Serum Autotaxin Levels Are Associated with Proteinuria and Kidney Lesions in Japanese Type 2 Diabetic Patients with Biopsy-proven Diabetic Nephropathy

Miho Shimizu1,2, Kengo Furuichi1,2, Tadashi Toyama1,2, Junya Yamahana2,3, Ryunosuke Ohkawa4, Koji Igarashi5, Junken Aoki6, Shuichi Kaneko2, Yutaka Yatomi4,7 and Takashi Wada1,8

Abstract

Objective   We evaluated the relationships between the serum autotaxin (ATX) levels and the clinical and pathological parameters, as well as the long-term renal outcome, in type 2 diabetic patients with biopsy-proven diabetic nephropathy.

Methods   In this retrospective single-center cohort study, serum samples were collected from 38 Japanese type 2 diabetic patients with biopsy-proven diabetic nephropathy at the time of renal biopsy. The serum ATX levels were measured using a specific sandwich enzyme immunoassay.

Results   A multivariate linear regression analysis revealed the urinary protein excretion to be independently associated with the serum ATX levels. In addition, patients with serum ATX levels above the median showed more advanced diffuse lesions, nodular lesions and arteriolar hyalinosis compared to those with serum ATX levels below the median. However, high serum ATX levels were not associated with any increase in the number of renal composite events [a need for dialysis or a 50% decline in the estimated glomerular filtration rate (eGFR) from baseline].

Conclusion   The serum ATX levels in type 2 diabetic patients with diabetic nephropathy were associated with proteinuria and diabetic kidney lesions, although the serum ATX levels were not identified to be a predictive indicator for the renal outcome.

Key words: autotaxin, diabetic kidney lesions, diabetic nephropathy, proteinuria, renal outcome

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Introduction

Diabetic kidney disease occurs in 20-40% of patients with diabetes and it is the leading cause of end-stage kidney disease in developed countries (1). Albuminuria and the glomerular filtration rate (GFR) are recommended to assess the progression of diabetic kidney disease in patients with diabetes (1). A historical cohort study of 4,328 Japanese type 2 diabetic patients with diabetic kidney disease showed that the association between macroalbuminuria and reduced estimated GFR (eGFR) was a predictor for renal outcome (2). In addition, our previous study conducted by evaluating 260 Japanese type 2 diabetic patients with...
biopsy-proven diabetic nephropathy showed that characteristic pathological lesions as well as macroalbuminuria (severe proteinuria) were closely related to the long-term outcomes [renal composite events (a need for dialysis, or a 50% decline in eGFR from baseline), cardiovascular events (cardiovascular death, nonfatal myocardial infarction, coronary interventions, or nonfatal stroke), and all-cause mortality] of diabetic nephropathy (3, 4). These observations suggest that biomarkers associated with diabetic kidney lesions may improve the prediction of risk for the progression of diabetic nephropathy.

Autotaxin (ATX) [ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2)] is an adipokine with lysophospholipase D activity which hydrolyzes lysophosphatidylcholine to lysophosphatidic acid (LPA) (5). LPA is a phospholipid mediator that regulates various cellular functions via specific G-protein-coupled receptors (5). Recently, the ATX-LPA pathway has been implicated in obesity and insulin resistance (6). In obese mice, the adipocyte-specific genetic deletion of ATX or pharmacological inhibition of LPA receptors improved glucose tolerance, whereas the administration of LPA impaired glucose tolerance (7, 8). However, the in vivo metabolic effects and underlying mechanisms in murine models remain controversial (9). In obese humans, ATX mRNA expression in visceral adipose tissue was associated with impaired glucose tolerance (10). In addition, upregulation of LPA and downregulation of ATX were shown in the vitreous fluid from patients with proliferative diabetic retinopathy (11). Interestingly, recent studies suggest that LPA may serve as a ligand for the receptor for advanced glycation end products, although its role is little known in diabetic microvascular complications (12).

Furthermore, experimental and clinical data supporting a pathophysiological role of the ATX-LPA pathway in kidney disease have been published. In experimental studies, LPA was shown to induce biological effects on various kidney cell types, including glomerular mesangial cells (13, 14), proximal tubular cells (14) and renal fibroblasts (15). It was also reported that the models of renal fibrosis induced by unilateral ureteral obstruction (UOU) and nephrotoxic serum injection showed that LPA and its LPA receptor play important roles in the development of renal fibrosis by modifying the connective tissue growth factor expression (16, 17). In addition, LPA receptor knockout mice and LPA receptor antagonist were shown to reduce UOU-induced renal fibrosis (16, 17). In clinical studies, patients with chronic renal failure have displayed the presence of increased plasma levels of LPA (18). In addition, the urinary lysophospholipase D/ATX activity levels and urinary lysophospholipase D/ATX antigen levels were reported to be closely correlated with the urinary protein concentration in patients with proteinuria (19, 20). However, no quantitative information regarding the serum ATX levels exists in diabetic nephropathy.

In serum and plasma, LPA is mainly produced via an ATX-mediated pathway. Previous studies demonstrated the plasma LPA levels to be closely correlated with the serum ATX levels (21, 22). While plasma LPA varies rapidly when the samples are not properly prepared, the serum ATX level remains relatively stable (23). Recently, an automated enzyme immunoassay for measuring serum ATX levels was developed, and its usefulness for clinical laboratory testing has been shown (21, 22, 24-31).

Based on these findings, the aim of this study was to determine the relationship between the serum ATX levels and diabetic nephropathy in type 2 diabetes. We hypothesized that the serum ATX level may be associated with diabetic nephropathy. To test this hypothesis, we determined the serum ATX levels and assessed their relationships with the clinical and pathological parameters as well as the long-term renal outcome in Japanese type 2 diabetic patients with biopsy-proven diabetic nephropathy. We found that the serum ATX levels in type 2 diabetic patients with diabetic nephropathy were associated with proteinuria and diabetic kidney lesions, although the serum ATX levels could not serve as a predictive indicator for the renal outcome.

Materials and Methods

Patients

A total of 38 type 2 diabetic patients with biopsy-proven diabetic nephropathy who were diagnosed at Kanazawa University Hospital between 1989 and 2010 were included in this study. The diagnosis of diabetes was based on the criteria of the Japanese Diabetic Society (32). The diagnosis of diabetic nephropathy was confirmed based on the histological characteristics, such as glomerular hypertrophy, thickened capillary basement membrane, diffuse mesangial expansion (sclerosis), nodular mesangial sclerosis, exudative lesion such as capsular drop or fibrin cap, mesangiolysis, capillary microaneurysm, or hyalinosis of afferent and efferent arterioles, using appropriate standards for renal biopsy specimens including light microscopy, electron microscopy and immunofluorescence examination. Patients with other glomerular diseases concomitant with diabetic nephropathy were excluded from this study. In addition, patients with liver diseases were not enrolled in this study, because the serum ATX levels are known to be elevated in patients with hepatic fibrosis (24-26). Renal biopsy was performed to make a precise diagnosis of kidney lesions with the written consent of each patient. The study protocol was approved by the Medical Ethics Committee of Kanazawa University (No. 1087) and the University of Tokyo (No. 2602).

Measurement of serum ATX levels

The concentrations of ATX were determined in serum samples that were collected at the time of renal biopsy (baseline) and stored at -20°C until the analyses were performed. The serum ATX levels were measured using a specific sandwich enzyme immunoassay that has been proven to be useful for clinical laboratory testing (21, 22, 24-31).
**Clinical examinations**

Age, sex, 24-h urinary protein excretion, serum creatinine, eGFR, duration of diabetes, presence of diabetic retinopathy, hemoglobin A1c, body mass index (BMI), systolic blood pressure (BP), diastolic BP and total cholesterol were used as the baseline clinical parameters at the time of renal biopsy. eGFR for Japanese patients was calculated using the following equation: eGFR (mL/min/1.73 m^2)=194x serum creatinine^{1.094} x Age^{0.203} (If female, x0.739) (33). The hemoglobin A1c was presented as the National Glycohemoglobin Standardization Program (NGSP) value according to the recommendations of the Japanese Diabetic Society (32).

**Pathological examinations**

For light microscopic examinations, the renal biopsy specimens were fixed in 10% phosphate-buffered formalin (pH 7.2), embedded in paraffin and sliced into 4-μm-thick sections. These specimens were stained with periodic acid-Schiff reagent (PAS), periodic acid silver methenamine (PAM), hematoxylin and eosin (H&E), and Mallory-Azan and then were examined by light microscopy. The severity of the diffuse lesions in the glomeruli was graded on a scale of 0 to 4 according to the description by Gellman et al. as follows: all glomeruli appear normal (grade 0); local lesions present within each glomerulus and focal lesions present within the kidney (grade 1); diffuse mesangial thickening within the glomerulus and generalized throughout the kidney (grade 2); narrowed capillary lumina and local obliteration (grade 3) and generally-narrowed lumina and ischemic glomeruli that appear to be hyalinized (grade 4) (3, 34, 35). Nodular lesions, exudative lesions and mesangiolysis in the glomeruli were simply identified by their presence or absence in each specimen (3, 35). The severity of interstitial fibrosis and tubular atrophy (IFTA) and interstitial inflammation were scored according to the description by Tervaert et al. (3, 36). The severity of IFTA was evaluated and graded on a scale from 0 to 3: absent (grade 0); <25% (grade 1); 25-50% (grade 2) and >50% (grade 3). The severity of arteriolar hyalinosis was also evaluated and graded on a scale from 0 to 3 according to the description by Takazakura et al. (3, 37) as follows: a normal appearance without PAS-positive deposits (grade 0); a light PAS-positive thickening observed but involving less than half of the circumference of the arteriole in many arterioles (grade 1); numerous moderately thickened vessel walls with PAS-positive deposition without apparent luminal narrowing (grade 2) and a heavy thickening of the majority of the vessel walls with luminal narrowing or obliteration (grade 3). The severity of arteriosclerosis was evaluated and graded on a scale from 0 to 2 according to the description by Tervaert et al. (3, 36) as follows: no intimal thickening (grade 0); intimal thickening less than the thickness of the media (grade 1) and intimal thickening greater than the thickness of the media (grade 2). All renal tissue specimens were examined by three nephrologists.

**Outcome**

The outcome for this study was the first occurrence of an event in the renal composite endpoint, defined as either a need for dialysis or a 50% decline in the eGFR from baseline. The patients were followed until the end of 2013 or death.

**Statistical analysis**

Data are expressed as the mean ± standard deviation (SD) for continuous variables or percentage for categorical data. A linear regression analysis was used to determine the clinical predictors of serum ATX levels. For a stepwise linear regression analysis, we included clinical parameters with a p value of <0.05 on a univariate analysis. To evaluate the effect of the serum ATX levels on the pathological parameters and renal composite events, the patients were divided into two groups according to whether the serum ATX levels were above or below the median value of the total cohort. The differences in the pathological parameters between groups were tested using either the Mann-Whitney U test or Chi-square test, as appropriate. The renal composite events were compared by the log-rank test and the Cox proportional hazards analysis. The Cox model was adjusted for the baseline clinical parameters.

All analyses were conducted using the SPSS version 23 software program (SPSS, Tokyo, Japan). A two-sided p value of <0.05 was considered to indicate statistical significance.

**Results**

**Baseline characteristics**

The baseline characteristics for the total cohort are shown in Table 1. The mean age was 53.6 years, and the proportion of males was 71.1%. The mean urinary protein excretion was 2.6 g/day. By using the definition and classification of chronic kidney disease [Kidney Disease: Improving Global Outcomes (KDIGO)] (38), the proportions with urinary protein excretion <0.15 [A1], 0.15-0.5 [A2] and ≥0.5 [A3] g/day were 18.4%, 13.2% and 68.4%, respectively. The mean serum creatinine was 1.2 mg/dL, and the mean eGFR was 60.1 mL/min/1.73 m^2. The proportions with eGFR ≥90 [G1], 60-89 [G2], 45-59 [G3a], 30-44 [G3b] and 15-29 [G4] mL/min/1.73 m^2 were 18.4%, 23.7%, 21.1%, 21.1% and 15.8%, respectively. Although the serum ATX levels exhibit a gender difference (22), there were no significant differences in the serum ATX levels between the male and female patients in this study.
Table 1. Clinical Characteristics at the Time of Renal Biopsy.

| Category                     | Value                  |
|------------------------------|------------------------|
| Age (years)                  | 53.6 ± 9.6             |
| Male (%)                     | 27 (71.1)              |
| Serum ATX (mg/L)             | 0.75 ± 0.27            |
| Kidney-related parameters    |                        |
| Urinary protein excretion (g/day) | 2.6 ± 2.6         |
| A1: <0.15 g/day              | 7 (18.4)               |
| A2: 0.15-0.49 g/day          | 5 (13.2)               |
| A3: ≥0.5 g/day               | 26 (68.4)              |
| Serum Cr (mg/dL)             | 1.2 ± 0.6              |
| eGFR (mL/min/1.73 m²)        | 60.1 ± 29.7            |
| G1: ≥90 mL/min/1.73 m²       | 7 (18.4)               |
| G2: 60-89 mL/min/1.73 m²     | 9 (23.7)               |
| G3a: 45-59 mL/min/1.73 m²    | 8 (21.1)               |
| G3b: 30-44 mL/min/1.73 m²    | 8 (21.1)               |
| G4: <30 mL/min/1.73 m²       | 6 (15.8)               |
| Diabetes parameters          |                        |
| Diabetes duration (years)    | 12.6 ± 8.5             |
| Diabetic retinopathy (%)     | 34 (89.5)              |
| HbA1c (%)                    | 8.3 ± 2.5              |
| Other clinical factors       |                        |
| BMI (kg/m²)                  | 23.6 ± 3.3             |
| Systolic BP (mmHg)           | 148.2 ± 18.9           |
| Diastolic BP (mmHg)          | 78.2 ± 12.5            |
| Total cholesterol (mg/dL)    | 230.2 ± 71.2           |

Data are expressed as means ± SDs, or number (%). ATX: autotaxin, BMI: body mass index, BP: blood pressure, eGFR: estimated glomerular filtration rate.

**Associations between the serum ATX levels and the clinical parameters**

To identify clinical parameters associated with the serum ATX levels, we used a linear regression analysis (Table 2). In the univariate regression analysis, urinary protein excretion was positively correlated with the serum ATX levels (r = 0.532, p = 0.001), whereas eGFR was negatively correlated with the serum ATX levels (r = -0.378, p = 0.019). In the multivariate regression analysis using a stepwise method, urinary protein excretion was identified as the only statistically significant independent predictor of the serum ATX levels (nonstandardized coefficient β = 0.055, p = 0.001).

**Associations between the serum ATX levels and the pathological parameters**

The pathological parameters were compared among the subgroups stratified by whether the serum ATX levels were above or below the median serum ATX level of the total cohort (0.711 mg/L). Diffuse lesions, nodular lesions, and arteriolar hyalinosis in patients with serum ATX levels above the median were more advanced compared to those in patients with serum ATX levels below the median (Table 3).

**Renal composite events according to the serum ATX levels**

The predictive effect of the serum ATX levels on the renal composite events associated with diabetic nephropathy were also compared among subgroups stratified by serum ATX levels above or below the median. During the average 7.1-year (SD 6.3 years) follow-up period, there were a total of 16 renal composite events. As shown in Figure, the cumulative incidences of the renal composite events were not significantly different among the two subgroups (p = 0.586, log-rank test). We next investigated the predictive effect of the serum ATX levels for renal composite events using the Cox proportional hazard regression. In the unadjusted model, the hazard ratio (HR) for renal composite events was 1.338 [95% confidence interval (CI) 0.468-3.828, p = 0.587] for patients with serum ATX levels above the median compared to those with serum ATX levels below the median. In the adjusted model adding the baseline clinical parameters (including age, sex, urinary protein excretion, eGFR, hemoglobin A1c, BMI, systolic BP, diastolic BP, and total cholesterol), the HR was 0.074 (95% CI 0.002-2.478, p = 0.146) for patients with serum ATX levels above the median compared to those with serum ATX levels below the median. In unadjusted and adjusted analyses, higher serum ATX levels were not significantly associated with a poor renal outcome.

**Discussion**

To the best of our knowledge, this is the first study to evaluate the relationships between the serum ATX levels and the clinical and pathological parameters, as well as the long-term renal outcome, in type 2 diabetic patients with biopsy-proven diabetic nephropathy. The serum ATX levels in type 2 diabetic patients with diabetic nephropathy were associated with increased proteinuria and advanced diabetic kidney lesions, although the serum ATX levels could not serve as a predictive indicator for the renal outcome.

The most compelling finding in this study was the fact that the serum ATX levels were associated with increased proteinuria. According to the multivariate regression analysis, urinary protein excretion was identified as the only statistically significant independent predictor of the serum ATX levels. Although an inverse correlation between the serum ATX levels and eGFR was shown in the univariate regression analysis, we speculated that a decreased eGFR was unlikely to be a convincing explanation for the association between the serum ATX levels and urinary protein excretion for the following reasons: First, circulating ATX is cleared by liver sinusoidal endothelial cells according to the findings of prior studies (24-26, 39). Second, the molecular weight of ATX, a 125-kDa protein, is not small enough to be filtered through the glomerular filtration barrier (5). In contrast, a previous study showed the serum ATX activity to remain unchanged even when urinary protein excretion decreased with treatment in a case with nephrotic syndrome (20). This discrepancy may be explained by the possibility that the measured serum ATX levels do not entirely reflect the serum ATX activity. The relationship between the serum ATX levels and urinary protein excretion should therefore be confirmed in larger populations.
The second finding in this study was that the serum ATX levels were associated with advanced diabetic kidney lesions. In the renal pathology of diabetic nephropathy, patients with serum ATX levels above the median showed more advanced diffuse lesions, nodular lesions, and arteriolar hyalinosis compared to those with serum ATX levels below the median. Our previous report which investigated 260 type 2 diabetic patients with biopsy-proven diabetic nephropathy showed that diabetic glomerular lesions (diffuse lesions, nodular lesions, exudative lesions, and mesangiolysis) and arteriosclerosis were associated with albuminuria (proteinuria) regardless of the eGFR categories (3). Thus, this result is consistent with the fact that serum ATX levels were independently related with urinary protein excretion in a multivariate regression analysis.

There is extensive evidence that urinary albumin excretion is a strong predictor of cardiovascular mortality in patients with type 2 diabetes (40), and the relationship between albuminuria and cardiovascular disease is widely considered to reflect the common underlying pathology of endothelial dysfunction (41, 42). Recently, phosphatidic acid phosphatase type 2B, an integral membrane protein known as lipid phosphate phosphatase-3 that inactivates LPA, was implicated in coronary artery disease by genome-wide association studies (43). LPA has been reported to promote endothelial permeability in culture models and mouse models with selective lipid phosphate phosphatase-3 deficiency (44, 45). In addition, it was reported that the endothelial barrier function was restored by the pharmacological or genetic inhibition of either LPA production by the circulating ATX or of G-protein-coupled receptor-dependent LPA signaling (45).

Meanwhile, previous studies have suggested that the renal endothelial dysfunction may contribute to the progression of diabetic kidney lesions. We previously reported that nodular-

Table 2. Univariate and Multivariate Linear Regression Analyses between the Serum ATX Levels and the Clinical Parameters.

| Parameters                      | Univariate       | Multivariate               |
|---------------------------------|------------------|---------------------------|
|                                 | r   | p     | Nonstandardized Coefficient β (95% CI) | p   |
| Age                             | 0.276 | 0.093 |                           |     |
| Male                            | -0.150 | 0.369 |                           |     |
| Urinary protein excretion       | 0.532 | 0.001 | 0.055 (0.025 – 0.084)     | 0.001 |
| eGFR                            | -0.378 | 0.019 |                           | 0.325 |
| Hemoglobin A1c                  | -0.305 | 0.063 |                           |     |
| BMI                             | -0.035 | 0.837 |                           |     |
| Systolic BP                     | 0.315 | 0.054 |                           |     |
| Diastolic BP                    | 0.069 | 0.681 |                           |     |
| Total cholesterol               | 0.054 | 0.749 |                           |     |

Values are shown only for significant differences in multivariate linear regression analyses. BMI: body mass index, BP: blood pressure, CI: confidence interval, eGFR: estimated glomerular filtration rate

Table 3. Associations between the Serum ATX Levels and the Pathological Parameters.

| Parameters               | Low serum ATX | High serum ATX | p     |
|--------------------------|---------------|----------------|-------|
| Diffuse lesion (Score)   | 2.2 ± 1.1     | 3.0 ± 0.8     | 0.024 |
| Nodular lesion (%)       | 7 (36.8)      | 14 (77.8)     | 0.012 |
| Exudative lesion (%)     | 6 (31.6)      | 9 (50.0)      | 0.254 |
| Mesangiolysis (%)        | 8 (42.1)      | 10 (55.6)     | 0.413 |
| IFTA (Score)             | 1.8 ± 1.1     | 2.4 ± 0.6     | 0.081 |
| Interstitial inflammation (Score) | 1.1 ± 0.6 | 1.2 ± 0.4 | 0.641 |
| Arteriolar hyalinosis (Score) | 2.1 ± 1.1 | 2.8 ± 0.4 | 0.031 |
| Arteriosclerosis (Score) | 0.9 ± 0.6     | 1.2 ± 0.5     | 0.252 |

Data are expressed as means ± SDs, or number (%). *p < 0.05 vs. low serum ATX group. IFTA: interstitial fibrosis and tubular atrophy

Figure. Kaplan-Meier curves for the cumulative incidences of renal composite events stratified by the serum ATX levels. Solid line, patients with serum ATX levels above the median (>0.711mg/L); dashed line, patients with serum ATX levels below the median (≤0.711mg/L). Differences between the groups were compared by the log-rank test.
like lesions resembling those seen in advanced human diabetic nephropathy were established through vascular endo-
theelial injury and mesangioysis by the administration of monocrotaline (46, 47). It was also reported that the endo-
theelial nitric oxide synthase deficient diabetic mice exhibited the distinct features of progressive diabetic nephropathy, in-
cluding pronounced albuminuria, nodular glomerulosclerosis, mesangioysis, and arteriolar hyalinosis (48). Although whether or not the ATX-LPA pathway promotes human dia-
betic kidney lesions, still remains to be established, we speculate that the higher serum ATX levels in this study may be related to the pathological progression of diabetic nephropathy based on these findings.

In a prognostic context, high serum ATX levels were not associated with increased renal composite events, as expected from the close relationship between serum ATX levels and clinical and pathological parameters. Our previous report which investigated 260 type 2 diabetic patients with biopsy-proven diabetic nephropathy showed that diabetic glomerular lesions, IFTA, and arteriosclerosis were the pathological determinants for renal composite events (3). Several other reports have found that diabetic kidney lesions were associated with the renal outcome, and such findings are in line with our results (49, 50). Based on these results, it is reasonable to predict the renal outcome of diabetic nephropathy by combination of clinical and pathological parameters. However, histological evaluations are not commonly applied in patients with diabetic nephropathy with a typical clinical course. Thus, an important issue is whether the markers related to diabetic kidney lesions improve the predictive power when added to clinical parameters. Even though we were unable to sufficiently assess how confounding factors influenced our results, our data suggested that the serum ATX levels could not serve in this role. Therefore, we presume that the serum ATX level can lead to kidney damage and consequently play a role in the pathogenesis of diabetic nephropathy, but it does not accelerate its progress-
ion directly.

There are several limitations associated with our study. First, because this was a retrospective analysis of collectable data, causal relationships could not be ascertained. Second, the present study was based on a cohort consisting of a rela-
tively small number of diabetic patients that all had undergone renal biopsies. The influence of selection bias may have placed constraints on the interpretation of the results, particularly related to the predictive effect of the serum ATX levels on the renal outcome. Third, it is possible that the long-term storage of the serum samples affected the results. Fourth, we could not investigate the urinary ATX levels, because urine samples were not available for all cases in the present study. Finally, the treatment contents and the time-
dependent changes of serum ATX levels during the follow-
up period were not evaluated.

In conclusion, we here presented the first data suggesting that the serum ATX levels were associated with in-
creased proteinuria and advanced diabetic kidney lesions in type 2 diabetic patients with diabetic nephropathy, although the serum ATX levels are not considered to be useful as a predictive indicator for the renal outcome. Further studies are required to elucidate the involvement of ATX in the progress-
ion and adverse outcomes of human diabetic nephropa-
thy.

Author’s disclosure of potential Conflicts of Interest (COI), Koji Igarashi: Employment, TOSOH.

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