Wilms' Tumor-1 (WT1) Protein in Urinary Exosomes Predicts Risk of Developing Proteinuria in Type-1 Diabetes

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Abstract

Objective: Previously, we reported presence of Wilms' tumor 1 (WT1) in urinary exosomes in ~50% diabetic (T1D) population without proteinuria as oppose to 100% in T1D population with proteinuria. Here, we investigated if T1D subjects with detectable WT1 in urinary exosomes (UE) progress to proteinuria faster in a 2 year follow-up study.

Method: 52 T1D subjects without proteinuria (age < 60 years) were enrolled. Urine and blood samples were collected for analysis. WT1 immunoblotting was performed on UE isolated by ultracentifugation of urine samples. Urine microalbumin, serum creatinine and glycated haemoglobin (HbA1c) were analyzed.

Results: WT1 protein was detected in UE of 28 out of 52 T1D subjects. The data from followed patients, at the end of two years of follow up, revealed that 4 subjects (out of total 19) progressed to proteinuria with urine microalbumin >20 µg/min in WT1-positive group as oppose to none in WT1 negative group. Moreover, binary logistic regression analysis revealed a significant relation of WT1 presence in UE with increase in urine microalbumin >5 µg/min as indicated by significant Odds ratio of 9.9 (95% CI of 92.686, p=0.04). Both groups had statistically similar diabetes duration, age, % HbA1c and estimated GFR at the baseline.

Conclusion: Thus, presence of WT-1 in urinary exosomes could potentially predict risk of developing proteinuria in type 1 diabetic patients.

Introduction

Diabetic nephropathy (DN) is one of the severe complications that affect population with diabetes [1]. The clinical hallmarks of DN includes an initial period of glomerular hyperfiltration, progressive albuminuria, hypertension, followed by a gradual decline in renal function concluding, after 5–15 years, with End-Stage Renal Disease (ESRD) [1-3]. On ESRD, the patients are left with the only choice of renal replacement therapy. This unnecessary pain and economical burden can be avoided by several interventions if the onset and course of development of renal pathology and dysfunction can be predicted at a very early stage of development. Presently, albuminuria is the most recommended screening test for onset and progression of DN. Although its presence is appropriate in patients with advanced diabetic nephropathy but it has limited ability to predict the earliest stages of DN.

This advocates an urgent need for early predictors of microalbuminuria (MA). In this regard, a number of circulating molecules have been proposed as MA predictors such as; homocysteine, aldosterone, B-type natriuretic peptide (BNP), serum mannose binding lectin, osteopontin (OPN) and plasma dipeptidyl peptidase 4 (DPP4) activity [4-7]. In addition, fractional excretion of potassium, and urine immunoglobulin G, Ceruloplasmin, and Transferrin levels were also associated with a risk of developing MA [8,9]. Being non-invasive, urine has unique advantages as biomarker source for example Tamm- Horsfall protein (THP), α-1 acid glycoprotein, clusterin and progranulin [10], however, it is limited by the presence of abundant-non-specific proteins that could mask potential marker present in trace amount [1,11]. This limitation could be overcome by enriching exosomes (UE), nanovesicles released by renal epithelial cells, from total urine samples [12]. UE contain molecules that faithfully reflect the physiological state of their respective cells of origin and thus, appear to serve as a promising tool as biomarker source for renal dysfunction and structural injury [13].

WT1 is known to regulate epithelial-mesenchymal transition process, important step in the pathogenesis and progression of diabetic nephropathy, by transcriptional activation of certain key genes [14]. Furthermore, WT-1 also regulates the expression of the major glomerular structural protein Podocalyxin that serve as early biomarker for DN and provide a molecular basis for the glomerular nephropathy [15]. Recently, we have reported detection of significantly higher level of WT1, a podocyte marker and transcriptional factor, in urinary exosomes of patients with proteinuria and its almost complete absence in non-diabetic healthy controls [16]. Notable, urinary exosomes from all the T1D patients with proteinuria studied were positive for WT1 whereas only ~50% of the patients without proteinuria had detectable WT1 [16]. Moreover, study by Zhou et al, 2013 has reported the detection of WT1 in animal model of Collapsing Glomerulopathy (CG), Focal Segmental Glomerulosclerosis (FSGS) and Steroid-Sensitive Nephrotic Syndrome (SSNS) patients followed by appearance of proteinuria and glomerular histological damage [17]. Taken together, it can be hypothesized that appearance of WT-1 in urinary exosomes may be a risk of developing proteinuria in diabetic

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population. To address this, we planned to follow type-1 diabetic patients with normolabuminuria/ without proteinuria for a period of two years.

52 T1D subjects without proteinuria were randomly enrolled for follow up study. Subjects over 60 years of age were excluded to minimize the effect of age related renal function decline. Spot urine samples, collected at the time of enrollment, were used for enrichment of urinary exosomes as described earlier [16]. All 52 samples of UE were positive for urinary exosomal marker proteins (Tumor susceptibility gene 101(TSG101) and Alix) indicating successful enrichment of UE from urine samples (Figure 1). Moreover, presence or absence of proteinuria does not affect the efficiency of exosomes enrichment as shown in Figure 1A and 1B. However, only 28 subjects (53%) had detectable levels of WT1 (WT1 positive group) in their UE while in 24 subjects WT1 was undetectable in UE (WT1 negative group). A similar proportion of WT positive diabetic population was found in our earlier study [16]. The patients were followed for a period of two years.

Among the enrolled 52 subjects, urine and blood samples could be collected from only 31 patients at the end of two years. Among them, 19 were from WT1 positive group and 12 from WT1- negative group. Urine microalbumin levels at the end of two years revealed that 4 subjects in WT1 positive group (total 19) progressed to microalbuminuria as oppose to none of the 12 in WT1 negative group (Figure 2, cut-off >20 μg/min shown by solid reference line). Baseline and follow up values of urine microalbumin of each subject in the two groups has been indicated in Figure 2. Moreover, WT1 positive
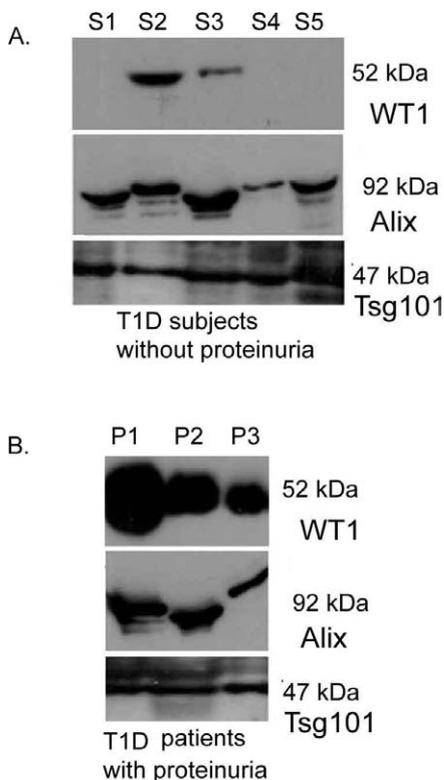
Figure 1: WT1 protein in urinary exosomes of diabetic patients. Representative immunoblots for WT1 in urinary exosome samples from (A) type 1 diabetic subjects (S1-S5) patients without proteinuria at the time of enrollment and (B) type 1 diabetic patients (P1-P3) with proteinuria. All exosomal preparations showed specific band for exosomal marker proteins, Alix and TSG101.
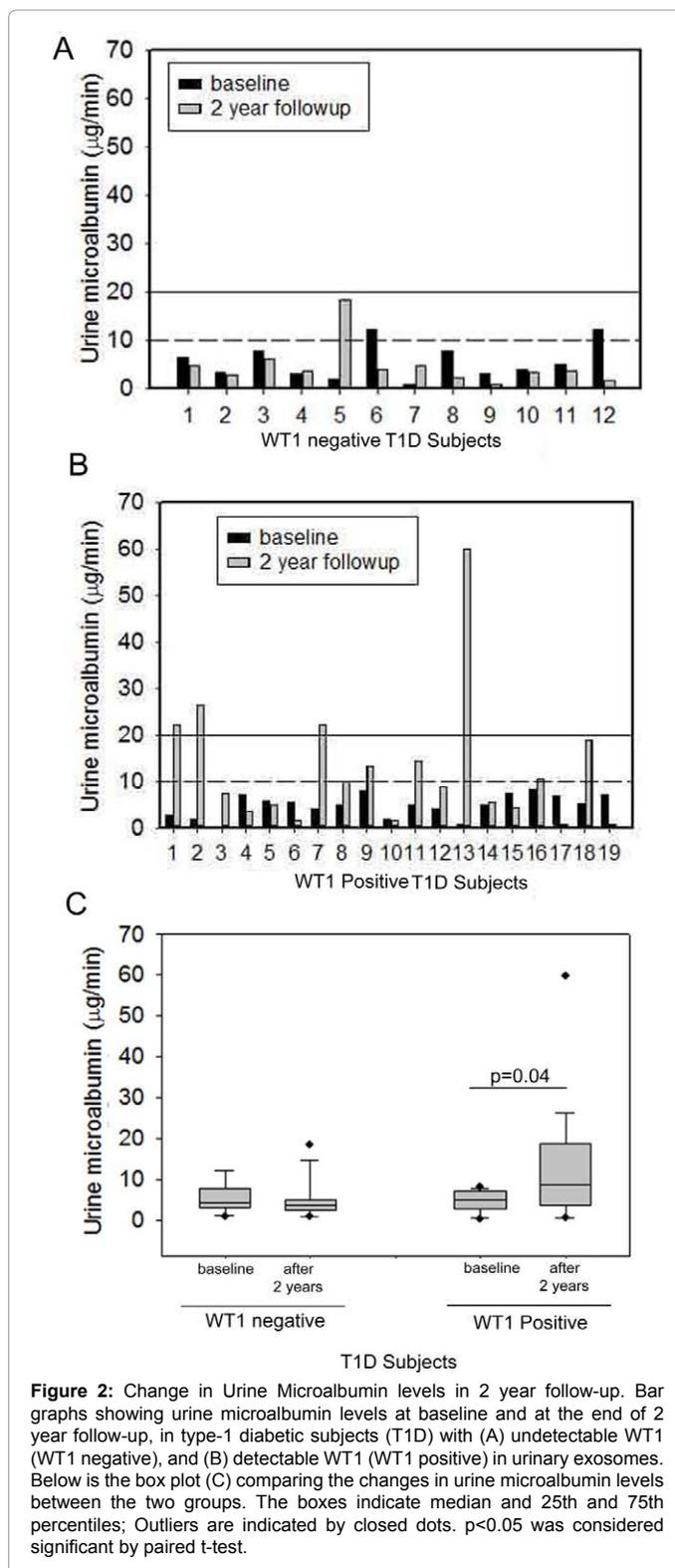
Figure 2: Change in Urine Microalbumin levels in 2 year follow-up. Bar graphs showing urine microalbumin levels at baseline and at the end of 2 year follow-up, in type-1 diabetic subjects (T1D) with (A) undetectable WT1 (WT1 negative), and (B) detectable WT1 (WT1 positive) in urinary exosomes. Below is the box plot (C) comparing the changes in urine microalbumin levels between the two groups. The boxes indicate median and 25th and 75th percentiles; Outliers are indicated by closed dots. p<0.05 was considered significant by paired t-test.

population showed significant increase in urine albumin at the end of 2 years of follow-up (p=0.04, Figure 2C).

Notably, degree of albuminuria well below the microalbuminuria cut-off has been reported as a risk factor for complications such as cardiovascular (CV) events in subjects with or without diabetes

Indices	WT1 Positive	WT1 Negative	p-value
N	19	12	-
Sex (male/female)	10/9	10/2	0.2*
Age (years)	25 ± 15	23 ± 16	0.844
Duration of Diabetes (years)	11 ± 9	9 ± 9	0.438
HbA1c (%)	8.2 ± 1.2	7.3 ± 1.2	0.068
Serum Creatinine (mg/dl)	0.85 ± 0.19	0.77 ± 0.21	0.371
Estimated Glomerular Filteration Rate (eGFR) (ml/min/1.73m2)	102 ± 28	105 ± 28	0.695
Urine microalbumin (µg/g)	4.8 ± 2.5	5.6 ± 3.7	0.4

All data represented as mean ± standard deviation of the means. P-values were calculated by Student t-test except for * which was calculated by Fisher exact test.

Table 1: Baseline characteristics of WT1 positive and WT1 negative diabetic subjects.
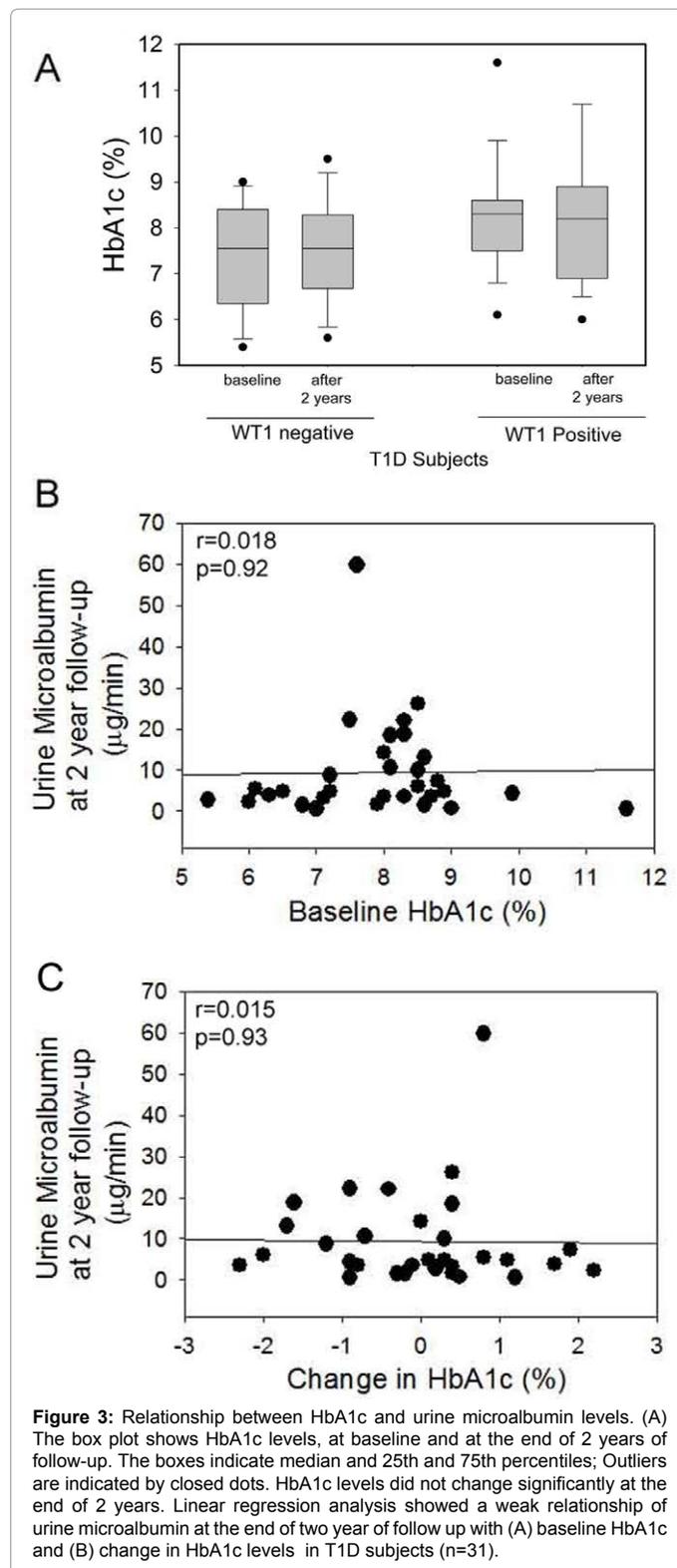
[18,19]. Moreover, urinary albumin excretion >4.8 µg/min has been reported as a strong and independent determinant of coronary heart disease and death [19]. Therefore, in our study we also analyzed the data using lower cut-offs of urine microalbumin value for proteinuria. With a cut-off urine microalbumin>10 µg/min (represented by dash line in Figure 2A and 2B), more number of subjects in WT1 positive group progressed to proteinuria (9 out of 19) relative to WT1 negative group (1 out of 12). We used Binary logistic regression analysis to test whether presence or absence of WT1 in urinary exosomes could predict proteinuria (increase in urine microalbumin>5 µg/min) in 2 year follow up using the following equation Logit P=-0.0953+(2.293* urine microalbumin). A significant Odds ratio of 9.9 with 95% CI of 92.686 was found (p=0.04) suggesting that detection of WT1 in urinary exosomes can predict proteinuria.

Poor glycemic control and diabetic duration could affect the development of proteinuria [20]. This does not appear to be the case in our study since both these parameters; including GFR (estimated Glomerular Filteration Rate eGFR) and urine microalbumin were not significantly different between the two groups at the time of enrollment (Table 1). Although, the baseline HbA1c was trending higher, but not significant (p=0.068) in WT1 positive group. Moreover, in both groups, HbA1c levels did not change significantly in 2 year follow up (Figure 3A). In addition, Linear Regression analysis was performed to determine any relationship between changes in HbA1c levels and urine microalbumin levels in our study (Figure 3B and 3C). Results revealed that baseline HbA1c, and also change in HbA1c levels had a weak association with urine microalbumin at the end of two year of follow up (r=0.02, p=0.9; n=31).

The increased packaging of WT1 in urinary exosomes found in a subset of diabetic subjects may reflect its altered regulation in podocytes and thus could predict the occurrence of proteinuria. This sound reasonable as WT1 has the ability to affect structural as well as the functional integrity of glomerular podocyte, one of the initial steps in the pathogenesis to proteinuria, by regulating the expression of key structural genes [21-24]. WT1 is a transcription factor which belongs to the Cys2-His2 zinc-finger family and reported to regulate the expression of many genes through DNA binding [15,24,25]. It plays crucial role in nephrogenesis and podocyte differentiation during embryogenesis [26]. Moreover, nphs1 and podocalyxin, podocyte specific key structural genes were shown to act downstream of WT1 [22]. Thus, we speculate that up-regulation of WT1 in podocytes in response in hyperglycemia-induced-injury in a subset of diabetic population may have resulted in its appearance in urinary exosomes.

In summary, we have shown that among a small cohort of type-

1 diabetic population, with similar duration of diabetes and % HbA1c, the odds of subjects developing proteinuria (as indicated by increase in urine microalbumin microalbumin>5 µg/min) is higher in WT1-positive group in two years compared to WT1 negative group. Thus, presence of WT1 in urinary exosomes could potentially

Figure 3: Relationship between HbA1c and urine microalbumin levels. (A) The box plot shows HbA1c levels, at baseline and at the end of 2 years of follow-up. The boxes indicate median and 25th and 75th percentiles; Outliers are indicated by closed dots. HbA1c levels did not change significantly at the end of 2 years. Linear regression analysis showed a weak relationship of urine microalbumin at the end of two year of follow up with (A) baseline HbA1c and (B) change in HbA1c levels in T1D subjects (n=31).

predict the risk of developing proteinuria in type-1 diabetic subjects. However, further studies, with large cohort and longer follow up, are warranted. Moreover, since 20-30% of type-1 diabetic patients develop nephropathy, the findings may be helpful in categorizing diabetic subgroup that may go on to develop this complication (Supplementary data).

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