by the hyperplasia tissues to glomerular capillary loops (3 points); mesangial cell proliferation; looping of capillaries; glomerular sclerosis; non-fibrous adhesion of cellular crescents; loop necrosis. Adhesion of fibrous or fibrous-cellular crescents were evaluated by the following criteria: no adhesion (0 point); lesion scope ≤30% (1 point); 30% < lesion scope <50% (2 points); lesion scope ≥50% (3 points). The percentage of affected area and glomerular area to total glumeral area was calculated using GQS-960 system. Tubulointerstitial scores were given based on the following criteria: 20 fields (×200) were selected under the electron microscope; glomerular dilation, necrosis and atrophy, tubulointerstitial edema and fibrosis, interstitial inflammatory cell infiltration and small artery lesions were observed. Each parameter was evaluated with points from 0 to 3; 0 point indicated no injury with lesion scope =0; 1 point indicated mild injury with lesion scope <15%; 2 points indicated moderate injury with 15% ≤ lesion scope <50%; 3 points indicated severe injury with lesion scope ≥50%. For each slide, the tubulointerstitial scores were 0-9 points. Histopathological scoring was performed by double-blind method. Two pathologists independently assigned scores to each parameter of glomerular and tubulointerstitial lesions according to the criteria.

Statistical analysis

Statistical analyses were performed using SPSS 13.0 software. Data were expressed as $\bar{x}\pm s$, and the means of two samples were compared using independent samples T test. Multiple-sample means were compared using one-way ANOVA. LSD test was used for data with homogeneity of variance, and Dunnett's *T*3 for data with heterogeneity of variance. Comparison of two or more rates (or percentages) was performed using chi-square test with α=0.05 in a two-tailed test. P<0.05 indicated statistically significant differences.

Results

Detection of CP-specific antibodies in the serum of IgAN group and healthy blood donor group

CP-specific antibodies IgG and IgA showed skewed distribution in both two groups. The differences in the percentages of acute CP infection (21.43% *vs.* 12.86%), previous infection (60.00% *vs.* 51.43%) and non-infection (18.57% *vs.* 35.71%) were not statistically significant between the two groups (χ^2=10.87, *P*=0.144). The incidence of persistent CP infection in IgAN group was higher than that of healthy blood donor group (18.57% *vs* 4.29%), and the difference was of statistical significance (χ^2=7.056, P=0.008). The morphological photos of each group were shown in **Figure 1**.

Relationship between CP infection and clinical classification of IgAN

The percentages of acute CP infection, previous infection and non-infection among different types of IgAN were not significantly different (χ^2=8.253, *P*=0.143, **Table 1**).

Comparison of incidence of persistent CP infection among different clinical subtypes of IgAN

As shown by χ^2 segmentation, the incidence of persistent CP infection among cases with severe proteinuria (including nephritic syndrome and non-renal severe proteinuria) and

Table 3. Renal pathological scores of cases with acute CP infection, previous infection and non-infection ($\bar{x}\pm s$)

Status of infection	*n*	Glomerular injury scores	Tubulointerstitial scores
Non-infection	13	4.54±3.67	6.69±3.92
Previous infection	42	4.97±3.23	7.48±4.63
Acute infection	15	5.53±3.16	9.53±3.52
F value		0.323	1.770
P		0.725	0.178

Table 4. Renal pathological scores of cases with persistent CP infection and non-persistent CP infection ($\bar{x}\pm s$)

Group	Cases	Glomerular injury scores	Tubulointerstitial scores
Persistent infection	13	6.92±3.23	11.23±4.75
Non-persistent infection	57	4.58±3.15[a]	6.98±3.88[b]

[a]*P*<0.05, [b]*P*<0.01, compared to persistent infection.

persistent renal insufficiency was higher than that among cases with non-severe proteinuria (including recurrent gross hematuria, asymptomatic abnormal urinalysis, and acute nephritic syndrome) (**Table 2**).

Relationship between CP infection and pathological changes of kidney tissues in IgAN group

IgAN cases with non-infection, previous infection and acute infection did not differ significantly in glomerular injury scores and tubulointerstitial scores (**Table 3**). The glomerular injury scores and tubulointerstitial scores of cases with persistent CP infection were both higher than those of cases with non-persistent infection. Cases with persistent CP infection showed aggravation of symptoms such as mesangial proliferation, stenosis and obstruction of capillary loops, interstitial edema, inflammatory cell infiltration and small artery lesions than those with non-persistent CP infection (**Table 4**).

Quantitative fluorescence PCR for detection of CP DNA in kidney tissues

Results of quantitative fluorescence PCR were analyzed by using GneneAmp 5700 SDS Software. Among 70 cases of IgAN, 15 cases were positive for CP DNA, with the level of 10^2-10^6 copies/mg; all 12 cases who died of accidents were negative for CP DNA (**Figure 2A**). The amplified products were separated by electrophoresis using 15 g/L agarose gel and 14 cases with IgAN showed specific 278 bp DNA bands; none of the 12 cases who died of accidents showed specific 278 bp DNA bands (**Figure 2B**).

Comparison of positive rate of CP DNA in kidney tissues among different clinical subtypes

χ^2 segmentation was adopted and the results showed that the positive rate of CP DNA among cases with severe proteinuria and persistent renal insufficiency was higher than that among cases with non-severe proteinuria (**Table 5**).

Comparison of pathological changes of kidney tissues among cases positive and negative for CP DNA

The glomerular injury scores and tubulointerstitial scores in cases positive for CP DNA (*n*=15) were higher than in negative cases (**Table 6**).

Relationship between persistent CP infection and positive CP DNA in kidney tissues

In χ^2 test, χ^2=33.39, *P*<0.01, indicating a correlation between persistent CP infection and positive CP DNA in kidney tissues. *P*=0.687 in McNemar's test, which confirmed the findings.

Discussion

Infectious diseases are independent risk factors of glomerular diseases in children and adults, and the question is which sources of infection are correlated with renal involvement. IgAN is a common type of glomerular disease, and the exact pathogenesis remains unknown. Few reports are published concerning the correlation between infection factors and occurrence of IgAN. It has been reported that mycoplasma pneumoniae, Staphylococcus spp., Haemophilus parainfluenzae, hepatitis B virus, and Plasmodium falciparum are associated with IgAN [8-12]. Most of the existing studies deal with clinical case reports, and it is still a disputed topic whether CP infection is directly related to IgAN.

We chose 70 healthy blood donors and detected CP-specific antibodies in the serum. It was found that the differences in percentages of

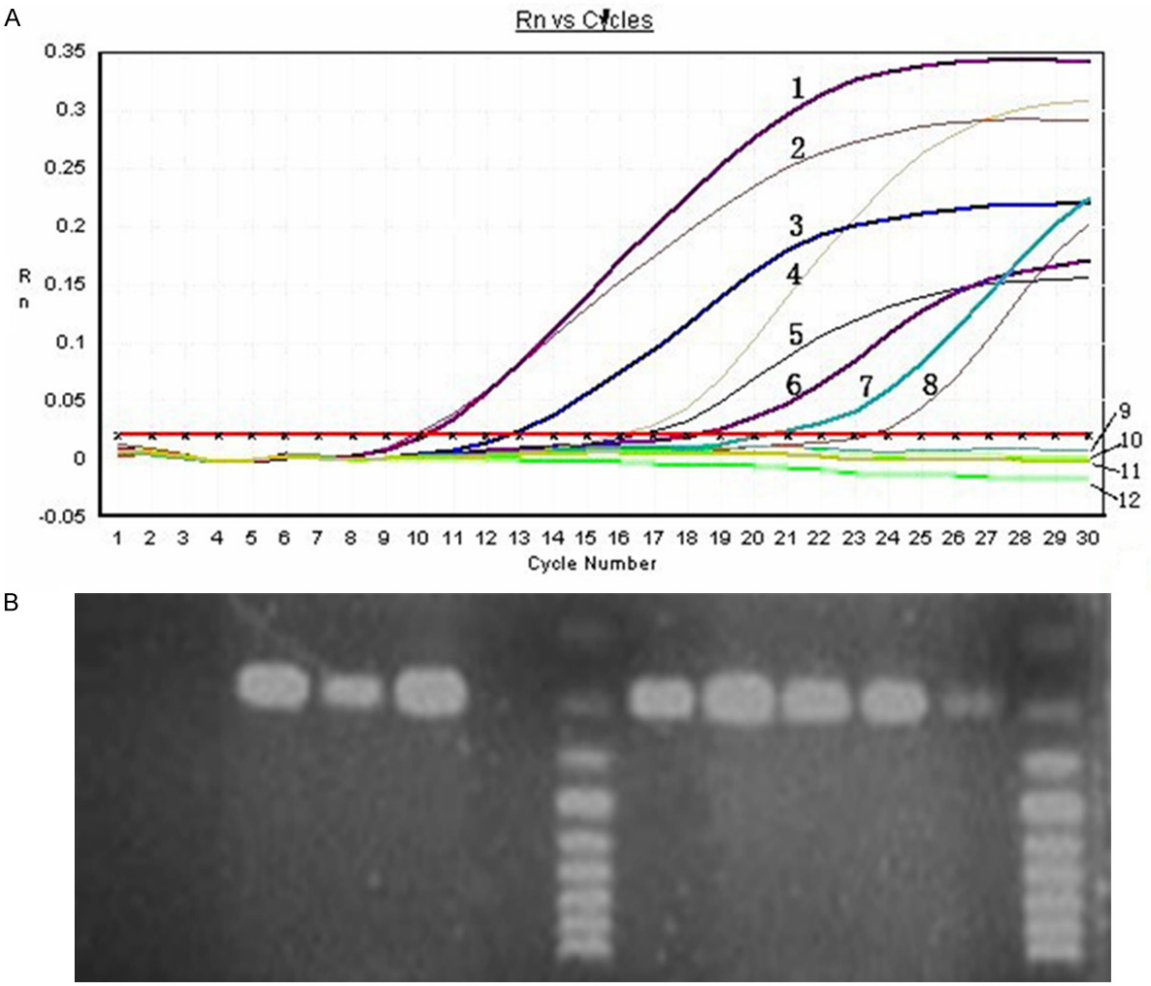

Figure 2. A. Dynamic curve of quantitative fluorescence PCR for detection of CP DNA in kidney tissues 1: 10^6 copies/μg; 2: 9.1×10^5 copies/μg; 3: 10^5 copies/μg; 4: 10^4 copies/μg; 5: 8.6×10^3 copies/μg; 6: 2.3×10^3 copies/μg; 7: 6.7×10^3 copies/μg; 8: 10^2 copies/μg; 9-12: negative. B. Agarose gel electrophoresis. 1, 6: Negative; 2: 2.1×10^2, no specific 278 bp bands; 3-5, 8-12: Specific 278 bp bands; 7, 13: DNA marker.

acute CP infection and previous infection between healthy controls and IgAN cases were not significant. The symptoms cased by CP were not easy to discern unless through specific immunological analysis. The incidence of persistent CP infection in IgAN group was obviously higher than that in the healthy donor group. Thus whether persistent or recurrent CP infection is a risk factor of IgAN needs further discussion.

No differences of statistical significance were found in the percentages of acute CP infection, previous infection and non-infection among cases with non-severe proteinuria, severe proteinuria and persistent renal insufficiency. However, the incidence of persistent CP infection in cases with severe proteinuria and persistent renal insufficiency was obviously higher than that in cases with non-severe proteinuria. This indicated the important role of persistent CP infection in the pathogenesis of IgAN. CP antibody titer, especially IgA antibody titer, usually shows a skewed distribution among the population. Therefore, it is not reasonable to use serum CP antibody titer alone to detect the relationship between CP infection and a specific disease. To overcome this defect, IIF was employed to compare serum CP-AB in cases with primary IgAN and healthy controls. Results showed that the two groups did not differ considerably in incidence of previous infection and acute CP infection, but the incidence of persistent CP infection in IgAN group was much high-

Table 5. Comparison of positive rate of CP DNA in kidney tissues among different clinical subtypes

Clinical subtype	*n*	CP DNA in kidney tissues	
		-	+
Non-severe proteinuria	47	41	6
Severe proteinuria	16	10	6[a]
Persistent renal insufficiency	7	4	3[a]

[a]*P*<0.05, compared to non-severe proteinuria.

Table 6. Comparison of pathological changes of kidney tissues among cases positive and negative for CP DNA ($\bar{x}\pm s$)

CP DNA in kidney tissues	*n*	Glomerular injury scores	Tubulointerstitial scores
Positive	15	6.80±3.12[a]	10.47±4.81[b]
Negative	55	4.53±3.16	7.04±3.94

[a]*P*<0.05, [b]*P*<0.01, compared to positive CP DNA in kidney tissues.

er than that in healthy controls. In 70 cases with primary IgAN, 15 cases were positive for CP DNA, while no CP DNA was detected in the kidney tissues of any cases who died of accidents. It was inferred that persistent CP infection was closely related to IgAN, but not so obviously related to previous infection and acute infection.

Cases positive and negative for CP DNA in the kidney tissues presented substantial differences in clinical manifestations and histopathological changes of kidney tissues. This coincided with the findings among cases having persistent CP infection and non-persistent CP infection. Persistent CP infection and positive CP DNA in kidney tissues are related to various clinical and histopathological indices. These two factors can reflect the severity of the disease, but it is not certain whether they serve as independent factors for predicting the prognosis of IgAN. Moreover, the positive rate of CP IgA antibodies in the serum is related to the detection rate of CP DNA in the kidney tissues. Therefore, CP IgA antibody can be an important indicator in diagnosis and outcome evaluation for diseases related to persistent CP infection.

Super-antigen event triggered by persistent CP infection may be one important reason for renal involvement [13]. Two conditions have to be met for an antigen to be pathogenic in IgAN: The antigen is observed at the sites where IgA antibodies are deposited in the glomeruli; the antigen can bind specifically to IgA that is deposited in the mesangium. We proved through real-time fluorescence PCR that CP DNA was present in the kidney tissues of some IgAN cases. However, we did not confirm the direct influence of the sites and status of CP on the pathological changes of the kidney tissues.

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Disclosure of conflict of interest

None.

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