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# Semi-Quantitative Non-Radioactive *In Situ* Hybridization and Its Clinical Application

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***In situ* hybridization (ISH) is widely used to determine the expression of specific mRNA in tissues. However, the results of ISH are influenced by the amount of retained RNA in the tissue. Estimation of retained RNA in the tissue is very important and the results of ISH should be interpreted taking into consideration the amount of retained RNA in samples. In the present study, we determined the amount of retained RNA by performing ISH for 28S rRNA. We also analyzed the results of ISH semi-quantitatively by computer image analysis. The method was applied by calculating the ratio of specific mRNA to 28S rRNA in order to standardize the results of ISH among different tissue samples. Our method is useful for comparing the expression of specific mRNA in different tissue samples with variable levels of retained RNA. Since the conditions of fixation and storage of tissues differ among samples, especially in clinical samples, our semi-quantitative method allows a more precise evaluation of gene expression in different samples.**

**Key words:** ***In situ* hybridization, Image analysis, Digoxigenin**

## I. Introduction

*In situ* hybridization (ISH) is a powerful tool for detecting specific mRNAs in tissue sections and is now widely used for the study of several types of factors such as cytokines and growth factors. Recently non-radioactive ISH has become very sensitive, especially with biotinylated tyramine [20]. For a full implementation of ISH for specific mRNAs in tissues, it is essential to use specimens with well-preserved RNA. Tissue samples retaining only small amounts of RNA would falsely underestimate the signal for specific mRNA. Therefore, it is important to evaluate the amount of RNA retained in the specimens.

There are two methods for estimation of the amount of RNA in tissue sections; the first requires staining of RNA with dyes such as acridine orange [11] and methyl green/pyronin Y [33], and the other is ISH. While staining with dyes is a reliable method for assessment of total RNA, it does not always provide information on RNA that can be reacted with a probe in tissue sections (hybridizable RNA). Using strongly fixed specimens, RNA in tissue is well stained with dyes but attempts to detect the RNA by ISH may yield false-negative results because of difficulties associated with the probe access to target mRNA. With respect to ISH as a method for evaluation of retained RNA in the tissue, the target molecules include poly (A) [16], β-actin and 28S rRNA. The probe for poly (A) of mRNA which does not contain G and C, results in having unsuitably low melting temperature (Tm) in the standard procedure. β-actin is widely expressed in tissues, however, its expression varies among cells, depending on the cell condition [31]. Thus, these molecules are not appropriate for the assessment of retained RNAs in tissue sections. In contrast, 28S rRNA is widely and abundantly distributed throughout the tissue and its expression is fairly constant across the same tissue [7].

In this study, we evaluated semi-quantitatively the level of 28S rRNA detected as a marker of retained RNA on histological sections using non-radioactive ISH combined with computer assisted image analysis. We also applied this technique to measure the level of specific mRNAs such as IL-6, platelet derived growth factor (PDGF), and transforming growth factor β (TGF-β), rela-

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tive to that of 28S rRNA. We also compared the level of specific mRNAs among renal samples from different patients with IgA nephropathy (IgAN), the most common form of chronic glomerulonephritis in Japan [6]. Our system is very useful for evaluating the expression of specific mRNAs in clinical samples, even when RNA is not well preserved.

## II. Materials and Methods

### *Materials*

Kidneys freshly obtained from male Wistar rats, and renal biopsy specimens obtained from 20 patients with IgAN were embedded in O.C.T. compounds (Miles Inc., Elkhardt, IN) and cryopreserved at −80°C until experimental use. The diagnosis of IgAN was confirmed by light microscopy, immunofluorescence staining and electron microscopy after excluding systemic diseases such as lupus erythematosus, liver cirrhosis and diabetes mellitus. No patient received steroids or immunosuppressive drugs prior to renal biopsy. Patients were divided into two groups according to the histopathological findings described elsewhere [28]: patients with mild lesions (10 patients) and patients with severe lesions (10 patients). The main characteristic features of mild lesions were the presence of minimal glomerular lesions or mild mesangial thickening with increased homogeneous periodic acid-Schiff reaction (PAS)-positive mesangial matrix, and mild and segmental hypercellularity. Severe lesions were characterized by the presence of diffuse mesangial thickening and mesangial cell proliferation. In certain glomeruli, capsular adhesion, fibrocellular crescents, glomerular sclerosis and hyalinosis were present in renal tissue from patients with severe lesions. In addition, widespread interstitial fibrosis and tubular atrophy were observed in these tissues. The clinical parameters of our patients are summarized in Table 1.

### In situ *hybridization (ISH)*

ISH was performed according to our protocol, described elsewhere [10]. In brief, tissue specimens were cut to a thickness of 4 $\mu$m and mounted on 3-aminopropyl-triethoxysilane-coated glass slides (Matsunami, Tokyo, Japan). Sections were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) and washed in PBS. After treatment of sections with 0.2 N HCl, they were digested with proteinase K (P-4914, Sigma Chemical Co., St. Louis, MO) at 37°C. Sections were put into prehybridization buffer and then hybridized overnight with a digoxigenin (DIG)-labeled oligonucleotide probe in the prehybridization buffer at 37°C. After washing, visualization of hybridized DIG-labeled probe was performed immunohistochemically as follows. After washing the slides, sections were pre-incubated with a blocking solution containing 20% normal swine serum (NSS; Dakopatts X901, Glostrup, Denmark), 5% fetal calf serum (FCS; Gibco BRL, Gaithersburg, MD) and 5% bovine serum albumin (BSA, Sigma) in PBS to prevent non-specific binding. After drainage, mouse monoclonal anti-DIG antibody (#1333 062, Boehringer Mannheim Biochemica, Mannheim, Germany) was overlaid on the sections, then the sections were incubated in methanol with 0.3% $H_2O_2$ to block the activity of endogenous peroxidase. Sections were then incubated sequentially with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibody (Dako P260, DAKO) and HRP-conjugated swine anti-rabbit antibody (Dako Z113) with triple washings in PBS with 0.075% Brij (Sigma 430AG-6) between each step. Visualization was performed by reacting with diaminobenzidine tetrahydrochloride in 0.05 M Tris-HCl, pH 7.6, and 0.03% $H_2O_2$, followed by mounting the sections. To evaluate the specificity of the signals, control experiments, including pretreatment of RNase, studies using a sense probe and competitive studies were performed as previously described by our laboratory [18].

To examine the effect of RNase treatment on the expression level of 28S rRNA, serial sections of rat renal tissue were incubated with different concentrations of RNase (0, 0.01, 0.1, 1.0 and 10 $\mu$g/ml) after proteinase K treatment. Sections were then hybridized with a probe for 28S rRNA in a similar manner. This was followed by computer analysis as described above.

### *Probe and labeling*

We selected 25 base sequences of human 28S rRNA, mRNA for human IL-6, and 30 base sequences of human

**Table 1.** *Clinical data of patients with IgA nephropathy.*

| | Age Years | Proteiuria g/day | Hematuria Index* | Serum creatinine mg/ml |
|---|---|---|---|---|
| Group M (n=10) | 29±12 | 0.68±0.58 | 2.3±1.3 | 0.86±0.18 |
| Group A (n=10) | 47± 7 | 1.67±2.44 | 2.1±1.0 | 1.53±0.66 |

Data are mean±SD. Group M: mild lesion, Group A: advanced stage lesion.

*Hematuria index: The severity of hematuria was graded as: grade 0, representing 0 to 5 erythrocytes per high-power field; grade 1, 5 to 30 erythrocytes per high-power field; grade 2, >30 erythrocytes per high-power field; and grade 3, representing macroscopic hematuria.

**Table 2.** *Comparison of the ratio of IL-6, PDGF and TGFβ mRNA to 28S rRNA in glomeruli between IgAN patients with deteriorating (Group I) and stable (Group II) renal function.*

| | IL-6 | PDGF | TGFβ |
|---|---|---|---|
| Group I (n=15) | 0.69±0.12 | 0.58±0.19* | 0.48±0.14* |
| Group II (n=30) | 0.61±0.12 | 0.31±0.12 | 0.23±0.15 |

Data are mean±SD. P*<0.05, compared with Group II. The expression of PDGF mRNA, TGFβ mRNA, not IL-6 mRNA are significantly higher in the three patients with deteriorating renal function (Group I) than in the three patients with stable renal function (Group II). N indicated the number of glomeruli examined in the present study.

TGF-$\beta$ and PDGF B-chain mRNAs. The 28S rRNA probe was complementary to bases 2313–2337 of human 28S rRNA [12] and also complementary to rat 28S rRNA [3]. The sequences of IL-6 and TGF-$\beta$ probes have already been reported in our previous studies [26, 32]. The sequence of IL-6 was complementary to bases 450–474 of human IL-6 cDNA [15] and the TGF-$\beta$ probe was complementary to bases 935–959 of human TGF-$\beta$ cDNA [8]. The PDGF B-chain probe, complementary to bases 983–1007 of human PDGF B-chain cDNA [4] was selected. Through a computer-assisted search, these sequences were significantly different from other known sequences deposited in the latest release of the gene data bank (GenBank). Each oligonucleotide probe was labeled by a DIG oligonucleotide tailing kit according to the standard protocol (Boehringer Mannheim Biochemica 1417 231).

### *Detection of probe nucleotides on nylon membranes*

Detection of probes on membranes was performed as reported previously [10]. Briefly, the DIG-labeled probe for 28S rRNA was diluted several times from 10 ng/$\mu$l to 1.0 pg/$\mu$l and spotted onto a nylon membrane (Hybond-N; Amersham, Aylesbury, UK). After the membrane was fixed by UV light irradiation (UV Stratalinker 2400; Stratagene, La Jolla. CA), it was stained using the same immunohistochemical method described in the ISH procedure in a sealed polyethylene bag. After visualization of the spotted probe on the membrane with diaminobenzidine tetrahydrochloride and $H_2O_2$, image analysis was performed to examine the density of each spot of DIG-labeled probe for 28S rRNA as described below.

### *Image analysis*

The image analysis of the stained section was performed using an Olympus BH-2 microscope connected by a Cannon CCD camera, and an Olympus SP-500 Image analyzer with Image Command program 5098 as described in detail previously [1]. The image was transformed into a matrix of 512 × 480 pixels per frame and each pixel had one of 256 gray levels; ranging from 0 (black) to 255 (absolute white). We measured the optical density of each cell in the glomerulus representing the sum of the gray values of all pixels corresponding to the cell. The sum of the density of the cells was defined as the signal density of the glomerulus. Since the background level was varied among tissues, it was subtracted from the staining intensity in each specimen. Furthermore, to minimize differences in staining between experiments, ISH for 28S rRNA was performed in sections from a normal rat kidney in every experiment and was used as a normal control.

ISH for 28S rRNA and IL-6 was performed in serial sections of renal biopsy specimens from IgAN patients. The density of 28S rRNA and IL-6 mRNA in the same glomerulus was calculated by image analysis as described above. Then, the ratio of IL-6 mRNA to 28S rRNA was calculated in 5 glomeruli in each section. ISH for 28 rRNA and IL-6 mRNA was performed in triplicate in each patient and we compared the ratio between patients with mild and severe lesions.

### *Clinical application of image analysis*

IL-6, PDGF and TGF-$\beta$ are thought to be involved in the development and progression of IgAN [32, 34, 36]. In a series of preliminary experiments, we examined whether the transcription of these cytokines differed between renal biopsy specimens from patients with stable disease and from those with deteriorating renal function during the clinical course using our semi-quantitative ISH. We selected three patients with mild lesions and normal serum creatinine levels at the time of renal biopsy, who showed worsening of renal function during clinical follow-up (>1.5 mg/dl of serum creatinine or increased to 200% of serum creatinine at the time of renal biopsy). Three IgAN patients with mild lesions and stable renal function during the clinical follow up were selected as control subjects. Using these samples, we performed ISH for IL-6, PDGF and TGF-$\beta$ mRNA and 28S rRNA. We examined five glomeruli and five areas of the interstitium in renal tissue from each patient and analyzed semi-quantitatively the expression of the above mRNAs and 28S rRNA and calculated the ratio of IL-6, PDGF and TGF-$\beta$ mRNAs

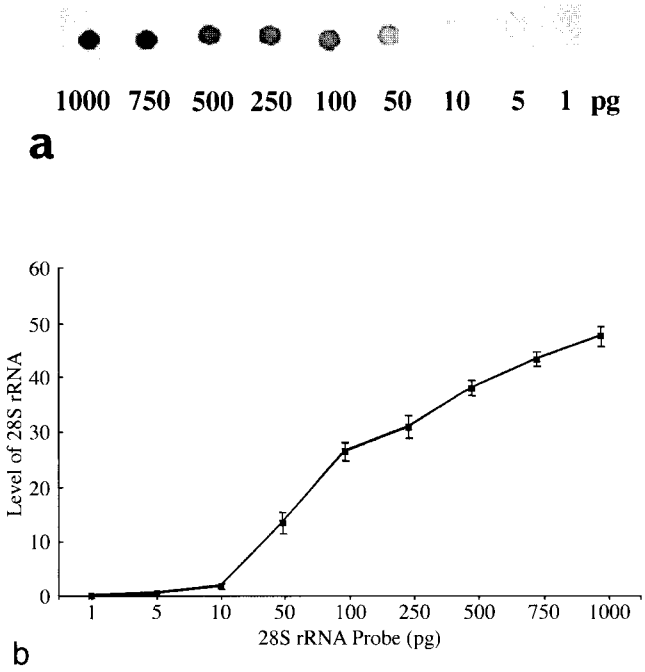


**Fig. 1.** Immunohistochemical detection of probe nucleotides on nylon membrane. **(a)** As little as 5 pg of DIG-labeled 28S rRNA antisense probe was detected on the membrane. **(b)** Quantitative image analysis of immunohistochemical detection of probe oligonucleotides on the membrane (n = 5). The level of detected probe nucleotides, which were fixed on nylon membrane increased linearly from 10 to 1,000 pg.

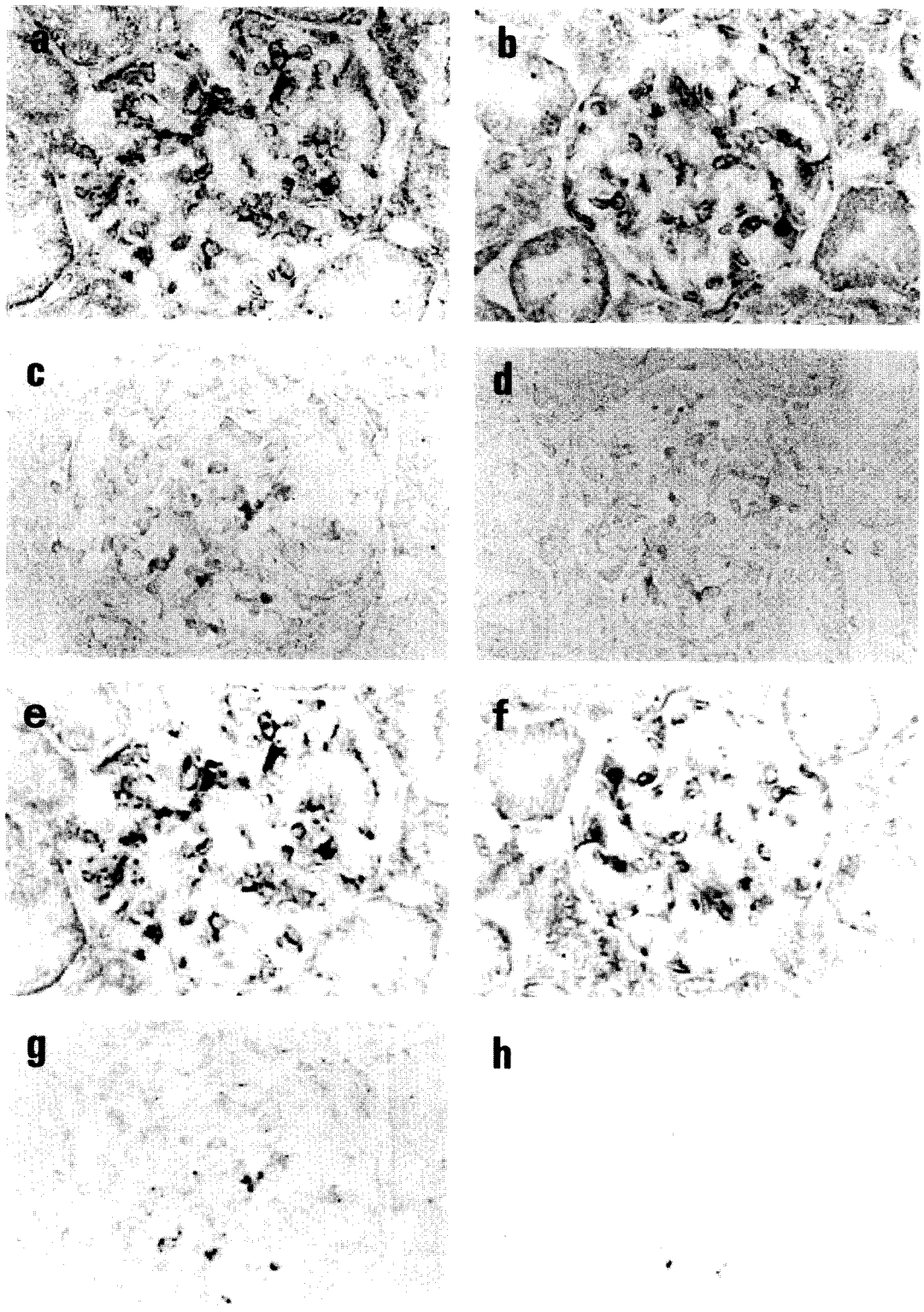


**Fig. 2.** Treatment of renal sections with RNase in ISH for 28S rRNA. **(a–d)**: Note that the expression of 28S rRNA was weaker with increased concentration of used RNase (Used RNase concentration, **a**; 0 $\mu$g/ml, **b**; 0.01 $\mu$g/ml, **c**; 1 $\mu$g/ml, **d**; 10 $\mu$g/ml). **(e–h)**: Digitized images of sections shown in **(a–d)**. Positively stained signal in the glomerulus appears gray in color. Image analysis more clearly demonstrates a positively stained signal compared to microscopic findings **(a–d)**. The signal for 28S rRNA became weaker with increased concentration of RNase, as shown in **(a–d)**.

relative to 28S rRNA in each group.

*Statistical analysis*

Data were expressed as mean±SD. The Mann-Whitney test was applied to examine differences between groups. A $p$ value of $<0.05$ denoted the presence of a significant difference.

## III. Results

*Immunohistochemical detection of probe nucleotides fixed on nylon membrane*

As little as 5 pg of DIG-labeled 28S rRNA antisense probe on the membrane could be detected immunohistochemically (Fig. 1a). The results of image analysis of each spot are demonstrated in Figure 1b, indicating that the optical density of the signal obtained in dot blot hybridization in each spot was proportional to the amount of probe spotted on the membrane from 10 pg to 1 ng (Fig. 1b).

*Treatment of renal sections with different concentrations of RNase*

Fresh frozen sections of rat kidneys were incubated with RNase at concentrations ranging from 0 to 10 μg/ml at 37°C for 5 min after proteinase K digestion in ISH for 28S rRNA. Without RNase treatment, 28S rRNA was expressed in glomerular cells. However, the signal for 28S rRNA became weaker with increased concentrations of RNase (Fig. 2a–d). Expression of 28S rRNA was hardly detected when sections were treated with 10 μg/ml of RNase. Figure 2e–h show representative digitized images of the sections. The signal was colored on the digitized image and, thus, even weakly stained signal on the microscopic image was clearly detected on the digitized image. In each digitized image, the density of positively stained signal for 28S rRNA in the glomerulus was measured by image analysis (Fig. 3). The density of 28S rRNA decreased proportionately to the concentration of RNase.

*ISH for 28S rRNA and IL-6 mRNA*

Expressions of 28S rRNA and IL-6 mRNA were detected by ISH on serial sections of renal tissues in IgAN. However, the staining intensity for 28S rRNA ISH varied from one patient to another, indicating the presence of variable degrees of RNA retention in the samples. As shown in Figure 4, some sections were stained strongly for 28S rRNA while others were stained only weakly regardless of the histological severity. In ISH for IL-6 mRNA, glomerular cells, especially cells in the area of mesangial proliferation, were positive. In general, as shown in Figure 5, the number of glomerular cells positive for IL-6 mRNA in renal sections was higher in patients with severe lesions than in those with mild lesions, while the staining intensity for IL-6 mRNA varied among patients. Since

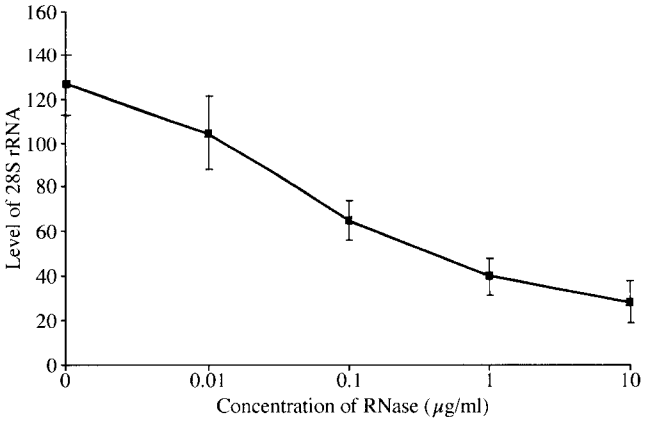


**Fig. 3.** Semi-quantitative image analysis of the density of positively stained signal for 28S rRNA in a representative glomerulus treated by various concentrations of RNase shown in Fig. 2. Measure of the signal density in all pixels and the sum of the density of gray values in the glomerulus was defined as the signal density in the glomerulus (we examined 10 glomeruli at each RNase concentration). Note that the level of 28S rRNA decreased linearly with increased concentration of used RNase.

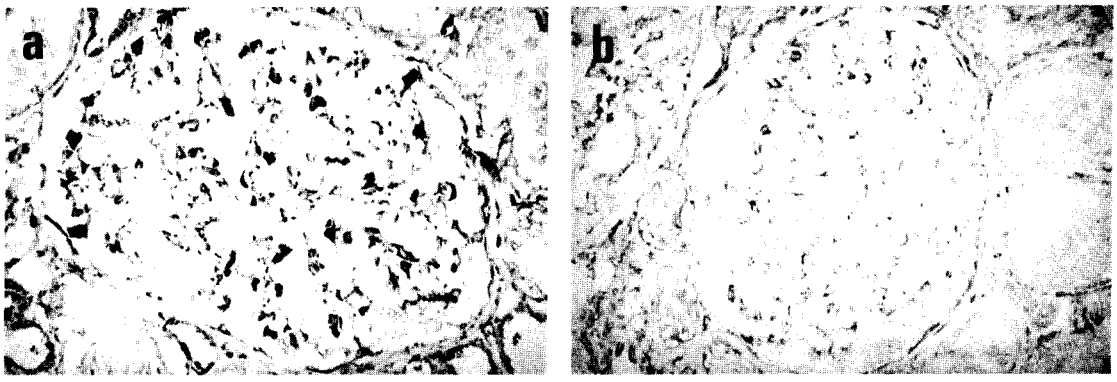


**Fig. 4.** *In situ* hybridization study for 28S rRNA in glomeruli of patients with IgA nephropathy. The signal for 28S rRNA varied among sections. **A**: A glomerulus stained strongly positive for 28S rRNA. **B**: A different glomerulus from another patient with IgA nephropathy showing weak staining for 28S rRNA.

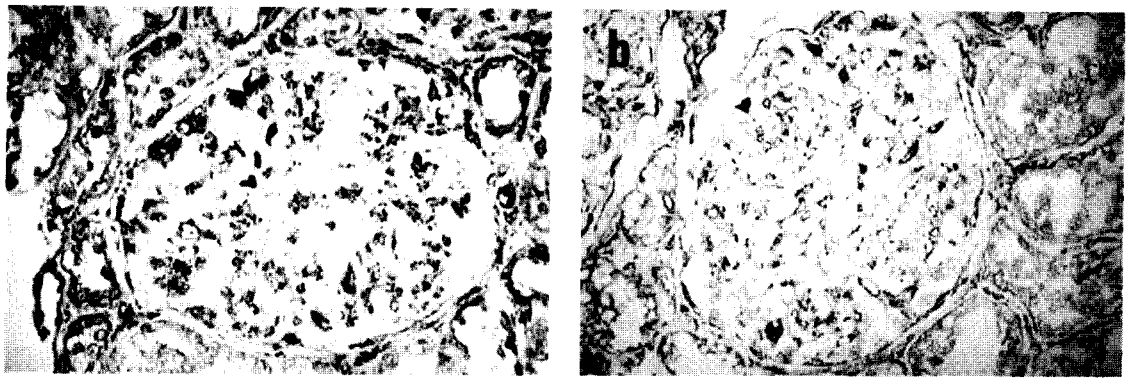


**Fig. 5.** *In situ* hybridization study for IL-6 mRNA in glomeruli of patients with IgA nephropathy. **a**: A glomerulus from a patient with severe tissue damage. **b**: A glomerulus from a patient with mild tissue damage. Note that the number of glomerular cells positive for IL-6 mRNA is higher in patients with severe tissue damage than in patients with mild lesions.

the level of 28S rRNA expression varied among tissues, calibration of the results of ISH for the specific mRNA was necessary. To compare the degree of IL-6 mRNA expression, we expressed the level of IL-6 mRNA expression relative to that of 28S rRNA (IL-6/28S ratio). Even when the degree of IL-6 mRNA expression was similar between two patients, the IL-6/28S ratio was different (Fig. 6). Furthermore, the mean ratio of IL-6 mRNA/28S rRNA in patients with severe lesions was significantly higher than in patients with mild lesions (Fig. 7).

We also examined the possible application of our semi-quantitative analysis system in clinical samples. Using our image analysis method, the expression levels of mRNAs of various cytokines was determined in tissues from two groups of IgAN patients. These patients had different prognosis in spite of normal serum creatinine levels at the time of renal biopsy. Some patients showed worsening of renal function during clinical follow-up while others had stable renal function during the clinical course. Figure 8 shows renal tissue from a representative patient in the former group stained with PAS. Only a mild degree of mesangial proliferation and expansion but no interstitial injury was found. In general, it was difficult to predict worsening of renal function based on microscopic findings. Our results showed that the expression levels of PDGF and TGF-$\beta$ mRNAs, not IL-6 mRNA, were significantly higher in patients with deteriorating renal function than in patients with stable renal function (Table 1).

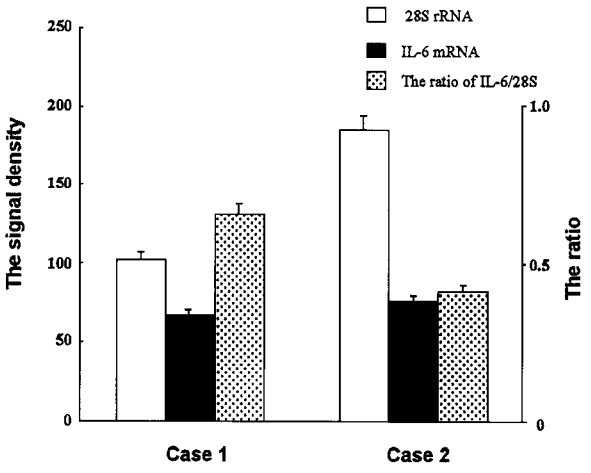


**Fig. 6.** Semi-quantitative analysis of the results of ISH for 28S rRNA, IL-6 mRNA in two patients with IgA nephropathy. The ratio of the expression level of IL-6 mRNA to that of 28S rRNA (IL-6/28S) was calculated. Seven glomeruli were examined in each patient. The level of expression of IL-6 mRNA was similar in the two patients, but the ratio of IL-6 mRNA/28S rRNA was different in these patients. Standardization using IL-6 mRNA/28S rRNA ratio demonstrated that the degree of IL-6 mRNA was higher in Case 1 than in Case 2.

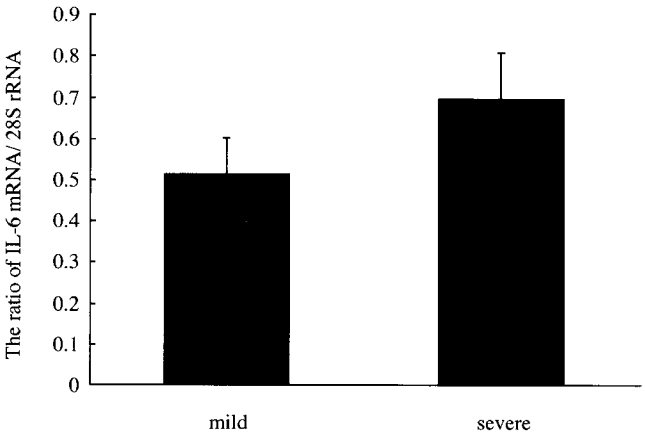


**Fig. 7.** Mean values of IL-6 mRNA/28S rRNA ratio in patients with mild lesions and patients with severe lesions. The ratio was significantly higher in patients with severe lesions than in those with mild lesions ($p < 0.05$). Data are mean $\pm$ SD of sixty glomeruli from 10 patients in each group.

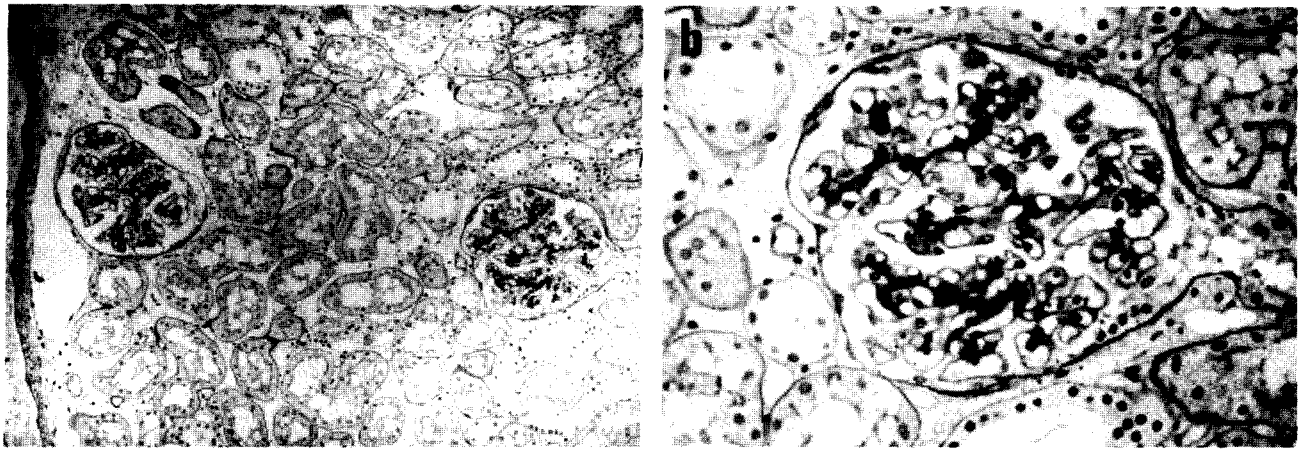


**Fig. 8.** Periodic acid-Schiff reaction (PAS) staining of renal tissue from a patient with IgA nephropathy who had a normal renal function at the time of renal biopsy but developed worsening of renal function during clinical follow-up. Note the mild degree of glomerular lesion and the lack of interstitial lesion (magnification for **a**, ×200 and **b**, ×400).

## IV. Discussion

In the present study, we described our method of semi-quantitative analysis based on non-radioactive ISH. Using image analysis, we determined the density of positive area by summating the density of each pixel in the digitized image. Furthermore, by computing the ratio of the level of specific mRNAs to that of 28S rRNA, as a suitable marker of retained RNA in tissue sections, we were able to compare the level of specific mRNAs in different tissue samples with variable levels of retained RNA. Thus, the presented ISH method seems to be very useful for semi-quantitative reporting of specific mRNAs.

To assess the results of ISH for specific mRNAs in the tissues, particularly in clinical specimens, it is essential to evaluate retained RNA in the tissue because RNA is easily broken down by RNase, which is ubiquitously present in the tissues and is difficult to be broken down. Since we usually have to compare specific mRNA levels among fresh and archival tissues stocked under different conditions, especially in clinical samples, the amount of retained RNA in each specimen should be evaluated. The retention of tissue mRNA was improved by the proper condition of fixation [30]. However, evaluation of retained RNA in tissue samples in ISH is not usually mentioned in many studies. As shown in the present study, the signal of 28S rRNA disappeared gradually when the specimens were treated with higher concentrations of RNase and the level of retained RNA was different among different samples. The most beneficial aspect of using 28S rRNA as a marker for retained RNA in specimens is the ability to assess not only retained RNA but also "hybridizable RNA" [19]. By performing ISH for 28S rRNA, one can evaluate the existence of retained RNA, capability of the probe to access the target mRNA and visualization of the haptenized probe by immunohistochemistry. This is a very unique feature of ISH for 28S rRNA and we confirmed that ISH for 28S rRNA is very useful as a positive control.

To evaluate the results of ISH semiquantitatively, we used image analysis in the present study. Few previous reports have described quantitative evaluation of mRNA in tissue sections. In radioactive ISH, the number of silver grains in cultured cells [5] and in tissue sections [2, 29] was counted. Recently, Jonker *et al.* [17] measured the grain density in autoradiography by using a computer densitometry system. Unfortunately, the signal is not always located in the identical cells when radioactive ISH is used for quantitative analysis, resulting in inadequate distinction of the true signal from the background. In non-radioactive ISH, two methods are available for quantitative analysis. The first measures the number or size of positively stained structures [9] while the second measures the intensity of the signal [25]. The latter method has been applied in several recent studies [14, 24]. In the present study, to determine the total amount of the signal, we calculated the product of the positively stained area and the degree of density of *each signal*. The staining intensity is influenced by numerous factors, such as duration of fixation, section thickness, efficiency of probe labeling, purification and concentration of probe, pretreatment and hybridization procedures and the used detection system [13, 23]. Since measurement of the signal intensity must be carefully standardized to minimize variability, we subtracted the level of background staining intensity from each signal density. Moreover, we performed ISH for 28S rRNA in the specimens from renal tissues of normal rats in each experiment as normal control. Using this method, section-to-section and experiment-to-experiment variability in the intensity are minimized. Since the recent progression of computer technology is remarkable, we can perform image analysis more easily by personal computer [22].

In ISH, it is important that the amount of retained RNA in the section is taken into account during the as-

sessment of expression of the specific mRNA. In quantitative analysis of Northern blot, it is classic to correct the results by comparing mRNA with those of house keeping genes, such as β-actin and 28S rRNA, whose expression is thought to be relatively constant. Applying this method to ISH, we calculated in the present study the ratio of the level of specific mRNA to that of 28S rRNA. Using this method, we were able to standardize the level of specific mRNA among different samples with variable levels of retained RNA. A weak expression of specific mRNA in ISH does not always indicate a low level of mRNA production if the tissue contains a small amount of retained RNA. Standardization is particularly important when clinical samples are used, in which the level of retained RNA varies widely from one tissue to another, because the condition of fixation and storage may differ among the samples. Since the amount of target mRNA varies, further studies are warranted for clarifying whether 28S rRNA is still a useful standard marker, when the amount of target mRNA is too small. Moreover, as in competitive RT-PCR, addition of a certain amount of sense oligonucleotide into the hybridization buffer might be another method for quantification of ISH. *In situ* RT-CR may also be able to quantify the expression of mRNA [35].

Several cytokines and growth factors are thought to be associated with the development and progression of IgAN [32, 34, 36]. Although we have recently reported that the expression of IL-6 mRNA correlates with the degree of mesangial cell proliferation and tubulointerstitial change in IgAN [27], semi-quantitative analysis of IL-6 mRNA as well as 28S rRNA was not performed in that study. In the present study, we compared the level of IL-6 mRNA in clinical samples of IgAN patients with two different levels of severity; mild and severe tissue injury. Although the degree of IL-6 mRNA was almost equal in different samples, the expression level of 28S rRNA was different among them. These results suggest that examination of IL-6 mRNA may only lead to misestimation of the expression of IL-6 mRNA. In consideration of retained RNA, we calculated the ratio of IL-6 mRNA to 28S rRNA in the present study and showed the relationship between IL-6 mRNA expression in glomeruli and the degree of glomerular injury. These results suggested that increased IL-6 expression may be important in the progression of IgAN.

Finally, we conducted a series of preliminary experiments in which we applied our semiquantitative ISH analysis to predict the prognosis of IgAN based on an estimation of the expression level of certain cytokines in renal tissues. Since the prognosis of IgAN is reported to be not as good as it was initially expected [21], it is important at the time of renal biopsy to identify patients who are at a high risk for developing end-stage renal diseases (ESRD). At present, however, it is difficult to predict the development of ESRD especially in patients with mild renal injury diagnosed only by microscopic histopathological examination. Using our method, we showed that the expression levels of TGF-β and PDGF mRNAs, not IL-6 mRNA, were higher in patients with deteriorating renal function than those with stable renal function. Although the number of patients examined in the present study was too small to draw definite conclusions, the results of our preliminary studies suggest that semiquantitative analysis of ISH may be clinically useful for predicting future development of ESRD at the time of renal biopsy.

In conclusion, ISH for 28S rRNA is helpful for assessing the level of retained RNA in tissue samples. Using computer analysis, the results of ISH can be expressed semi-quantitatively and the computed ratio of specific mRNA to 28S rRNA allows comparison of the level of specific mRNA in different tissues irrespective of the variable level of retained RNA. Our method may allow a better understanding of gene expression in clinical samples as well as samples of experimental models.

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