Morphologic Analysis of Urinary Podocytes in Focal Segmental Glomerulosclerosis

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Abstract

Background The development of glomerulosclerosis in FSGS is associated with a reduction in podocyte number in the glomerular capillary tufts. Although it has been reported that the number of urinary podocytes in FSGS exceeds that of minimal-change nephrotic syndrome, the nature of events that promote podocyte detachment in FSGS remains elusive.

Methods In this study, we provide detailed, morphologic analysis of the urinary podocytes found in FSGS by examining the size of the urinary podocytes from patients with FSGS, minimal-change nephrotic syndrome, and GN. In addition, in urinary podocytes from patients with FSGS and minimal-change nephrotic syndrome, we analyzed podocyte hypertrophy and mitotic catastrophe using immunostaining of p21 and phospho-ribosomal protein S6.

Results The size of the urinary podocytes was strikingly larger in samples obtained from patients with FSGS compared with those with minimal-change nephrotic syndrome and GN (P=0.008). Urinary podocytes from patients with FSGS had a higher frequency of positive immunostaining for p21 (P<0.001) and phospho-ribosomal protein S6 (P=0.02) than those from patients with minimal-change nephrotic syndrome. Characteristic features of mitotic catastrophe were more commonly observed in FSGS than in minimal-change nephrotic syndrome urinary samples (P=0.001).

Conclusions We posit that the significant increase in the size of urinary podocytes in FSGS, compared with those in minimal-change nephrotic syndrome, may be explained by hypertrophy and mitotic catastrophe.


Introduction

FSGS is a condition that manifests in the loss of integrity of the kidney filtration barrier, resulting in severe proteinuria, and is often ascribed to the reduction in podocyte number (1). It has been reported that there are elevated numbers of podocytes that are shed into the urine in FSGS and active GN. Conversely, in minimal-change nephrotic syndrome (MCNS) and inactive GN, almost no urinary podocytes are observed (2–10).

To date, there is no documented morphologic analysis of urinary podocytes in FSGS. Ultrastructural examination by transmission electron microscopy has revealed enlarged podocytes within the glomerulus in the collapsing/cellular variant of FSGS and diabetic nephropathy, which is likely due to hypertrophy (11–14). Hypertrophic podocytes, observed in the FSGS cellular lesion of the glomeruli, were reported to have significantly higher expression of p21 (15). Furthermore, as a compensatory mechanism after the depletion of podocytes, protective roles of the mammalian target of rapamycin complex 1 (mTORC1)—which phosphorylates ribosomal protein S6 and induces the hypertrophy of the remaining podocytes—have been reported (16,17).

The toxic forms of podocyte injury cause dysfunction of the glomerular filtration barrier, which requires DNA synthesis, chromosome segregation, rearrangement of the cytoskeleton to assemble the mitotic spindle, and cell division. But terminally differentiated podocytes have a limited capacity to divide, resulting in aneuploid podocytes and disruption of the actin-dependent foot process attachment, which is referred to as mitotic catastrophe (MC) (15). In our assessment of morphologic changes of urinary podocytes in patients with diabetic nephropathy, we previously reported that urinary podocytes demonstrated characteristic features of MC (18). However, the morphologic changes of urinary podocytes in other forms of podocytopathies remain unknown. In this study, compared with urine samples obtained from patients with

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MCNS, we found that the urinary podocytes from patients with FSGS demonstrate robust hypertrophy along with MC, mirroring what was observed in diabetic nephropathy, thus providing new insights into the pathophysiology of FSGS.

Materials and Methods

Patients’ Urine Samples

From August 2017 to April 2020, fresh urine samples, voided in the morning, were obtained from 25 patients who were admitted to the Department of Pediatric Nephrology, Tokyo Women’s Medical University. The patients presented with primary FSGS (eight patients), MCNS (nine patients), and GN (eight patients). GN consisted of IgA nephropathy (three patients), IgA vasculitis with nephritis (two patients), ANCA-associated GN (one patient), lupus nephritis (one patient), and infection-related GN (one patient). All of the patients were diagnosed on the basis of clinical manifestations and renal biopsy specimens. At the time of collecting the urine samples for this study, all patients had proteinuria (urinary protein-creatinine ratio [UPCR] ≥0.20) and their eGFRs were >60 ml/min per 1.73 m². Eight of the nine patients with MCNS had frequently relapsing nephrotic syndrome and steroid-dependent nephrotic syndrome.

We also analyzed urine samples from five of the nine patients with MCNS, once they were in remission, and from six healthy children. All participants and/or their guardians provided informed consent to be enrolled in the study. This study was conducted with the approval of the Tokyo Women’s Medical University Hospital Ethics Committee (number 4528).

Urine Sediment Preparation and Staining

Urine samples (10 ml) were centrifuged at 1750 × g for 5 minutes and the urine sediments were fixed in TACAS Amber (Medical & Biologic Laboratories, Nagoya, Japan) immediately. The urine sediments were placed on TACAS slides (slides for liquid sample cytology; Medical & Biologic Laboratories), and fixed in 90% ethanol within 2 days (19). Slides were incubated with mouse anti-human podocalyxin mAb (PHM5; Merck Millipore, Darmstadt, Germany) at a dilution of 1:100 for 60 minutes. After washing with PBS, the slides were incubated with goat anti-mouse IgG, Superclonal recombinant secondary antibody, Alexa Fluor 488 (Thermo Fisher Scientific, Waltham, MA) at a dilution of 1:100 for 60 minutes. After being washed with PBS, 4',6-diamidino-2-phenyindole (Thermo Fisher Scientific) was added for 30 minutes and, after washing with PBS, coverslips were mounted. The slides were examined under a fluorescence microscope (model BX-53 equipped with a DP73 digital camera; Olympus, Tokyo, Japan). Nucleated podocalyxin-positive cells were defined as urinary podocytes (5).

The number of urinary podocytes were counted and expressed as the number of urinary podocytes per 1 mg of urinary creatinine (U-creatinine) observed under an immunofluorescence microscope (400-fold magnification) (9). The size of urinary podocytes was defined as the area (micrometers squared) per urinary podocyte (micrometers squared per cell), analyzed with ImageJ software, using the picture taken under an immunofluorescence microscope (400-fold magnification).

The number of urinary podocytes and the size of urinary podocytes were compared statistically among diagnoses. In patients with FSGS, the relationships between the size of urinary podocytes and clinical data (UPCR and disease duration) were analyzed statistically.

Immunofluorescence Study of p21 and Phospho-Ribosomal Protein S6

To evaluate the expression of p21 and phospho-ribosomal protein S6, we performed immunofluorescence studies in the urinary podocytes from patients with FSGS and MCNS. The slides were incubated with rabbit anti–p21 antibody polyclonal 10355-1-AP (Proteintech, Rosemont, IL), at a dilution of 1:100, or rabbit anti–Phospho-S6 Ribosomal Protein (Ser235/236) antibody monoclonal D572.2E (Cell Signaling Technology, Beverly, MA), at a dilution of 1:100, and mouse anti–Wilms tumor 1 transcription factor (WT-1) antibody monoclonal clone 6F-H2 (Merck Millipore), at a dilution of 1:100 for 60 minutes. After being washed with PBS, goat anti-rabbit IgG, Superclonal recombinant secondary antibody, Alexa Fluor 568 (Thermo Fisher Scientific) and goat anti–mouse IgG, Superclonal recombinant secondary antibody, Alexa Fluor 488 were added at dilutions of 1:1000 for 60 minutes. Nucleated WT–1-positive cells were defined as podocytes. All urinary podocytes expressing p21 in the nuclei were counted in all patients with FSGS and MCNS. All urinary podocytes expressing phospho-ribosomal protein S6 in the nuclei were counted in three patients with FSGS and two patients with MCNS. The proportions of urinary podocytes expressing p21 and phospho-ribosomal protein S6 were compared between FSGS and MCNS.

Morphologic Analysis of Urinary Podocyte Nuclei

To evaluate morphologic changes in urinary podocyte nuclei in FSGS and MCNS samples, hematoxylin-eosin staining was performed after the immunofluorescence studies using anti-podocalyxin antibodies. The characteristic feature of MC was defined as follows: (1) enlarged podocytes with irregular nucleus, (2) multinucleation, (3) presence of micronucleus (round DNA aggregates close to the nucleus) or mitotic spindle, and (4) denucleation (invisible nucleus) (18). All urinary podocytes showing MC were counted in all patients, and the proportions of urinary podocytes showing MC were compared between FSGS and MCNS.

To further evaluate ultrastructural changes in urinary podocytes by electron microscopy, urinary sediments from a patient with FSGS were fixed with 2% paraformaldehyde. Horseradish peroxidase–based, pre-embedding labeling by PHM5 was performed before electron microscopic examination to identify the podocytes. The specimen was observed by transmission electron microscopy using a JEOL 1400 (Jeol Ltd., Tokyo, Japan). Digital images were captured using the SightX Viewer (Jeol Ltd.).

Morphologic Analysis of Glomerular Podocyte Nuclei

We examined whether glomerular podocytes in kidney biopsy specimens have characteristic features of MC in three patients with FSGS whose urine samples were obtained within a month from the kidney biopsies.
All of the pathologic images were observed by Y.S., T.Y., and M.H. T.Y. was blinded to the clinical diagnosis of patients.

Statistical Analyses
Continuous variables were shown as medians (interquartile range [IQR]), and were compared using the Wilcoxon rank sum tests or ANOVA. Categoric variables were compared using the Fisher exact or Pearson test, where appropriate. Statistical analysis was performed using JMP Pro 14.0.0 software (SAS Institute Inc., Cary, NC), and a P value of <0.05 was considered statistically significant.

Results
Clinical Patient Characteristics
We observed no significant differences in age and sex among control patients and those with FSGS, MCNS, and GN. Disease duration, serum creatinine levels, and UPCRs were also similar among patients with FSGS, MCNS, and GN (Table 1). The variants of FSGS were categorized according to the Columbia classification as follows: collapsing variant (three patients), cellular variant (four patients), and not otherwise specified (one patients).

Urinary Podocyte Characteristics in Patients with FSGS, MCNS, and GN
The number (IQR) of urinary podocytes controlled for by U-creatinine concentration in each group was 8.0 (5.5-36.5) cells/mg U-creatinine, 4.0 (2.0-7.5) cells/mg U-creatinine, and 19.0 (12.0-23.8) cells/mg U-creatinine in FSGS, MCNS, and GN groups, respectively (Figure 1). The Wilcoxon rank sum test between the three groups showed a significant difference with P=0.008. Moreover, the Wilcoxon rank sum tests between each pair of diseases were as follows: FSGS versus MCNS, P=0.08; FSGS versus GN, P=0.43; and MCNS versus GN, P=0.002 (Figure 1A). The number of urinary podocytes in five patients in remission of MCNS was <0.3 (IQR, <0.3-1.2) cells/mg U-creatinine. In normal controls, no urinary podocytes were observed in their urine sediments (<0.3 cells/mg U-creatinine).

Next, we analyzed the correlation between the number of urinary podocytes and UPCR. The number of urinary podocytes was positively correlated with UPCR in all patients (P<0.001, r=0.67; Figure 1B), and in patients with FSGS (P=0.03, r=0.77; Figure 1C) and in patients with MCNS (P=0.002, r=0.87; Figure 1D). No correlation was observed between the number of urinary podocytes and UPCR in patients with GN (P=0.94; Figure 1E).

Urinary Podocytes from Patients with FSGS Demonstrate Increased Size
Because urinary podocytes were observed in patients with FSGS, MCNS, and GN, we then further characterized the size (IQR) of the podocytes as follows: 65.9 (38.5-104.3) μm², 54.0 (44.3-92.3) μm², and 230.1 (128.6-481.2) μm² in MCNS, GN, and FSGS samples, respectively. Compared with MCNS and GN, there was a striking increase in the size of urinary podocytes in FSGS samples (Figure 2A). Representative images of urinary podocytes immunostained with the podocyte-specific marker, podocalyxin, also reveal an increase in podocyte size (Figure 2B). Because the size of urinary podocytes varied among patients with FSGS, the distribution of the podocyte size was compared between the groups. As shown in Table 2, 40% of urinary podocytes were >200 μm² in patients with FSGS, whereas almost no urinary podocytes exceeded 200 μm² in patients with MCNS and GN.

In patients with FSGS, there were no significant correlations between the size of urinary podocytes and UPCR (5.5 [IQR, 0.7-10.3]; P=0.67) and disease duration (38 [IQR, 3-75] months; P=0.44) (Figure 3).

Nuclear Cyclin Kinase Inhibitor (p21) and Phospho-Ribosomal Protein S6 Expressions Are Increased in FSGS Urinary Podocytes
Next, we wanted to identify the potential mechanism behind the increased size of FSGS urinary podocytes. For this aim, we queried whether differential upregulation of cyclin kinase inhibitor would be observed, because this gene has previously been shown to be essential in inducing glomerular hypertrophy (14). We also evaluated the expression of phospho-ribosomal protein S6, a downstream target of mTORC1 which is directly associated with cell size regulation (16,17).

Coimmunostaining with the podocyte-specific marker WT-1 and p21 demonstrated robust immunoreactivity in the urinary podocytes of patients with FSGS when compared with those from patients with MCNS (Figure 4). The analysis demonstrated that, in urinary podocytes from patients with FSGS, 64% of podocyte nuclei (89/139 cells) stained positively for p21 compared with 7% from those in...
patients with MCNS (18/261 cells) ($P<0.001$). Further, coimmunostaining with WT-1 and phospho-ribosomal protein S6 resulted in intense immunoreactivity in the urinary podocytes of patients with FSGS when compared with those from patients with MCNS (Figure 5). Staining for phospho-ribosomal protein S6 was positive in 42% of FSGS urinary podocytes (63/150 cells) compared with 20% from those in MCNS (7/35 cells) ($P=0.02$).

FSGS Urinary Podocytes Demonstrate MC

To examine whether an increase in MC was observed in the FSGS samples to account for the increase in podocyte loss, we examined the specific features of MC: enlarged podocytes with irregular nuclei, multinucleation, presence of a micronucleus, and denucleation. In comparison with MCNS, urinary podocytes from patients with FSGS demonstrated a striking increase in the number of urinary podocytes demonstrating characteristic features of MC ($P<0.001$; Figure 6). Representative images of MC are shown in Figure 7. Electron microscopic examination revealed mitotic features in the nucleus of urinary podocytes from patient with FSGS (Figure 7I). Interestingly, we failed to observe the characteristic features of apoptosis, such as discrete fragments and budding of the cell to form apoptotic bodies, in urinary podocytes from this FSGS sample (20).

MC in FSGS Glomerular Podocytes

We examined whether characteristic features of MC are observed in glomerular podocytes in three patients with FSGS whose urine samples were obtained within a month from kidney biopsies. The median size of urinary podocytes was 119.4, 142.9, and 531.3 $\mu$m$^2$ in each patient, respectively.

Figure 1. The number of urinary podocytes was significantly correlated with UPCR in patients with FSGS and MCNS. (A) The number of urinary podocytes was significantly higher in patients with GN than in those with MCNS. There was no significant difference in the number of urinary podocytes between those with MCNS and FSGS. There were significant correlations between urinary protein-creatinine ratio (UPCR) and the number of urinary podocytes (B) across all groups ($P<0.001, r=0.67$), (C) in patients with FSGS ($P=0.03, r=0.77$), and (D) in patients with MCNS ($P=0.002, r=0.87$). (E) The number of urinary podocytes did not correlate with UPCR in patients with GN ($P=0.94$). MCNS, minimal-change nephrotic syndrome; U-creatinine, urinary creatinine.
Bowman’s space in one of 11 glomeruli, one of 15 glomeruli, and one of 33 glomeruli in each patient, respectively (Figure 8).

**Discussion**
We believe this study is the first detailed, morphologic evaluation of urinary podocytes from patients with FSGS.

![Figure 2](image)

**Table 2. The distribution of urinary podocytes stratified by the size**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Number of Cells ≥200 μm² (%)</th>
<th>Number of Cells &lt;200 μm² (%)</th>
<th>Total, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSGS</td>
<td>300 (40)</td>
<td>454 (60)</td>
<td>754 (100)</td>
</tr>
<tr>
<td>MCNS</td>
<td>0 (0)</td>
<td>595 (100)</td>
<td>595 (100)</td>
</tr>
<tr>
<td>GN</td>
<td>51 (4)</td>
<td>1374 (96)</td>
<td>1425 (100)</td>
</tr>
</tbody>
</table>

Pearson test showed a significant difference in the distribution of the size of urinary podocytes (P<0.01). MCNS, minimal-change nephrotic syndrome.
Previous reports have suggested that the number of urinary podocytes was higher in active GN than in MCNS (7), which was validated in our study. We also observed that patients with active GN and FSGS had a comparable number of urinary podocytes in our study. A correlation between the progression of glomerulosclerosis and the number of urinary podocytes from several kidney diseases has been postulated (2). Nakamura et al. (8) have previously reported that, in patients with MCNS who have achieved remission within 6 months, their urine was devoid of podocytes, whereas the entire cohort of patients with FSGS did have urinary podocytes. In contrast, we detected urinary podocytes in patients with MCNS, although the number of urinary podocytes trended lower than in patients with FSGS. One possibility to explain this observation is that, in our study, the patients with MCNS were highly dependent on steroids. Wickman et al. (21) described that urinary podocyte mRNAs showed close association with proteinuria in patients with MCNS and that urinary podocyte mRNAs could be useful for monitoring the risk of progressive depletion of podocytes, which leads to glomerulosclerosis. Our results also showed that the number of urinary podocytes was significantly correlated with urinary protein excretion in patients with MCNS and FSGS. These findings suggest that podocyte detachment may be associated with the amount of proteinuria in MCNS and FSGS.

Further implications gained from our study are that the size of urinary podocytes in FSGS, which was significantly larger than those in MCNS, may be useful to provide an initial diagnosis differentiating FSGS and MCNS, especially in patients contraindicated for kidney biopsy. However, it should also be noted that there is some heterogeneity in the size of podocytes in patients with FSGS and whether this form of analysis could be used clinically motivates further investigation.

Kikuchi et al. (22) have previously advocated for “podo-metrics,” i.e., analyzing the number, size, density, and glomerular volume of podocytes in glomeruli from renal biopsy specimens, and estimating the loss of podocytes in urine from the urinary podocin mRNA/creatinine ratio. They reported that podocytes shed from the basement membrane after podocyte injury resulted in residual podocyte hypertrophy (22). It has been reported that, when the hypertrophic stress exceeds the applicable range of podocytes, hypertrophic podocytes would be observed in both the urine and glomeruli of patients with FSGS (1,23–26).

![Figure 3](image1.png)

*Figure 3.* The size of urinary podocytes were not correlated with the backgrounds of patients in FSGS. There were no significant correlations between the size of urinary podocytes and (A) UPCR ($P=0.67$) and (B) disease duration ($P=0.44$) by ANOVA.

![Figure 4](image2.png)

*Figure 4.* The representative images of urinary podocytes of FSGS showing p21 expression. Urinary podocytes of FSGS showed p21 expression in the nuclei, whereas those of MCNS were negative for p21. Merged images with a higher magnification are also shown. Original magnification, 400×. Scale bars, 50 μm. DAPI, 4’,6-diamidino-2-phenylindole; WT-1, Wilms tumor 1 transcription factor.
Mature podocytes are terminally differentiated, growth-arrested cells losing mitotic activity (11). Each phase of the cell cycle is controlled by specific cyclin-dependent kinases, which are inactivated by p21 (27,28). The hypertrophic podocyte observed in the FSGS cellular lesion in the glomeruli was suspected to have re-entered the cell cycle (15). Wang S. et al. (29) reported that, although glomerular cells were negative for p21 in normal controls, podocytes occasionally showed nuclear staining for p21 in the cellular lesion of FSGS (30). Therefore, it has been suspected that hypertrophic podocytes were observed due to arrest at the G1/S check point by p21 (31,32). It has also been reported that the mTORC1 signaling pathway mediates compensatory hypertrophy of podocytes and that excessive upregulation of mTOR causes podocyte depletion, which is observed as increased expression of phospho-ribosomal protein S6 (16,17). In addition, phospho-ribosomal protein S6 has been reported to regulate the expression of genes associated with the cell cycle, such as those encoding cyclin D1 and cyclin E1, at both translational and transcriptional levels (33). The frequent positive immunostaining for p21 and phospho-ribosomal protein S6 in FSGS urinary podocytes in our study may suggest that an increased size in podocytes is associated with podocyte hypertrophy, evidenced by aberrant regulation of the cell cycle. Further studies with increased numbers of patients are required to confirm this hypothesis.

It has also been reported that glomerular podocytes show MC in adriamycin nephropathy from FSGS model mice (32). Although the podocytes enter the mitotic cycle after injury, they are not able to complete mitosis nor assemble efficient mitotic spindles due to the complex actin cytoskeleton resulting in MC (11). In addition, the appearance of binucleated or micronucleated cells and cytoskeleton disruption in podocytes were reported to be driven by p21 (15). In this study, characteristic features of MC were frequently observed in urinary podocytes and were also observed in some glomerular podocytes, which may suggest that MC.

Figure 5. | The representative images of urinary podocytes of FSGS showing phosphoribosomal protein S6 expression. FSGS urinary podocytes showed phospho-ribosomal protein S6 expression in the cytoplasm, whereas those of MCNS were negative for phospho-ribosomal protein S6. Merged images with a higher magnification are also shown. Original magnifications, 400×. Scale bars, 50 μm. DAPI, 4’,6-diamidino-2-phenylindole; p-rpS6, phospho-ribosomal protein S6.

Figure 6. | The proportion of urinary podocytes with characteristic features of mitotic catastrophe in patients with FSGS and MCNS. Urinary podocytes showing characteristic features of mitotic catastrophe were more frequently observed in patients with FSGS (26/101 cells, 26%) than in those with MCNS (1/55 cells, 2%) (P<0.001). The features of mitotic catastrophe in FSGS consisted of multinucleation, denucleation, enlargement with irregular nucleus, and micronucleus in 15 (15%), five (5%), three (3%), and three (3%) cells, respectively.
is involved in the pathogenesis of an increased size of urinary podocytes in patients with FSGS.

The limitations inherent in our study are as follows. The association of the histopathologic findings in the glomerulus and the number and size of urinary podocytes were evaluated in a small number of patients. Additionally, patients with genetic FSGS and secondary FSGS were not included in this report. Lastly, the sample size will need to be increased. Nevertheless, these findings provide further impetus to address these limitations.

In conclusion, we provide evidence that the size of urinary podocytes in FSGS is significantly larger than those in MCNS, which may be explained by both hypertrophy and MC in a p21-dependent manner. The morphologic study of urinary podocytes may also provide useful information in distinguishing between FSGS and MCNS, which will be critical to personalize therapeutic options.

Disclosures
All authors have nothing to disclose.

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Figure 7. The representative images of urinary podocytes showing mitotic catastrophe in FSGS. Urinary podocytes in FSGS showed characteristic features of mitotic catastrophe. A and B, enlarged and irregular nucleus; C and D, multinucleation; E and F, presence of micronucleus; G and H, denucleation. Hematoxylin eosin staining (A, C, E, G) and immunofluorescent staining of podocalyxin (B, D, F, H) Original magnifications, 400-fold. Electron microscopy revealed mitotic feature of the nuclei in the urinary podocyte in an FSGS case. The image was created using a 3×3 montage system to produce high quality image data. Original magnifications, 6000-fold (I).

Figure 8. Glomerular podocytes showing mitotic catastrophe in FSGS. Some podocytes in the Bowman’s space showed multinucleations in the kidney specimens obtained from three patients with FSGS. The arrowheads highlight the podocytes showing multinucleations. Periodic acid-Schiff staining. Original magnifications, 400×. Scale bars, 50 μm.
Author Contributions
T. Ando, S. Ishiwa, K. Ishizuka, N. Kaneko, Y. Shirai, A. Shiratori, and T. Yabuuchi were responsible for data curation; M. Hara and M. Hattori reviewed and edited the manuscript; M. Hara, M. Hattori, K. Miura, and Y. Shirai conceptualized the study; S. Horita, H. Nakayama, H. Seino, Y. Shirai, and T. Yokoyama were responsible for resources; K. Miura and Y. Shirai wrote the original draft, and were responsible for formal analysis and investigation; Y. Shirai was responsible for visualization; and all authors approved the final version of the manuscript.

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