Reviewer’s report

Title: Validation of a newly proposed histopathological classification in Japanese patients with anti-neutrophil cytoplasmic antibody-associated glomerulonephritis

Version: 3 Date: 25 January 2013

Reviewer: Arda Goceroglu

Reviewer’s report:

Major Compulsory Revisions

Methods ‘Statistical analysis’

1) The authors mentioned in their previous response that they could not perform competing risk analysis in their statistical software and that they, in order to avoid competing risk for ESRD, excluded the 5 patients with death before ESRD in the Kaplan-Meier renal survival analysis and Cox regression multivariate analysis in accordance with the report by Berden et al [8]. The authors should reconsider this. Berden et al did not exclude the patients with death before ESRD. Since the authors are unable to perform competing risk analysis, they should include the patients with death before ESRD in their Kaplan-Meier renal survival analysis and Cox regression multivariate analysis and treat them as censored (at the moment of death). These patients can not be excluded. The authors should reconsider these analyses, include these patients and censor them at the moment of death.

In accordance with this also reconsider the sentence ‘Third, although the patients with death before ESRD were excluded in the renal survival analysis, we included the cases with missing follow-up as censored. Thus, renal survival could have been overestimated’ in the discussion.

Results ‘Clinical and histopathological study’

2) The authors mention that 23 patients developed ESRD during follow-up and that 3 of these had ESRD at the moment of diagnosis. Does this mean that these 3 presented with ESRD? If this is the case, the authors should change this to: ‘Three patients had ESRD at the moment of diagnosis. During follow-up 20 patients developed ESRD.’ This change must then be reconsidered throughout the manuscript. The 3 patients which present with ESRD at the moment of diagnosis can not be seen as patients that develop ESRD during follow-up.

Since this manuscript looks at predicting ESRD with the classification and #-SMA, patients with ESRD at moment of presentation/diagnosis can not be included in the analyses, because in these patients you are not looking at prediction but coincidence. So these patients should be described in the manuscript, but they should not be taken into account in case of analyses regarding predicting ESRD during follow-up.

3) ‘There was no significant difference between crescentic and mixed classes,
particularly during the early to mid-term follow-up.’ In the previous answers of the authors, they mention that this was based on the fact that in Figure 2 the Kaplan-Meier survival curves of crescentic and mixed class cross near the points of 12 and 36 months. Thus they estimated that the two classes did not significantly differ. Although this reasoning is understandable (when looking at figure 2) it is better to perform statistical analysis to compare these 2 classes with each other (univariate Cox regression with 1 of the 2 as reference category). Since the authors perform immunohistochemie because it was not possible differentiate crescentic and mixed class in terms of renal outcome, they should provide statistical analysis showing this insignificance (in stead of estimating).

Minor Essential Revisions

Abstract

1) Conclusion: ‘The new histopathological classification may be appropriate for the patients with ANCA-associated glomerulonephritis in our Japanese cohort. #-SMA positivity also might be a prognostic factor for ESRD.’ change into ‘The new histopathological classification tended to be associated with ESRD in our Japanese cohort with ANCA-associated glomerulonephritis. #-SMA positivity might be an additional prognostic factor for ESRD.’

Methods ‘Treatment’

2) ‘In this present study, 83 (81.4%) cases were received methylprednisolone pulse therapy, and 89 (87.3%) cases were administered oral prednisolone.’ change into ‘In this present study, 83 (81.4%) cases received methylprednisolone pulse therapy, and 89 (87.3%) cases were administered oral prednisolone.’

3) ‘Furthermore, 29 (28.4%) cases were treated with cyclophosphamid (CYC): Fourteen (13.7%) cases were treated with intravenous CYC pulse therapy (IV-CY), 8 (7.8%) cases were treated with oral CYC therapy, and 4 (3.9%) cases were treated with both IV-CY and oral CYC.’ If you count the numbers: 14+8+4=26. The authors should reconsider the numbers since this does not make up 29.

4) ‘Twenty-one (20.6%) cases were treated with mizoribine (MZB), and 3 (2.9%) cases were treated with azathioprine (AZA).’ change into ‘Twenty-one (20.6%) cases were treated with mizoribine (MZB), 3 (2.9%) cases were treated with azathioprine (AZA), and 1 (1.0%) case was treated with gusperimus hydrochloride.’

Methods ‘Patient parameters and outcomes’

5) ‘We investigate the short term and long term renal outcome. Long term renal outcome: We defined the development of ESRD during follow-up as a primary endpoint. Short term renal outcome: The definition of secondary endpoint is serum creatinine doubling and/or the development of ESRD at the time of 1 year after diagnosis. ESRD is defined as requiring permanent renal replacement therapy.’ This part is not clear to me.
The authors should only mention their primary and secondary outcome, leaving out the separation in long term and short term renal outcome. This makes this part confusing. The authors should also mention eGFR at 1 year as secondary outcome.

Methods ‘Renal histopathology’

6) ‘Samples with > 50% normal glomeruli were classified as focal, those with > 50% cellular crescent glomeruli were classified as crescentic, and those with > 50% globally sclerotic glomeruli were classified as sclerotic. The other cases, and those with < 50% normal, cellular crescent, or globally sclerotic glomeruli were classified as mixed.’ change into ‘Samples with # 50% normal glomeruli were classified as focal, those with # 50% cellular crescent glomeruli were classified as crescentic, and those with # 50% globally sclerotic glomeruli were classified as sclerotic. The other cases, those with < 50% normal, cellular crescent, and globally sclerotic glomeruli were classified as mixed.’

The authors should mention at the end of this paragraph ‘Discrepancies between the observers were resolved by conference to achieve consensus.’

Methods ‘Immunohistochemical analysis’

7) ‘Of 50 cases with crescentic and mixed classes, 16 cases could not be performed immunohistochemical study because the rest paraffin embedded samples from renal biopsy were too small.’ change into ‘Of the 50 cases with crescentic or mixed class, immunohistochemical analysis could not be performed on 16 cases, because the paraffin embedded samples from renal biopsy were too small.’

8) ‘in samples from 17 34 patients with crescentic and mixed classes for’ change into ‘on samples from 34 patients with crescentic or mixed class for’

9) ‘Charactereistics’ change into ‘Characteristics’

10) ‘For -SMA, CD68 and cytokeratin, the cytoplasmic staining in a cell was positive. For WT1, the nuclear and/or cytoplasmic staining in a cell was positive.’ This sentence is unclear. Do the authors mean: ‘For #-SMA, CD68 and cytokeratin, the cytoplasmic staining in a cell was considered as positive. For WT1, the nuclear and/or cytoplasmic staining in a cell was considered as positive.’? The authors should reconsider this sentence.

11) The authors should reconsider the sentence “For WT1, the nuclear and/or
cytoplasmic staining in a cell was positive.' This, because in case of a positive WT1 the nucleus always has to be positive, and sometimes the cytoplasm can also be positive. Normally the case of cytoplasm staining without nucleus staining does not occur. Therefore particularly the ‘and/or’ part of this sentence should be reconsidered.

12) ‘Reproducibility was assessed by blinded replicate countings of immunopositive cells performed by 2 readers (n = 20); the intraclass correlation coefficients with #-SMA, WT1, CD68 and cytokeratin were r = 0.86, 0.77, 0.91, 0.87, respectively (all P < 0.001).’ Are these 2 readers different from the two investigators (T.I. and A.Y.) mentioned above? Do the authors mean with ‘n=20’ how many samples of the 34 were recounted? The authors should reconsider this sentence and describe these 2 aspects more accurately. The authors should also ask a statistician whether the P-value should be mentioned in case of intraclass correlation coefficients.

The authors should mention at the end of this paragraph ‘Discrepancies between the observers were resolved by conference to achieve consensus.’

Results ‘Clinical and histopathological study’

13) The authors mention that 23 patients developed ESRD during follow-up and that 3 of these had ESRD at the moment of diagnosis. Does this mean that these 3 presented with ESRD? If this is the case, the authors should change this to: ‘Three patients had ESRD at the moment of diagnosis. During follow-up 20 patients developed ESRD.’ This change must then be reconsidered throughout the manuscript. The 3 patients which present with ESRD at the moment of diagnosis can not be seen as patients that develop ESRD during follow-up.

14) The authors added the following part, which is very informative:
‘Table 2 shows the clinical and histopathological findings between four histopathological classes. Age and gender were not significantly different between four classes. PR3-ANCA positive patients were seen in focal class (n = 3) and in crescentic class (n = 2), however PR3-ANCA positive patients were not present in mixed or sclerotic classes. Serum CRP was higher in the focal and mixed classes than in the mixed and sclerotic classes. Proteinuria in focal class was lower than the other three classes. The rate of normal glomeruli was higher in the mixed class than in the crescentic class, but the proportion of sclerotic glomeruli was higher in the mixed class than in the crescentic class.’

The authors should reconsider this part, regarding the linguistic errors and some small mistakes (example: ‘in the focal and mixed classes than in the mixed and sclerotic classes’).

15) ‘However, the Kaplan-Meier renal survival curve was almost similar to these of Berden et al. report’ change into ‘However, the Kaplan-Meier renal survival curve was almost similar to the curve of Berden et al.’

16) ‘In the Cox regression multivariate analysis, new classification system and eGFR at diagnosis tended to be prognostic factors for ESRD in the total follow-up
In the multivariate Cox regression analysis, the new classification system and eGFR at diagnosis tended to be prognostic factors for ESRD during the follow-up period.

Results ‘Immunohistochemical study’

17) ‘By contrast, #SMA immunoreactivity differed substantially between each glomerulus.’ change into ‘By contrast, #SMA immunoreactivity differed substantially between each normal glomerulus.’

18) ‘As shown in Figure 3A, there was no immunoreactivity for #SMA in the normal glomerulus, except in the glomerular vascular pole and surrounding Bowman’s capsule. On the other hand, Figure 3B shows marked immunoreactivity for #SMA in a normal glomerulus.’ change into ‘As shown in Figure 3A, there was no immunoreactivity for #SMA in this normal glomerulus, except in the glomerular vascular pole and surrounding Bowman’s capsule. On the other hand, Figure 3B shows marked immunoreactivity for #SMA in a normal glomerulus.’

19) The mean rate of #SMA-positive glomeruli per normal glomeruli was 82.9%.

This sentence is not clear to me. Do the authors mean that the mean proportion of #SMA-positive normal glomeruli per biopsy 82.9% is? The authors should reconsider this sentence.

20) The authors should describe in the manuscript what the cut-off of 83.3% is based on.

Discussion ‘Clinical and histopathological findings’

21) ‘Although the Kaplan–Meier survival curves were similar to those of the study by Berden et al., however, there was no significant difference between the crescentic and mixed classes, particularly in early 1 to mid-term follow-up.’ change into ‘Although the Kaplan–Meier survival curves showed the same distribution as the study by Berden et al., the histopathologic classification was not associated with ESRD during follow up in our cohort in the multivariate analysis.’

22) ‘We think there are several possible explanations for why the proposed classification did not completely differentiate the crescentic and mixed classes in our cohort.’ change into ‘We think there are several possible explanations for why we did not find an association between the classification and developing ESRD.’

23) The authors should mention that the histopathologic classification was associated with eGFR at 1 year, which was also the case in the study of Berden et al.

Discussion ‘Immunohistochemical findings’

24) The author should start this paragraph with: ‘There was no significant difference regarding eGFR at 1 year and the development of ESRD between the
crescentic and mixed classes. Therefore we performed immunohistochemical staining on biopsies from 34 patients with crescentic or mixed class to examine whether immunohistochemical factors are useful to predict eGFR at 1 year and ESRD in ANCA-associated glomerulonephritis.'

25) The authors mention ‘We performed immunohistochemical staining of biopsies from 34 patients to examine whether immunohistochemical factors are useful to predict eGFR at 1 year and ESRD in ANCA-associated glomerulonephritis.’, but nothing is mentioned about immunohistochemical factors and eGFR at 1 year. The authors only focus on ESRD in the manuscript. The authors should reconsider this sentence (or add the eGFR at 1 year analysis), and also the sentence in the results ‘Because histopathological evaluation by light microscopy could not differentiate the crescentic and mixed classes in terms of eGFR at 1 year and ESRD, we conducted immunohistochemical studies to examine whether it could estimate the renal outcome.’

26) ‘On the other hand, #-SMA immunoreactivity tended to differ between patients with or without ESRD in crescentic and mixed classes (Figure 3A, 3B). The proportion of #-SMA-positive glomeruli per normal glomerulus in patients with ESRD group was much higher than that in patients without ESRD (98.5% versus 74.3%, P < 0.05).’ The authors should reconsider this part in their discussion:
• Figure 3 (A and B) does not support the statement made here.
• This part discusses results which are not presented in the results part. The authors should remove this part, since they also removed this part in the results (see author’s comment on previous review, n°33)
• The authors should focus more on the Kaplan-Meier (figure 3C) in this part of the discussion and recall and discuss their findings on whether aSMA differentiates the crescentic and mixed class in terms of the development of ESRD.

27) ‘However, #-SMA positivity was not correlated with renal survival at 1 to 3 year after diagnosis. Therefore, further studies are needed to clarify whether immunohistochemical staining of #-SMA is a useful predictor of the early phase of renal prognosis.’ change into ‘However, #-SMA positivity was not associated with renal survival. Therefore, further studies are needed to clarify whether immunohistochemical staining of #-SMA is a useful predictor for renal prognosis.’

The authors should at between these 2 sentences that it could be due to the low power of the #-SMA analysis (n=34), since the survival analysis did not reach significance but came close to it (p=0.11)

28) ‘Further verification by the prospective study is desired.’ change into ‘Further verification by a prospective study is desired.’ The authors should also explicitly mention the retrospective character of the study as a limitation.

Conclusion
29) ‘The histopathological classification system proposed by Berden et al. [8] is a simple method, and tends to be correlated with the incidence of ESRD in Japanese patients with ANCA-associated glomerulonephritis.’ change into ‘The histopathological classification system proposed by Berden et al. [8] is a simple method, and tends to be correlated with the incidence of ESRD in Japanese patients with ANCA-associated glomerulonephritis in our cohort.’

30) ‘#-SMA immunopositivity shows trend toward an association with the renal survival rates of patients classified 1 as crescentic or mixed classes.’ change into ‘#-SMA immunopositivity shows a trend toward an association with the renal survival rates of patients classified as crescentic or mixed class’

31) ‘Further verification of #-SMA staining to the ANCA-associated glomerulonephritis is desired’ change into ‘Further verification of the histopathologic classification and #-SMA staining for ANCA-associated glomerulonephritis is desired’.

32) The authors should mention in the conclusion that the histopathologic classification was associated with eGFR at 1 year. This should also be mentioned in the abstract.

Whole manuscript

33) The authors should check their manuscript for linguistic corrections (misspellings, construction of some sentences).

Discretionary Revisions

Methods ‘Patients’

1) ‘Histological confirmation was the findings of necrotizing vasculitis and pauci-immune crescentic glomerulonephritis’ change into ‘Histological confirmation of renal involvement was the finding of necrotizing vasculitis and pauci-immune crescentic glomerulonephritis in the renal biopsy.’

Results ‘Clinical and histopathological study’

2) ‘On the other hand, there was no significant difference between patients treated with or without CYC (data not shown).’ change into ‘In addition, there was no significant difference between patients treated with or without CYC (data not shown).’

Whole manuscript

3) The authors use a lot of commas when writing down statistical output, examples: (median, 41.0 months, IQR, 20.0-63.8) and (classification system, P = 5 0.0986, Crescentic vs. Focal, hazard ratio (HR), 2.61, 95% confidence interval (CI), 6 0.53-20.1, Mixed vs. Focal, HR, 4.43, 95% CI, 0.96-32.2, Sclerotic vs. Focal, HR, 8.70, 7 95% CI, 1.32-75.8, and eGFR, P = 0.0547, HR, 0.97, 95% CI,
0.93-1.00, respectively. It does not look very clear to me. My suggestion to the authors is to note the same statistical output differently.

**Level of interest:** An article of importance in its field

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** Yes, and I have assessed the statistics in my report.

**Declaration of competing interests:**

I declare that I have no competing interests.