

Peer Review File

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Reviewer comments

Comment 1: The title of this article is “Low serum IgG4 levels were observed in IgA Nephropathy”. That may not be suitable and not attractive for readers. Please exchange another title that contains critical message and novelty of the present study.

Reply 1: thanks for your suggestion. We have changed our title as “Low serum IgG4 level: a potential diagnostic biomarker for IgA Nephropathy”. Please let us know if you have better advise on the title, we would appreciate and would like to consider your suggestions.

Changes in the text: we have modified our title as advised (see Page 1, line 1)

Comment 2: Authors mentioned that low serum IgG4 levels due to decreased IgG4+B cells and Th2 expressions may hinder to suppress the IgA-IgG immunocomplex formation on glomerulus and complement activity, leading to the development of active mesangial proliferative glomerulonephritis (GN). That could be attractive and intriguing proposal to let us presume another pathogenesis of IgAN other than Galactose-deficient IgA1 (Gd-IgA1)-related multi-hit theory (Suzuki H. Clin Exp Nephrol. 2019). Meanwhile, in contrast to the expectation, obtained results from the present study showed that the serum IgG4 levels and IgG4/IgG were comparable among IgAN patients with different severity. Please discuss more and explain about the reason why authors got discrepancy between the above-mentioned presumption (expectation) and obtained results. Otherwise, description in Page 14, Line 1-3 seems be exaggerated although those description were not assertive.

Reply 2: we made further data analysis. Although there was no significant difference in IgG4 levels and IgG4/IgG (%) when stratifying IgAN participants by hypertension, proteinuria, eGFR, and Oxford score separately, after comprehensive assessment of

disease severity based on the combination of proteinuria, eGFR, and Oxford score, severe IgAN displayed lower IgG4 levels than mild IgAN ($P = 0.039$). Participants with higher risk ($> 50\%$) of renal progression demonstrated lower IgG4 levels than participants with lower risk ($\leq 15\%$) of progression ($P = 0.019$). Additionally, we measured the serum Gd-IgA1 levels in 100 IgAN patients and found a negative correlation between serum Gd-IgA1 and IgG4 levels in IgAN (Supplementary Fig. S3). Moreover, we detected the serum IgG4 and IgG levels in 16 patients with Henoch-Schönlein purpura nephritis (IgA vasculitis) which is similar to IgAN in pathological manifestations and mesangial Gd-IgA1 deposition (1). Low serum IgG4 levels which were comparable with IgAN were observed in Henoch-Schönlein purpura nephritis (Supplementary Fig. S2). These results indicated that IgG4 might be involved in the pathogenesis of IgAN.

Otherwise, we agree with you in the exaggerated description in previous Page 14, Line 1-3, we have modified the expression as “Whether abundant IgG4 could prevent IC formation by competing for similar antigens in IgAN remains to be shown”.

Changes in the text: the measurement of disease severity and risk prediction were added in the method section (see Page 9, line 1-11). We added some data on results, Table 2, supplementary Fig. S2 and supplementary Fig. S3 (see Page 14, line2-8, Table 2; Supplementary file, Page 3 and Page 4). We made more discussion and explanations in the text (see Page 15, line13-14; Page 16, line14-17; Page 17, line1-3). Conclusions were modified correspondingly (see Page 4, line 5-6 and Page 19, line 10-11). We have modified the exaggerated description as advised (see Page 17, line 13-14)

Comment 3: As far as I ascertain, abnormal T-cell-mediated immunity is related to the pathogenesis of IgAN by affecting the aberrant IgA1 galactosylation process. In a recent review by Ruszkowski et al. (Ruszkowski J et al. Clin Exp Nephrol. 2019), IgAN was usually characterized by higher proportions of circulatory Th2, not Th1. It was reported that Th2-related IL-4 amplifies Gd-IgA1 formation by altering terminal glycosylation of secreted IgA1 in an experimental in vitro study (Suzuki H et al. J Biol Chem 2014). In other word, activated Th2 or Th2 derived cytokines might also play a

pivotal role on the development of IgAN, which was incompatible with your results; activation of Th1/2 balance was higher in IgAN, and Th2-related IL-4 were lower in patients with IgAN compared to the healthy control (HC) or the disease control (DC) group. Please discuss more and explain this conflict point between your results and previously reported results.

Reply 3: thanks for your intriguing comments, the comments encouraged us to think more deeply about the results, and enlightened us on the writing of discussions. We have read the review by Ruszkowski et al. (Ruszkowski J et al. Clin Exp Nephrol. 2019) carefully (2), and found that the opinion “IgAN was usually characterized by higher proportions of circulatory Th2, not Th1” was based on one research article (Lichuan Yang et al. Int Urol Nephrol 2017) (3). We read Lichuan’s article and found that the inconsistent results may be attributed to methodological differences. Firstly, we excluded patients with allergic diseases, usually characterized by higher Th2 cells and Th2-dominated inflammation (4,5), In contrast, allergic diseases were not displayed in the exclusion criteria of Lichuan et al. Secondly, Th was defined as CD3⁺CD8⁻ cells in the present study, but it was defined as CD3⁺CD4⁺ by Lichuan et al. It was reported that surface CD4 expression could be interfered by PMA used during the Th cells’ detection (6). Finally, when calculating the ratio of Th1 and Th2, we set Th cells' count as the denominator, while the denominator used in Lichuan et al. was unclear, and appeared to be the count of PBMCs in the review of Ruszkowski et al.

Similar to our results, Clara et al. observed higher percentages of effector memory Th1 cells in the peripheral blood of IgAN patients (7). Higher Th1 related IFN- γ has been reported in the glomeruli and tubules of Henoch-Schönlein purpura nephritis, and the staining grades were positively correlated with the urinary protein/creatinine ratio (8). At an mRNA level, increased expression of Th1 transcriptional factor T-bet and decreased Th2 transcriptional factor GATA3 have been displayed in IgAN patients' urine (9). In animal studies, the bone marrow Th1 was elevated in commencing IgAN while Th2 was elevated in quiescent IgAN (10).

IL-4 has been reported to induce the production of Gd-IgA1 in an experimental in vitro study (Suzuki H et al. J Biol Chem 2014)(11), suggesting that Th2 may participate in

the pathogenesis IgAN and it is worth further research. However, besides of Th2, many other cells have been shown to produce IL-4, such as basophils, mast cells, and eosinophils (12). We did not detect the serum IL-4 level. In our study, IL-4 was detected as a marker of Th2, and it was expressed in the cytoplasm before detection, so it is different from serum IL-4 concentrations.

Changes in the text: we added some discussion and explanation in the text (see Page 18, line 4-15; Page 19, line 6-8).

Comment 4: In the present study, authors mentioned that low serum IgG4 and IgG4/IgG seem to be remarkable diagnostic biomarkers for IgAN. In the comparison of serum IgG4 levels between the study groups, authors selected the minimal change disease (MCD) or primary membranous nephropathy (PMN) as DC. However, the selection might be unconvincing. As you know, patients with PMN have serum anti-PLA2R Antibody (that is consisted of IgG4), thus serum IgG4 level in patients with PMN could be definitely high when comparing other kidney diseases. Thus, I wonder that authors just got positive results when comparing the data of HC as negative control and PMN as positive control. Furthermore, as you mentioned in the background, IgAN is the most common GN, whereas MCD or PMN is typical glomerular disease that present nephrotic syndrome (NS), not GN. Therefore, to establish the strong evidence that low serum IgG4 level has a distinctive diagnostic marker for IgAN, please consider to select not only kidney diseases exhibiting NS but also other GN exhibiting glomerular IgA deposition such as post infectious glomerular disease or lupus nephritis, or IgA vasculitis, or hepatic IgA nephritis, and so on. Otherwise, it may be hard to propose that low IgG4 has a potential of diagnostic value for IgAN.

Reply 4: thanks for your professional advice. We added 17 patients with lupus nephritis which exhibited glomerular IgA deposition in the disease control, the results on comparison among groups and ROC curve analysis were consistent with our previous description. 16 patients with IgA vasculitis (also known as Henoch-Schönlein purpura nephritis or secondary IgAN) were also included, their IgG4 levels were comparable with that in primary IgAN, which indicated that the low IgG4 level may be associated

with the essence of IgAN. We did not include hepatic IgA nephritis, because most of our patients with hepatic nephritis manifested as membranous nephropathy rather than IgA nephritis at kidney biopsy.

Changes in the text: we added some data in Table 1, Fig. 1, and Fig. 2. We described the results associated with IgA vasculitis in Page 16, line 15-17 and Page 17, line 1.

Comment 5: In the section of Background, authors implied the possibility of the involvement of serum IgG4 in the immune-mediated pathogenesis of IgAN by referring the previous report (Lai KN et al. Nat Rev Dis Primers 2016). This might be important information, thus please consider to search more supportive reports or explanation.

Reply 5: we added additional supportive reports and explanations in the background as follows. The immune complex (IC) containing Gd-IgA1 in IgAN can promote the proliferation of human mesangial cells (13), while IgG4 has been reported to inhibit the IC formation through preventing cross-linking of antigens in autoimmune myasthenia gravis (MG) (14). The activation of complement can further aggravate the proliferation of mesangial cells and excessive production of inflammation mediators in IgAN (15), whereas IgG4 has been demonstrated to suppress the complement activation through competing with IgG1 or IgG3 for similar antigens in Bullous pemphigoid (16).

Changes in the text: we have added some text as advised (see Page 5, line 16-17; Page 6, line 1-6)

Comment 6: If it's OK with you, please increase the sample number of analysis for IgG4+B/B cells (n = 21) and Th2/Th cells (n=12) in Figure 3 in order to improve the evidence. Compared to the total number of enrolled IgAN patients (n=112) in the present study, the number of patients who received the analysis for FACS might be low.

Reply 6: although it is hard to collect a large amount of qualified fresh blood in a short time, we have tried our best to increase the sample size of flow cytometry to 35-36 individuals in each group.

Changes in the text: we added some data in Fig. 3.

References:

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