Assessment of Serum Small Mothers Against Decapentaplegic 2 (SMAD2) in Type 2 Diabetic Patients with Early and Chronic Diabetic Nephropathy

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Abstract
Background: Diabetic nephropathy (DN) is one of the most frequent and severe complications of diabetes mellitus and is associated with increased morbidity and mortality in diabetic patients. Transcription factor small mothers against decapentaplegic (Smad) family has key roles in cell fate decision in transmitting extracellular signals to the nucleus through transforming growth factor beta(TGF-β) receptors to activate downstream target gene transcription(Gomes et al., 2021). Smad proteins are a group of molecules that function as intracellular signal transducers downstream of the receptors of the TGF-b superfamily. The goal of this study is to investigate the diagnostic utility of Serum Small Mothers Against Decapentaplegic 2 (SMAD2) as a predictor biomarker in Type 2 Diabetic Patients with Early and Chronic Diabetic Nephropathy. The current study was designed as case control study, involved 120 individuals, 60 patients with type 2 diabetes mellitus (T2DM) group and 60 participants who are apparently healthy was used as control group. The patients group were subdivided into three equal groups according to their urinary albumin to creatinine ratio (ACR): 20 Type 2 Diabetic patients with normoalbuminuria; included patients with urinary albumin to creatinine ratio (uACR) <30 mg/g creatinine, 20 Type 2 Diabetic patients with microalbuminuria; included patients with urinary albumin to creatinine ratio (uACR) = 30-299 mg/g creatinine, Type 2 Diabetic patients with macroalbuminuria; included patients with urinary albumin to creatinine ratio (uACR) more than 300 mg/g. The results of the present study found that the Serum SMAD2 levels in T2DM with macroalbuminuria and in T2DM with microalbuminuria were much higher than T2DM with normoalbuminuria and control groups with highly statistically significant difference (P-value <0.001). Also, serum SMAD2 showed a positive correlation with ACR (r=0.703, P=0.001) and serum creatinine(r=0.673, P=0.001).So SMAD2 revealed a good diagnostic profile with sensitivity 66.70 %, specificity 90 %, positive predictive value (PPV) 91%, negative predictive value (NPV) 39%, and accuracy 63 % at a cutoff value of 2.995 ng/ml. In conclusion, this study revealed the importance of Small Mothers Against Decapentaplegic 2 (SMAD2) as a predictor for early stages of DN in T2DM which is powered by their correlation with albuminuria and it's high specificity and sensitivity through ROC analysis
Keywords: Diabetic nephropathy, albuminuria, SMAD2.

1. Introduction

Diabetic nephropathy (DN) is one of the most frequent and severe complications of diabetes mellitus and is associated with increased morbidity and mortality in diabetic patients(Valencia and Florez, 2017). DN represents the leading cause of chronic kidney disease (CKD) and end-stage renal disease (ESRD) worldwide, accounting for nearly 50% of all ESRD cases that required treatment with dialysis or renal transplantation and approximately 40% of patients with T2DM and 30% of those with T1DM will eventually develop CKD(Vallon and Thomson, 2020).

The molecular pathophysiology of DN involves complex interactions between hyperglycemia-induced metabolic, hemodynamic and inflammatory factors. These factors alter the function and morphology of blood vessel walls and interact with adjacent cells leading to renal endothelial dysfunction, which plays a crucial role in the development of DN(Motawi *et al.*, 2018). Early changes in DN include increases in kidney size, glomerular volume, and glomerular filtration rate (GFR), followed by the accumulation of glomerular extracellular matrix, increased urinary albumin excretion, glomerular sclerosis and tubular fibrosis. Late-stage overt DN is clinically characterized by proteinuria, hypertension and progressive renal insufficiency(Guo *et al.*, 2015).

So, now, investigations are in search of ideal biomarkers to identify DN in the stage of functional damage so that measured can be taken to prevent the progression or retard the disease process.

Transcription factor small mothers against decapentaplegic (Smad) family has key roles in cell fate decision in transmitting extracellular signals to the nucleus through transforming growth factor beta(TGF-β) receptors to activate downstream target gene transcription(Gomes *et al.*, 2021). Smad proteins are a group of molecules that function as intracellular signal transducers downstream of the

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receptors of the TGF-b superfamily. Eight different Smad proteins have been identified in mammals, divided into three subfamilies based upon their function: receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads), and inhibitory Smads (I-Smads)(Zhu *et al*., 2004). R-Smads are activated by the type I receptor serine kinase through phosphorylation. This family consists of Smad1, Smad2, Smad3, Smad5, and Smad8. Smad1, Smad5, and Smad8 mediate signaling for bone morphogenetic proteins (BMP) and anti-Mu¨ llerian hormone pathways , while Smad2 and Smad3 act in the TGF-b and activin pathways. Smad4 is the Co-Smad, which positively regulates all the above pathways(Zhong *et al*., 2021). In contrast to R-Smads and Co-Smad, I-Smads, including Smad6 and Smad7, bind to the intracellular domain of type I receptors. They compete with R-Smads for activation by the type I receptors, resulting in inhibition of TGF-b superfamily signaling. Smad6 inhibits BMP signaling, while Smad7 inhibits TGF-b and activin signaling(Zhong *et al*., 2021). For TGF-b and active in signaling, phosphorylation and activation by the active type II and type I receptor complex causes Smad2 and Smad3 to form heterooligomers with Smad4 and translocate the entire complex into the nucleus. There they interact with different cellular partners, bind to DNA, and regulate transcription of various downstream response genes. \

The goal of this study is to investigate the diagnostic utility of Serum Small Mothers Against Decapentaplegic 2 (SMAD2) as a predictor for early stages of kidney disease in Type 2 Diabetic Patients[1]

2. Subjects and Methods

The current study was designed as case control study, samples collection in outpatient's clinic at Diabetes and Endocrine Center in Marjan Medical City in Hilla city, Babylon province from October 2021 until May 2022. It's involved 120 individuals, 60 patients with type 2 diabetes mellitus (T2DM) group and 60 participants who are apparently healthy was used as control group their age ranged (40-70) years old, all patients were diagnosed by physicians and according to American Diabetic Association criteria (Association, 2015b). The patients group were subdivided into three equal groups according to their urinary albumin to creatinine ratio (ACR): 20 Type 2 Diabetic patients with normoalbuminuria; included patients with urinary albumin to creatinine ratio (uACR) <30 mg/g creatinine, 20 Type 2 Diabetic patients with microalbuminuria; included patients with urinary albumin to creatinine ratio (uACR) =30–299 mg/g creatinine, Type 2 Diabetic patients with macroalbuminuria; included patients with urinary albumin to creatinine ratio (uACR) more than 300 mg/g [2].

The study samples include whole blood, serum, and fresh urine. Five milliliters of blood were obtained from each participant in this study after overnight fasting. Each blood samples divided into two parts: The first part, two milliliters were collected into an EDTA tube for measuring glycated hemoglobin (HbA1c) by A1Care™ Analyzer. The second part, three milliliters of the blood sample was left for 20 minutes into a plain tube at room temperature. After coagulation, serum was separated by centrifugation at 1000xg for 10 minutes. Serum was used for the measurement of fasting serum glucose (FSG), creatinine, and urea by using available commercial kits supplied by LINEAR(SPAIN). The rest of serum stored at -40Co for the subsequent assay of serum SMAD2 which measured by enzyme-linked immunosorbent assay method (Bioassay Technology Laboratory, China).. Ten milliliters of freshly morning urine samples were collected into a clean container and divided into two aliquots. Part one: Used for general urine examination which includes macro-examination using urine strip kit supplied by Human diagnostics, and microscopic. Part two: Urine sample was centrifuged to remove particulate matter and used for the determination of urinary albumin and urinary creatinine by using turbidimetric test with kits supplied by HUMAN(Germany). It is important to compare the amount of albumin in sample of urine against its concentration of creatinine. ACR was determined by dividing microalbumin level in mg/l over urine creatinine level in gram per liter. ACR was expressed in mg albumin/gm creatinine. Body mass index(BMI) was calculated in all subjects according to ratio depend on weight and height obtained by applying a mathematical equation, in which the weight in kilogram was divided by the square height in meter. eGFR was calculated by using the Chronic Kidney Disease Epidemiology Collaboration (CKD–EPI) equation: [2].

3. Data Analysis

Statistical analysis was carried out using SPSS version 23. Categorical variables were presented as frequencies and percentages. Continuous variables were presented as (Means±SD). Student t-test was used to compare means between two groups. ANOVA test was used to compare means between three groups or more. Pearson chi-square and fisher-exact test were used to find the association between categorical variables. Pearson correlation coefficient was used to assess the relationship between two continuous variables. Receiver operating characteristic (ROC) curve was used to evaluate the diagnostic value of SMAD2 in the context of early detection of nephropathy in patients with type 2 diabetes mellitus. The area under the curve(AUC) provides a useful tool to compare different biomarkers. Whereas an AUC value close to 1 indicates an excellent diagnostic and predictive marker. *p*-value of ≤ 0.05 was considered as significant[3].

4. Results

Comparison of baseline characteristics among different groups

Table 1 demonstrate the clinical characteristics of

patients and control groups. The results for age and BMI showed that there was no significant difference (P>0.05) between the study groups. This matching is important to eliminate any effects on the results that may arise from the difference in these characteristics. The results of the present study were shown the mean concentration of FBG and HbA1c levels were significantly increased in T2DM patients with macroalbuminuria and microalbuminuria compared to those with normoalbuminuria and the control group, P(<0.001). DM duration was significantly (P < 0.001) longer in T2DM patients with macroalbuminuria compared to those with microalbuminuria and normoalbuminuria. In addition, urinary albumin to creatinine ratio (ACR) recorded higher levels in T2DM patients with macroalbuminuria and microalbuminuria as compared with the control group. In contrast, eGFR was significantly lower in T2DM patients with macroalbuminuria as compared with those with normoalbuminuria and the control group, P(<0.001)

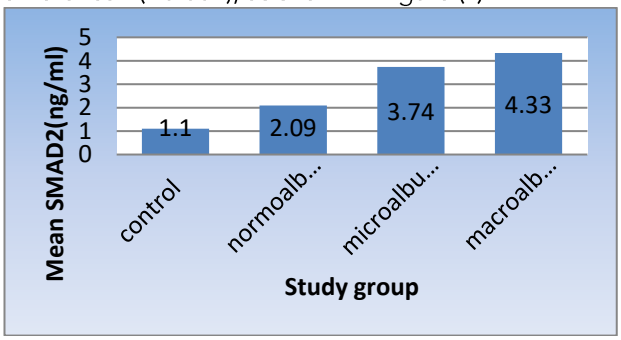
Table 1: Comparison of clinical characteristics among different groups

T2DM				
Macroalbuminuria N=20 Mean±SD	Microalbuminuria N=20 Mean±SD	Normoalbuminuria N=20 Mean±SD	Control group N=60 Mean±SD	Biochemical parameters
14/6	12/8	10/10	33/27	Gender(M/F)
53±9.4[a]	52±5.5[a]	50±6.6[a]	48±4.46[a]	AGE (year)
28.1±3.5[a]	28.4±3.0[a]	28.6±3.2[a]	26.2±3.5[a]	BMI(kg/m^2)
9.4±2.0 [c*]	14.9±3.4 [b*]	13.7±4.4 [b*]	4.4±0.68 [a*]	FBG(mmol/l)
9.0 ±1.3[c*]	8.7 ±1.2[c*]	10.5± 2.7[b*]	4.3± 0.4[a*]	HBA1C(%)
13.7± 5.4[b]	8.9± 3.8[a]	6.6 ±2.88[a]	-	Duration (years)
10.1± 2.9[c*]	8.2± 1.3[b*]	5.4 ±1.1[a*]	4.7± 0.8[a*]	Blood urea(mmol/l)
120.5± 21.0[c*]	78.5 ±16.5[b*]	76.0± 21.8[ab*]	63.0± 8.5[a*]	Serum creatinine(μmol/l
56.5 ±14.2[b*]	86.9 ±17.1[a*]	88.9± 14.6[a*]	92.2 ±5.0[a*]	eGFR (ml/min/1.73/m^2)
585.6± 167.2[c*]	132.4 ±63.6[b*]	15.6± 6.4[a*]	10.5± 3.2[a*]	ACR (mg/g)

Abbreviations: FBG, fasting blood glucose; HBA1C: glycated haemoglobin; BMI, body mass index; GFR, glomerular filtration rate; ACR, albumin to creatinine ratio. p-value <0.05 is significant , by one way ANOVA test . Different letters (a ,b and c) mean significant difference between studied groups , Similar letters means no significance difference between studied groups (Tukeys).

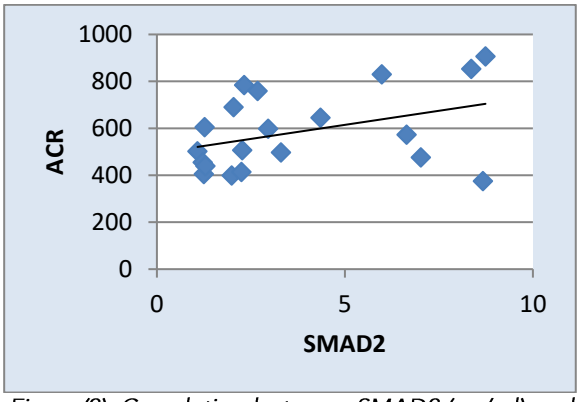
Comparison of serum SMAD2 values according to albuminuria:

Serum SMAD2 levels (Mean±SD) in T2DM with Normoalbuminuria, T2DM with Microalbuminuria, T2DM with Macroalbuminuria and control groups were, (2.09±0.68), (3.74±1.18), (4.33±1.10), (1.10±0.31) respectively with highly significant difference P(<0.001), as shown in figure (1):

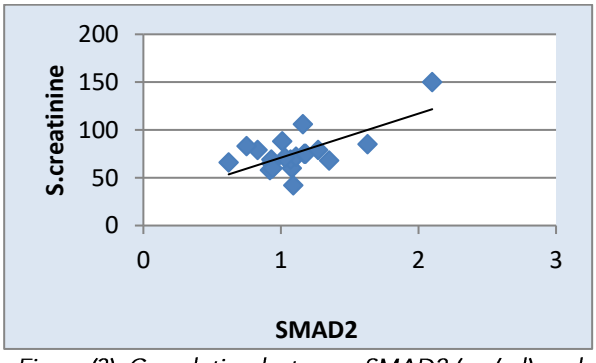


Figure(1): Serum SMAD2 level according to studied groups

Also, serum SMAD2 showed a positive correlation with ACR (r=0.703, P=0.001) and serum creatinine(r=0.673, P=0.001). As shown in figures (2,3) respectively.

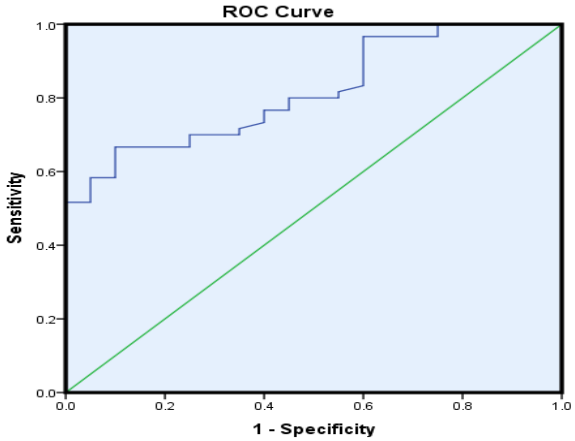


Figure(2): Correlation between SMAD2 (ng/ml) and ACR(mg/g) (r=0.703, P=0.001).



*Figure(3): Correlation between SMAD2 (ng/ml) and S.creatinine(mg/dl) (r=0.673**,P=0.001).*

Receiver operating characteristic curves (ROC) revealed that for early detection of DN, A good diagnostic profile of serum SMAD2 revealed a good diagnostic profile with sensitivity 66.70 %, specificity 90 %, positive predictive value (PPV) 91%, negative predictive value (NPV) 39%, and accuracy 63 % at a cutoff value of 2.995 ng/ml. As shown in figure(4).



Figure(4): ROC curve for sensitivity and specificity of SMAD2 to predict diabetic nephropathy

5. Discussion

The most common microvascular consequence of diabetes is DN. Diabetes affects 20% to 40% of T2DM patients and might progress to ESRD, impacting T2DM patients' mortality and morbidity. Early identification, however, is vital in improving clinical care, and therapeutic intervention from the earliest stages of DN is critical to avoid progression to ESRD and dialysis (Balducci et al., 2014).

Microalbuminuria, also known as ACR (30-299 mg/g creatinine), has been considered the gold standard diagnostic and prognostic biomarker for the past three decades. It was the first and most widely used clinical measure of DN and is linked to cardiovascular risk in T2DM patients (Association, 2020). Microalbuminuria, on the other hand, may not be a sensitive or specific predictor of DN due to several limitations. First, only approximately 30 % patients with incipient nephropathy(micro albumin) progress to macro albuminuria after ten years of follow up(Rossing, Hougaard and Parving, 2005). Second, some patients do not progress to overt nephropathy but stay at Microalbuminuria or even regress to Normoalbuminuria (Satirapoj, 2018). Third, some patients without Microalbuminuria display advanced renal pathological changes, indicating that Microalbuminuria may not be an optimal marker for "early" detection of DN. Furthermore, some patients may be presented with decreased glomerular filtration rate whereas Normoalbuminuria, indicating that albuminuria is not a perfect marker for early detection (Roux et al., 2018).

The results of the present study pointed out a significant difference in the levels of SMAD2 in T2DM patients with Macroalbuminuria and T2DM patients with Microalbuminuria as compared to those with Normoalbuminuria and control groups. These results suggesting that SMAD2 may be involved in the development of DN.

Furthermore, there were a significant difference between T2DM patient with Normoalbuminuria as compared with control group in SMAD2. By comparison with SMAD2,

The TGF-β/Smad pathway plays a critical role in renal fibrosis and also inflammation. Researchers have suggested the TGF-β/Smad pathway as a potential therapeutic target for treating chronic kidney diseases(Lan and Chung, 2012). A study done by (Zuo et al., 2019) showed that TGF-β/Smad pathway plays a critical role in the renal fibrosis process by promoting expression of key components of the ECM by promote the synthesis of adhesive proteins, collagen, and the proteoglycans found in extracellular matrix; it can also attenuate the decrease in protease synthesis, prevent decomposition of newly synthesized ECM, disrupt the balance between ECM synthesis and degradation, and accelerate the development of renal fibrosis. In rodents with diabetic nephropathy, the intracellular Smad pathway is significantly activated, transducing the TGF-β signal (Wang et al., 2021).

The presrnt study showed the levels of SMAD2 were higher in T2DM patients with Macroalbuminuria and Microalbuminuria than the normal control group, indicating that the Smads signaling pathway was activated during the development of diabetic kidney disease. In the TGF-β/Smad signaling pathway, TGF-β binds to the TβRII receptor and phosphorylates it and then forms a TGF-β complex by binding to TβRI receptor. The complex further activates the downstream signaling proteins Smad2 and Smad3 and is finally transported to the nucleus and directly combines with DNA, regulating the regulation of transcription of target genes(Ren et al., 2022). Also, the current results are in agreement with previous study pathway proteins (pSmad2) was significantly increased in the diabetic renal tissues (Bian et al., 2022).

In conclusion, this study revealed the importance of Small Mothers Against Decapentaplegic 2 (SMAD2) in DN pathogenesis which is powered by it's correlation with albuminuria and it's high specificity and sensitivity through ROC analysis, and thus the possibility for using it as biochemical marker in DN was suggested.

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