Original Article

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The Relationship among Homocysteine, Bilirubin, and Diabetic Retinopathy

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Background: Diabetic retinopathy is a common microvascular complication of diabetes mellitus (DM) and the leading cause of blindness in adults. Homocysteine, a risk factor with toxic effects on vascular endothelial cells, and bilirubin, a protectant with antioxidant and anti-inflammatory properties on the vasculature, have been reported to be linked to vaso-occlusive disorders. Therefore, the author of the present study investigated the association between the levels of plasma homocysteine and serum total bilirubin and the incidence of diabetic retinopathy as a chronic microvascular complication in patients with type 2 diabetes mellitus (T2DM).

Methods: A total of 102 patients with T2DM who visited our hospital from January 2009 to January 2010 were assessed.

Results: Of the 102 patients, the prevalence of diabetic retinopathy was 67 cases (65.7%) according to clinical ophthalmic examination. The duration of DM ($P<0.001$), age ($P=0.003$), fasting blood glucose ($P=0.045$) and urine albumin-creatinine ratio ($P=0.015$) in univariate analysis and plasma homocysteine level ($P=0.038$), duration of DM ($P=0.001$), and total bilirubin level ($P=0.012$) in multiple logistic regression analysis were statistically significantly associated with the incidence of diabetic retinopathy.

Conclusion: The present study indicates that homocysteine and bilirubin may be useful biomarkers for increased risk of diabetic retinopathy since retinopathy in patients with T2DM was linked to higher plasma homocysteine level and decreased serum total bilirubin level.

Keywords: Bilirubin; Diabetes mellitus; Homocysteine; Retinopathy

INTRODUCTION

The incidence of diabetes mellitus (DM) and its complications are increasing rapidly due to an aging population and the Westernization of life styles [1]. Diabetic retinopathy is a common microvascular complication that develops in the early period of DM and induces visual impairment and even blindness in adults. Thus early diagnosis and appropriate treatments are important [2]. Diabetic retinopathy is caused primarily by microvascular injury and the duration of DM, hyperglycemia, hypertension, and hyperlipidemia have been shown to be risk factors for diabetic retinopathy [3]. Recent studies have reported that homocysteine was linked to vaso-occlusive disorders in the eye and the level of plasma homocysteine may be a useful biomarker or a novel risk factor for increased risk of diabetic retinopathy in patients with type 2 diabetes mellitus (T2DM) [4,5]. Few studies exist on the relationship between bilirubin and microvasculopathy, such as diabetic retinopathy. However, bilirubin may exhibit strong antioxidant and anti-inflammatory properties on the vasculature [6,7]. Therefore, the authors of the present study suggest that bilirubin may have an endogenous protective effect on the retinal vasculature in patients with T2DM.

In the present study, the association among the level of plasma homocysteine as a risk factor, serum total bilirubin as an endogenous protectant and the presence of diabetic retinopa-

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thy as a chronic common complication and peripheral microvascular disorder in patients with T2DM was investigated.

METHODS

Study population

The present study was conducted on 102 patients with T2DM who were confirmed with insulin secretion test in the Department of Internal Medicine and underwent fundoscopic examinations for the assessment of retinopathy in the Department of Ophthalmology in Daegu Medical Center from January 2009 to January 2010. Patients were excluded from the present study if they had a history of chronic alcohol drinking, hepatobiliary abnormalities, or any other acute disease. The patients with blood levels greater than three times the normal level of aminotransferase (AST) or alanine aminotransferase (ALT), or with problems in the evaluation of diabetic retinopathy due to other reasons were excluded. The present study was conducted under participant voluntary consent after explanation of the purpose, methods, and course.

Study design and assessments

A detailed questionnaire was completed for each of the 102 participating patients. Information obtained included age, gender, smoking history, history of alcohol consumption or hepatobiliary disorders, duration of DM, and history of hypertension or cardiovascular diseases. Additionally, height, waist circumference, blood pressure, fasting blood glucose (FBG), 2-hour postprandial blood glucose (2PPBG), C-peptide, and insulin levels were obtained. Fasting venous blood was drawn on the day of examination via a venipuncture from the patient's antecubital vein and baseline biochemical profiles including serum total bilirubin, AST, ALT, glycosylated hemoglobin (HbA1c), creatinine, C-reactive protein (CRP) and lipid profiles were analyzed using the ARCHITECT c8000 (Toshiba, Tokyo, Japan). The standard reference range of total bilirubin is 3.4 to 20.5 μmol/L. For plasma homocysteine measurement, blood samples were collected in tubes containing EDTA and plasma homocysteine concentrations were analyzed using a HITACHI 7600 (Hitachi, Tokyo, Japan). The standard reference range of plasma homocysteine is 5.0 to 15.0 μmol/L.

All patients underwent full ophthalmologic examinations through dilated pupils including fundus with a binocular indirect ophthalmoscope (Keeler Instruments Inc., Broomall, PA, USA) and slit lamp biomicroscope using 90-diopter lens (Carl

Zeiss, Jena, Germany). Diabetic retinopathy was clinically classified by an ophthalmologist as cases without diabetic retinopathy; proliferative diabetic retinopathy (PDR) with neovascularization, scars, vitreous hemorrhage, or retinal detachment; and non-proliferative diabetic retinopathy (NPDR) with increased capillary permeability, capillary obstruction and distention, microaneurysm formation, cotton wool spots, or hard exudate. Non-proliferative retinopathy was divided into mild, moderate and severe NPDR.

Data analysis

Statistical analysis was performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) and the baseline characteristics of participants were presented as the mean±standard deviation and n (%). Data were compared using an independent-sample Student's t-test for continuous data, odds ratio (OR) and chi-square test for categorical variables, and the Mann-Whitney U test for nonparametric data with an irregular distribution. Multiple logistic regression analysis was performed to assess independent associations between the presence of key risk factors of diabetic retinopathy including statistically significant factors in univariate analysis and suggested biomarkers such as plasma homocysteine and total bilirubin levels. A P value less than 0.05 was considered statistically significant.

RESULTS

Baseline characteristics

The baseline clinical and laboratory characteristics of the present study population are shown in Table 1. Among the participants, 54 were male and 48 were female, and the mean age was 59 years. The average duration of DM was 9.3 years. The laboratory data showed a mean HbA1c of 7.9%, FBG of 8.4 mmol/L, 2PPBG of 13.2 mmol/L, homocysteine of 18.3 μmol/L, and total bilirubin of 11.7 μmol/L.

Clinical characteristics in patients with T2DM according to the incidence of retinopathy

Characteristics of the participants according to the incidence of retinopathy are shown in Tables 2 and 3. The number of patients with diabetic retinopathy was 67 (65.7%; 62 cases of NPDR and 5 cases of PDR) and the number of patients without diabetic retinopathy was 35 (34.3%). As expected, the age of patients with diabetic retinopathy was significantly higher than that of patients without retinopathy ($P<0.01$) and pa-

Table 1. Patient baseline clinical and laboratory characteristics

Parameter	Patients without diabetic retinopathy ($n=35$)	Patients with diabetic retinopathy ($n=67$)
Age, yr	55±12	62±11
BMI, kg/m^2	25.2±3.9	24.0±3.7
Waist/Hip ratio	0.940±0.017	0.941±0.019
DM duration, yr	2 (0.0-15.0)	10 (0.0-30.0)
Smoking, n (%)	9 (25.7)	23 (34.3)
Hypertension Hx, n (%)	16 (45.7)	37 (55.2)
CVA Hx, n (%)	1 (2.9)	3 (4.5)
SBP, mm Hg	120 (100-160)	127 (110-190)
DBP, mm Hg	80 (60-90)	80 (50-120)
Hemoglobin, g/dL	13.5 (11.7-18.0)	13.1 (8.4-16.1)
HbA1c, %	7.1 (5.6-12.8)	7.4 (5.0-15.6)
FBG, mmol/L	7.5 (4.7-12.9)	7.9 (4.7-17.5)
2PPBG, mmol/L	11.5 (6.8-22.3)	12.6 (6.3-30.9)
C-peptide, mmol/L	1.0 (0.3-3.1)	0.8 (0.4-8.3)
Total cholesterol, mmol/L	4.8 (2.6-6.7)	4.6 (2.8-9.0)
Triglyceride, mmol/L	1.7 (0.6-6.1)	1.5 (0.5-7.1)
HDL-C, mmol/L	1.2 (0.9-2.6)	1.2 (0.5-2.4)
Non-HDL, mmol/L	3.5 (0.9-5.4)	3.4 (1.8-7.7)
AST, IU/L	25.1 (14.0-98.0)	22.0 (11.0-75.0)
ALT, IU/L	32.1 (10.0-84.0)	20.0 (8.0-75.0)
Homocysteine, µmol/L	13.7 (6.0-65.0)	17.1 (7.0-57.3)
Total bilirubin, µmol/L	13.7 (6.8-51.3)	10.3 (3.4-25.7)
CRP, mg/dL	0.1 (0.0-18.8)	0.1 (0.0-18.8)
Urine ACR, µg/mg	0.1 (0.0-5.9)	0.8 (0.0-73.4)

Values are presented as mean±standard deviation or median (min-max).
BMI, body mass index; DM, diabetes mellitus; CVA, cerebrovascular accident; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycosylated hemoglobin; FBG, fasting blood glucose; 2PPBG, 2-hour postprandial glucose; HDL-C, high density lipoprotein cholesterol; AST, aminotransferase; ALT, alanine aminotransferase; CRP, C-reactive protein; ACR, albumin-creatinine ratio.

Table 2. Clinical characteristics in type 2 diabetic patients according to the incidence of diabetic retinopathy

Parameter	Patients without diabetic retinopathy ($n=35$)	Patients with diabetic retinopathy ($n=67$)	P value
Age, yr	55±12	62±11	0.003[a]
Gender			
Male	19 (54.3)	35 (52.2)	0.884[b]
Female	16 (45.7)	32 (47.8)	
DM duration, yr	3.4±4.3	10.5±7.5	0.000[c]
BMI, kg/m^2	25.2±3.9	24.0±3.7	0.112[a]
Waist/Hip ratio	0.940±0.017	0.941±0.019	0.851[a]
Hypertension	16 (45.7)	37 (55.2)	0.361[b]
Smoking	9 (25.7)	23 (34.3)	0.373[b]
CVA	1 (2.9)	3 (4.5)	1.000[d]
SBP, mm Hg	123.7±12.9	127.5±15.1	0.215[a]
DBP, mm Hg	76.0±6.5	79.9±10.9	0.059[a]

Values are presented as mean±standard deviation or number (%).
DM, diabetes mellitus; BMI, body mass index; CVA, cerebrovascular accident; SBP, systolic blood pressure; DBP, diastolic blood pressure.
[a]Independent-samples t-test, [b]Mann-Whitney test, [c]Chi-square test, [d]Fisher's exact test.

min-creatinine ratio (ACR) was 4.3 in the retinopathy group and 0.7 in the group without retinopathy, with the diabetic retinopathy group showing significantly higher values ($P<0.01$). The level of plasma homocysteine in the group with retinopathy was an average of 19.2 µmol/L and it was higher in comparison with the average of 16.3 mmol/L in the group without retinopathy; however, the difference was not statistically significant ($P>0.05$) (Table 3). There were no significant differences between the two groups in gender, smoking, body mass index, blood pressure, waist to hip ratio, HbA1c, CRP, cholesterol concentrations, or renal function (Tables 2 and 3). There were no significant differences between the two groups in current medication except that the number of patients who took angiotensin II receptor blockers (ARBs) was higher in the group with retinopathy than in the group without retinopathy (49, 73.1% vs. 18, 51.4%, $P<0.05$).

Independent risk factors for diabetic retinopathy

To examine the independent associations between the key risk factors and the presence of diabetic retinopathy, a multiple logistic regression analysis was performed with the significant variables in univariate analysis including age, DM duration,

tients with diabetic retinopathy had a significantly longer duration of DM, by 7.2 years on average ($P<0.01$) (Table 2). In addition, FBG of the retinopathy group was on average 8.7 mmol/L, and was significantly higher than the average of 7.7 mmol/L in the group without retinopathy ($P<0.05$). Total bilirubin was 10.1 µmol/L in the retinopathy group and 15.1 µmol/L in the group without retinopathy and the retinopathy group had significantly lower bilirubin levels ($P<0.01$). Urine albu-

Table 3. Laboratory characteristics in type 2 diabetic patients according to the incidence of diabetic retinopathy

Parameter	Patients without diabetic retinopathy (*n*=35)	Patients with diabetic retinopathy (*n*=67)	*P* value[a]
HbA1c, %	7.5±1.6	8.1±2.2	0.165
C-peptide, mmol/L	1.2±0.7	1.0±1.0	0.547
FBG, mmol/L	7.7±2.0	8.7±2.8	0.045
2PPBG, mmol/L	11.9±4.0	13.7±5.6	0.102
Total cholesterol, mmol/L	4.7±0.9	4.8±1.1	0.808
Triglyceride, mmol/L	2.0±1.2	1.9±1.3	0.831
HDL-C, mmol/L	1.3±0.4	1.3±0.4	0.748
Non-HDL, mmol/L	3.4±0.9	3.5±1.1	0.723
Creatinine, mg/dL	1.0±0.2	1.3±1.2	0.098
CRP, mg/dL	1.2±3.7	0.8±2.6	0.515
Homocysteine, μmol/L	16.3±11.7	19.2±8.4	0.165
Total bilirubin, μmol/L	15.1±8.0	10.1±4.1	0.000
Urine ACR ratio, μg/mg	0.7±1.4	4.3±11.6	0.015

HbA1c, glycosylated hemoglobin; FBG, fasting blood glucose; 2PPBG, 2-hour postprandial glucose; HDL, high density lipoprotein cholesterol; CRP, C-reactive protein; ACR, albumin-creatinine ratio.
[a]Mann-Whitney test.

Table 4. Adjusted odds ratio for associations between key risk factors and the incidence of diabetic retinopathy with logistic regression model

	Adjusted OR	95% CI	*P* value
Age, yr	1.0	1.0-1.1	0.074
DM duration, yr	1.2	1.1-1.4	0.001
FBG, mmol/L	1.3	1.0-1.6	0.067
Homocysteine, μmol/L	1.1	1.0-1.1	0.038
Total bilirubin, μmol/L	0.9	0.7-1.0	0.012
Urine ACR, μg/mg	1.0	0.8-1.3	0.683

OR, odds ratio; CI, confidence interval; DM, diabetes mellitus; FBG, fasting blood glucose; ACR, albumin-creatinine ratio.

FBG, total bilirubin, and urine ACR, with homocysteine as the biomarker as used in previous studies [4,5]. The analysis showed that variables independently associated with diabetic retinopathy were a longer duration of DM with an OR of 1.2 (95% confidence interval [CI], 1.1 to 1.4), homocysteine with an OR of 1.1 (95% CI, 1.0 to 1.1), and total bilirubin with an OR of 0.9 (95% CI, 0.7 to 1.0) (Table 4).

DISCUSSION

Diabetic retinopathy is a common ophthalmic complication that occurs primarily in the microvasculature of the retina and has been estimated to account for 5% of all cases of global blindness [8]. Diabetic retinopathy is also known to be common in patients with newly diagnosed T2DM [9].

The change in the retina of patients with DM includes formation of retinal capillary microaneurysms, increased vascular permeability, vascular occlusion, proliferation of new blood vessels, fibrosis of tissues in the surface of the retina and optic disk, and the contraction of fibrovascular proliferations and the vitreous [10]. Several hypotheses have been tested on the cause and pathogenesis of diabetic retinopathy. In non-proliferative retinopathy, prolonged hyperglycemia may produce microaneurysms because of the selective decrease of cells in the vicinity of the retinal microvasculature and may also induce thickening of the vascular fundus membrane, endothelial proliferation, microvascular dilatation due to decreased endothelin-1 synthesis, increased vascular permeability, and vascular occlusion in microvascular endothelial cells. In addition, prolonged hyperglycemia may increase blood flow and viscosity, resulting in an increase of shear stress against the vascular wall, as well as injury of the vascular wall, in severe cases, it is known to induce macular edema [11]. However, the main pathogenesis of proliferative retinopathy is known to be neovascularization caused by several factors including insulin-like growth factor released from a retinal ischemic area [11].

Recently, four biochemical pathways including enhanced glucose flux through the polyol pathway, increased intracellular formation of advanced glycation end-products, activation of protein kinase C isoforms, and stimulation of the hexosamine pathway have been suggested for eye disorders caused by hyperglycemia-induced vascular injury in diabetes. These pathogenic mechanisms also appear to be associated with superoxide overproduction by the mitochondrial electron transport chain [12].

A longer duration of diabetes appears to be the main risk factor for diabetic retinopathy, and aging, hyperglycemia, hyperlipidemia, proteinuria, severe obesity, early onset of diabetes, alcohol drinking, genetic factors, and various hormones including growth hormone have been reported as additional risk factors for diabetic retinopathy [13-16]. Similarly, the present study showed that a longer duration of DM and aging in the group with retinopathy were statistically significant risk

factors for diabetic retinopathy in comparison to the group without retinopathy (Table 2). In addition, high FBG level and high urine ACR appear to be significant risk factors for diabetic retinopathy, and total bilirubin level is inversely associated with the incidence of diabetic retinopathy (Table 3). Regarding medications, there were more patients taking ARBs in the group with retinopathy, which may be due to the medication being used as preventative treatment for a relatively high ACR. A longer duration of diabetes was statistically significantly associated with diabetic retinopathy not only in univariate analysis but also in logistic regression analysis, and thus it was also confirmed to be an independent risk factor in the present study (Tables 2 and 4).

Based on the results from the present study, the author suggest that plasma homocysteine and serum total bilirubin can be used as biomarkers of diabetic retinopathy. Homocysteine is an amino acid containing a sulfur group produced during the metabolism of methionine. Dietary methionine is converted to S-adenosyl methionine, and then demethylated to S-adenosyl homocysteine, which is hydrolyzed to homocysteine and adenosine [17]. The level of total plasma homocysteine varies widely depending on age, gender, genetics, geographic and environmental factors. In particular, homocysteine may be increased primarily in hereditary diseases and with deficiencies of folic acid and vitamins B12 and B6, which are required for homocysteine metabolism [18,19]. Recent studies have shown that homocysteine is associated with an increased risk of chronic vascular complications of diabetes including coronary artery diseases, stroke, and peripheral arterial diseases, and these mechanisms can be explained by direct toxic effects on vascular endothelial cells and an indirect effect on normal methylation in endothelial cells [17,20]. In particular, hyperhomocysteinemia causes toxic effects on vascular endothelial cells directly or indirectly through the impairment of vascular endothelial cells, promotion of platelet activation, enhanced coagulability, generation of reactive oxygen species with cellular toxicity, decreased antioxidant actions, and vascular smooth muscle cell proliferation [19,21-24]. Recent studies have reported that elevated homocysteine level may be associated with the onset of retinopathy as a chronic microvascular complication of diabetes [3-5,25]; however, the results were not confirmed and the issue remains controversial [17,20,26]. In the present study, the levels of plasma homocysteine in the group with retinopathy were higher in comparison to those in the group without retinopathy in univariate analysis,

although statistical significance was not shown (Table 3). Homocysteine level was added to the key factor list in univariate analysis for multiple logistic regression analysis since several previous studies have reported homocysteine level as a novel biomarker of diabetic retinopathy [3,4]. The homocysteine level was independently associated with diabetic retinopathy with an OR of 1.1 according to multiple logistic regression analysis (Table 4). Consequently, homocysteine level may be a useful biomarker for increased risk of diabetic retinopathy.

Bilirubin is the metabolic end-product of heme degradation and is considered a toxic substance with adverse effects such as jaundice [27]. However, recent reports have suggested that bilirubin may exert anti-atherosclerotic effects through antioxidant actions [7], anti-inflammatory effects on the vasculature [28,29], and cytoprotectant properties [30]. These properties are explained by the mechanism of bilirubin inhibition of inflammatory cytokine-induced endothelial cell expression of vascular cell adhesion molecules [28], inhibition of transmigration of monocytes [31], prevention of the oxidation of low density lipoprotein [32], inhibition of inflammation and dysfunction of endothelial cells [29], inhibition of proliferation of vascular smooth muscles [33], as well as prevention of the formation of thrombi [7,34]. Notably, oxidative stress has been implicated in the pathogenesis of microvascular and macrovascular complications of diabetes [35]. A direct pathological connection of serum bilirubin with microvascular disorders has not been fully established. However, higher serum levels of bilirubin are inversely associated with the incidence of not only macrovascular diseases, such as coronary arterial disease [36-38], but also peripheral arterial disease through the anti-atherogenic and antioxidant properties of bilirubin [7]. Recently published studies have suggested that elevated serum bilirubin may provide protection from coronary microvascular dysfunction and coronary flow reserve impairment by improving microvascular integrity [39,40]. In the present study, the correlation between levels of serum total bilirubin and diabetic retinopathy as a chronic microvascular complication was investigated. The results showed an inverse association of bilirubin level with retinopathy not only in univariate but also multiple logistic regression analysis (Tables 3 and 4), which indicates that bilirubin may be an important biomarker of diabetic retinopathy as an endogenous protectant from microvascular complications due to antioxidant and anti-inflammatory properties.

There were several limitations in the present study. First, examinations of dietary deficiencies of folic acid and vitamins

involved in homocysteine metabolism were not performed. Second, testing for anti-hepatitis C virus or performing an ultrasonogram on the hepatobiliary system which may determine the bilirubin level, was not conducted in the present study. Third, the study was cross-sectional in a small group, and thus additional long term prospective studies are needed in a larger population.

In summary, including homocysteine and bilirubin levels in predictions of diabetic retinopathy may be clinically useful since retinopathy in patients with T2DM was linked to higher plasma homocysteine level and decreased serum total bilirubin level in the present study. Additional studies are necessary to confirm these results and evaluate related mechanisms.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGMENTS

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