# Urinary WT1-positive cells as a non-invasive biomarker of crescent formation

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**Urinary WT1 positive cells as a non-invasive biomarker of crescent formation.**

**Running headline:** Relevance of urinary WT1 positive cells in crescent formation

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**Abstract**

**Objective:** The purpose of this study was to assess the relationship between urinary WT1-positive cells (podocytes and active parietal epithelial cells) and WT1-positive cells in renal biopsy to investigate whether urinary WT1-positive cells are useful for detection of crescent formation.

**Methods:** Fifty-two patients with kidney disease were investigated (15 cases with crescentic lesion and 37 cases with non-crescentic lesion) for immunoenzyme staining using anti-WT1 antibody for urine cytology and renal biopsy. Numbers of WT1-positive cells in urine and renal biopsy were counted, respectively.

**Results:** There was no correlation between urinary WT1-positive cells and WT1-positive cells in renal biopsy. However, the number of urinary WT1-positive cells in patients with crescentic lesion was significantly higher than in patients with non-crescentic lesion. In addition, the best cut-off value to detect patients with crescentic lesions using urinary was 5 cells/10-mL (AUC = 0.735).

**Conclusions:** The results of our study suggest urinary WT1-positive cells can be used to detect patients with crescentic formation using 5 cells/10-mL cutoff value.

**Key words:** Urine cytology, Liquid-based cytology, WT1, immunocytochemistory, Crescent formation

## Introduction

Podocytes (glomerular epithelial cells) line the outside of glomerular capillaries and, thus, face Bowman's capsule and primary urine.[1–3] Podocytes form part of the filtration barrier together with capillary endothelial cells and the glomerular basement membrane (GBM), to ensure selective permeability of the glomerular capillary wall.[4–6] In glomerular injury or aging, podocytes retract and broaden their foot processes, and may detach from the GBM.[5,7] Detachment of podocytes and loss into urine has been implicated in the development of proteinuria, progression of glomerular diseases, glomerulosclerosis, and crescent formation.[8–10]

Parietal epithelial cells (PECs), which have polygonal cytoplasm resembling squamous epithelial cells, cover the inner aspect of Bowman's capsule.[11] PECs, located within the parietal epithelium predominantly at the vascular pole, share properties with visceral podocytes with regard to size, shape and interdigitating foot process pattern.[11–13] It has been reported that PECs proliferate and shed in urine during active glomerular disease.[14]

Cellular crescents result from glomerular epithelial cell overgrowth filling up Bowman's space, which compromises glomerular function. The cellular composition of crescents has been debated because crescentic cells are often positive for both podocyte and PEC markers in immunohistochemistry.[7,15,16] However, recent research using lineage-tracing studies performed in mice has clearly shown that PEC proliferation leads to a significant increase in

the number of cells within crescents.[16,17] Although, crescent formation most frequently occurs in necrotizing crescentic glomerulonephritis, it also occurs in several glomerulopathies including IgA nephropathy, lupus nephritis and diabetes mellitus.[16] Crescent formation is a poor prognostic factor for any glomerular disease; therefore, early detection and early treatment are important. However, there have been few studies of non-invasive biomarkers that could detect all forms of crescent formation.[18]

It is now well established from a variety of studies that podocytes and PECs are present in the urine of patients with various glomerular diseases.[5,8–10,14] Data from several studies have indicated that not both podocytes and active PECs begin to express podocyte markers such as WT1, which is essential for nephrogenesis during kidney development, in various glomerular diseases and crescentic nephritis.[7,14,15,19] Previously, we reported the usefulness of urine cytology based on SurePath™ combined with immunoenzyme staining with an anti-WT1 antibody to identify urinary podocytes and active PECs; indeed, we found WT1 immunoenzyme staining of urine cytology could be used to detect some forms of kidney disease.[6] The purpose of this study was to assess the relationship between urinary WT1-positive cells (podocytes and active PECs) and renal biopsy WT-1 positive cells (podocytes, PECs and crescentic cells) to determine whether urinary WT1-positive cells are useful for detection of crescent formation.

## Material and Methods

*Patients and urine samples*

Fifty-two kidney disease patients who underwent renal biopsy (21 males and 31 females, age 51.0 ± 17.9 years) were recruited for this study. In these 52 patients, 15 cases were associated with crescent formation. Histological diagnosis of patients is shown in Table 1. Urine samples from these patients were obtained just before biopsy.

*Sample preparation*

Urine cytology slides were prepared following SurePath manual protocols.[6] Briefly, 10 mL of fresh urine homogenized before dispensing was centrifuged in a conical tube at 600 × *g* for 10 minutes. The supernatant was discarded and the sediment resuspended in 5–10 mL of CytoRich Red™ (Becton, Dickinson). After a 30-minute fixation, specimens were centrifuged at 600 × *g* for 10 minutes and the supernatant discarded. Six mL of distilled water was added to the sediment and specimens were centrifuged at 600 × *g* for 5 minutes. The supernatant was again discarded. Another 300 μL of distilled water was added to the sediment and specimens were resuspended by vortexing for 10 seconds. After resuspension, each specimen was transferred into plastic chambers and mounted on slides for 10 minutes using gravity sedimentation and electrical adhesion. The slide rack was then turned upside down to discard the supernatant and the inside of the plastic chambers were rinsed with 95% alcohol.

The slide rack was turned upside down, the plastic chambers were removed, and slides were immediately placed in 95% alcohol.

*Immunocytochemistry*

SurePath slides were incubated with 0.3% hydrogen peroxide in methanol for 15 minutes to block endogenous enzyme activity. After blocking, SurePath slides were incubated with anti-WT1 antibody (dilution 1:1000, clone 6F-H2; DakoCytomation, Glostrup, Denmark) for 1 hour at room temperature. Then, Histofine Simple Stain MAX-PO (MULTI) (Nichirei Biosciences, Tokyo, Japan) was applied for 30 minutes at room temperature. Antibodies were visualized using a DAB substrate kit (Nichirei Biosciences). Counterstaining was performed with Mayer's hematoxylin. Number of WT1-positive cells in a total field of each SurePath smear were counted under a light microscope (10× and 40× magnifications) by two authors (T.F. and H.O.) blinded to the patients' diagnoses.

*Immunohistochemistry*

Three-micron-thick paraffin-embedded renal biopsy sections were evaluated with anti-WT1 antibody (dilution 1:200, DakoCytomation). These renal biopsy slides were used for a two-step antigen retrieval method. Briefly, slides were first activated by protease (Nichirei Biosciences) at room temperature for 10 minutes and then heated in citrate buffer at

98ºC for 20 minutes. Subsequent processing was the same as for immunocytochemistry.

We previously reported that with heat treatment alone, both nuclei and cytoplasm of podocytes were positive for WT1.[6] Therefore, in this study, the cytoplasm was destroyed by proteolytic enzyme treatment to facilitate the counting of podocytes. WT1-positive nuclei were counted in renal corpuscle and crescentic lesion by two authors (T.F. and H.O.) blinded to the patients' diagnoses.

*Statistical analysis*

Spearman's rank correlation coefficient was employed to assess correlation between urinary WT1-positive cells and WT1-positive cells in renal biopsy. A Mann–Whitney U test was used to assess correlation between urinary WT1-positive cells and crescent formation. In addition, a receiver operating characteristic (ROC) curve was used to determine the best cut-off value of urinary WT1-positive cells for detection of crescent formation. These statistical tests were defined as $P$-value $< 0.05$. Statistical analysis was performed using R (developed by Ihaka R and Gentleman R) (version 2.8.1).

*Ethical considerations*

Informed consent was obtained from each patient. This study was approved by the Ehime Prefectural University of Health Sciences (13-002) and Kagawa University Hospital

(H26-111).

## Results

*Immunocytochemistry (urinary WT1-positive cells)*

Urinary WT1-positive cells exhibited moderate to strongly positive staining of cytoplasm and were negative for nuclei. The morphology of these cells was observed as round to oval-shaped, with a diameter of 20–50 μm and a clear or vacuolated cytoplasm. WT1-positive cells showed a variety of morphologies ranging from anucleate to multinucleated cells. Occasionally, cast encasement of WT1-positive cells was present (Figure 1a-c).

*Immunohistochemistry (renal biopsy)*

WT1 immunohistochemistry showed strongly positive nuclei for podocytes and PECs (Figure 2A). In contrast, WT1-positive nuclei in cellular and fibrocellular crescents were moderately to weakly positive (Figure 2b). In addition, nuclei in fibrous crescents were negative for WT1 (Figure 2c).

*Correlation between urinary WT1-positive cells and WT1-positive cells in renal biopsy*

There was no correlation between urinary WT1-positive cells and WT1-positive cells in renal corpuscle and crescentic lesion (Spearman correlation = -0.133, $P = 0.346$) (Figure 3).

*Correlation between urinary WT1-positive cells and crescent formation*

The number of urinary WT1-positive cells in patients with crescent formation was significantly higher than in patients without crescentic formation (P = 0.007) (Figure 4).

*Correlation between WT1-positive cells in renal biopsy and crescent formation*

The number of WT1-positive cells in renal corpuscle and crescentic lesion in patients with crescentic formation was significantly lower than in patients without crescentic formation (P = 0.049) (Figure 5)

*Cutoff value of urinary WT1-positive cells*

The best cut-off value of urinary WT1-positive cells to differentiate patients with crescentic lesion from patients without crescentic lesion was 5 cells/10-mL (sensitivity 73.3%, specificity 64.9%). Urinary WT1-positive cells produced an AUC of 0.735 (Figure 6).

## ***Discussion***

As cytoplasm was degraded by a protease in this study to facilitate counting of WT1-positive cells in renal biopsy, WT1 was positive only in the nuclei of podocytes, PECs and crescent cells. In contrast, urinary WT1-positive cells showed positive staining of the

cytoplasm and were negative for nuclei. The primary reason for this discrepancy is destruction of the cytoplasm by protease during immunohistochemistry, whereas urinary cells were fixed with alcohol for immunocytochemistry (Figures 1 and 2). Alcohol-based fixatives reportedly yield less reproducible staining for antibodies to nuclear epitopes.[20] Additionally, nuclear expression of WT1 is only detectable in formalin-fixed material.[21] In the current study, the fixative employed for urine cytology was CytoRich Red, an alcohol-based fixative including isopropanol and methanol.[22] Several studies have reported that WT1 is involved not only in transcriptional regulation within the nucleus, but also in RNA metabolism and translational regulation in the cytoplasm. The binding of WT1 to splicing factors and murine IGF-II mRNA has been demonstrated *in vitro*.[23,24] Moreover, nucleocytoplasmic shuttling of WT1 and the association of WT1 with actively translating polysomes have been reported.[25] Western blot analysis of nuclear and cytoplasmic fractions prepared from two WT1-expressing cell lines, M15 (mouse mesonephros) and AC29 (mouse mesothelioma), revealed that WT1 was predominantly nuclear, as detected by WT1 antibody; however, both cytoplasmic fractions were also positive for WT1.[25] For these reasons, urinary WT1-positive cells showed positive cytoplasmic staining and were negative for nuclei in this study. Considering positive cytoplasmic staining ensures detection of WT1-positive cells in urine cytology.

The morphological features of urinary WT1-positive cells observed in this study were

consistent with previous reports using anti-WT1 and anti-PDX antibodies, the latter of which is a podocyte marker.[5,9,14,26,27] Furthermore, cast encasement of urinary WT1-positive cells observed in this study suggests that these cells were derived from the nephron, as such casts are formed as the molds for lumens of renal tubules and collecting ducts. It has been demonstrated that both podocytes and active PECs are positive for WT1 and PDX.[5,7,14,15] In our study, podocytes, PECs and crescent cells in renal biopsy were WT1-positive. Therefore, urinary WT1-positive cells are possibly derived not only from podocytes and proliferative PECs, but also crescent cells.

In the present study, WT1-positive cells exhibited various sizes and shapes, including both mono- or multinucleated cells. If injury to the podocyte is severe, the podocyte may detach from the GBM into the urine. Alternatively, serious injury can cause podocytes to undergo apoptosis.[28] The remaining podocytes increase their size to cover the GBM in denuded areas where neighboring podocytes have detached or died.[29] Although podocytes are considered terminally differentiated cells, they can re-enter the cell cycle in some circumstances.[29] Moreover, expression of cell cycle proteins such as cyclins A and D has been reported in several glomerular diseases,[30–32] as has strong up-regulation of p21 and p27 in podocytes of glomerular disease model rats and human crescentic glomerulonephritis.[26,33,34] These findings suggest that podocytes resist entering the cell cycle, but they proceed at least until the mitosis phase under stress or injury. However, as podocytes cannot assemble mitotic spindles,

cytokinesis is impossible. Consequently, the podocyte becomes multinucleated as the result of cytokinesis dysfunction.[29] In addition, PECs are also WT1-positive; thus, urinary WT1-positive cells can exhibit various morphologies.

The results of this study demonstrated no significant correlation between urinary WT1-positive cells and WT1-positive cells in renal biopsy. Notably, as the number of cases in this study was small, various disorders such as IgA nephropathy, membranous glomerulonephritis, minimal change disease, etc. were evaluated without classification according to diagnosis. Furthermore, the severity of each patient was not classified. If initial damage to the podocyte is mild, it leads to the effacement of its foot process but may not cause the cell to detach from the GBM. Foot process effacement may be a protective strategy to prevent detachment of podocytes from the GBM and limit the loss of podocytes into the urine.[28] In severe glomerular disease, many podocytes detach from the GBM, whereas podocytes do not detach from GBM in minimal change disease or during the early stages of various glomerular diseases. In this study, it is reasonable to suppose that since there was a mixture of various diseases and severity, no correlation between urinary and renal biopsy WT1-positive cells was observed. Additionally, WT1-negative cells present in fibrous crescent could also be a reason for the lack of correlation between urine and renal biopsy. Similar to our result, Yu D *et al*. reported that an increase of urinary podocytes is not associated with a significant decrease in the number of glomerular WT1-positive cells in

three rat models of renal disease.[27] However, as WT1-positive nuclei were counted in renal biopsy in both our and the previous study, there is a possibility that multinucleated WT1-positive cells have been counted as multiple cells.

The results of this study indicate that the number of urinary WT1-positive cells in patients with crescentic lesion was significantly higher than in patients without crescentic lesion. Additionally, the best cut-off value of urinary WT1-positive cells to differentiate patients with crescentic lesion from patients without crescentic lesion was 5 cells/10-mL. We revealed that crescent formation can be detected with moderate accuracy using this cutoff value (AUC = 0.735). Crescentic glomerulonephritis is a unique glomerular disease in which the space of the Bowman capsule is filled up by the proliferation of predominantly PECs.[16,28] Although mechanisms underlying crescent formation are still not fully understood, it has been reported that both fibrin and plasma are involved. Fibrin formation or the leakage of several plasma components at the site of glomerular vascular injury promotes PEC proliferation and, thus, a cellular crescent is formed.[35,36] Nitta K *et al*. reported that the expression of p27 was low in cellular crescents, whereas podocytes were positive for p27 within the same section.[34] This phenomenon indicates that podocytes entered the cell cycle; although, as previously mentioned, they cannot complete cytokinesis. As such, podocytes exhibit various morphologies such as hypertrophy and multinucleation, depending on the timing of cell cycle arrest. Regardless, this causes detachment from the GBM and cell death, in a process known

as mitotic catastrophe.[16,29] In crescentic glomerulonephritis, there is the potential for many podocytes to detach from the GBM as a result of mitotic catastrophe. It is also possible for proliferative PECs constituting a crescent to detach into the urine. For these reasons, we hypothesized that urinary WT1-positive cells significantly increased in crescentic glomerulonephritis.

To the contrary, the number of WT1-positive nuclei in renal biopsies from patients with crescentic lesions was significantly lower than in patients without crescent formation. We believe the reason for this result is weakly positive and negative WT1 expression of nuclei in fibrocellular and fibrous crescent cells. Cellular crescents can progress to fibrocellular and fibrous crescents over time, as the result of accumulating extracellular matrix. This process might represent an epithelial–mesenchymal transition (EMT) in PEC characterized by loss of epithelial adhesion and gain of secretory capacity for extracellular matrix of mesenchymal cells.[16] In EMT, an intermediate phenotype is shown during the transition from epithelial to mesenchymal cells, such that intermediate phenotypes co-express epithelial and mesenchymal markers.[37] Therefore, we interpret weakly positive WT1 expression in fibrocellular crescent cells to be an intermediate phenotype, and negative WT1 expression in fibrous crescents to be completely transmuted mesenchymal cells.

In conclusion, the results of this study suggest that urinary WT1-positive cells can be used to detect crescentic glomerular nephritis by setting a 5 cells/10-mL cutoff value. However, a

limitation of this study was the small numbers of patients with and without crescentic nephritis. Therefore, further research is necessary to more closely examine potential links between urinary WT1-positive cells and crescentic nephritis.

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**Figure legends**

Fig 1. Urinary WT1-positive cells. WT1 immunocytochemistry (× 400).
(a, b) Mono- and multi-nucleated WT1-positive cell surrounded by urothelial cells and leukocytes.
(c) Cast encasement of a WT1-positive cell.

Fig. 2. WT1-positive cells in renal biopsy. WT1 immunohistochemistry (× 200).
(a) Nuclei of podocytes and parietal epithelial cells show strongly positive WT1 expression.
(b) Nuclei of cells in cellular crescent show weakly positive WT1 expression.
(c) Nuclei of cells in fibrous crescent are negative for WT1.

Fig. 3. Correlation between urinary WT1-positive cells and WT1-positive cells in renal biopsy.

Fig. 4. Correlation between urinary WT1-positive cells and crescent formation.

Fig. 5. Correlation between WT1-positive cells in renal biopsy and crescent formation.

Fig. 6. ROC curve for cutoff-value of urinary WT1-positive cells.

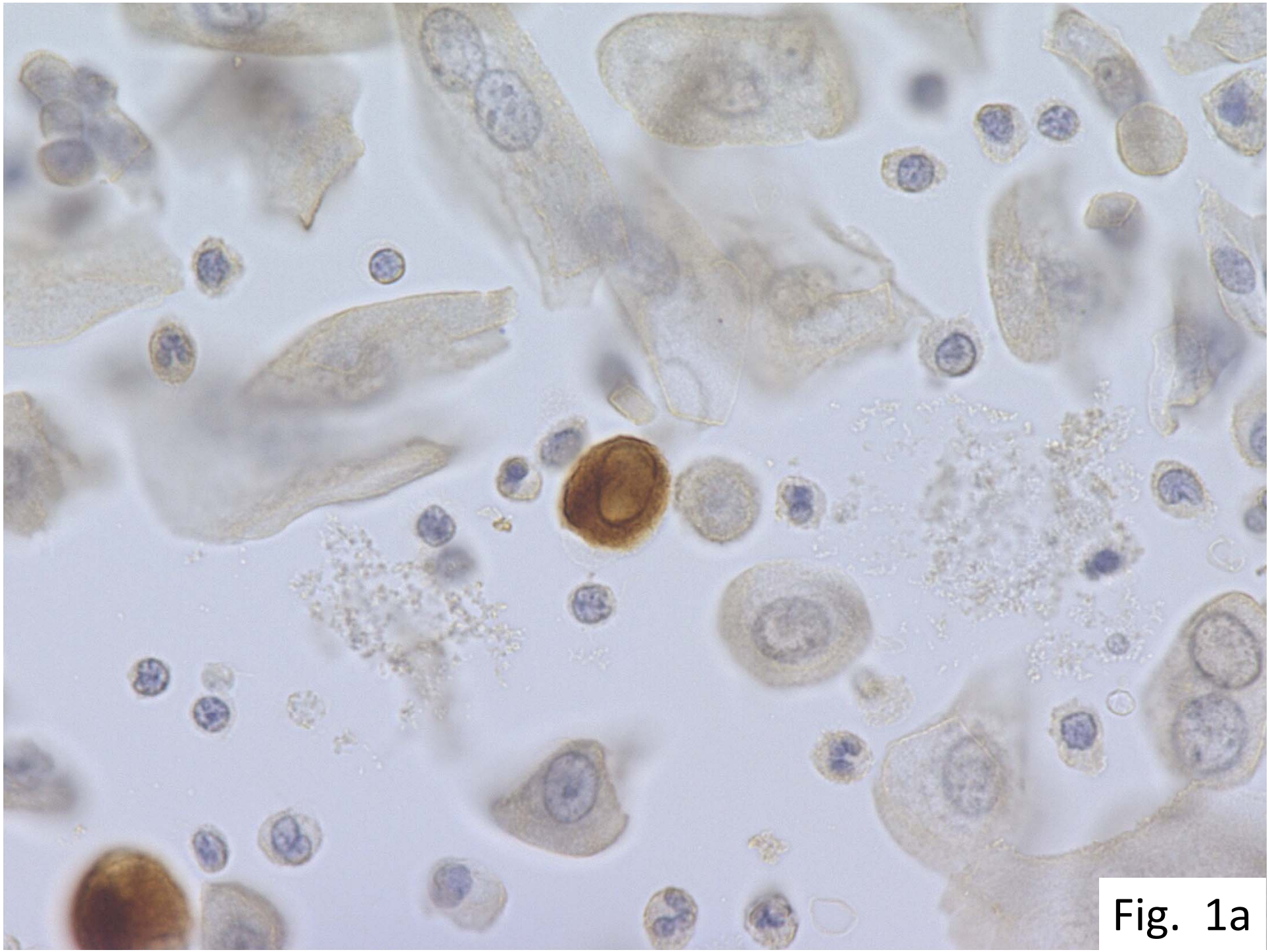

Fig. 1a

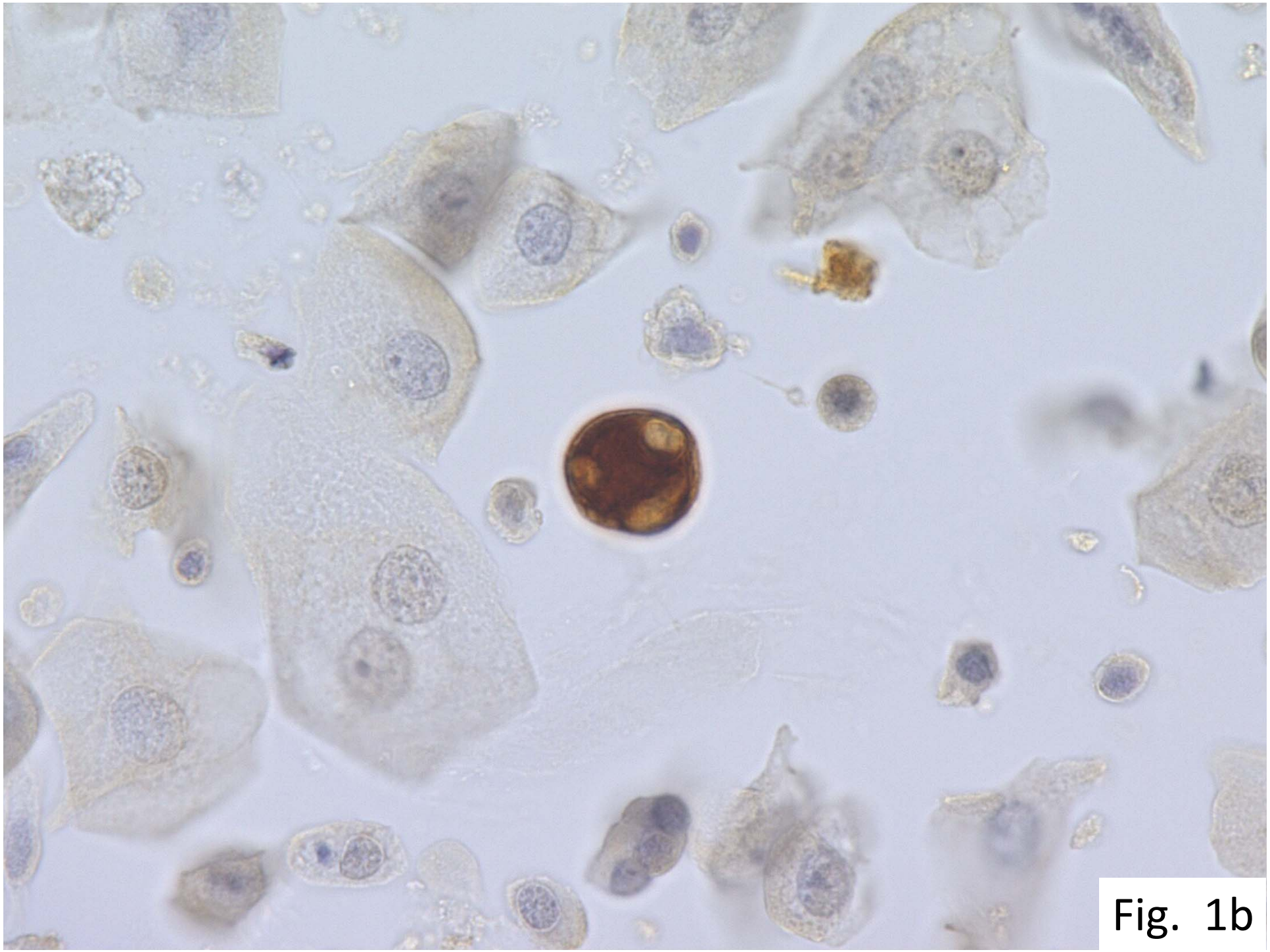

Fig. 1b

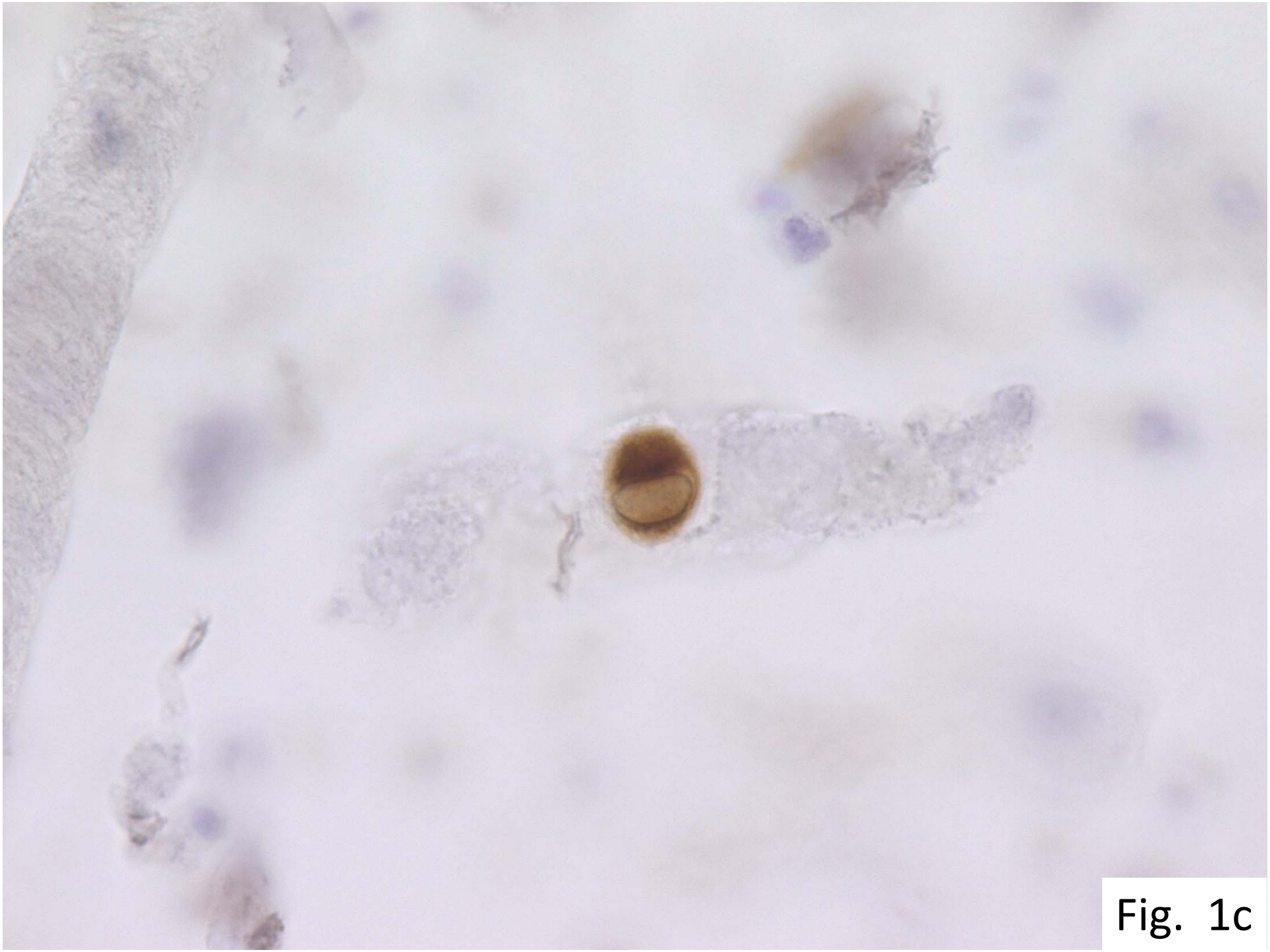

Fig. 1c

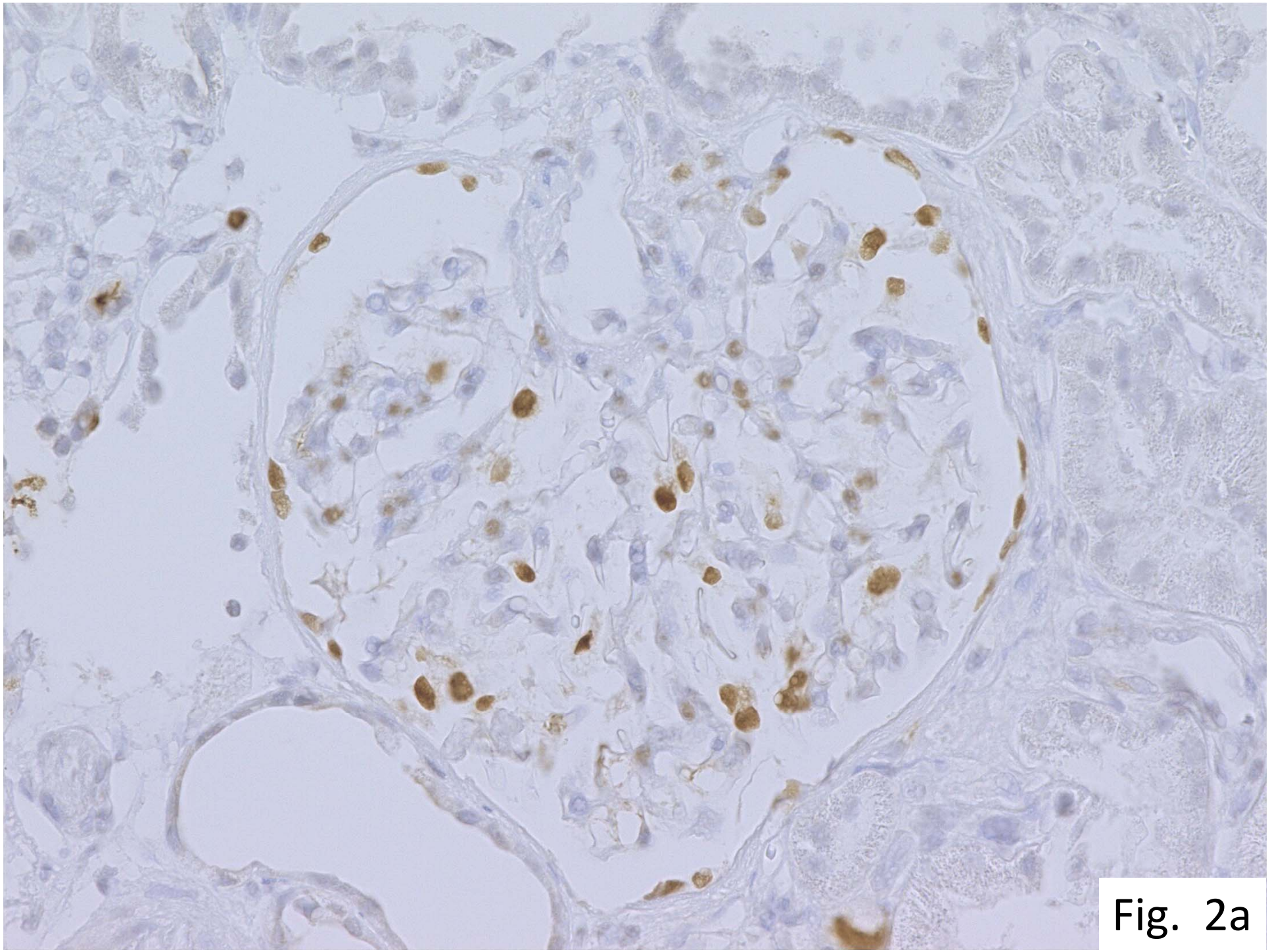

Fig. 2a

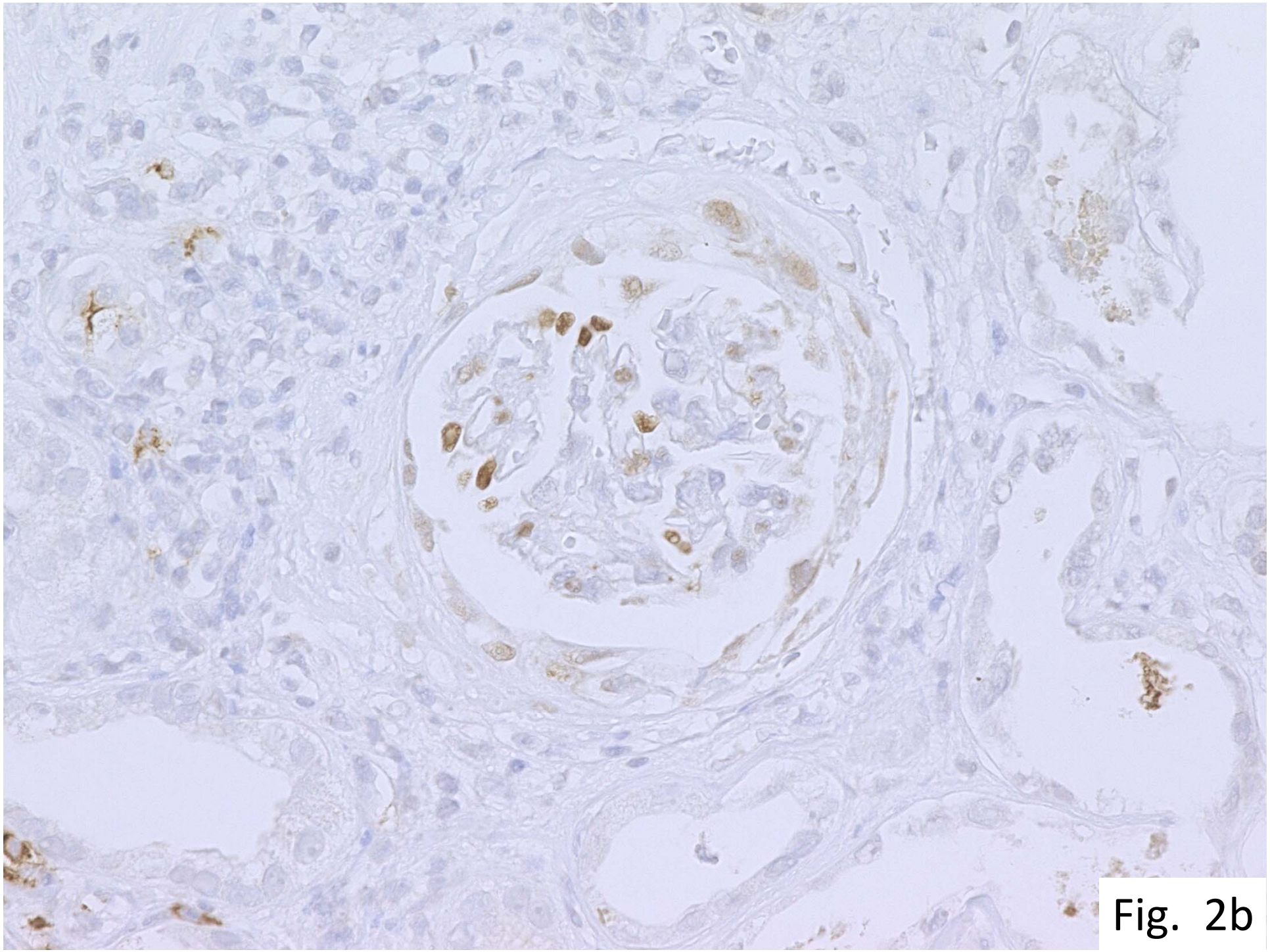

Fig. 2b

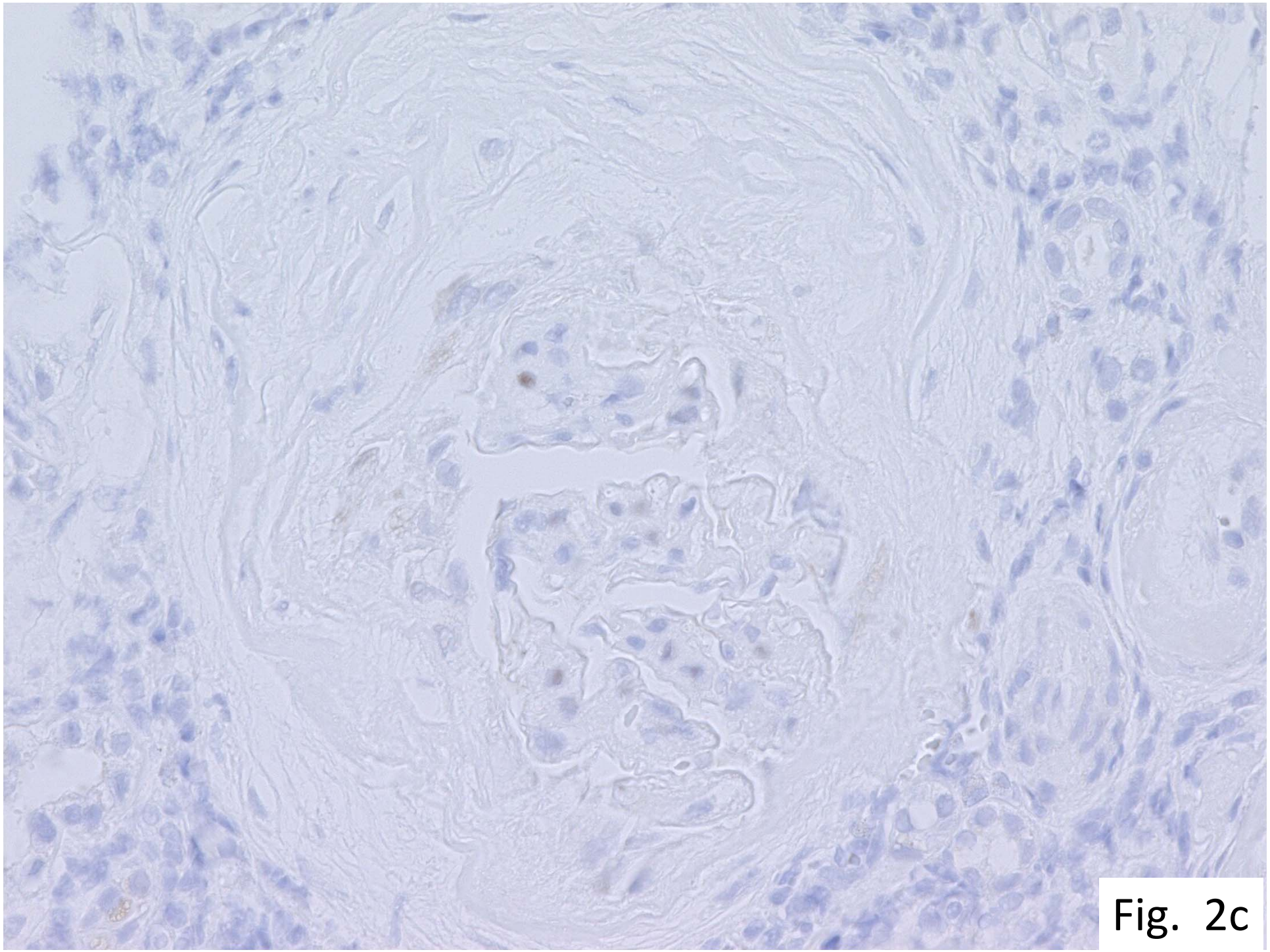

Fig. 2c

Fig. 3. Correlation between urinary WT1-positive cells and WT1-positive cells in renal biopsy.

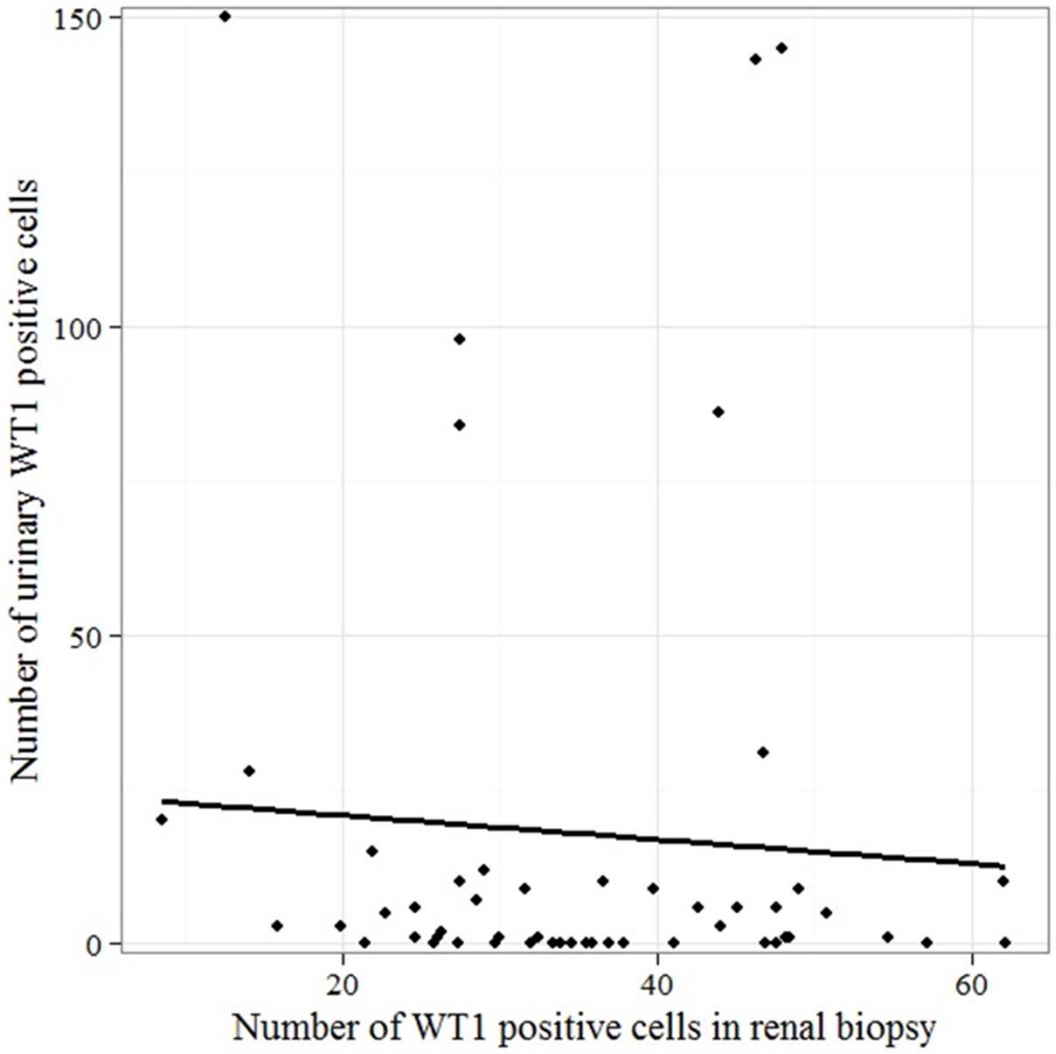

Fig. 4. Correlation between urinary WT1-positive cells and crescent formation.

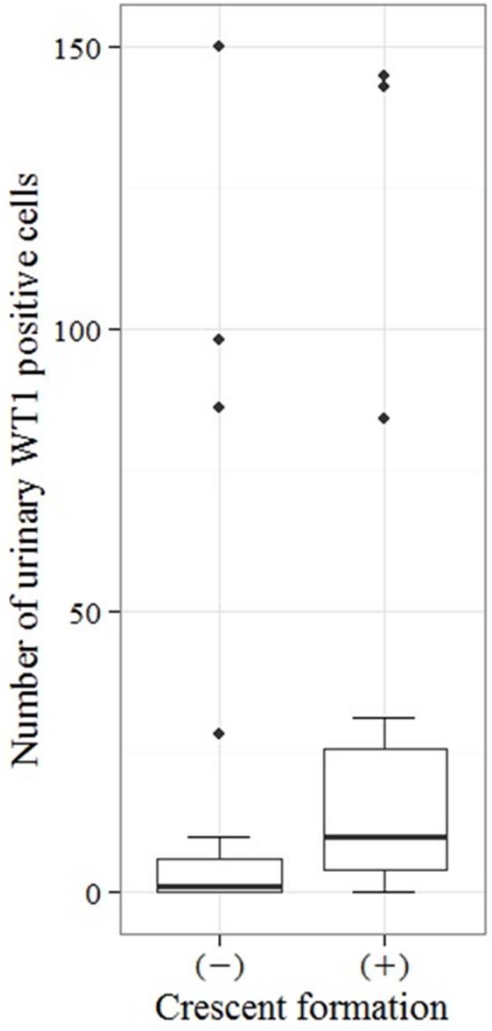

Fig. 5. Correlation between WT1-positive cells in renal biopsy and crescent formation.

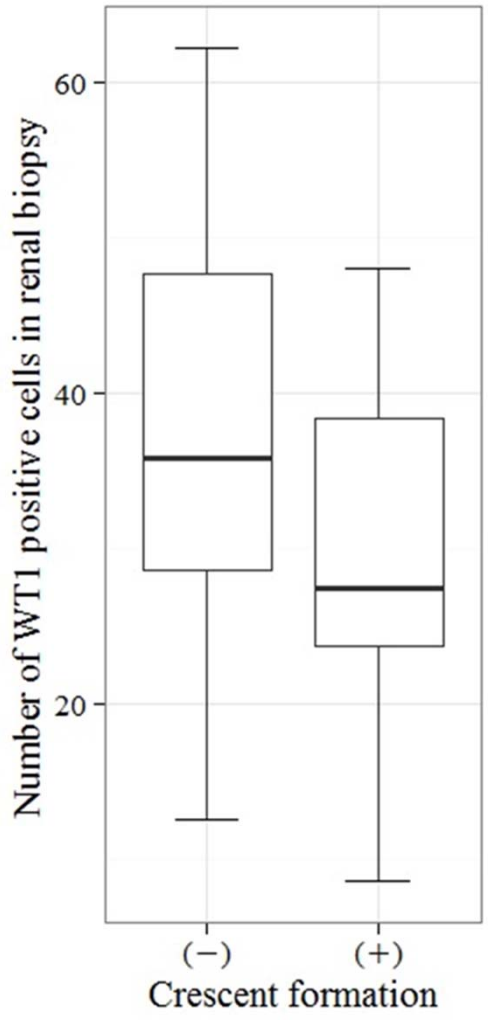

Fig. 6. ROC curve for cutoff-value of urinary WT1-positive cells.

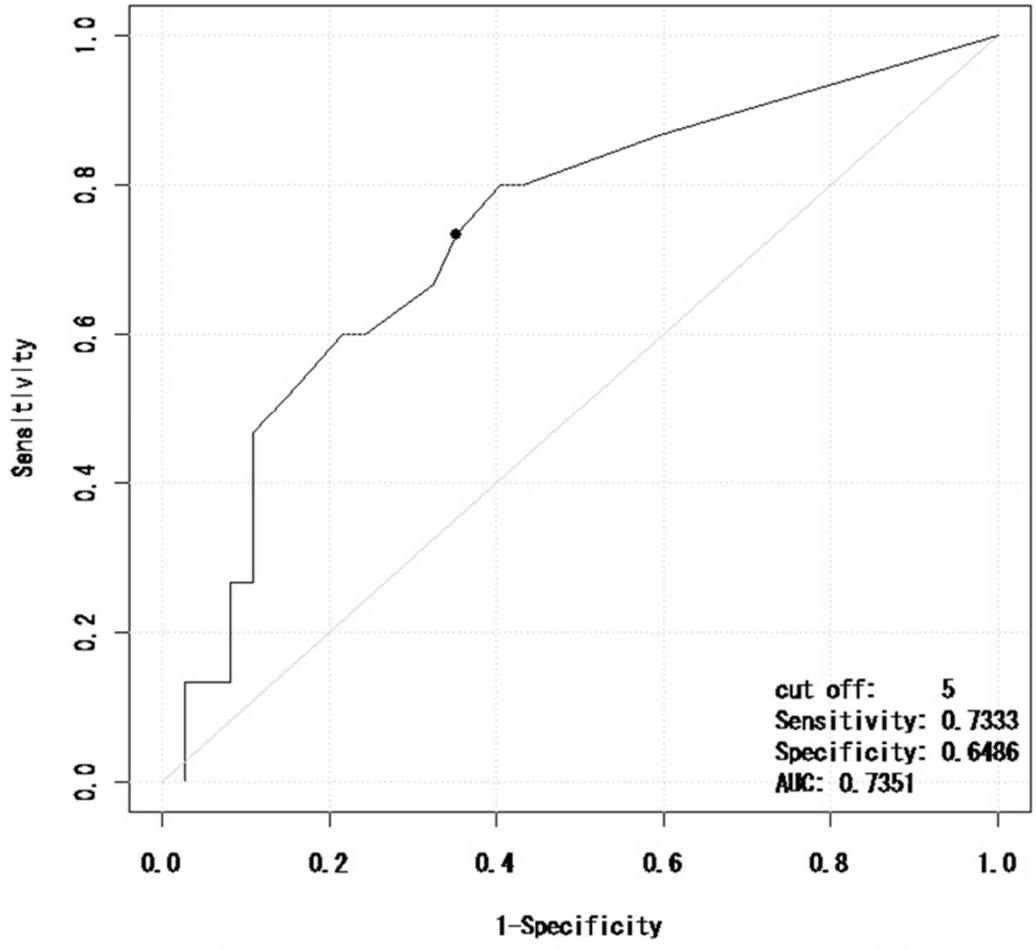

**Table 1.** Frequency of crescent formation in 52 cases of kidney disease

| Histologic diagnosis | n | Crescent formation | |
|---|---|---|---|
| | | (+) | (−) |
| IgA nephropathy | 20 | 9 | 11 |
| Membranous glomerulonephritis | 7 | 0 | 7 |
| Diabetic glomerulopathy | 5 | 2 | 3 |
| Minor glomerular abnormalities | 5 | 0 | 5 |
| Tubulointerstitial nephritis | 5 | 0 | 5 |
| Nephritis of Henoch-Schönlein purpura | 3 | 2 | 1 |
| Crescentic and necrotizing glomerulonephritis | 1 | 1 | 0 |
| Alport's syndrome | 1 | 1 | 0 |
| Amyloidosis | 1 | 0 | 1 |
| Lupus nephritis | 1 | 0 | 1 |
| Monoclonal immunoglobulin deposition disease | 1 | 0 | 1 |
| Nodular glomerulosclerosis | 1 | 0 | 1 |
| Obesity related glomerulopathy | 1 | 0 | 1 |
| Total | 52 | 15 | 37 |