ORIGINAL ARTICLE ÖZGÜN ARAŞTIRMA

Serum Mindin, Nephrin and Podocalyxin Levels in Patients with Type 1 Diabetes: Are These New Markers to Detect the Development of Nephropathy?

Tip 1 Diyabet Hastalarının Serum Mindin, Nephrin ve Podokaliksin Düzeyleri: Nefropati Gelişimini Saptamada Yeni Markerlar Olabilir mi?

Irmak Tanal Şambel* (0000-0002-4035-9785), Erdal Eren (0000-0002-1684-1053), Cengiz Bozyigit*** (0000-0003-2215-6973), Emre Sarandöl*** (0000-0002-2593-7196)**

*University of Health Sciences Turkey, Antalya Training and Research Hospital, Clinic of Pediatrics, Antalya, Turkey
**Bursa Uludağ University Faculty of Medicine, Department of Pediatric Endocrinology, Bursa, Turkey
***Bursa Uludağ University Faculty of Medicine, Department of Biochemistry, Bursa, Turkey

Abstract

Introduction: Type 1 diabetes mellitus (DM) is a chronic disease that develops as a result of absolute insulin deficiency. DM is accompanied by chronic complications which are associated with a high risk of morbidity and mortality. In the present study, we aimed to understand whether serum mindin, nephrin (NPHS1) and podocalyxin (PODXL) are useful biomarkers in the determination of nephropathy in patients with type 1 DM and to understand any correlation between serum mindin, PODXL, nephrin levels and hemoglobin A1c (HbA1c) levels and 24 hour urinary albumin excretion of patients. We investigated serum PODXL, nephrin and mindin levels in pediatric patients with type 1 DM.

Materials and Methods: Forty patients with type 1 DM along with controls, all in the pubertal stage, were included in the study. Serum mindin, nephrin and PODXL levels were measured using commercial ELISA kits. In the DM group, the mean age, gender distribution, follow-up time, and HbA1c levels were evaluated.

Results: There was no significant difference in the serum mindin levels between the DM and control groups (p=0.053). When the serum nephrin levels were compared between the two groups, the nephrin levels were significantly lower in the DM group (p=0.016). The serum PODXL levels of the DM group were significantly lower when compared to the control group (p=0.014).

Conclusion: We found that the blood levels of nephrin, PODXL were decreased in the DM group. These markers can be excreted in urine and may be sensitive markers for DM. This is the first study in the literature to evaluate PODXL, mindin, nephrin biomarkers in pediatric patients with type 1 DM.

Keywords
Diabetes mellitus, mindin, podocalyxin, nephrin, diabetic nephropathy

Anahtar kelimeler
Diabetes mellitus, mindin, podokaliksin, nefrin, diyabetik nefropati

Received/Geliş Tarihi : 21.02.2022
Accepted/Kabul Tarihi : 01.06.2022

DOI:10.4274/jcp.2022.34635

Address for Correspondence/Yazışma Adresi: Irmak Tanal Şambel MD, University of Health Sciences Turkey, Antalya Training and Research Hospital, Clinic of Pediatrics, Antalya, Turkey
Phone: +90 507 571 66 09
E-mail: tanalirmak@gmail.com

Öz

Giriş: Tip 1 diabetes mellitus (DM) mutlak insülin eksikliği ile gelişen kronik bir hastalıktır. DM yüksek mortalite ve morbidite ile sonuçlanabilen kronik komplikasyonlarla seyreder. Bu çalışmada serum mindin nefrin (NPHS1) ve podokaliksin (PODXL) düzeylerinin tip 1 DM hastalarında nefropatinin erken dönemde saptanmasında yararlı bir biyobelirteç olup olamayacağını ve serum mindin, PODXL, nefrin düzeyleri ile serum hemoglobin A1c (HbA1c) düzeyleri ve 24 saatlik idrarda albümin atılım düzeyleri arasında bir ilişki olup olmadığını araştırmayı hedefledik.

Gereç ve Yöntem: Tip 1 DM tanısı almış hastaların serum mindin, PODXL, nefrin düzeylerini çalıştık. Hasta grubu olarak tip 1 DM tanısı almış pubertal evredeki 40

hasta; kontrol grubu olarak pubertal evredeki ek bilinen hastalığı olmayan 40 hasta çalışmaya dahil edildi. Serum mindin, PODXL, nefrin düzeyleri ELISA kitleri kullanılarak ölçüldü. DM grubunda yaş ortalaması, cinsiyet dağılımı, takip süresi ve HbA1c düzeyleri değerlendirildi.

Bulgular: Kontrol ve hasta grubunun serum mindin düzeyleri arasında anlamlı fark saptanmadı (p=0,053). Hasta grubunun serum nefrin ve PODXL düzeyleri kontrol grubuna göre anlamlı olarak düşük saptandı (p=0,016; p=0,014).

Sonuç: Serum nefrin ve PODXL düzeylerini hasta grubunda düşük olarak saptadık. Bu belirteçlerin idrarla atılıyor olmaları ve serum düzeylerinin düşük olması diyabetik nefropatinin gelişimi için ilerde sensitif belirteçler olabileceğini bize gösterebilir.

Introduction

Diabetes mellitus (DM) is the most common chronic disease occuring in childhood and is a condition where absolute insulin deficiency develops as a result of the destruction of pancreatic beta cells (1). Microvascular and macrovascular complications may develop in the long-term as a result of poor glycemic control in patients with DM (2-4). One significant long-term complication is diabetic nephropathy (DN), which can result in end-stage renal failure (2-4). Detection of microalbuminuria is the most widely used laboratory tool for DN, however it is known to be affected by various factors; such as fever, dehydration, urinary tract infection, hypertension and heavy exercise. In previous studies, approximately 30% of diabetics patients with renal dysfunction have normoalbuminuria, while renal morbidity is increased in the high normal range of urine albümin excretion (5). The presence of microalbumin in urine may provide clues about damage to all three components of the glomerular filtration barrier: the endothelium, glomerular basement membrane, and podocytes and it is not a reliable marker that structural damage might precede microalbumin excretion. However, the other podocyte-specific proteins indicate only damage to podocytes, independent of the other two components of the glomerular filtration barrier (6).

The 24 hour collection of urine can be difficult due to patient compliance and is also time consuming. For these reasons, diagnosis can be delayed and a workday can be lost. Furthermore pediatric patients experience problem with timed and correct collection of 24 hour urine. Estimation of albumin in spot urine is no longer regarded as a reliable test, as it only provides instant information and this is not as accurate as that which is provided in 24-hour collection of urine. Thus, there is ongoing research to identify cheaper and more sensitive markers to better detect the development of nephropathy.

Mindin, a member of the F-mindin family, is an extracellular matrix protein synthesized in the basal lamina. Mindin has many functions in cells and acts similarly to integrin, which plays a crucial role in podocyte damage (7). It has been found that cytokines, such as transforming growth factor-beta and vascular endothelial growth factor, hyperglycemia, integrins, and extracellular proteins cause an increase in glomerular expression of mindin, fibrosis in glomeruli, and nephropathy (7-9). Similarly, mindin levels may increase in hyperglycemic situations. In recent studies, it was found that mindin mRNA expression was increased in diabetic mice (10). In addition, urinary mindin levels were reported to have increased in patients with type 2 DM who had developed DN (10).

Nephrin is a transmembrane protein located on the lateral surface of the glomerular podocyte feet. Nephrin provides complete physical barrier and makes up the structure of the filtration diaphragm. It was found that the expression of nephrin mRNA and protein increased in kidneys of patients with proteinuric nephropathy (7,11).

Podocalyxin (PODXL) is an anionic transmembrane protein found in the apical membranes of glomerular podocytes. PODXL is an O-glycolyzed and sialyzed type 1 transmembrane protein normally secreted by kidney podocytes, hematopoietic precursor cells, vascular endothelium and neurons. PODXL is one of the important proteins of the charge barrier of the glomerular basement membrane and plays a critical role in regulating the permeability of the glomerular filtration barrier (12). In a group of patients with type 2 DM and overt proteinuria, the level of urinary PODXL levels was found to be higher than those of the healthy control group (13).

To the best of our knowledge, markers that indicate podocyte inflammation, such as mindin, nephrin, and PODXL have not been previously investigated in children and adolescents with type 1 DM. In this study, we aimed to understand whether serum mindin, nephrin and PODXL are a useful biomarker in the determination of nephropathy in patients with type 1

DM and to understand any correlation between serum mindin, PODXL, nephrin levels and hemoglobin A1c (HbA1c), urea, creatinine levels and 24-hour urinary albumin excretion of patients. We chose to study these biomarkers in a serum, considering that we can get faster and more accurate results than urine due to the difficulties associated with collecting 24-hour urine in pediatric patients.

Materials and Methods

Participants

This study was performed on 40 patients with type 1 DM who were followed up in the pediatric endocrinology outpatient clinic of Bursa Uludağ University Faculty of Medicine between January 2016 and December 2017. The control group consisted of 40 healthy children without any endocrinological or renal disease who attended the pediatric outpatient clinic of the same university during the same period. This study was carried out after obtaining permission from the parents of the patients and controls and approval from the Bursa Uludağ University Faculty of Medicine Ethics Committee with the (approval number: 2016-16/1, date: 19.08.2016).

Patients with type 1 DM aged 10-18 (pubertal period), who had been followed up for at least two years following diagnosis of DM, were included in the study. Patients were questioned about their diagnosis date, medications, and any complications. Type 1 DM patients with nephropathies, syndromic cases, patients that were in pubertal stage 1 and those with a diagnosis of DM for less than two years were excluded from the study. The blood pressures of all patients was measured and they were all found to be normal. The HbA1c levels were classified as good/moderate (<9%) or poor control (>9%). Patients were subdivided according to diabetes duration time as <5 years and >5 years.

The control group consisted of age-matched individuals who attended the outpatient clinic for growth and development monitoring or vaccination with no complaint or known disease.

Statistical Analysis

Blood samples were taken using 0.18x40 mm needles (Becton Dickinson, USA) to obtain serum from the antecubital vein (Vacutainer, Becton Dickinson, USA) following 8-10 hours of fasting. The samples were centrifuged at 3,000x rpm for 10 minutes (1200 NF Core, Turkey) and divided into portions. The portioned serum samples were stored at -80 °C until study. Mid stream urine was collected in the morning was used for microalbumin analysis.

HbA1c was analyzed using high-performance liquid chromatography-specifically, the Boronate affinity chromatography method on a Premier Hb9210 (Trinity Biotech, Ireland) device. The serum mindin levels were measured using the micro-ELISA method with a MyBioSource human SPONDIN-2 ELISA kit. The serum nephrin levels were measured using the micro-ELISA method with the Fine Test human NPHS1 ELISA kit. The serum PODXL levels were measured using the micro-ELISA method with the Fine test human PODXL ELISA kit. By dissolving the lyophilized standard present in the kit, different concentrations were obtained with the diluent. The absorbance of the samples studied in accordance with the kit procedure was measured at 450 nm on the FLASHScan® S12 (Analytik Jena, Germany) device. Using the formula obtained with the help of the standard curve graph, the concentrations in ng/mL corresponding to all absorbances were calculated (Mindin kit range: 0.05-38 ng/mL, sensitivity: <0.78 ng/mL) (Nephrin kit Range: 3.12-200 pg/mL, sensitivity: <1.875 pg/mL) (PODXL kit range: 0.156-10 ng/mL, sensitivity: <0.094 ng/mL).

The SPSS package program (version 11.5) was used for statistical analyses. The compliance of the data with the normal distribution curve was evaluated using the Shapiro-Wilk test. Normally distributed data were presented as mean ± standard deviation. For continuous variables, the Student's t-test was used for the normally distributed data and the Mann-Whitney U test for the data that did not meet the normality assumption. Categorical data were compared using the chi-square test. The Spearman correlation test was conducted to investigate whether there was a correlation between the data. Results with $p<0.05$ was considered statistically significant.

Results

Of the 80 subjects who constituted the DM and control groups, 43 (53.7%) were girls and 37 (46.2%) were boys. Of the patients with type 1 DM, 17 (42.5%) were girls and 23 (57.5%) were boys while the control

group comprised 26 (65%) girls and 14 (35%) boys. The mean age of the DM group was 14.5 years, and that of the control group was 13.3 years (p=0.11) (Table 1). All of the DM patients and controls were in the pubertal stage. The HbA1c value was <9% in 12 patients and ≥9% in 28. The mean HbA1c level of the patients was calculated as 9.94±1.92% (Table 1). In the DM group, the mean duration of the disease was 7.64±3.05 years. In this group, nine (22.5%) patients had been followed up for DM for less than five years and 31 (77.5%) had been followed up for DM for more than five years, and the mean ages of these subgroups were 14 and 14.6 years, respectively.

The median serum mindin level was 5.38 pg/mL (3.66-9.82 pg/mL) in the DM group and 6.34 pg/mL (4.76-9.89 pg/mL) in the control group. A difference was found in the serum mindin level between the two groups, but it was not statistically significant (p=0.053) (Figure 1). The median serum mindin value of the patients with an HbA1c of <9% was 5.38 pg/mL (3.66-8.59 pg/mL), and that of the patients with an HbA1c of ≥9% was 5.38 pg/mL (4.20-9.82 pg/mL), indicating no significant difference (p=0.34) (Table 2). The median serum mindin level of the patients with a DM duration of <5 years was 5.38 pg/mL (4.89-7.63 pg/mL), and that of the patients with a DM duration of ≥5 years was 5.3 pg/mL (3.66-9.82 pg/mL), revealing no significant difference (p=0.97) (Table 3).

The median serum PODXL values were 0.28 pg/mL (0.24-1.26 pg/mL) and 0.36 pg/mL (0.15-0.91 pg/mL) for the DM and control groups, respectively. When the serum PODXL levels were compared between the two groups, they were significantly lower in the DM group (p=0.014) (Figure 1). The median serum PODXL value of the patients with HbA1c of <9% was 0.28 pg/mL (0.16-0.63 pg/mL), which did not significantly differ from the value of the patients with HbA1c ≥9% [0.29 pg/mL (0.14-1.26 pg/mL)] (p=0.69) (Table 2). Concerning the evaluation according to disease duration, the median serum PODXL value was determined as 0.33 pg/mL (0.26-1.26 pg/mL) for the patients with a DM duration of <5 years and 0.28 pg/mL (0.14-0.86 pg/mL) for those suffering from DM for ≥5 years. There was no significant difference in the serum PODXL levels between these two subgroups (p=0.075) (Table 3). There was no significant correlation between the DM duration and the serum mindin, nephrin, and PODXL levels of the patient

Table 1. Characteristics of the cases

	Diabetes mellitus patients	Controls
Age (year)	14.5±2.08	13.3±1.02
Gender (famele/male)	17/23	26/14
HbA1c (%)	9.94±1.92	-

HbA1c: Hemoglobin A1c

Table 2. Comparison of the serum mindin, nephrin, podocalyxin levels of moderately and poorly controlled DM cases according to HbA1c value

HbA1c	(n)	Mindin (pg/mL)			Nephrin (ng/mL)			Podocalyxin (pg/mL)		
%	Total	Median	Min.	Max.	Median	Min.	Max.	Median	Min.	Max.
<9	12	5.38	3.66	9.82	1.89	1.18	43	0.28	0.16	0.63
≥9	28	5.38	4.20	9.82	1.85	0.42	13	0.29	0.14	1.26
p	-	0.34	-	-	0.59	-	-	0.69	-	-

HbA1c: Hemoglobin A1c, DM: Diabetes mellitus, Min: Minimum, Max: Maximum

Table 3. Comparison of the serum mindin, nephrin, podocalyxin levels of the DM cases with a disease duration of <5 years and ≥5 years

DM duration	(n)	Mindin (pg/mL)			Nephrin (ng/mL)			Podocalyxin (pg/mL)		
	Total	Median	Min.	Max.	Median	Min.	Max.	Median	Min.	Max.
<5	9	5.38	4.89	7.63	3.71	0.94	43	0.33	0.26	1.26
≥5	31	5.38	3.66	9.82	1.83	0.42	7.86	0.28	0.14	0.86
P		0.97			0.106			0.075		

DM: Diabetes mellitus, Min: Minimum, Max: Maximum

group (r=0.034, -0.116, and -0.055, respectively; p=0.834, 0.475, and 0.735, respectively) (Table 3).

The serum nephrin levels were found to be low in the DM group. Accordingly, a negative correlation was found between the HbA1c levels and the serum nephrin levels (p=0.041, r=-0.32) (Figure 2) while a correlation wasn't detected between the HbA1c levels and the serum PODXL (p=0.69, r=0.064) and mindin levels (p=0.93, r=0.014) (Figure 2). The serum PODXL and mindin levels were low in the DM group. As in nephrin, we expected negative correlations between HbA1c and the levels of mindin and PODXL but in contrast, HbA1c did not correlate with these two proteins. However, it is not possible to draw any definitive conclusion concerning these results due to the insufficient number of patients.

The urea, creatinine and 24-hour urine albumin levels of the patients were measured. There was a significant difference in serum urea level between the patient group (n=40, mean 25.3±7.24 mg/dL) and the control group (n=40, mean 21±4.45 mg/dL) (p=0.002). Serum urea level in the patient group was found to be significantly higher than the control group. There was a significant difference in serum creatinine level

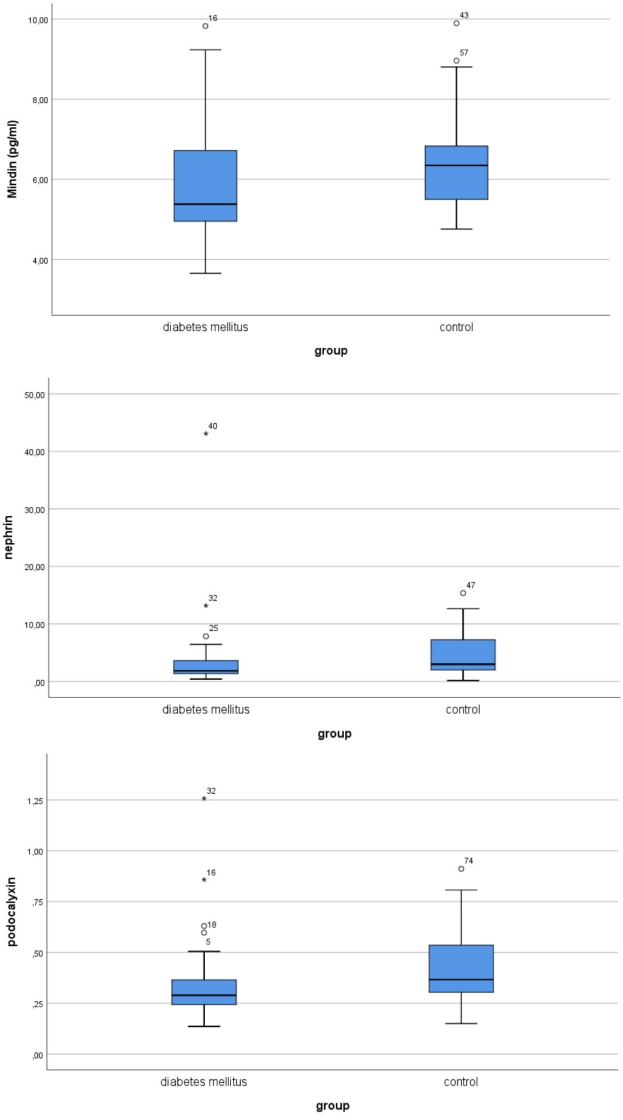

Figure 1. Serum mindin, nephrin, podocalyxin levels of the study groups (box-and-whisker diagram).

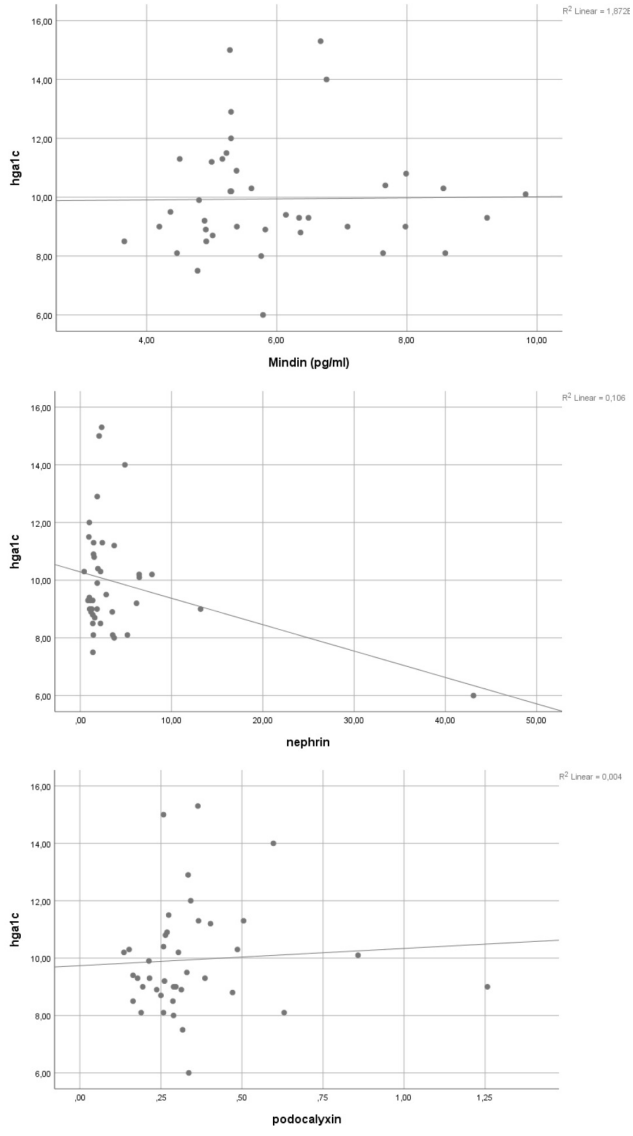

Figure 2. Correlation analysis with scatter plot HbA1c level and the serum mindin, nephrin and podocalyxin values.
HbA1c: Hemoglobin A1c

between the patient group (n=40, mean 0.70±0.082 mg/dL) and the control group (n=40, mean 0.61±0.86 mg/dL) (p<0.0001). Serum creatinine levels were found to be significantly higher in the patient group compared to the control group. Only 4 patients had microalbuminuria in a check of their last 24-hour urine albumin levels. When blood nephrine, PODXL and mindin levels of patients were correlated with albumin level in 24-hour urine, no significant difference was found (p=0.34, p=0.72, p=0.13).

Discussion

In the present study we found that serum PODXL and nephrin levels were significantly lower in type 1 DM patients (p=0.014 and p=0.016, respectively). Serum mindin levels were also lower with a significance of p=0.05. These results show that the serum levels of PODXL, nephrin, and mindin -which are extracellular matrix proteins known to have increased expression if the podocyte feet of the kidney are damaged- were lower. In our study serum nephrin, mindin and PODXL levels did not correlate with disease duration.

The fact that the patient group had no microalbuminuria yet their urea and creatinine values were found to be significantly higher compared to the control group, and that nephrin and PODXL levels were found to be significantly lower in the patient group compared to the control group made us think that nephrin and PODXL may be sensitive proteins in predicting renal damage before microalbuminuria develops.

Murakoshi et al. (10) showed that mindin mRNA expression increased in the glomeruli of mice with DM. Mindin is also excreted in the urine of patients with type 2 DM and DN in correlation with age. All the cases included in our study were in the pubertal stage, and no linear increase was observed in the serum mindin levels. Kahvecioglu et al. (14) found that serum mindin was high in the type 2 DM group. In the same study, it was demonstrated that the serum mindin levels in patients with type 2 DM had a close relationship with podocyte damage, although not as much as urine and tissue mindin levels Dogan et al. (15) showed in a study that serum mindin levels were significantly higher in patients undergoing hemodialysis treatment when compared to the population with normal renal functions.

Jim et al. (11) found that the synthesis of nephrin was significantly lower in the renal biopsy materials of the patients who developed DN compared to the control group. They also detected nephrinuria in 54% of the type 2 DM cases that were normoalbuminuric. Kostovska et al. (16) found urinary nephrin significantly higher in normoalbuminuric patients with type 2 DM compared to healthy subjects. These results led them to believe that damage in podocytes is present in patients before the appearance of microalbuminuria. Yang et al. (17) showed a downregulation of nephrin in the endocapillary proliferative lesion segment in children with Henoch-Schönlein purpura nephritis. They suggested that down-regulation of nephrin in the endocapillary proliferation segment is a potential molecular mechanism of nephrotic-range proteinuria. In our study, the serum nephrin levels of the DM patients were determined to be significantly lower compared to the control group (p=0.016) as shown in other studies. Urine nephrin excretion can increase due to renal loss; however, we were not able to confirm this in our study.

Kanno et al. (18) measured the urinary PODXL level in children with glomerular disease. The level of urine PODXL was significantly higher in the urine of the patients with glomerular disease compared to the controls. Wang et al. (19) found that patients with DN had a lower renal expression of PODXL and a higher urinary PODXL/urinary creatinine ratio than healthy subjects. The patients with DN in the low PODXL expression group had a longer diabetes disease course, lower plasma albumin and estimated glomerular filtration rate, higher HbA1c, 24 hour urinary protein, serum creatinine, and urinary PODXL/urinary creatinine ratio, and more severe glomerular, tubulointerstitial, and renal interstitial inflammation than patients in the high expression group. El-Ashmawy et al. (20) showed that serum PODXL levels were significantly higher in patients with type 2 DM and peripheral arterial disease compared with subjects with type 2 DM without any vascular complications. This study suggest that PODXL is released from an injured endothelium through a mechanism which may be similar to that of urinary PODXL release from injured podoctyes. In our study we found that serum PODXL levels were lower in the DM group and serum urea and creatinine levels were higher in the DM group whose serum PODXL levels were lower than

the control group. In our study, the low levels of serum PODXL in the DM group led us to consider that their urinary excretion might be high. However, since it was not possible to examine the levels of urinary PODXL in our study, we cannot make a definitive comment about this issue. This study was the first to investigate serum PODXL levels in pediatric patients with type 1 DM.

This was the first study to examine serum mindin, nephrin and PODXL levels in pediatric patients with DM. However, our study consisted of DM patients aged between 10 and 18 years, who had not yet developed nephropathy, a factor which could have affected our results. In addition, it would be better to examine the follow-up data of these patients. Further studies should be conducted to clarify the precise mechanism of these proteins.

Strengths of our study

The cases included in the study were in the pubertal period and this is the first study in type 1 DM patients in the pubertal period. In the literature, the levels of mindin, nephrin, and PODXL markers in the urine were previously examined, but there is no study in which the serum levels of these three parameters were analyzed at the same time. Our study is essential in terms of being the first to examine serum mindin, PODXL, and nephrin levels in type 1 DM patients aged 10-18 years. More significant results can be obtained by analyzing the serum and urine nephrin levels in the follow-up of these patients in 10 or 20 years.

Study Limitations

The limitations of this study include the small sample size, the low rate of developing DN among the DM patients aged 10-18 years, and the urine levels of mindin, PODXL, and nephrin not being simultaneously examined.

Conclusion

The results indicate that the low levels of serum mindin, nephrin, and PODXL may indicate podocyte damage in the early period of DM. However, this information needs to be confirmed by further studies analyzing the urine levels of these markers.

Ethics

Ethics Committee Approval: This study was carried out after obtaining permission from the parents of the patients and controls and approval from the Bursa Uludağ University Faculty of Medicine Ethics Committee with the (approval number: 2016-16/1, date: 19.08.2016).

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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