ASSOCIATION BETWEEN RETINOL-BINDING PROTEIN AND RENAL FUNCTION AMONG ASIAN SUBJECTS WITH TYPE 2 DIABETES MELLITUS: A CROSS-SECTIONAL STUDY

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Abstract. Retinol-binding protein 4 (RBP4) has been suggested as new adipokine, possibly linking obesity to type 2 diabetes mellitus (T2DM). Since the kidneys are the main site of RBP4 degradation and since renal failure is a frequent co-morbid condition with diabetes mellitus, we evaluated the association among RBP4, renal function and T2DM in an Asian population. RBP4 serum levels were analyzed in 110 subjects (50 with T2DM) using an enzyme-linked immunosorbent assay (ELISA). Based on a cut-off estimated glomerular filtration rate (eGFR) of 60 ml/min per 1.73 m^2 (calculated according the abbreviated MDRD formula which uses serum creatinine level, age and gender) and on the T2DM status, subjects were assigned to four subgroups: Group A - controls with an eGFR > 60 ml/min per 1.73 m^2, Group B - controls with an eGFR < 60 ml/min per 1.73 m^2, Group C- T2DM subjects with an eGFR >60 ml/min per 1.73 m^2, and Group D - T2DM subjects with an eGFR <60 ml/min per 1.73 m^2. In both the T2DM and control groups, RBP4 levels were higher in subjects with an eGFR < 60 ml/min per 1.73 m^2 than in subjects with an eGFR >60 ml/min per 1.73 m^2. However, the difference was only significant between the control groups (p <0.05). After adjusting for age, gender, BMI, eGFR and the presence of T2DM, eGFR, not T2DM, was associated with plasma RBP4 levels (p<0.05). These results suggest among Asians the eGFR, but not the presence of T2DM, is a major determinant of RBP4 serum levels. The eGFR should be taken into account when evaluating the role of RBP4 in the pathogenesis of insulin resistance and T2DM.

Keywords: retinol-binding protein 4, renal function, type 2 diabetes mellitus, Asian subjects

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INTRODUCTION

Once considered a disease of wealthy nations, type 2 diabetes mellitus (T2DM) is a truly global affliction. The worldwide incidence of T2DM is expected to increase by approximately 70% in the next 20 years

(King *et al*, 1998). Approximately 80% of patients with T2DM are obese; both of these conditions are associated with insulin resistance (King *et al*, 1998; Korc, 2003). Adipose tissue plays a crucial role in the pathogenesis of T2DM since it produces a variety of adipokines that can regulate energy metabolism and insulin sensitivity (Kershaw and Flier, 2004). Retinol-binding protein 4 (RBP4) had been initially studied as an adipokine linking obesity and insulin resistance (Yang *et al*, 2005). This adipokine along with retinol is thought to impair insulin signalling in muscle and induce hepatic glucose secretion (Yang *et al*, 2005; Graham *et al*, 2006). Several studies investigating RBP4 and insulin resistance in humans are controversial (Yang *et al*, 2005; Graham *et al*, 2006; Janke *et al*, 2006; Sell and Eckel, 2007; van Eynatten *et al*, 2007). Circulating RBP4 has been reported to be elevated not only in insulin-resistant mice but also in humans with obesity and T2DM (Yang *et al*, 2005; Cho *et al*, 2006; Graham *et al*, 2006). However, differences in circulating RBP4 concentration have not been found to occur in normal-weight, overweight, and obese women (Janke *et al*, 2006). Associations between RBP4 and diabetic risk factors, such as the HOMA-IR score, fasting plasma glucose levels, and $HbA_{1c,}$ have been described (Raila *et al*, 2007; van Eynatten *et al*, 2007; Ziegelmeier *et al*, 2007).

Unlike other adipokines, RBP4 is predominantly synthesized in hepatocytes and secreted into the circulation bound to transthyretin (TTR). Binding to TTR increases the molecular weight of RBP4 from approximately 21 kDa to approximately 76 kDa, preventing its loss through filtration by the renal glomeruli (Malpeli *et al*, 1996; Monaco, 2000). Beside hepatocytes and adipose tissue, the kidneys are also thought to be an important site of RBP4 synthesis and metabolism (Goodman, 1980; Janke *et al*, 2006). In patients with chronic renal failure, elevated of RBP4 has been reported due to impaired catabolism of retinol-RBP4 complex in the proximal tubule (Gerlach and Zile, 1991a,b).

Whether elevated of RBP4 levels are a cause or a consequence of renal dysfunction remains to be elucidated. In this study we evaluated the association between RBP4 levels and estimated glomerular filtration rate (eGFR), as recommended by the National Kidney Foundation as the best overall indicator of kidney function, and compared it with different factors of metabolic syndrome, and its relationship to the magnitude of insulin resistance among T2DM patients and non-diabetic subjects.

MATERIALS AND METHODS

Subjects

This study was conducted among 50 patients with T2DM (19 men and 31 women, median age 54 years, age range 42-77 years) and 60 controls (4 men and 56 women, mean age 56 years, age range 42-76 years). Patients with a previous history of chronic disease of the kidneys, pancreas, liver, with other known existing diseases, active inflammatory disease or who were receiving insulin were excluded. The protocol was approved by the ethics committee of Burapa University, Chon Buri, Thailand and all participants signed a consent form. A cut-off eGFR of 60 ml/min per 1.73 m^2, estimated by a simplified MDRD study group formula (MDRD Study Group, 2002), was used to classify the subjects into four subgroups, namely, Group A - controls with an eGFR >60 ml/min per 1.73 m^2, Group B - controls with an eGFR < 60 ml/min per 1.73 m^2, Group C- T2DM subjects with an eGFR

> 60 ml/min per 1.73 m^2, and group D - T2DM subjects with an eGFR < 60 ml/min per 1.73 m^2.

Laboratory

Anthropometric measurements and fasting venous blood were taken from all subjects. The body mass index (BMI, kg/m^2) and waist-to-hip ratio (WHR) were calculated. Fasting blood glucose (FBG) and lipid levels were measured on the same day as the blood was collected; the plasma was the stored at -70°C for insulin determination. Serum insulin levels were determined by radioimmunoassay using a commercial RIA kit (LINCO Research, St Charles, MS). The FBG was measured using an enzymatic colorimetric method as previously reported (Chanchay *et al*, 2006). The homeostasis model for the assessment of insulin resistance (HOMA-IR) score was calculated as the fasting insulin level (μU/ml) x fasting glucose level (mmol/l) divided by 2.25 (Matthews *et al*, 1985). The total cholesterol (TC) level was determined using a colorimetric method (Chanchay *et al*, 2006). Triglyceride (TG) levels were determined using an enzymatic colorimetric test with a lipid clearing factor (GPO-PAP method) (Ashwood and Burtis, 1999). Chylomicrons, very-low-density lipoprotein cholesterol (VLDL-C), and low-density lipoprotein cholesterol (LDL-C) were removed from the blood plasma using a precipitation method in which phosphotungstic acid and magnesium chloride were added to the plasma. After centrifugation of the remaining plasma, high-density lipoprotein cholesterol (HDL-C) was determined in the same way as total cholesterol (Ashwood and Burtis, 1999). LDL-C was calculated according to the formula LDL-C = TC – HDL-C – (TG/5) mg/dl. Blood urea nitrogen (BUN) and creatinine (Cr) levels were determined using an enzymatic colorimetric method described elsewhere (Ashwood and Burtis, 1999). Plasma levels of TTR and RBP4 were determined by ELISA using polyclonal rabbit anti-human antibodies (DakoCytomation, Hamburg, Germany) as previously described (Raila *et al*, 2004, 2007). A simplified MDRD study group formula was used to calculate the estimated glomerular filtration rate (eGFR), based on age, sex, and serum creatinine concentration: $eGFR = 186.3 \times [\text{serum creatinine (mg/dl)}]^{-1.154} \times [\text{age (years)}]^{-0.203} \times 0.742$ (if female) (Kuan *et al*, 2005).

Statistical analysis

Since the data were not normally distributed, nonparametric statistics were used. Data were expressed as medians and ranges. Possible differences between two independent groups were tested using the Mann-Whitney *U* rank-sum test (two-tailed). Kruskal-Wallis analysis of the variance for multiple comparisons was performed to test variable differences among the subgroups. The correlation among the variants was calculated using the Spearman's rank correlation for nonparametric methods with SPSS version 11.5 (Chicago, USA). Linear regression analysis was performed to predict the effect of type 2 diabetes and the eGFR on the levels of RBP4. A p-value < 0.05 was considered statistically significant.

RESULTS

Biometric and biochemical data of the study groups

The biometric and biochemical data for the T2DM and control subjects are shown in Table 1. The ages differed significantly among the four groups. The T2DM and control subjects with eGFR > 60 ml/min per 1.73 m^2 were younger than those subjects who had eGFR levels < 60 ml/min per 1.73 m^2 (p <0.05).

Table 1
Medians and ranges for biometric and biochemical data, transthyretin (TTR), and RBP4/TTR molar ratio among non-diabetic (nT2DM) and diabetic (T2DM) patients classified with an eGFR cut-off point of 60 ml/min per 1.73 m^2.

	nT2DM		T2DM		
Parameters	eGFR > 60 ml/min	eGFR < 60 ml/min	eGFR > 60 ml/min	eGFR < 60 ml/min	*p*-value
Age (years)	52 (42-76)[a]	64 (48-75)[b]	54 (42-72)[a,c]	61 (42-77)[b,c]	0.002
Sex (M/F)	3/47	1/9	12/20	7/11	
BMI (kg/m^2)	24.6 (19.0-35.1)	26.7 (22.-32.0)	28.5 (19.3-42.7)	28.0 (21.7-37.1)	0.072
WHR	0.87 (0.78-0.98)[a]	0.85 (0.83-1.01)[a]	0.90 (0.86-1.03)[b]	0.92 (0.83-0.99)[b]	0.000
TG (mg/dl)	114 (44-397)[a]	153 (59-453)[a]	129 (56-327)[b]	180 (89-300)[b]	0.009
Total-C (mg/dl)	220 (159-427)[a]	200 (178-352)[a]	245 (170-473)[b]	269 (186-386)[b]	0.003
LDL-C (mg/dl)	148 (40-359)	125 (46-259)	166 (131-288)	184 (67-296)	0.057
HDL-C (mg/dl)	45 (27-83)	44 (32-58)	43 (28-67)	41 (15-59)	0.827
FBS (mg/dl)	79 (61-120)[a]	87 (74-113)[a]	169 (131-288)[b]	209 (133-387)[b]	0.000
Insulin (μU/dl)	14.7 (2.2-48.7)	14.1 (3.1-24.8)	18.3 (4.6-48.5)	12.9 (4.1-42.2)	0.133
HOMA	3.0 (0.6-10.6)[a]	3.5 (0.6-6.9)[a]	5.8 (1.5-29.1)[b]	4.9 (1.0-14.9)[b]	0.000
SBP (mm Hg)	126 (86-206)	134 (120-160)	134 (100-174)	140 (119-160)	0.076
DBP (mm Hg)	80 (50-130)	80 (68-90)	80 (60-111)	80 (68-98)	0.662
BUN (mg/dl)	12 (6-19)[a]	16 (11-23)[b,c]	12 (5-42)[a]	13 (2-28)[a,c]	0.003
Cr (mg/dl)	0.80 (0.6-1.0)[a]	1.10 (1.0-1.7)[b]	0.89 (0.5-1.3)[a]	1.45 (1.0-34.0)[b]	0.000
eGFR (ml/min/1.73 m^2)	83 (60-123)[a]	53 (33-59)[b]	82 (60-186)[a]	48 (29-59)[b]	0.000
TTR (μg/ml)	255.9 (105.3-871.0)	182.8 (93.1-603.5)	189.4 (135.6-501.4)	185.7 (115.9-430-6)	0.769
RBP4/TTR ratio	0.49 (0.14-2.24)	0.95 (0.25-1.72)	0.65 (0.39-1.51)	0.66 (0.29-0.88)	0.054

BMI, body mass index; WHR, waist to hip ratio; TC, total cholesterol; TG, triglyceride; LDL-C, low density-lipoprotein cholesterol; HDL-C, high-density-lipoprotein cholesterol; FBS, fasting blood sugar; HOMA, homeostasis model of the insulin resistance; SBP, systolic blood pressure; DBP, diastolic blood pressure; BUN; blood urea nitrogen; Cr, creatinine; eGFR, estimated glomerular filtration rate; TTR, transthyretin; RBP4, retinol-binding protein 4

Significant difference tested using the Kruskal-Wallis H test.

[a,b,c]Indicates a significant difference within the same row.

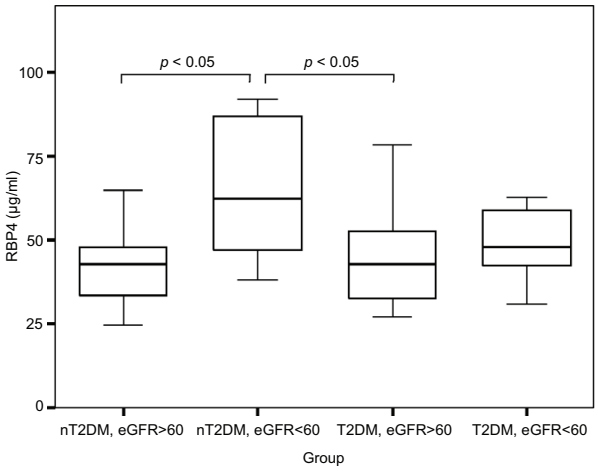
WHR but not BMI differed significantly between T2DM and control subjects (p <0.001). Levels of TG and TC were significantly different among the four groups: the highest levels were in subjects with T2DM (p <0.05). No significant differences in LDL-C and HDL-C levels were observed. FBG levels were significantly higher in both T2DM groups; the highest levels were found in T2DM subjects with eGFR <60 ml/min per 1.73 m^2 (p <0.001). Although significant differences in insulin levels were not present among the four groups, the HOMA-IR scores were significantly higher in the T2DM groups (p <0.001). BUN (p <0.05), serum Cr (p <0.01), and eGFR (p < 0.01) differed significantly among the four groups.

Biochemical variables of the RBP4-TTR complex in plasma

Plasma RBP4 levels were significantly different among the four groups (p < 0.05). The highest mean plasma RBP4 level was found in the control group with an eGFR < 60 ml/min per 1.73 m^2 (62.5 µg/ml) (p <0.05) (Fig 1). In the T2DM groups, the mean RBP4 levels were 11% higher among subjects with an eGFR < 60 ml/min per 1.73 m^2 than with an eGFR > 60 ml/min per 1.73 m^2 (47.9 *vs* 43.0 µg/ml), but this difference was not statistically significant (Fig 1). Other biochemical parameters of the RBP4 transport complex, including circulating TTR levels and the RBP4/TTR molar ratios were not significantly different (Table 1).

Linear regression and correlation analysis

To predict the effect of T2DM and eGFR on RBP4 levels, linear regression analysis was used in a separate model. After values were adjusted for the confounding effects of age, gender, and BMI, the eGFR value was significantly associated with RBP4 levels (p = 0.015) but not



nT2DM, non-diabetes; T2DM, diabetes; eGFR, estimated glomerular filtration rate (ml/min per 1.73 m^2)

Fig 1–Retinol-binding protein 4 (RBP4) levels in the plasma of diabetic and non-diabetic subjects classified by eGFR. Significance was tested using the Mann-Whitney *U*, Wilcoxon rank-sum W test (two-tailed). Overall p-value = 0.023.

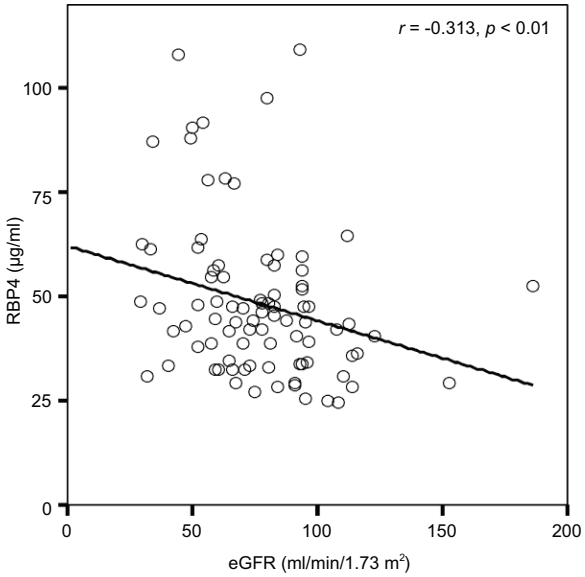


Fig 2–The correlation between retinol-binding protein 4 (RBP4) levels and estimated glomerular filtration rate (eGFR) among all subjects, based on the Spearman's rank correlation method.

Table 2
Linear regression analysis using RBP4 level as a dependent variable; including age, gender, BMI, and the presence of type 2 diabetes (A) or age, gender, BMI, and GFR (B).

Model	Standard error	Standardized β	*p*-value
(A)			
Age	0.155	0.157	0.117
Gender	4.316	-0.179	0.066
BMI	0.388	-0.072	0.449
Type 2 diabetes	4.031	0.000	0.999
(B)			
Age	0.225	0.008	0.974
Gender	5.402	-0.128	0.273
BMI	0.467	-0.092	0.422
eGFR	0.075	-0.279	0.015

RBP4, retinol-binding protein 4; BMI, body mass index; eGFR; estimated glomerular filtration rate.

Table 3
Correlation coefficients for the parameters and transthyretin (TTR) -retinol-binding protein 4 complex with biometric and biochemical variables[a].

Parameters	RBP4	TTR	RBP4/TTR
BMI	0.000	-0.171	0.147
WHR	-0.041	0.009	0.003
TC	0.020	0.076	0.041
TG	0.329	-0.025	0.230[b]
LDL-C	-0.070	0.045	-0.028
HDL-C	-0.159	-0.084	-0.068
FBS	0.027	-0.028	0.084
Insulin	0.014	-0.030	0.046
HOMA	0.020	-0.047	0.090
SBP	-0.172	-0.021	-0.044
DBP	-0.171	0.021	-0.075
BUN	0.220[b]	-0.024	0.236[b]
Cr	0.345[c]	0.009	0.197
eGFR	-0.313[c]	0.031	-0.274

BMI, body mass index; WHR, waist to hip ratio; TC, total cholesterol; TG, triglyceride; LDL-C, low-density-lipoprotein cholesterol; HDL-C, high-density-lipoprotein cholesterol; FBS, fasting blood sugar; HOMA, homeostasis model of the insulin resistance; SBP, systolic blood pressure; DBP, diastolic blood pressure; BUN; blood urea nitrogen; Cr, creatinine; eGFR, estimated glomerular filtration rate; TTR, transthyretin; RBP4, retinol-binding protein 4.
[a]All participants were included; correlation was calculated using the Spearman's rank test
[b]Correlation was significant at $p<0.05$ (2-tailed)
[c]Correlation was significant at $p<0.01$ (2-tailed)

with the presence of T2DM (Table 2). To determine whether TTR, RBP4 and the RBP4/TTR molar ratio were correlated to other biometric or biochemical data, the Spearman's rank correlation analysis was carried out. A significant correlation for each parameter with the others was observed when all participants were included (Table 3). TTR had no correlation with the other parameters. RBP4 levels were positively correlated with BUN (p <0.05) and Cr (p <0.01), and negatively correlated with eGFR (p <0.01). The negative correlation between RBP4 and eGFR is shown in Fig 2 (r = 0.313, p <0.05). No association between the presence of T2DM and the insulin resistance, HOMA-IR score, or RBP4 level was observed. The RBP4/TTR ratio was positively correlated with TG and BUN (both p <0.05).

DISCUSSION

RBP4 has been suggested as an adipokine, providing a link between obesity and insulin resistance (Muoio and Newgard, 2005; Yang *et al*, 2005; Tamori *et al*, 2006). This theory is supported by the finding of elevated RBP4 levels in the serum of subjects with obesity, impaired glucose tolerance, and T2DM and in non-obese and non-diabetic subjects with a strong family history of T2DM (Cho *et al*, 2006; Graham *et al*, 2006; Lee *et al*, 2007). Other studies have not found an association between RBP4 levels and adiposity, glucose disposal rate, or GLUT4 mRNA expression in adipose tissue (Bajzova *et al*, 2008), which had led to even more controversy on this topic (Janke *et al*, 2006). These inconsistent findings may indicate other processes independent of adipocyte metabolism, T2DM or insulin resistance influence the homeostatic control of RBP4 levels. Hepatocytes and adipocytes are the main sites for RBP4 synthesis. The kidneys are the main site for RBP4 catabolism and influence RBP4 homeostasis (Goodman, 1980; Usuda *et al*, 1983; Gerlach and Zile, 1991a,b; Marino *et al*, 2001). The maintenance of total body retinol homeostasis is regulated by glomerular filtration and subsequent reabsorption of RBP4 by proximal tubular cells (Marino *et al*, 2001). Renal dysfunction due to an impairment in GFR may directly affect RBP4 levels.

In the present study, in both the T2DM and control groups, RBP4 levels were higher among subjects with eGFR <60 ml/min per 1.73 m^2 than in subjects with eGFR >60 ml/min per 1.73 m^2. Linear regression analysis confirmed the eGFR, but not the presence of T2DM, was a significant predictor of the RBP4 level. These findings indicate the eGFR values in our Thai cohort are a major determinants of plasma RBP4 levels. Our findings are consistent with a previous study by Jaconi *et al* (1995) who found RBP4 levels were elevated in patients suffering from chronic kidney disease and were nearly fourfold higher among hemodialysis patients than among control subjects (Ziegelmeier *et al*, 2007). The results of our study confirm and expand these data, by showing significantly higher RBP4 levels among both diabetic and non-diabetic individuals with eGFR <60 ml/min per 1.73 m^2.

Microalbuminuria has been suggested as a major determinant of elevated RBP4 levels along with higher levels of urine RBP4 among T2DM patients with eGFR values >60 ml/min per 1.73 m^2 (Raila *et al*, 2007). However, the limited data regarding urinary protein and urinary RBP4 excretion in our study does not allow us to confirm these findings; further investigation is needed. We found RBP4

levels did not significantly differ between T2DM patients and control subjects with comparable eGFR values. RBP4 levels were correlated with BUN and eGFR. These findings show RBP4 levels are strongly related to renal function. Interestingly, the highest RBP4 levels in our study were present among control subjects with eGFR < 60 ml/min per 1.73 m^2. This controversial finding may be due to the hypoglycemic agent used in the T2DM group, which could depress the RBP4 plasma level, as demonstrated by Möhlig *et al* (2008) for metformin in T2DM patients.

In contrast to the findings of some studies (Graham *et al*, 2006; Aeberli *et al*, 2007; Qi *et al*, 2007; Reinehr *et al*, 2008), we found no association between RBP4 levels and parameters of the metabolic syndrome, including BMI, WHR, blood lipid levels, and the T2DM biomarkers FBG, insulin levels, and the HOMA-IR score. Differences in age, genetic background, diet, and lifestyle, might conceal this association, but no associations between the parameters of metabolic syndrome, except for triglyceride levels, have been reported among Asian populations (Silha *et al*, 2007).

This study analyzed the associations between RBP4 levels and renal failure and T2DM among Asians, in contrast to previous studies carried out mostly among Caucasian subjects. The results of the present study among Asians shows impairment of eGFR as a marker of renal function, rather than the parameters of T2DM, is the major determinant of RBP4 levels among both diabetics and non-diabetics. Therefore, renal function should be considered in studies regarding the biological importance of RBP4 in patients with T2DM and metabolic syndrome.

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