

RESEARCH ARTICLE

Immunochemical assay to detect α -tubulin in urine for the diagnosis of kidney injury

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Abstract

Backgrounds: Shortening of primary cilia in kidney epithelial cells is associated with kidney injury and involved with the induced level of α -tubulin in urine. Therefore, rapid detection and quantification of α -tubulin in the urine samples could be used to the preliminary diagnosis of kidney injury.

Methods: Cellulose-based nanobeads modified with α -tubulin were used for the detection probe of competitive immunochemical (IC) assay. The concentration of α -tubulin in the urine samples was determined by IC assay and compared with the amount determined by Western blotting analysis.

Results: The relationship between α -tubulin concentration and the colorimetric intensity resulted from IC assay was determined by logistic regression, and the correlation coefficient (R^2) was 0.9948. When compared to the amount determined by Western blotting analysis, there was a linear relationship between the α -tubulin concentrations measured by the two methods and the R^2 value was 0.823.

Conclusions: This method is simple, rapid, and adequately sensitive to detect α -tubulin in patient urine samples, which could be used for the clinical diagnosis of kidney injury.

KEYWORDS

cellulose nanobeads, immunochemical, kidney injury, rapid kit, α -tubulin

1 | INTRODUCTION

The primary cilium is a small cellular projection found on most eukaryotic cell membranes.¹ The disruption of the kidney primary cilia is associated with kidney injury such as autosomal recessive polycystic kidney disease² and transplantation of kidney.³ It was demonstrated that the disrupted cilia were excreted in the urine upon detachment from the tubular cells.^{4,5} The cilium is comprised of microtubules, which have a major structural role in eukaryotic

cilia. Therefore, the release of microtubule proteins into urine can be associated with diseases and post-operative acute injury of kidney.^{6]}

For this reason, the release of the primary ciliary proteins into the urine can be used as a non-invasive biomarker for diagnosing patients of kidney diseases including patients who undergo kidney transplantation.⁷ Therefore, a rapid and non-invasive diagnostic strategy is required to determine the prognosis of these patients.

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Immunochromatography (IC) can be efficiently used for rapid detection of protein biomarkers. In IC assay, the antibody for a target protein is usually immobilized on the surface of gold nanoparticles to quantify the amount of proteins bound on the IC strips. The purple color intensity of the gold nanoparticle on the IC strips determines the amount of target protein in the samples.^{8,9} However, gold nanoparticles aggregate easily in buffer solutions, due to the electronic property of gold nanoparticle surfaces.¹⁰

In this study, we used a cellulose nanobeads-based IC strip, which allows for colorimetric quantification of α -tubulin. Cellulose nanobeads are highly stable, which have demonstrated improved performance in the lateral flow applications, compared with gold nanoparticles.¹¹ Few studies have been reported to use cellulose nanobeads as a detection probe of IC assay until now.^{12,13} The hydroxyl group of the glucose units in cellulose beads are expected to induce stability of them in buffer solution, allowing for the use in the quantitative IC assay. We had previously reported that the cellulose nanobeads could replace the gold nanoparticles in IC assay.¹³

In this study, we prepared IC assay platform to determine the α -tubulin level in the urine of patients with kidney injury. The samples were collected from the patients before/after transplantation of kidney, and the concentration of α -tubulin was determined in each sample from a patient. It was expected that the α -tubulin concentration in urine could be increased after transplantation of kidney. We compared the concentration determined by IC assay with the amount determined by Western blotting analysis. Based on the detection range and correlation to the result of Western blotting, the IC assay platform prepared in this study was evaluated as a diagnostic tool for kidney injury.

2 | MATERIALS AND METHODS

2.1 | Materials

Cellulose nanobeads (NanoAct™) were purchased from Asahi Kasei. The α -tubulin (Cat. No., Ag18034) was purchased from Proteintech. Chicken IgY (Cat. No. AGCIG-0100) and goat anti-chicken IgY (ABGAC-0500) were purchased from Arista. Monoclonal mouse anti- α -tubulin antibody (Cat. No. T6793) and secondary antibody conjugated with horseradish peroxidase (HRP) were purchased from Sigma-Aldrich.

2.2 | Preparation of detection probes for competitive immunochromatography

The cellulose nanobead-based detection probes were prepared as shown in Figure 1A. The mixture of 100 μ L of 0.2% cellulose nanobeads with 1 μ g α -tubulin in phosphate-buffered saline (PBS, pH 7.4) was incubated for 30 minutes at 25°C. Further, 5 μ g IgY was added to the mixture and incubated for 30 minutes. The mixture was centrifuged at 12 000 *g* for 3 minutes to obtain the cellulose nanobead precipitate. To the precipitate, 100 μ L of PBS was added and centrifuged at the same condition to remove unbound proteins. The precipitated beads were resuspended using 100 μ L PBS and were used as probe to detect α -tubulin in IC assay.

The morphology of cellulose nanobeads was analyzed using a field emission scanning electron microscope (FE-SEM, S-4800, Hitachi). The size distribution of the beads was analyzed by dynamic light scattering (DLS, ZetaSizer NanoZS, Malvern Instrument).

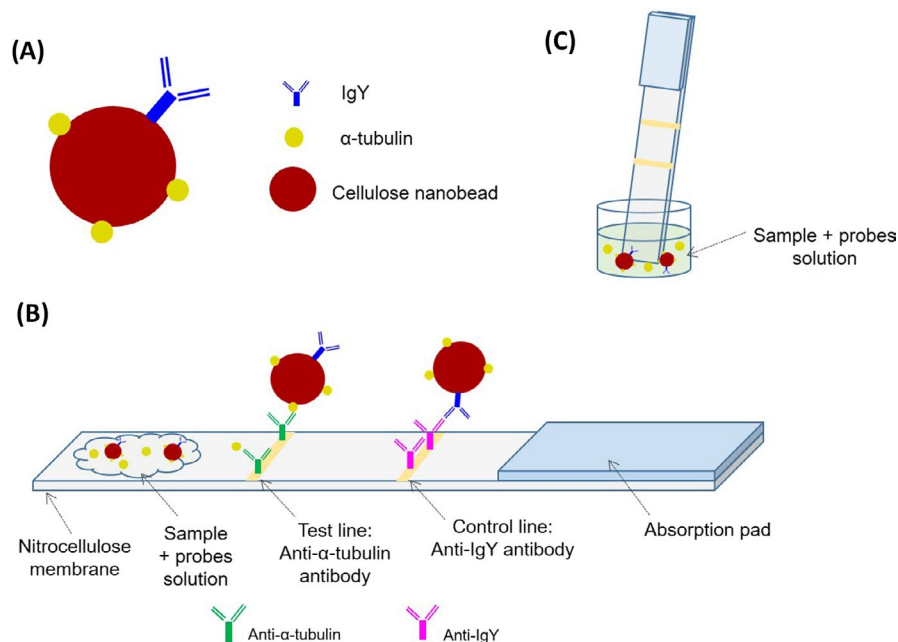


FIGURE 1 Schematic representation of immunochromatographic (IC) assay prepared in this study. A, Modification of detection probes using cellulose nanobeads. B, Design of IC assay platform. C, Method for running samples through IC strips

2.3 | Preparation of immunochromatographic assay strip

The IC assay strip was prepared as shown in Figure 1B. The test strip consisted of a nitrocellulose membrane and an absorbent pad. Anti- α -tubulin antibody (diluted to 1:100 of ascites fluid) and anti-IgY (0.1 mg/mL) were sprayed on the membrane to form test and control lines on the assay strip, respectively, using a custom-made spraying machine for IC.

2.4 | Quantitative relationship between standard α -tubulin and color intensity by immunochromatographic assay

We prepared various concentrations (0, 0.37, 1.1, 3.3, 10, and 30 μ g/mL) of the α -tubulin standard solution. IC assay was performed using the prepared strips as described above. The standard solution (5 μ L) was diluted with 35 μ L PBS, and 3 μ L cellulose nanobead solution was mixed, which was prepared as a probe for IC assay as described above. Then, the mixture was added to a 96-well plate. One end of IC strips was immersed in each standard solution, and the solution was allowed to run along the strips (Figure 1C).

The intensity of the colored lines, formed by the competitive binding of cellulose nanobeads with standard proteins, was normalized to the intensity of control solution, which was prepared only

with cellulose nanobeads and without the patient sample. Based on the digitalized intensity of test lines, the quantitative relationship between the concentration and color intensity was evaluated. We generated a regression curve for α -tubulin standard solution from 3 independent experiments. The color intensity of test and control lines was analyzed using an image analysis software (UN-SCAN-IT gel, Ver. 6.1, Silk Scientific Co.).

2.5 | Quantitative analysis of α -tubulin level in patient urines

We analyzed 28 urine samples obtained from 9 patients who underwent kidney transplantation for quantifying the α -tubulin level using IC assay. For the collection of patient samples, informed consent was obtained from all individual participants included in the study. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee (Institutional Review Board [IRB] of Kyungpook National University Hospital, College of Medicine, Approval No. KNUH 2013-04-026) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

The patient sample (5 μ L) was mixed with 35 μ L PBS and added to a 96-well plate. Further, 3 μ L of a cellulose nanobead probe was added to each sample. One end of IC strips was immersed in the sample solution. The intensity of test line formed by patient samples

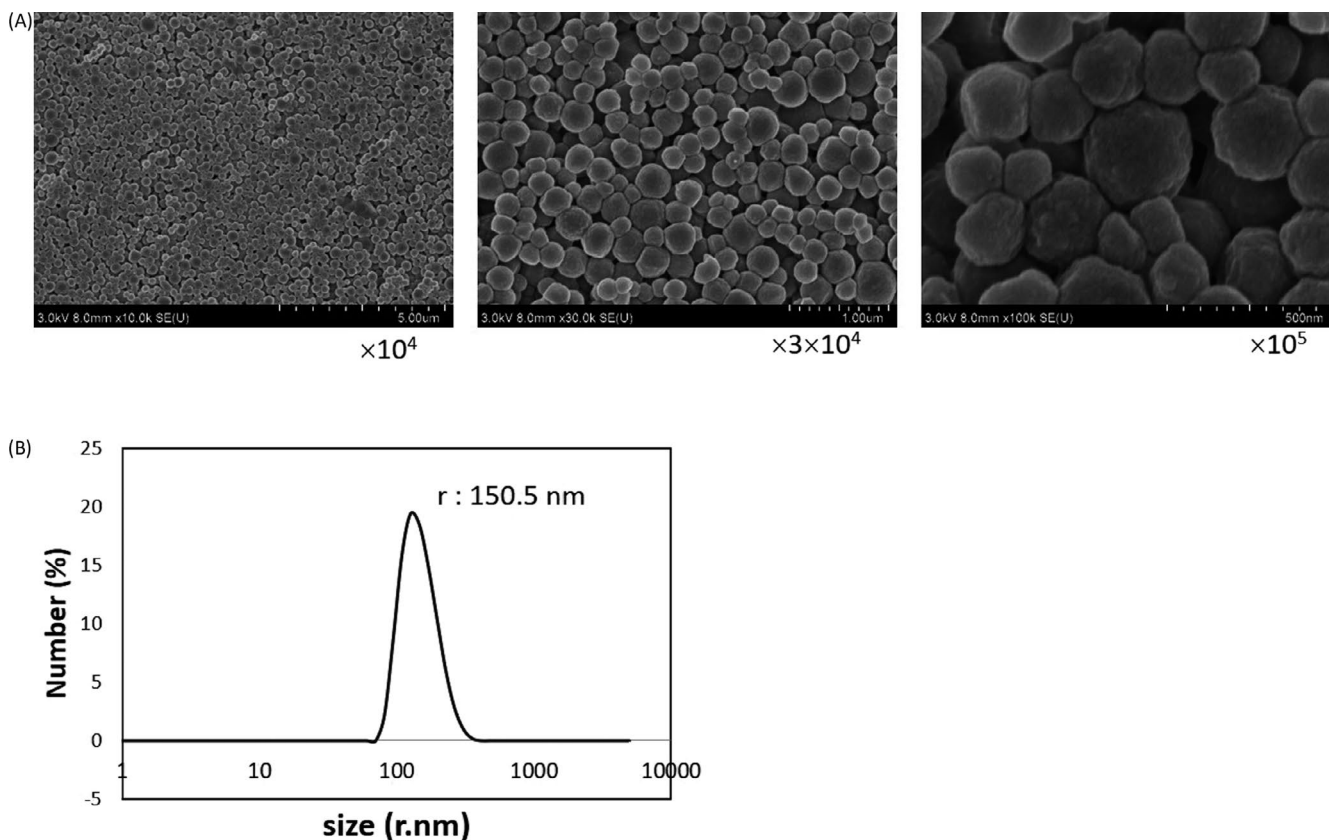


FIGURE 2 Properties of cellulose nanobeads. A, Morphology of nanobeads analyzed by FE-SEM. B, Size distribution of nanobeads analyzed by DLS

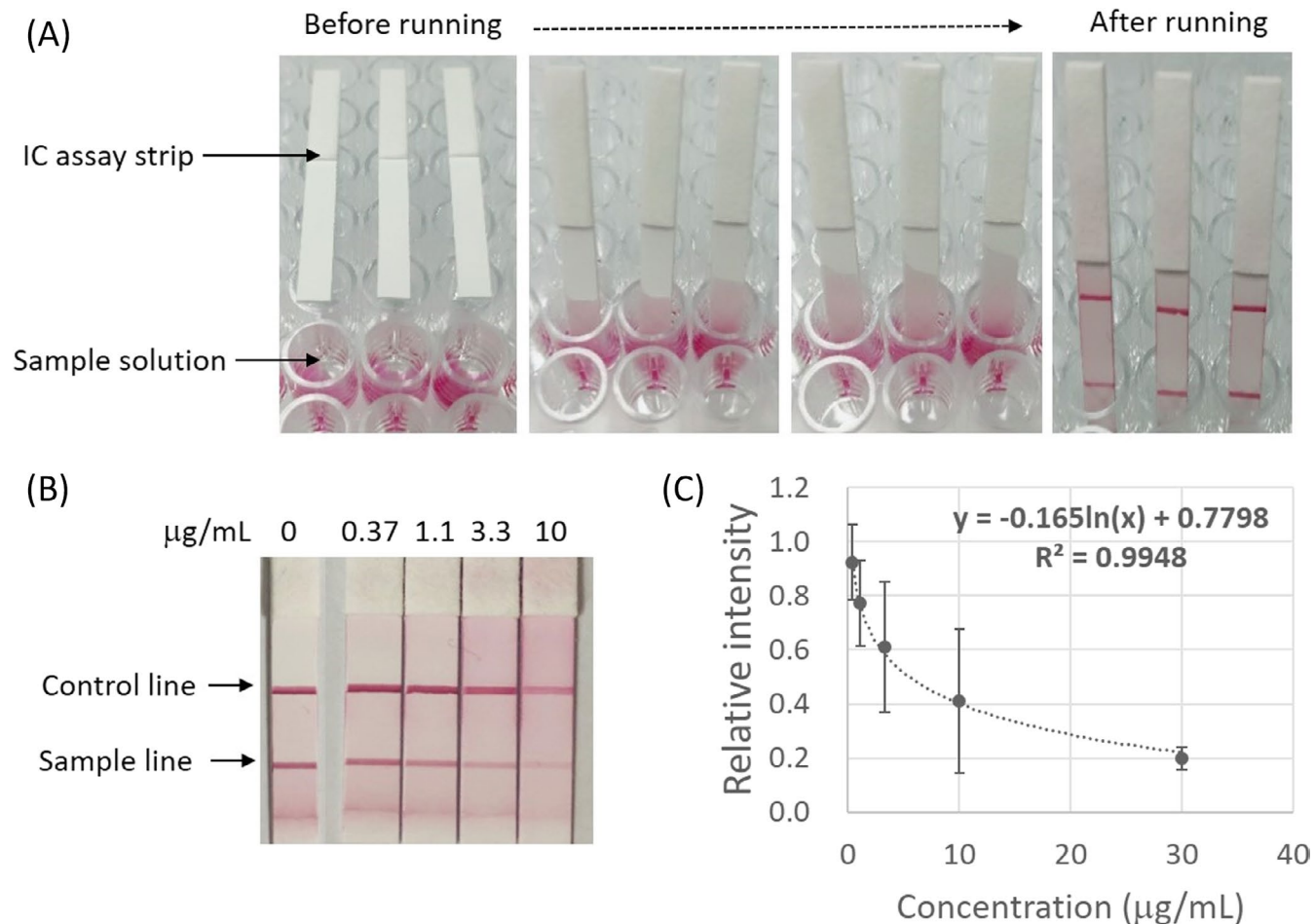


FIGURE 3 Immunochromatographic (IC) assay result for α -tubulin standard protein. A, IC platform and running method used in this study. B, Representative image of IC assay result using standard α -tubulin. C, Standard curve for the quantification of α -tubulin by IC assay

was normalized compared with that of the control solution, which was prepared only with cellulose nanobeads and without patient samples. The α -tubulin level in the patient urine was determined based on the regression curve of the standard solution.

2.6 | Western blotting

The amount of α -tubulin in patient samples was analyzed by Western blotting, and the result was compared with that analyzed by IC assay. Western blotting was performed following standard protocols. Briefly, urine samples (30 μ L) were resolved via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel and electroblotted onto a nitrocellulose membrane using a transfer apparatus (Trans-Blot SD semi-dry transfer cell, Bio-Rad) following the manufacturer's instructions. Anti- α -tubulin antibody and secondary antibody conjugated with HRP were used to detect the proteins on the membrane. The enhanced chemiluminescence (ECL) substrate was purchased from Thermo Fisher Scientific, and the intensity of ECL was observed using a ChemiDoc™ system (Bio-Rad) under the same condition of ECL development for all samples. The intensity of each band was analyzed using an image analysis software (UN-SCAN-IT gel, Ver. 6.1, Silk Scientific Co.).

3 | RESULTS

3.1 | Properties of the cellulose nanobeads

The morphology of the cellulose nanobeads was analyzed by FE-SEM. As shown in Figure 2A, the probes had a homogenous spherical shape. The radius of the polymer beads was 150.5 nm (Figure 2B). The nanobeads consisted of cellulose, which are composed of hundreds to thousands of D-glucoses. In the structure of cellulose, there is a clear segregation into polar (OH) and nonpolar (CH) patches, which gives a clear amphiphilicity.¹⁴ The hydroxyl groups on the surface of cellulose nanobeads would make them highly dispersible in PBS solution. In addition, the hydrophobic properties of the glucopyranose plane can stack proteins in cellulose chains via hydrophobic interactions. Therefore, proteins (α -tubulin and IgY used in this study) can be immobilized on the surface of cellulose nanobeads (Figure 1A).

3.2 | Standard curve for the quantification of α -tubulin

The strips for IC assay of α -tubulin were prepared by line-spraying the anti- α -tubulin antibody and anti-IgY antibody (Figure 1B). The

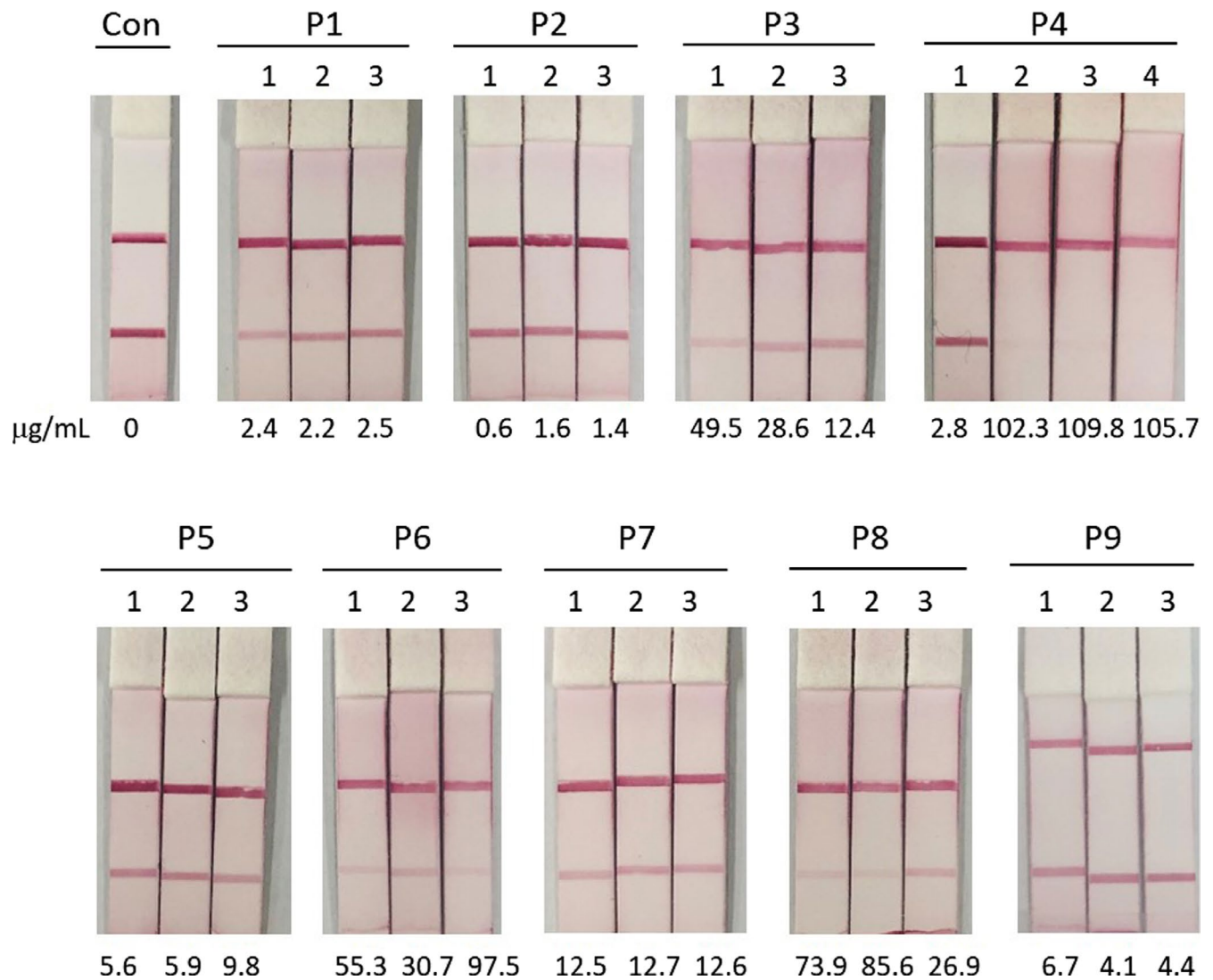


FIGURE 4 Quantitative detection of α -tubulin in patient urine samples by immunochromatographic (IC) assay. Below each strip, the calculated concentration of α -tubulin by IC is provided using standard regression curve shown in Figure 3C. Patient number was given from P1 to P9. Strip no. 1, the samples collected before transplantation; strip no. 2 and strip no. 3, the samples collected at 24 and 48 h post-transplantation, respectively; and strip no. 4, the sample collected at 72 h post-transplantation

mixture of α -tubulin standard proteins and detection probes was placed in each well of the 96-well plate at various concentrations of α -tubulin. One end of IC strips was immersed in a well as shown in Figure 1C, and the standard solution of α -tubulin was allowed to run along the strip for 1-2 minutes. Figure 3A shows the process of sample running at several time steps.

Because the probes were coated with the α -tubulin, the binding of the probes to the test line sprayed by anti- α -tubulin antibody would be competitive with the binding of α -tubulin in standard solution. Therefore, higher the standard concentration, lower the color intensity on the test line of the strip. The image of each strip in different concentrations of α -tubulin is shown in Figure 3B.

IgY on the surface of probes made them to associate with anti-IgY on the control line; thus, all standard protein solutions including

0 μ g/mL α -tubulin developed colorimetric responses on the control line.

When the average intensity from 3 independent experiments was plotted against the concentration of α -tubulin, the standard curve exhibited a logarithmic fit (Figure 3C). The coefficient of determination, R^2 , was 0.9948, which indicated that the regression model of standard curve had a good fit to determine the concentration of protein in samples.

3.3 | Determination of α -tubulin concentration in the urine sample of patients with kidney injury

The urine samples were collected from the patients with kidney injury, who had undergone kidney transplantation. We collected the urine sample from 9 patients before and after transplantation. The

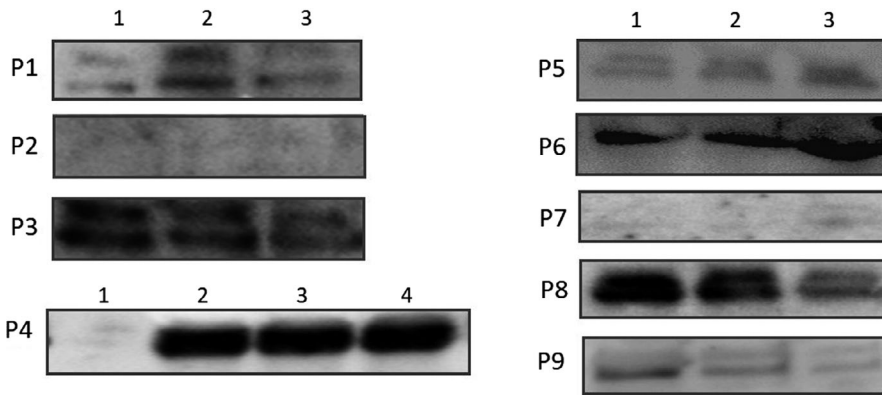


FIGURE 5 Western blotting analysis of α -tubulin in the same volume (30 μ L) of patient urine samples. Patient number was given from P1 to P9. Lane no. 1, the samples collected before transplantation; lane no. 2 and lane no. 3, the samples collected at 24 and 48 h post-transplantation, respectively; and lane no. 4, the sample collected at 72 h post-transplantation

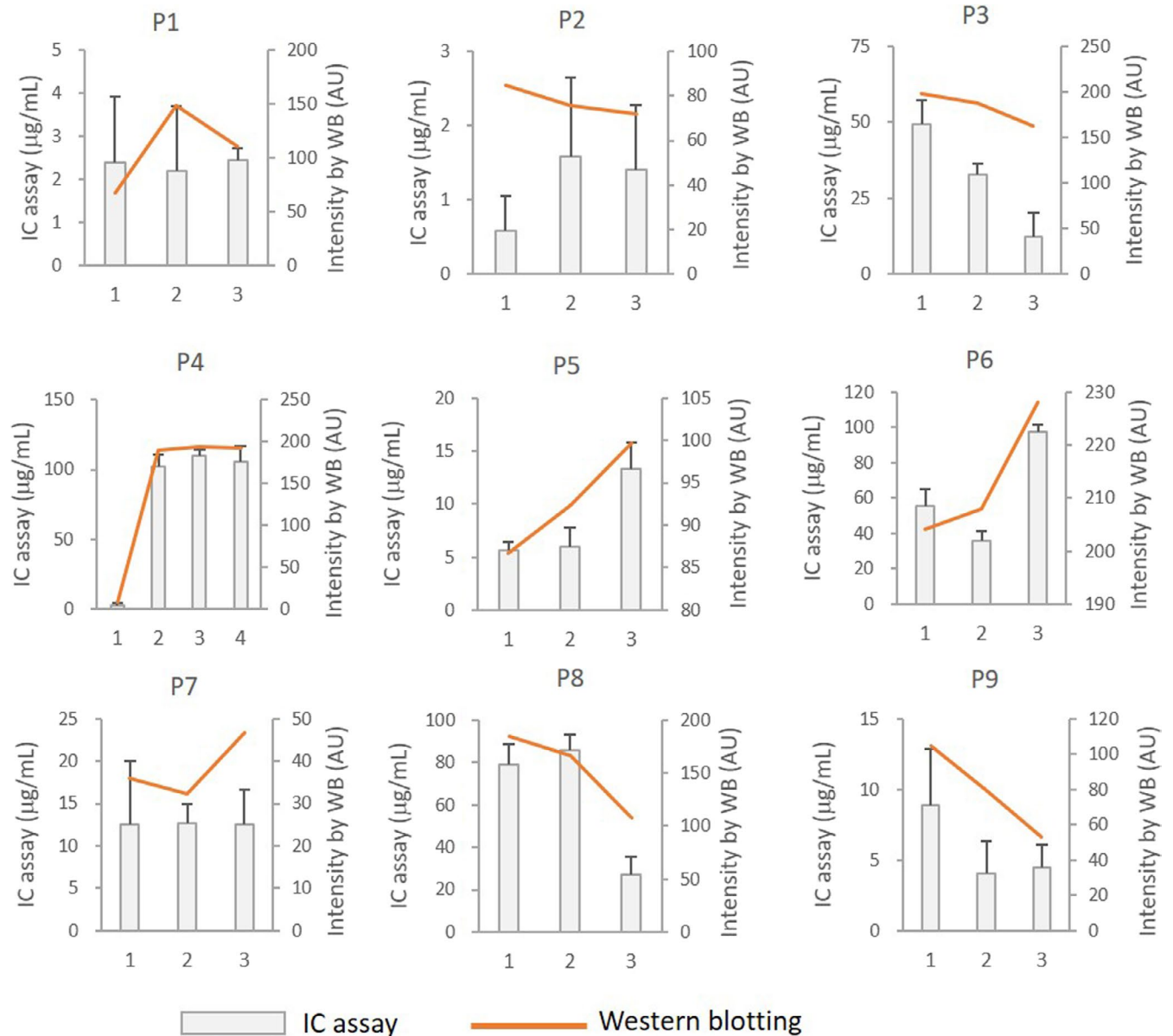


FIGURE 6 Comparison of α -tubulin expression determined by immunochromatographic (IC) assay and Western blotting. Patient number was given from P1 to P9. 1, the samples collected before transplantation; 2 and 3, the samples collected at 24 and 48 h post-transplantation, respectively; and 4, the sample collected at 72 h post-transplantation

concentration of α -tubulin in the urine sample was determined by IC assay, using the standard curve for the quantification of α -tubulin, as shown in Figure 3C.

The image of the strips following the sample running is shown in Figure 4. Below each strip, the calculated concentration of α -tubulin by IC assay is provided by the average of 3 independent experiments. Strip no. 1 in each patient (P1-P9) represents the samples collected before transplantation of kidney. Strip no. 2 and strip no. 3 represent the samples collected at 24 and 48 hours post-transplantation, respectively. For P4 patient, the samples were collected at 24, 48, and 72 hours post-transplantation; thus, strip no. 4 represents the sample collected at 72 hours post-transplantation.

The concentrations of α -tubulin in P1, P2, P5, and P7 samples were <10 $\mu\text{g}/\text{mL}$ and lower than those in other patient samples. In P3, P8, and P9 samples, we observed time-dependent decrease in α -tubulin concentration after renal transplantation, compared with that measured before transplantation. However, the concentration of α -tubulin in P4, P5, and P6 urine samples increased time-dependently after transplantation. Especially for P4 samples, the α -tubulin concentration was increased by approximately 50-fold after transplantation compared with that before transplantation. In P1 and P7 urine samples, the level of α -tubulin did not exhibit a discrete pattern after transplantation.

3.4 | Comparison of immunochromatography and Western blotting methods for quantification of α -tubulin

The amount of α -tubulin in the patient samples was also analyzed by Western blotting (Figure 5). Most of all, Western blotting also revealed that there was a dramatic increase in the α -tubulin level in no. 2, 3, and 4 urine samples collected from P4 patient, compared with that of no. 1 sample. The α -tubulin level determined by Western blotting was quite similar to that of IC assay. Additionally, for the α -tubulin level in P2 sample, which was determined to be as low as ~ 1 $\mu\text{g}/\text{mL}$ (0.6 – 1.6 $\mu\text{g}/\text{mL}$) by IC assay, we could not observe any band in the Western blotting analysis. In other samples in which α -tubulin was quantified approximately ~ 10 $\mu\text{g}/\text{mL}$ by IC assay, P1, P5, P7, and P9, the intensity of α -tubulin was relatively lower than that of other samples. In most of samples, α -tubulin was observed in a doublet on Western blot (P1, P3, P5, P7, P8, and P9). It was suspected that the lower band in the doublet represented the $\alpha 3$ subunit of tubulin.¹⁵

The amount of α -tubulin determined by Western blotting is compared with that of IC assay, and shown in Figure 6. As shown in Figure 6, the pattern of α -tubulin level determined by IC assay in each patient sample was similar to those determined by Western blotting. The increased pattern of α -tubulin expression was observed in P4, P5, and P6 by both IC assay and Western blotting methods. In P3, P8, and P9 samples, the α -tubulin level was determined to be decreased by both IC and Western blotting method. In P1 and P7 samples, the α -tubulin expression did not show any discrete pattern by both analysis methods.

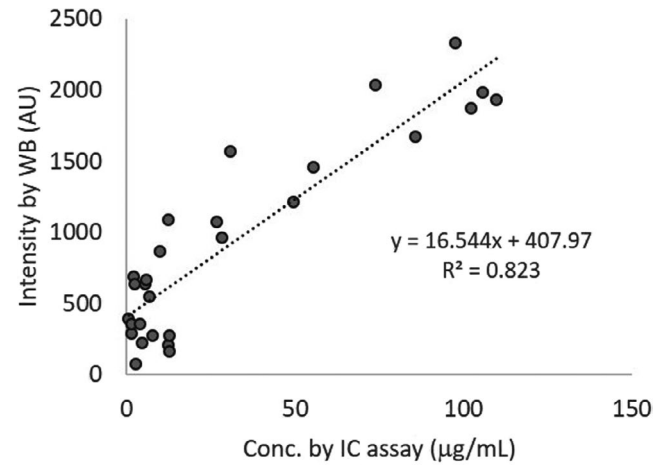


FIGURE 7 Relationship of the α -tubulin concentration determined by immunochromatographic (IC) assay and expression levels analyzed by Western blotting

In Figure 7, the relationship of α -tubulin level in patient urine samples determined by IC assay and Western blotting analysis is provided. It was observed that a linear relationship between the amount measured by the two methods and the R^2 value of the correlation was 0.823. Taken together, the IC assay developed in this study could be a rapid method to detect α -tubulin in human urine samples, and the quantitative values determined by IC assay was correlated to the amount determined by Western blotting analysis, which has been widely used as a reliable test method to determine the protein levels in biological samples.

4 | DISCUSSION

Most mammalian cells possess nonmotile primary cilium, which projects from the apical surface to the internal lumen of the tissues, and it consists of a microtubule in the core and a surrounding cilia membrane.¹⁶ Upon cutting from the tubular and parietal cells, they drop into the ultrafiltrate, drain into the bladder, and are excreted in the urine.¹⁷

Recently, new evidence has been reported that the primary cilium has an important effect on signaling pathways in the cell and is associated with various types of diseases.¹⁸ In the kidney, the length of primary cilia is dynamically altered during the normal cell cycle.⁴ Furthermore, the length of primary cilia is dynamically altered in the renal cells, as a result of various kidney diseases and acute injury.¹⁹ Recent data have been reported that the shortening of the primary cilia is associated with fragmentation of cilia with clear breaks in α -tubulin.²⁰ The breakdown product of fragmented primary cilia, α -tubulin, was found to be released into the urine.²¹ These data revealed that α -tubulin from the shortened primary cilia could be related to the progression of kidney diseases and acute injury. Therefore, the alteration of cilia proteins in the urine could be a biomarker of diseases and acute injury occurred in kidney, and α -tubulin is proposed

as a representative molecule to be checked to monitor the alteration of cilia length.

Human and animal studies have demonstrated that the detection of primary cilia fragments or ciliary proteins in the urine and the alteration of primary cilia length in the kidney cells could be useful for the diagnosis of kidney diseases.³ For examples, it was reported that the increases in α -tubulin were observed in the urine of mice at 4 hours following kidney ischemia.²² The patients with kidney ischemia/reperfusion injury also exhibited enhanced α -tubulin concentration, and α -tubulin level was highly variable among patients.²³

In this study, three among nine patients (P4, P5, and P6) showed increased levels of α -tubulin by both IC assay and Western blotting analysis. However, three among nine patients (P3, P8, and P9) showed decreased level of α -tubulin following the transplantation of kidney. Two patients (P1 and P7) did not show any noticeable alteration of α -tubulin levels. The decreased concentration of α -tubulin in urine samples following transplantation of kidney could be originated from the difference of patients' symptoms and condition of transplantation. However, the dramatic increase in α -tubulin in P4 patient was observable, which was expected in the patients who had a severe damage in kidneys by a transplant operation.

Although the above evidence implies that the measuring of cilia proteins such as α -tubulin may be useful for the diagnosis of kidney disease or acute injury, a rapid detection method that can be used for clinical samples has not been developed. Furthermore, to provide sufficient evidence for the relationship of the acute injury in kidneys and α -tubulin, more comprehensive studies should be performed with a large number of patient samples.

In Figure 7, the relationship between the concentration of α -tubulin determined by IC assay and Western blotting is provided. For the determination of α -tubulin concentration by IC assay, standard curve prepared in Figure 3C was used. A conventional image analysis was performed to determine the relative level of α -tubulin by Western blotting and IC assay.²⁴ IC assay has been usually used as a qualitative diagnosis method,²⁵ and fluorescence probes are recently introduced for quantitative determination of protein concentration.²⁶ The linearity of the relationship (R^2) between the concentration determined by IC assay and Western blotting was 0.823, which shows that the IC assay prepared in this study could determine the concentration of α -tubulin without using fluorescence probes.

It has been controversial on the loading control of Western blotting, including for urine proteins.²⁷ The loading control which could be used conventionally in Western blotting, such as β -actin, was reported to be differently expressed from sample to sample in urine.²⁸ For this reason, loading a constant amount of proteins could be a method to compare the concentration of a target protein in each sample.^{29,30} In our present study, the level of α -tubulin released into urine is important; therefore, we used same volume of urine samples to compare the α -tubulin concentration between samples from a same patient, to avoid a skewing result.

Immunochromatography assay developed in this study can quantitatively determine the α -tubulin level in the urine sample preliminarily. The platform and method for the quantitative detection of

α -tubulin provided in this study will lead to improved approaches for the post-operative therapy following kidney transplantation.

5 | CONCLUSION

The IC method provided in this study used colored cellulose nanobeads, instead of gold nanoparticles; thus, it has a potential to improve the stability and sensitivity of the detection kit. The color intensity was observed clearly and could determine the protein amount quantitatively. This method is simple, rapid, and adequately sensitive to detect α -tubulin in urine samples collected from kidney patients, which could be used for the clinical diagnosis of kidney injury.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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