Reviewer’s report

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

Version: 2 Date: 16 October 2012

Reviewer: Arda Gocergolu

Reviewer’s report:

The authors of this manuscript recognize the importance to validate the histopathologic classification of ANCA-associated glomerulonephritis in different cohorts. In their manuscript Iwakiri and colleagues validated this classification in a Japanese cohort. In addition they performed immunohistochemical staining. For both they investigated the predictive value for the development of ESRD during follow up. This study provides important information on the usefulness of this classification in a Japanese cohort regarding the prediction of ESRD. This kind of information is needed and hopefully will lead to classification refinements in the future. However, this manuscript could in general benefit from the suggestions listed below.

- Major Compulsory Revisions

The author must respond to these before a decision on publication can be reached. For example, additional necessary experiments or controls, statistical mistakes, errors in interpretation.

Methods

1) Patients: It is mentioned that the patients received methylprednisolone with or without immunosuppressants after diagnosis. Could the authors mention the numbers of patients receiving immunosuppressants and what types of immunosuppressants (CYC, AZA, MMF, MTX etc.)? Depending on the numbers it could be an option to include the therapy as a covariate in the multivariate analysis, for example: immunosuppressants yes/no.

2) Immunohistochemical analysis: It is not clear how the positive cells have been counted. This is not an easy job, especially regarding the synaptopodin staining, which stains slit pores of podocytes. It is hard to score these stainings by counting positive cells, like the authors mention. I would recommend to perform a WT1 staining for podocytes, which makes it much easier to count the positive cells. The authors should comment on how they exactly counted the positive cells, and provide a picture of a synaptopodin staining as a figure with the given score.

3) Statistical analysis: The authors used the Mann-Whitney test and the Fisher’s exact test (both non-parametric) when comparing 2 groups with each other. In case of comparing >2 groups they used the one-way analysis variance test,
which is a parametric test. Since the groups in comparing >2 groups are smaller than the smallest group in comparing 2 groups, I would recommend to also use a non-parametric test for the >2 groups comparison, namely the Kruskal-Wallis test. It should also be mentioned here that a post hoc analysis was performed, in stead of mentioning this only in the legend of figure 1.

4) Statistical analysis: I assume that the patients in the Kaplan-Meier survival analysis and the Cox regression were censored for last visit and death. The authors should mention this explicitly in this section. Since 12 patients died without ESRD, it would be statistical more feasible to perform a competing risk analysis, which considers death without ESRD as a competing event of ESRD.

5) Additional paragraph: A paragraph should be added to the methods, called: ‘Patient parameters and outcomes’. In this paragraph the following should be described: the clinical and histological variables collected for each patient, the primary endpoint of this study (ESRD), the secondary endpoint of this study (eGFR at 1 year), and a clear definition of ESRD. In the results it is mentioned that ESRD is defined by requiring maintenance hemodialysis therapy, but later on (results, paragraph 2) 2 patients who had a doubling in SCr were also taken into account as ESRD.

Results ‘Clinical and histopathological study’

6) Additional table: The authors should add a baseline table, showing and comparing the baseline characteristics (like in table 1) between the patients of the four histopathologic classes. This gives an overview regarding the baseline characteristics per histopathologic class.

Results ‘Immunohistochemical study’

7) The authors start this paragraph with ‘Because histopathological evaluation by light microscopy could not differentiate the crescentic and mixed class in terms of renal outcome, we conducted immunohistochemical studies to examine whether it could estimate the renal outcomes.’ After this sentence it is unclear throughout the whole paragraph which patient group they are looking at (all the patients or only the patients with crescentic and mixed class). They stained samples of 79 patients. Is this a selection out of the total of 102? Is it conceivable that this selection influences the prognostic value? The total number of patients with crescentic or mixed class is 50. In the text the authors write about ‘patients’ (all 79 patients?), but the figure they refer to (figure 3C) only describes the patients with crescentic and mixed class.

It is also unclear whether the authors only looked at the normal and morphologically normal glomeruli, or at all glomeruli (even the ones with glomerular lesions) as mentioned in Methods, Immunohistochemical analysis. It looks like they did both, but they don’t distinguish between them clearly.

The authors should rewrite this part of the results. They should look at all four classes (in this case also mention the distribution over the classes) or only at the patients with crescentic or mixed class. It is also possible to do both, but if they choose to do this, they should describe the four and two classes separately. It is
important to clearly describe which patients (and how many) are being analysed and why. The authors should also clearly distinguish whether they are looking at only the normal glomeruli, the morphological normal glomeruli, or all glomeruli (including the ones with lesions). They can choose to look at only (morphological) normal glomeruli or all glomeruli. It is also possible to take both into account in the analysis, as long as it is clearly mentioned. In the discussion should be described why they chose to look at a specific group of glomeruli or all glomeruli.

In accordance to the changes above, the ‘immunohistochemical findings’ part in the discussion, conclusion, and figures 3 and 4 should be reconsidered. In the current version it is unclear whether the prognostic significance of aSMA, mentioned by the authors, counts for the whole group or a subgroup.

Discussion

8) Immunohistochemical findings: The authors should add to the discussion that they only performed univariate analysis for the relation between aSMA positive glomeruli and ESRD. They did not perform a multivariate Cox regression for aSMA as they did for the histopathologic classification. Why not? Actually, the Kaplan Meier curve of the classification (figure 2) shows a significant relation with the development of ESRD. This is not shown by the Kaplan Meier curve of the aSMA positivity (figure 3). In the conclusion the authors describe that the histopathologic classification is not useful for Japanese patients, but that aSMA could be useful. This does not seem to be supported by these results. The authors should perform a multivariate Cox regression analysis of aSMA positivity of the glomeruli, and reconsider their discussion and conclusion regarding these results.

9) Additional paragraph: The authors should clearly state the limitations of their study in a paragraph in the discussion.

Conclusion

10) First sentence: The authors conclude that the classification is not well correlated with renal prognosis in Japanese patients. It should be mentioned that this has only been investigated for eGFR at 1 year and the development of ESRD during follow-up. The authors extrapolate their results to all Japanese patients, although they only looked at a small cohort within the Japanese ANCA Vasculitis population. The multivariate Cox regression showed a p-value of 0.0590 for the classification corrected for the other variables. It could be that the classification would reach significance if the cohort was larger. The authors should limit their conclusion to their cohort and discuss the power-issue (as described above) in their discussion.

11) Second sentence: This conclusion is not supported by the Kaplan-Meier survival plot (figure 3C). Reconsider this sentence in accordance with the adaptations described above regarding the results of the immunohistochemical study.

Total manuscript
12) The discussion and conclusions are not well balanced and adequately supported by the data. At the moment the article overemphasizes the differences between Berden et al. and the results of this article. Since the data do not seem to differ that much, especially regarding the survival plot of the four classes and the near significant p-value of the classification in the multivariate Cox regression, the authors should not only focus on the differences but also mention the similarities between their results and the results of Berden et al. The authors emphasize on the prognostic significance of aSMA staining. They have not yet demonstrated the superior prognostic value of the aSMA staining. The authors should temper this emphasize in the discussion and conclusion, or provide more data and explanation on why aSMA is a better predictor for ESRD than the classification. It is unclear how the results presented in fig. 4, although statistically significant, show that ESRD can be predicted on the basis of aSMA positivity.

- Minor Essential Revisions

The author can be trusted to make these. For example, missing labels on figures, the wrong use of a term, spelling mistakes.

# = a linguistic correction

Abstract

1) Methods: 'We conducted immunohistochemical staining for #-smooth muscle actin (SMA), synaptopodin, CD68, and cytokeratin to examine whether the new classification can predict renal prognosis.' This sentence is not correct, since the classification has nothing to do with immunohistochemical staining. The authors should reconsider this sentence.

2) Results: The authors did not mention the results of the multivariate Cox regression in the abstract. The authors should mention these results in the abstract.

Introduction

3) Paragraph 1: 'however, no active lesions such as fibrous crescent formation, and focal or global glomerular sclerosis are usually observed' # 'however, lesions such as fibrous crescent formation, and focal or global glomerular sclerosis, indicating non active lesions, are also observed'

4) Paragraph 1: 'Several studies have determined the clinical and histopathological predictors of renal outcomes, and shown that low levels of serum creatinine (Scr) at diagnosis and a high percentage of normal glomeruli were better predictors of renal outcome, whereas a high percentage of sclerotic glomeruli was a worse predictor for renal outcome' # 'Several studies have determined the clinical and histopathological predictors of renal outcome, and shown that low levels of serum creatinine (Scr) at diagnosis and a high percentage of normal glomeruli were predictors for a better renal outcome, whereas a high percentage of sclerotic glomeruli was a predictor for a worse
renal outcome

5) Last paragraph: ‘The classification system was based on glomerular pathology and four general categories of disease: focal, crescentic, sclerotic, and mixed’ # ‘The classification system is based on glomerular pathology and distincts four classes: focal, crescentic, mixed, and sclerotic’

Methods

6) Patients: ‘Between January 2000 and March 2010, we enrolled 122 patients with renal biopsy-confirmed ANCA-associated glomerulonephritis across six institutions in Japan’ # ‘We enrolled 122 patients diagnosed with biopsy-confirmed ANCA-associated glomerulonephritis between January 2000 and March 2010 from six institutions in Japan’

7) Patients, second to last sentence: judgment # judgement.

8) Renal histopathology: ‘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. Other cases, those with < 50% normal, cellular crescentic, and globally sclerotic glomeruli were classified as mixed.’ # ‘Samples with > 50% normal glomeruli were classified as focal, those with > 50% cellular crescentic glomeruli were classified as crescentic, and those with > 50% globally sclerotic glomeruli were classified as sclerotic. The other cases, those with < 50% normal, cellular crescentic, and globally sclerotic glomeruli were classified as mixed.’

9) Renal histopathology: Nothing is mentioned about the agreement between the 2 nephropathologists. The authors should mention the interobserver variation (%) and how this is solved?

10) Immunohistochemical analysis: Nothing is mentioned about the agreement between the 2 investigators who scored the immunoreactivity and whether they were blinded. The authors should mention the interobserver variation (%), how this variation was solved, and whether the investigators were blinded to the patients’ characteristics.

11) Immunohistochemical analysis: ‘For all markers, the cytoplasmic staining > 50% in each cell was defined as positive’ # ‘For all markers, a cytoplasmic staining of > 50% in a cell was defined as positive’
12) Statistical analysis: In the manuscript (text, tables and figures) the authors only provide significant P-values. The authors should accompany all the significant P-values with a 95% confidence interval. In case of the Cox regression the hazard ratio should also be mentioned.

Results ‘Clinical and histopathological study’
13) First paragraph, first sentence: The median follow-up and range should be mentioned.

14) Table 1: Some small adaptations should be made to this table to make it clearer.
   • ESRD (n=23) # ESRD during follow-up (n=23)
   • The brackets around the numbers in the row ‘sex’ can be removed.
   • Mention the 95% CI for the 2 significant variables

15) Paragraph 1: the authors mention that SCr is measured and renal biopsies are collected before starting immunosuppressive therapy or hemodialysis. Do the authors mean temporary hemodialysis immediately after diagnosis or maintenance dialysis which means that some patients had ESRD at the moment of diagnosis?

16) Paragraph 1: the authors mention the median SCr, eGFR and proteinuria at time of renal biopsy in the text. The authors should also mention the distribution (SD or range) of these variables in the text.

17) Paragraph 1: ‘The 102 patients were then categorized into the four classes according to the newly proposed histopathological classification, including focal in 46 patients, crescentic in 32 patients, mixed in 18 patients, and sclerotic in six patients.’ # ‘The 102 patients were then categorized into four classes according to the newly proposed histopathological classification: 46 patients with focal, 32 patients with crescentic, 18 patients with mixed, and six patients with sclerotic class.’

18) Paragraph 1: There is an improvement of the eGFR between the moment of diagnosis and 1 year later, especially in the crescentic class. The authors should comment on the improvement of eGFR after 1 year follow up.

19) Figure 1: Some small adaptations should be made to this figure to make it clearer.
   • Rename the title: eGFR at 1 year later # eGFR at 1 year
   • The figure shows a range for each dot. The authors should mention in the legend of the figure which proportions these ranges cover?
   • It seems that the position of the dots in the figure on the eGFR scale do not match with the numbers described in the text (results, first paragraph). For example: the dot of the ‘mixed class’ group in the graph of eGFR at diagnosis is on the eGFR scale on the level of 20. In the text the authors describe that the eGFR at baseline of the ‘mixed class’ group is 16.5. The authors should check
this for both graphs of figure 1 and refine both graphs according to the numbers described in the text.

20) Paragraph 2: The word ‘rate’, which is used 2 times in this paragraph, should be replaced by the word ‘proportion’. Also change this in the figures and discussion accordingly.

21) Paragraph 2: ‘The renal mortality rate increased with sequential category’ # ‘The proportion of patients who developed ESRD during follow up increased with sequential category’

22) Paragraph 2: The authors use different terms for ESRD throughout the text/tables/figures, like ‘renal mortality rate’ in this paragraph. They should use ESRD throughout the whole text/tables/figures for clarity and consistency. The words ‘renal survival’ regarding the Kaplan-Meier curve can stay as they are in the text and in figure 2.

23) Paragraph 2: The authors mention that the renal prognosis was not significantly different between the crescentic and mixed class, particularly during the early to mid-term follow-up. On the long term follow up the prognosis of these 2 classes seem to differ (see figure 2). The authors do not show the insignificance of this difference in the text. The authors should provide a statistical output (p-value) showing this insignificance.

24) Table 2: Change the title ‘Rate of renal outcomes according to histopathological class’ # ‘Proportions of ESRD according to the histopathological classes’

25) Figure 2
• Change the title ‘Renal survival in follow up period’ # ‘Renal survival during follow up’
• Change ‘renal death’ in the figure (top right) to ‘ESRD’.
• Change the first 2 sentences of the legend of this figure: ‘Renal survival rates in total follow-up period. Overall, 23/102 patients became dialysis dependent during the total follow-up period.’ # ‘Renal survival during the follow-up period. Overall, 23/102 patients developed ESRD during the total follow-up period.’
• ‘Real survival rates in the crescentic and mixed classes were similar, especially in the early to mid-term’ # ‘Renal survival in the crescentic and mixed classes were similar, especially in the early to mid-term’
• The Kaplan-Meier curve does not start at 100% for the crescentic and sclerotic class. This means that there were patients who had ESRD at the moment of diagnosis. Is this true? If this is the case, the authors should mention in the results how many patients had ESRD at the moment of diagnosis. I assume that these patients (if there were any) are not included in the count of 23 patients who developed ESRD during follow up and were excluded from the multivariate Cox regression?

26) Table 3: The authors should mention the hazard ratios and the 95% CI’s for
all the variables in this table.

Results ‘Immunohistochemical study’

27) First paragraph: ‘Therefore, we performed immunohistochemical staining of samples from 79 patients’ # ‘Therefore, we performed immunohistochemical staining on samples from 79 patients’

28) First paragraph: The authors should describe more clearly what they mean with ‘a morphologically normal glomerulus’.

29) First paragraph: The word ‘rate’ (two times used in this paragraph) should be changed to ‘proportion’. Also change this in the figures and discussion accordingly.

30) The last sentence: This sentence is confusing. Should the author have written morphologically normal glomeruli in this sentence?

31) Figure 3 legend: The title of figure 3 seems not appropriate. It mentions aSMA expression in normal and crescentic glomeruli. In A and B only normal and morphological normal glomeruli are shown. In C we see the survival of different aSMA expressions. According to the text this should be the expression in all glomeruli. The authors should clarify this (in accordance with the point stated under ‘Major Compulsory Revisions’).

32) Figure 3C
   • The group of patients is split on the basis of an aSMA positive ‘rate’ above or below 82.9%. Why did the authors choose to take the mean ‘rate’ of #SMA positive glomeruli per morphologically normal glomeruli as the cut off point?
   • The survival analysis shows a p-value of 0.1008 in the log rank test. Is this number correct? You would expect a significant difference when looking at the difference in survival between the 2 groups.

33) Figure 4: Why did the authors choose to analyse the data this way, and not like in figure 3 with a Kaplan-Meier survival analysis? The authors should make a Kaplan-Meier survival curve of the data in this figure. Although this figure shows a significant difference in aSMA expression in normal glomeruli between the patients who did and did not have ESRD during follow up, it does not take their follow up time into account. It is better to take the follow up time into account in the analysis, since a patient with a follow up of 1 year has another weight in the analysis than a patient with a follow up of 5 years.

Discussion

34) Clinical and histopathological findings, paragraph 1, second sentence: The authors mention that the ‘investigated clinical and histopathological predictors of renal outcome were conflicting’. Regarding these studies ‘conflicting’ is a too negative word. These studies showed different results, but with some overlap, like the predictive value of normal glomeruli, glomerulosclerosis and eGFR at baseline. The authors should reconsider this sentence.
Next to this, not all these publications proposed a classification system. That is why the authors should change the word ‘these’ in the next sentence to ‘the’.

35) Clinical and histopathological findings, paragraph 1: The authors should recall the significant result of figure 2 and that it shows the same survival distribution as the survival plot of Berden et al.

36) Clinical and histopathological findings, paragraph 2: A space should be added between ‘can’ and ‘not’ in the sentence ‘However, we cannot explain whether the mechanism of glomerular injury was due to the different type of ANCA.’ After this sentence the authors should mention that this could be the case, since patients with MPO-ANCA show more active and chronic lesions than PR3-ANCA patients. The authors can find these results in the following manuscript: Hauer et al., KI, 61:80-89,2002, and should also refer to this manuscript.

37) Clinical and histopathological findings, paragraph 3: Only in this paragraph the word ‘strategies’ should be changed to ‘strategy’, and ‘glomerulonephritis’ into ‘vasculitis’.

38) Clinical and histopathological findings, paragraph 4: In the last 2 sentences the authors discuss the proportion of normal glomeruli and sclerotic glomeruli in the crescentic and mixed class. These numbers should be mentioned in the results (also for the other classes) and can also be mentioned in the additional table that should be added (mentioned under ‘Major Compulsory Revisions’). This additional table actually covers the points discussed in this paragraph.

39) Immunohistochemical findings, paragraph 1: The sentence ‘On the other hand, #-SMA immunoreactivity differed markedly between patients (Figure 3)’ refers to figure 3, but this figure does not support this statement. The authors should reconsider this sentence or the referral to figure 3.

40) Immunohistochemical findings, paragraph 1: In this paragraph the authors present the aSMA score as aSMA negative glomeruli. In the results they present it as aSMA positive glomeruli. The authors should be consistent regarding this for clarity.

41) Immunohistochemical findings, paragraph 1: If the authors decide to look at whether aSMA differentiates the crescentic and mixed class in terms of the development of ESRD, then this result should be recalled in the first paragraph of the discussion.

42) Immunohistochemical findings, paragraph 2: ‘We thought that morphologically normal glomerulus under light microscopy’ # ‘We thought that a morphologically normal glomerulus under the light microscope’

43) Immunohistochemical findings, paragraph 2: In the second to last sentence the authors mention ‘However, #-SMA positivity was not correlated with renal
outcome at 1 year after diagnosis.' Where does this data come from? If it is based on the Kaplan-Meier survival plot (figure 3C), why is the sentence then restricted to 1 year?

Conclusion
44) Last sentence: change the word ‘may’ to ‘might’, because might is more reserved and fits better with the results of this study.

Throughout the whole manuscript
45) The authors use the term ‘renal outcomes’ throughout the manuscript. This should be corrected to ‘renal outcome’. Since renal outcome is a very broad term, the authors should specify this term in their manuscript into ESRD or eGFR at 1 year, depending on what they investigated and what they refer to. The same counts for ‘renal prognosis’.

46) The authors should use ESRD throughout the whole text/tables/figures for clarity and consistency. The words ‘renal survival’ regarding the Kaplan-Meier curve can stay as they are in the text and in figure 2. Synonyms like renal mortality and renal death should be avoided.

- Discretionary Revisions

These are recommendations for improvement which the author can choose to ignore. For example clarifications, data that would be useful but not essential.

# = a linguistic correction

Methods
1) Patients, last sentence: The University of Miyazaki ethical committee approved this study (Approval number: 2010-751). # The ethical committee of the University of Miyazaki approved this study (Approval number: 2010-751).

Results ‘Immunohistochemical study’
2) Figure 3C: ‘aSMA positive rate’ # ‘aSMA positivity’

Discussion
3) Clinical and histopathological findings, paragraph 2, first sentence: Add the word ‘possible’ between ‘several’ and ‘explanations’.

4) Immunohistochemical findings, paragraph 1: The authors mention the percentages aSMA glomeruli and a p-value ‘(25.7% versus 1.5%, P < 0.001)’. The authors do not have to mention this, since it has already been mentioned in the results.

5) Immunohistochemical findings, paragraph 1: The following 2 sentences ‘Notably, glomeruli often appeared normal under light microscopy, but #-SMA
positive or negative glomeruli could be detected after careful observation.’ and
‘The expression of #-SMA is considered to be a useful marker for myofibroblast
differentiation in several disease settings [21-24].’ would fit better in the second paragraph.

**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