Journal of Diabetes and Its Complications 33 (2019) 651–656

Contents lists available at ScienceDirect

Journal of Diabetes and Its Complications

journal homepage: WWW.JDCJOURNAL.COM

Leucine-rich α-2-glycoprotein predicts proliferative diabetic retinopathy in type 2 diabetes

Xiao Zhang [a,1], Sharon L.T. Pek [a,1], Subramaniam Tavintharan [b,c], Chee Fang Sum [b,c], Su Chi Lim [b,c,d], Keven Ang [a], Darren Yeo [a], Tang Wern Ee [e], Chee Chew Yip [f], Neelam Kumari [f,*]

[a] Clinical Research Unit, Khoo Teck Puat Hospital, Singapore
[b] Diabetes Centre, Khoo Teck Puat Hospital, Singapore
[c] Department of Medicine, Khoo Teck Puat Hospital, Singapore
[d] Saw Swee Hock School of Public Health, National University of Singapore, Singapore
[e] National Healthcare Group Polyclinics, Singapore
[f] Department of ophthalmology and Visual Sciences, Khoo Teck Puat Hospital, Singapore

ARTICLE INFO

Article history:
Received 6 September 2018
Received in revised form 8 May 2019
Accepted 26 May 2019
Available online 31 May 2019

Keywords:
LRG1
Angiogenesis
T2DM
DR
PDR

ABSTRACT

Aim: We aim to examine the association of plasma leucine-rich-α-2-glycoprotein 1 (LRG1) with diabetic retinopathy (DR) in type 2 diabetes.
Methods: At baseline visit, plasma LRG1 levels were assessed using ELISA. Central arterial stiffness was estimated by carotid-femoral pulse wave velocity (PWV). At follow-up visit (median = 3.2 years), digital color fundus photographs were assessed for DR. DR severity was categorized into non-proliferative DR (NPDR) and proliferative DR (PDR).
Results: DR was diagnosed in 396 (32.8%) of 1206 patients. DR has higher LRG1 than non-DR (19.5 ± 11.3vs.16.9 ± 8.9 μg/ml, $p \ll 0.001$). After adjustment, LRG1 was not associated with DR (OR = 1.2, [95% CI, 0.96–1.30], $p = 0.16$). LRG1 was higher in PDR ($n = 107$) than NPDR ($n = 270$) (23.2 ± 15.4vs.18.1 ± 8.9 μg/ml, n = 270, $p \ll 0.001$). After adjustment, with 1-SD increase in LRG1, the relative risk of NPDR and PDR was 0.99 ([0.83–1.18], $p = 0.91$) and 1.42 ([95% CI, 1.14–1.76], $p = 0.002$) (p-trend = 0.01), respectively. We didn't observe significant improvement in AUC after adding LRG1 into the model. Baseline PWV mediated 12.0% of the association between LRG1 and PDR ($p = 0.03$).
Conclusion: Baseline plasma LRG1 is associated with PDR, suggesting it maybe a promising biomarker for prediction for advanced proliferative stages of DR. The mediation result indicates the potential benefit of ameliorating central arterial stiffness to prevent PDR.

© 2019 Published by Elsevier Inc.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a rapidly evolving global health issue and Asia is the epicenter of this worldwide epidemic. Globally, the number of people with T2DM will double from 171 million in 2000 to 366 million by 2030.[1] In Singapore, the prevalence of T2DM has been predicted to increase from 7.3% in 1990 to 15% in 2050.[2] Diabetic retinopathy (DR) is a highly specific microvascular complication of diabetes and remains a leading cause of avoidable blindness in the working age group individuals worldwide.[3,4] Similar to T2DM, the number of DR and vision-threatening DR has been projected to rise to 191.0 million and 56.3 million, respectively by 2030.[5]

DR is categorized into early non-proliferative (NPDR) and late proliferative stages (PDR), the latter stage together with diabetic macular edema that can develop at any stage are the primary causes of irreversible visual loss in individuals with diabetes (vision-threatening DR). The proliferative stage of DR is characterized by abnormal growth of new vessels in the retina and remains asymptomatic until these fragile and misdirected vessels results in pre-retinal or vitreous hemorrhage or tractional detachment with consequential loss of vision.[6] Therefore, it is critical to identify novel angiogenic factors promoting neovascularization for screening and controlling pathogenic angiogenesis in ocular disease among diabetic patients.

Leucine-rich alpha-2-glycoprotein (LRG1), a highly conserved member of the leucine-rich repeat family of proteins, is a novel pro-angiogenic factor.[7] Wang et al. demonstrated up-regulated levels of LRG1 in retinae with pathogenic retinal and choroidal vasculature in mouse models, as well as in vitreous fluid from PDR patients.[7] Consistently, Chen et al. reported higher LRG1 levels in the vitreous as well

* Corresponding author at: Department of ophthalmology and Visual Sciences, Khoo Teck Puat Hospital, 90 Yishun Central, 768828, Singapore.
E-mail address: kumari.neelam@ktph.com.sg (N. Kumari).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.jdiacomp.2019.05.021
1056-8727/© 2019 Published by Elsevier Inc.

as plasma of PDR patients in a small study among Chinese population.[8] However, both these studies are limited by small sample size and univariate examination of LRG1 with PDR without taking into account the effect of any confounding factors. Moreover, the cross-sectional design precludes any inference about causal or mediation connections among LRG1, DR and other DR risk factors.[9]

It is believed that microvascular complications of diabetes, particularly DR and chronic kidney disease (CKD), may progress in a parallel manner possibly due to shared risk factors, such as poor glycaemia control and systolic hypertension.[10,11] Indeed, our research group has reported associations between plasma LRG1 level with peripheral arterial disease[9] and CKD progression,[12] suggesting that LRG1 might also be a potential risk biomarker for DR. Moreover, antibody blockade of LRG1 reduced lesion sizes of choroidal neovascularization in mouse models, thereby suggesting that LRG1 might be a risk factor for neovascularization.[7] Taken together, LRG1 may represent both a potential screening tool and a therapeutic target for controlling angiogenesis in DR.

In this large and multi-ethnic T2DM Asian cohort in Singapore, we aim to 1) evaluate the association of baseline plasma LRG1 level with the presence and severity of DR after taking into account the effect of confounding factors; 2) examine whether central arterial stiffness mediates the association between LRG1 and DR.

2. Materials and methods

2.1. Study population and design

The Singapore Study of Macro-angiopathy and Micro-vascular Reactivity in Type 2 Diabetes (SMART2D) is a completed cross-sectional study conducted between August 2011 and February 2014 including a total of 2057 adults aged 21–90 years with T2DM.[13] The participants were recruited consecutively from a secondary hospital and a neighboring primary-care public outpatient clinic in equal proportion in Singapore. Diagnosis of T2DM was based on American Diabetes Association criteria. The exclusion criteria included: Type 1 diabetes, pregnant subjects, subjects with active inflammation (e.g. systemic lupus erythromatosis) and cancer, subjects taking non-steroid anti-inflammation drugs on the same day of clinical/vascular/biomedical assessment, subjects on oral steroids equivalent to ≫5 mg/day prednisolone, those who could not fulfill the informed consent process and those who wore a pacemaker or any device that may be affected by electric current. After phlebotomy, those with fasting glucose ≪4.5 mmol/l or ≫ 15.0 mmol/l, and subjects with Hemoglobin A1c (HbA1c) ≫ 108 mmol/mol (≫ 12%) were also excluded from the study.[13] 1206 patients have been followed up for a median of 3.2 years (range 2.1–5.8 years) from September 2014. This study has been approved by the National Healthcare Group Domain Specific Review Board (NHG-DSRB). Individual written informed consent was obtained prior to enrollment in the study.

2.2. Assessment of DR at follow-up

45 degree single field photos, centered on the macula, were taken using non-mydriatic camera (pupils were not dilated) for both eyes in all study subjects (TRC-NW 200, Topcon Co Japan) at follow-up visit. Therefore, all DR cases including prevalent DR at baseline (no assessment) and incident prospective DR at follow-up were included in our study. For each eligible eye, two digital color fundus photographs were captured and assessed by a fellowship trained retina specialist in a masked fashion to minimize any possible bias. The photographs were not graded and labeled as ungradable if more that 50% of the retinal photographs were not clearly visible. DR was considered present if any characteristic lesions as defined by Early Treatment Diabetic Retinopathy Study were present. The minimum criterion for diagnoses of DR was presence of at least one definite micro aneurysm and/or retinal hemorrhage. DR severity was further categorized into NPDR and PDR.[14] DR was classified as NPDR based on the presence of one or more of the following features: micro aneurysms, hemorrhages, hard or soft exudates, venous beading, and intraretinal microvascular abnormalities. DR was classified as PDR if there was neovascularization, pre-retinal hemorrhages, vitreous hemorrhage, or panretinal laser photocoagulation scars. The severity of DR in the worse affected eye was used for retinopathy grading. Intra-rater reliability was excellent with an observed percentage agreement of 92.4% for the presence of DR (kappa = 0.79) and 89.9% for the severity of DR (kappa = 0.74).

2.3. Baseline plasma LRG1 measurement

Fasting blood samples were obtained and plasma aliquots were stored at −80 °C until analysis. LRG1 level was measured in plasma samples with 4–6 freeze-thaws cycles by a sandwich enzyme-linked immunosorbent assay kit (Immuno-Biological Laboratories, Hamburg, Germany). The intra-assay coefficient of variation was 3.0% to 4.9%, inter-assay coefficient of variation was 4.2% to 5.1%, and sensitivity was 0.17 mg/ml.

2.4. Clinical and biochemical measurement

Body mass index (BMI) was calculated as body weight (kg)/height $(m)^2$. Blood pressure was measured using a mercury sphygmomanometer on the right arm using appropriate cuff sizes. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were calculated from the average of three most consistent readings. HbA1c was measured based on monoclonal antibody agglutination reaction using a point-of-care immunoassay analyzer (DCA Vantage Analyzer; Siemens, Erlangen, Germany) certified by National Glycohemoglobin Standardization Program. High-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein-cholesterol (LDL-C) were quantified by enzymatic method using Kodak Ektachem chemistry slides. The intra- and inter-assay coefficients of variation were 5.7% and 7.7%, respectively. The sensitivity reported by the manufacturer was 4.12 pg/ml. Urinary albumin-to-creatinine ratio (ACR) was determined by urinary creatinine measured by enzymatic method on Roche/Hitachi cobas c system (Roche Diagnostic GmbH, Mannheim, Germany) and albumin measured by a solid-phase competitive chemiluminescent enzymatic immunoassay with a lower detection limit of 2.5 μg/ml (Immulite; DPC, Gwynedd, UK). Estimated glomerular filtration rate (eGFR) was calculated based on a widely used Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation in patients with diabetes.[15] CKD was defined by the presence of eGFR ≪60 ml/min/1.73m^2 or kidney damage as indicated by ACR ≥ 30 mg/g.[16] Plasma level of pigment epithelium-derived factor (PEDF), an important angiogenic inhibitor, was measured and quantified by ELISA (Biovendor Laboratory Medicine, Modrice, Czech Republic). Arterial stiffness was estimated by carotid-femoral pulse wave velocity (PWV) using SphygmoCor® (AtCor Medical, Sydney, Australia) and expressed as meters per second.[9] Microvascular endothelial vasodilation was quantified using laser Doppler flowmetry (Lisca PIM 1.0; Lisca Development AB, Linkoping, Sweden) to assess cutaneous perfusion accompanied by iontophoresis of acetylcholine (Ach) (endothelium-dependent) or sodium nitroprusside (NaNP) (endothelium- independent), respectively. Vasodilation was presented as percentage change from baseline.[9] Data collections and measurements at baseline and follow-up visits were performed using the same protocol.

2.5. Statistical analysis

Standard descriptive statistics were used to describe the characteristics of T2DM patients. Normally distributed continuous data were expressed as means and standard deviations (SDs). Skewed variables were expressed as median and inter-quartile range (IQR) and nature log (ln)-transformed before data analyses. Differences between groups

were compared by *t*-test, Wilcoxon rank-sum test or χ^2 test where appropriate.

Univariate analysis was performed for individual clinical and biochemical parameters to verify associated factors (Table S1). Variables that were statistically significant in univariate analysis ($p \ll 0.1$) or with putative roles in the pathobiology of DR were added into multivariable logistic regression models. Logistic regression was used to examine the association of LRG1 with the presence of DR. Multinomial logistic regression was used to examine the associations of LRG1 with the severity of DR. Ordinal logistic regression models were used to estimate the overall trend of the above associations. The following baseline variables were incorporated as covariates: age, gender, ethnicity, T2DM duration, SBP, HBA1c, smoking status, TG, HDL, CKD, endothelial-dependent and independent function, and usage of renin-angiotensin system (RAS) medications and insulin. Performance in prediction of DR was assessed by area under curve (AUC).

The mediation effect on the association between LRG1 and DR was tested using a series of hierarchical regressions adjusting for potential confounders. According to Baron and Kenny,[17] mediation is demonstrated based on the following criteria: 1) the independent variable (LRG1) is significantly associated with the mediator (PWV), 2) the independent variable is significantly associated with the dependent variable (DR), 3) the mediator is significantly associated with the dependent variable, 4) the association between the independent and dependent variable must be attenuated when the mediator is included in the regression model (Fig. 1). The total effect (c) can be divided into the direct (c') and indirect effect effected mediated by PWV (a x b). Therefore, the size of the mediation effect was evaluated by indirect effect divided by total effect, using the equation of a x b/(a x b + c'),[18] where a (a = 0.29, $p \ll 0.001$) is the coefficient relating the independent variable (LRG1) to the mediator (PWV), b (b = 0.14, $p = 0.002$) is the coefficient relating the mediator (PWV) to the dependent variable (DR), and c' (c' = 0.30, $p = 0.005$) is the coefficient relating the independent variable (LRG1) to the dependent variable (DR) while adjusting for the mediator. The bootstrap analysis of 500 replications was adapted to estimate the mediation effect and its corresponding 95% confidence interval.[18] All statistical analysis was performed using STATA version 14.0 (STATA Corporation, College Station, Texas, USA). A two-tailed p value of ≪0.05 was considered as statistically significant.

3. Results

3.1. LRG1 level and the presence of DR

DR was found in 396 (32.8%) of the 1206 patients. Table 1 compares baseline characteristics of T2DM patients stratified by the presence of DR in univariate analysis. DR patients have longer T2DM duration, higher HbA1c, SBP, PWV and PEDF level, worse kidney function and endothelial function compared with non-DR patients. More DR patients used RAS medications and insulin than non-DR patients. LRG1 level

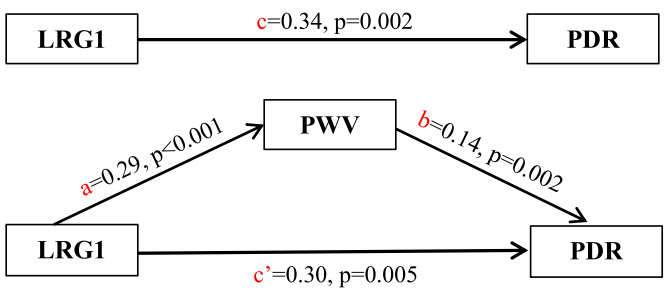

Fig. 1. Mediation effect through PWV. PWV at baseline as a mediator of the relationship between baseline LRG1 and PDR at follow-up (12.0% mediation effect, $p = 0.03$) after adjustment for age, T2DM duration, SBP, HBA1c, insulin usage and CKD.

Table 1
Baseline characteristics of individuals with T2DM stratified by presence of DR ($n = 1206$).

Variables	DR (396)	Non-DR (810)	All (1206)	P-value[a]
LRG1 (μg/ml)	19.5 ± 11.3	16.9 ± 8.9	17.7 ± 9.8	**≪0.001**
Entry age (yrs)	56.4 ± 9.6	55.9 ± 10.6	56.1 ± 10.3	0.41
Duration of T2DM (yrs)	15.4 ± 9.4	8.8 ± 7.1	11.0 ± 8.5	**≪0.001**
Male gender (%)	215 (54.3)	409 (50.6)	624 (51.8)	0.22
Ethnicity, n (%)				
Chinese	191 (48.2)	455 (56.2)	646 (53.6)	
Malays	97 (24.5)	142 (17.6)	239 (19.8)	
Indians	99 (25.0)	184 (22.7)	283 (23.5)	
Others	9 (2.3)	28 (3.5)	37 (3.1)	**0.01**
Hba1c (%)	8.4 ± 1.4	7.5 ± 1.2	7.8 ± 1.3	**≪0.001**
Current/former smokers, n (%)	76 (19.2)	114 (14.1)	190 (15.8)	**0.02**
RAS medication, n (%)	286 (72.2)	411 (51.3)	697 (58.2)	**≪0.001**
Insulin (%)	226 (57.2%)	170 (21.2%)	396 (33.0%)	**≪0.001**
BMI (kg/m^2)	27.7 ± 4.9	27.8 ± 5.4	27.8 ± 5.2	0.85
HDL-C (mM)	1.26 ± 0.3	1.30 ± 0.3	1.29 ± 0.3	0.09
LDL-C (mM)	2.7 ± 0.8	2.8 ± 0.8	2.7 ± 0.8	0.14
TG (mM)[b]	1.4 (1.0–1.9)	1.4 (1.1–1.9)	1.4 (1.0–1.9)	0.10
SBP (mmHg)	143.8 ± 19.2	136.2 ± 16.1	138.7 ± 17.5	**≪0.001**
DBP (mmHg)	79.4 ± 9.8	78.8 ± 9.3	79.0 ± 9.4	0.29
PWV (m/s)	10.7 ± 3.1	9.0 ± 2.4	9.6 ± 2.8	**≪0.001**
PEDF (ng/ml)[b]	16.4 (13.5–20)	14.8 (12.1–18.0)	15.2 (12.5–18.7)	**≪0.001**
Ach (%)	106.7 ± 84.4	131.0 ± 96.9	123.0 ± 93.6	**≪0.001**
NaNP (%)	66.7 ± 52.1	82.9 ± 56.6	77.6 ± 55.7	**≪0.001**
eGFR (ml/min/1.73 m^2)	80.0 ± 30.5	92.3 ± 21.3	88.3 ± 25.4	**≪0.001**
ACR (mg/g)[b]	69 (187–432)	13 (4–41)	21 (6–84.5)	**≪0.001**
CKD, n (%)[c]	280 (70.1)	296 (36.5)	576 (47.8)	**≪0.001**

HbA1c, hemoglobin A1c; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, total triglycerides; RAS (renin–angiotensin system) medication, angiotensin-converting-enzyme or angiotensin receptor blockers; PWV, pulse wave velocity; PEDF, pigment epithelium-derived factor; Ach (Acetylcholine), endothelial-dependent induced change in micro-circulatory perfusion; NaNP (Sodium Nitroprusside), endothelial-independent induced change in micro-circulatory perfusion; LRG1, Leucine-rich alpha-2-glycoprotein; eGFR, estimated glomerular filtration rate; ACR, albumin-to-creatinine ratio; CKD, chronic kidney disease.

[a] Statistical significance ($P \ll 0.05$) in univariate analysis was shown in bold.

[b] expressed as median (inter-quartile range).

[c] eGFR ≪60 ml/min/1.73m^2 AND/OR ACR ≥ 30 mg/g.

were significantly higher in DR than non-DR patients (19.5 ± 11.3 vs. 16.9 ± 8.9 μg/ml, $p \ll 0.001$).

In univariate analysis, 1-SD increase in LRG1 level were associated with the presence of DR (OR = 1.30, 95% CI, 1.15–1.46, p ≪ 0.001). Table 2 shows that the association was attenuated to non-significant in fully adjusted model (OR = 1.12, 95% CI, 0.96–1.30, $p = 0.16$).

3.2. LRG1 level and the severity of DR

In 396 DR cases, there were 270 NPDR and 107 PDR (19 ungradable). LRG1 level was significantly higher in patients with PDR (23.2 ± 15.4 μg/ml) than NPDR (18.1 ± 8.9 μg/ml, $p \ll 0.001$) and non-DR patients (p ≪ 0.001). LRG1 level in NPDR is marginally higher than that in non-DR patients ($p = 0.05$). PEDF levels in PDR (17.0 (13.8–21.4) ng/ml, p ≪ 0.001) and NPDR (16.3 (13.4–19.7) ng/ml, p ≪ 0.001) patients were significantly higher than non-DR patients (14.8 (12.1–18.0) ng/ml). There is no significant difference between NPDR and PDR ($p = 0.12$).

Table 2 shows the association of LRG1 with the severity of DR in multivariable analysis. After adjustment, with 1-SD increase in LRG1, the relative risk of having NPDR and PDR was 0.99 (95% CI, 0.83–1.18, $p = 0.91$) and 1.42 (95% CI, 1.14–1.76, $p = 0.002$) (p-trend = 0.01), respectively.

The AUC of ROC analysis of LRG1 for the prediction of PDR was 0.64 (95% CI, 0.58–0.69) (Fig. S1). The positive and the negative predictive value was 66.7% and 88.7%, respectively. Fig. S1 also shows predictive

Table 2
The association of LRG1 with the presence ($n = 1206$) and severity of DR ($n = 1187$).

Variables	DR (396)			NPDR (270)			PDR (107)		
	OR	(95%CI)	P[a]	OR	(95%CI)	P[a]	OR	(95%CI)	P[a]
LRG1 (μg/ml)	1.12	(0.96–1.30)	0.16	0.99	(0.83–1.18)	0.91	1.42	(1.14–1.76)	**0.002**
Age (yrs)	0.98	(0.97–0.99)	**0.03**	0.99	(0.97–1.01)	0.21	0.97	(0.94–0.99)	**0.02**
Male gender	0.95	(0.67–1.34)	0.76	0.94	(0.64–1.38)	0.77	0.84	(0.48–1.46)	0.53
Ethnicity									
Chinese	Ref.			Ref.			Ref.		
Malays	1.43	(0.97–2.10)	0.07	1.77	(1.16–2.69)	**0.01**	0.89	(0.48–1.67)	0.72
Indians	1.57	(1.08–2.27)	**0.02**	1.78	(1.19–2.65)	**0.01**	0.98	(0.53–1.82)	0.96
Others	0.62	(0.25–1.55)	0.31	0.90	(0.35–2.30)	0.83	0.18	(0.02–1.45)	0.11
T2DM duration (yrs)	1.07	(1.05–1.09)	**≪0.001**	1.06	(1.04–1.08)	**≪0.001**	1.11	(1.08–1.14)	**≪0.001**
SBP (mmHg)	1.02	(1.01–1.03)	**≪0.001**	1.02	(1.01–1.03)	**≪0.001**	1.02	(1.01–1.03)	**0.01**
Hba1c (%)	1.44	(1.27–1.63)	**≪0.001**	1.52	(1.331.73)	**≪0.001**	1.22	(1.01–1.49)	**0.04**
Current/former smokers	1.62	(1.07–2.45)	**0.02**	1.85	(1.19–2.88)	**0.01**	1.31	(0.66–2.62)	0.44
LnTG (mM)	0.80	(0.57–1.11)	0.19	0.70	(0.48–1.02)	0.06	0.86	(0.51–1.42)	0.56
HDL-C (mM)	1.25	(0.76–2.03)	0.37	1.15	(0.67–1.97)	0.60	1.39	(0.64–3.00)	0.41
RAS medication (%)	1.14	(0.82–1.57)	0.44	1.12	(0.79–1.59)	0.53	1.12	(0.65–1.95)	0.68
Insulin (%)	1.88	(1.34–2.65)	**≪0.001**	1.81	(1.24–2.63)	**0.002**	2.16	(1.28–3.67)	**0.004**
Ach (%)	0.999	(0.997–1.001)	0.42	0.999	(0.997–1.001)	0.52	0.998	(0.994–1.001)	0.27
NaNP (%)	0.996	(0.993–0.999)	**0.03**	0.996	(0.993–1.00)	0.05	0.997	(0.992–1.002)	0.38
CKD	2.74	(1.99–3.79)	**≪0.001**	2.25	(1.59–3.20)	**≪0.001**	5.64	(3.04–10.44)	**≪0.001**

LRG1, Leucine-rich alpha-2-glycoprotein; SBP, systolic blood pressure; HbA1c, hemoglobin A1c; TG, total triglycerides; HDL-C, high density lipoprotein cholesterol; RAS, renin–angiotensin system; Ach, Acetylcholine; NaNP, Sodium Nitroprusside; CKD, chronic kidney disease.

[a] Statistical significance ($P \ll 0.05$) in multivariate analysis adjusted for age, gender, ethnicity, T2DM duration, SBP, HBA1c, smoking status, TG, HDL, CKD, endothelial-dependent and independent function, and usage of RAS medications and insulin was shown in bold.

ability of variables that are significantly associated with PDR (age, T2DM duration, SBP, HBA1c, insulin usage and CKD), and combination of these variables with LRG1. We observed slightly improvement in AUC when LRG1 (0.84, 95% CI, 0.80–0.88) was added to the model consisting of the above variables (0.83, 95% CI, 0.79–0.88, $p = 0.11$).

3.3. Mediation effect between LRG1 and PDR

Because LRG1 was associated with PDR only, we examined whether PWV is a mediator for the association between LRG1 and PDR. Fig. 1 shows mediation analysis after adjustment for age, T2DM duration, SBP, HBA1c, insulin usage and CKD. There was significant association of LRG1 with PWV ($a = 0.29$, $p \ll 0.001$), and PWV with PDR ($b = 0.14$, $p = 0.002$). LRG1was significantly associated with PDR ($c = 0.34$, $p = 0.002$). When LRG1 and PWV were simultaneously included in the model, the association between LRG1 and PDR was attenuated, but remains significant ($c' = 0.30$, $p = 0.005$). The partial indirect effect through PWV can be expressed as ????/(???? + ??') = $(0.29 \times 0.14)/[(0.29 \times 0.14) + 0.30] = 12.0\%$. The bootstrap method estimated that the indirect, direct and total effects are 0.02 (95%CI, 0.002–0.04, $p = 0.03$), 0.16 (95%CI, 0.06–0.26, $p = 0.002$), and 0.18 (95%CI, 0.08–0.28, $p = 0.001$), individually.

4. Discussion

This is the first study to demonstrate that plasma LRG1 levels were significantly associated with PDR in T2DM patients after taking into account the effect of confounding factors. Further mediation analysis shows that central arterial stiffness partially mediated the association between LRG1 and PDR, accounting for 12% of the association.

LRG1 was shown to localize exclusively in the vasculature of various human tissues including the eye.[7] Previous studies have demonstrated higher LRG1 level in vitreous fluid from PDR than non-DR patients.[7,8] However, it is uncertain whether the increase in vitreous of LRG1 is the result of increased local production induced by hypoxia or leakage from the systemic circulation under diabetic conditions.[7] Measurement of LRG1 levels in vitreous fluid may have limited clinical application because vitreous fluid can only be obtained invasively in a subgroup of DR patients undergoing surgery as part of their clinical management. Thus, LRG1 level in easily and inexpensively collected samples, such as plasma, may represent a potential screening tool for DR.

To our knowledge, only one study has measured plasma level of LRG1 in a Chinese population ($n = 86$), including healthy controls, non-DR T2DM patients, NPDR, and PDR.[8] Compared with non-DR patients, there was slight increase in plasma LRG1 level in NPDR, and a significantly higher LRG1 level in PDR (1.4-fold).[8] Our result in a much large-scale cohort agrees very well with the finding, showing marginally significant increase of LRG1 in NPDR than non-DR, and a 1.4-fold increase of LRG1 in PDR than non-DR. Although both studies measured plasma LRG1 levels using ELISA, plasma level of LRG1 was different, possibly because of differences between the Chinese and our population in terms of BMI (24.6 vs. 27.8 kg/m^2) and fasting blood glucose (10.5 vs. 8.1 mmol/l). To date, all studies of vitreous and plasma LRG1 levels are cross-sectional, and therefore, it remains unclear whether changes in plasma LRG1 level represent cause or consequence of DR.

Although the exact mechanism underlying LRG1 and PDR is not clear, we proposed several possibilities based on our mediation analysis and previous reports. First, we found plasma level of LRG1 was associated with PWV,[9] and higher PWV was associated with the presence and severity of DR in our previous cross-sectional studies in SMART2D,[19] suggesting that arterial stiffness might have an etiologic implication in any stage of DR. However, it has not been fully clarified whether PWV acts as a confounder or as a mediator. Our study clarified the mediating role of PWV, the "gold-standard" measurement for arterial stiffness, in the relationship between LRG1 and PDR. Central arterial stiffness can aggravate systemic vasculopathy by propagating elevated systolic and pulse pressures forward, thereby accentuating global vascular injury. If this process affects other vascular-bed systemically, it may be potentially harmful to retinal microcirculation with consequential development of DR.[20] Second, enhanced angiogenesis is known as a feature of advanced DR, such a PDR. The process of angiogenesis is dependent on the dynamic balance between angiogenic stimulators and inhibitors.[21] In our study, we found higher plasma level of PEDF, an angiogenic inhibitor, in PDR (17.0 (13.8–21.4) ng/ml, $p \ll 0.001$, $n = 107$) and NPDR (16.3 (13.4–19.7) ng/ml, $p \ll 0.001$, $n = 270$) compared with non-DR patients (14.8 (12.1–18.0) ng/ml, $n = 810$). Our results suggests that higher level of PEDF occurs probably as a secondary response to counteract the activity of the angiogenic stimulators (such as LRG1); however, such anti-angiogenic effect may be limited to early stages like

NPDR.[22,23] Studies on other angiogenic factors, such as vascular endothelial growth factor, are needed to provide a more complete understanding on the relationships of LRG1, angiogenesis and PDR. Third, inflammation, an essential process interlinked with angiogenesis, is also implicated in any stage of DR, including PDR with neovascularization.[24] LRG1 has been proposed to be implicated in the elevation of inflammatory pathways, possibly through induction of other pro-inflammatory cytokines.[25,26] Future studies involving cytokine analysis will be needed to clarify the regulatory mechanisms of LRG level in PDR patients.

In this study, we also demonstrated that age, duration of diabetes, SBP, HbA1c, usage of insulin and CKD at baseline are also risk factors for DR, which agrees with previous studies among Asians with T2DM.[27–29] The use of insulin therapy reflects insufficient β-cell insulin secretory response and is also often a proxy for disease duration and burden among T2DM patients.[30] Previous studies have consistently demonstrated the associations of worse kidney function with increased risk of DR among T2DM patients, suggesting that DR and CKD may progress in a parallel manner.[10] We also observed significantly increased CKD with DR severity (36.5% vs. 65.6% vs.83.2% for non-DR vs. NDR vs. NPDR, $p \ll 0.001$), suggesting that DR is a proxy for total disease burden in T2DM patients. Taken together, T2DM patients with usage of insulin, worse kidney function, and poor blood pressure and glycemic control, deserve global vascular risk factors reduction.

Our findings have potentially important clinical implications. First, early detection through eye examination is key to successful treatment for DR. Given the current recommendation for diabetic patients is annual dilated retinal examination as the standard of care, only 24.7% diabetic patients with visual symptoms attended annual eye examination in Singapore.[31] Screening for PDR by biomarkers, such as LRG1, may be a simple and inexpensive tool for screening and treatment. Second, the discovered mediating effect may offer new opportunities for clinical interventions to reduce PDR risk by intervention at the modifiable mediator. For example, amelioration of arterial stiffness through life-style modification (i.e., smoking, salt intake) or pharmacotherapy, may contribute to reduction in advanced blinding stages of DR. Thirdly, Wang et al. have demonstrated reduced lesion sizes of choroidal neovascularization by antibody blockade of LRG1 in mouse model. It is a strong experimental evidence that LRG1 is a potential therapeutic target for controlling angiogenesis in DR.[7]

The strengths of this study include accurate DR status obtained by fundus imaging, complete patient information, and a prospective study design. Compared with DR status obtained by self-report and fundus imaging, the overall accuracy of self-report DR status is 73.2% and 74.6% in our and others studies.[32] Previous studies with small sample size and incomplete patient information were unable to examine the association with statistical adjustment. In our study, problems of inaccurate or incomplete data could be overcome. The prospective study design allows better inference about mediation.

Several limitations have also been identified. First, our findings should be interpreted with caution because of small sample size for PDR patients. We did not observe significant improvement in the risk prediction for PDR when LRG1 was incorporated in the risk prediction model, possibly because of the limited sample size in PDR patients. Second, we captured non-mydriatic single-field but not ultra-widefield fundus photographs, and therefore it is possible that some cases of PDR were not detected. Finally, our study was based on diabetes patients in a secondary hospital, whose conditions may be different from diabetes patients at national level. For example, Singapore National Health Survey in 2010 reports better profiles than ours, such as lower fasting plasma glucose level and less poor glycemic control. Therefore, whether our findings can be extrapolated to a national level remains to be determined.

In summary, our results demonstrated that higher plasma level of LRG1 were significantly associated with PDR in T2DM patients, suggesting that LRG1 may be used as a potential biomarker for PDR. The associations were partially mediated by PWV, suggesting that amelioration of central arterial stiffness may contribute to prevention of PDR.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jdiacomp.2019.05.021.

Declaration of Competing Interest

No.

Acknowledgements

This work was supported by Singapore National Medical Research Council Grant [PPG/AH(KTPH)/2011] and [NMRC/CIRG/1398/2014]. The funder has no role in study design, data collection, analysis, interpretation and manuscript writing.

References

1. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004;27:1047-53.
2. Phan TP, Alkema L, Tai ES, et al. Forecasting the burden of type 2 diabetes in Singapore using a demographic epidemiological model of Singapore. BMJ Open Diabetes Res Care 2014;2, e000012.
3. Klein BE. Overview of epidemiologic studies of diabetic retinopathy. Ophthalmic Epidemiol 2007;14:179-83.
4. Cheung N, Mitchell P, Wong TY. Diabetic retinopathy. Lancet 2010;376:124-36.
5. Zheng Y, Ley SH, Hu FB. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. Nat Rev Endocrinol 2018;14:88-98.
6. Lee R, Wong TY, Sabanayagam C. Epidemiology of diabetic retinopathy, diabetic macular edema and related vision loss. Eye and vision 2015;2:17.
7. Wang X, Abraham S, McKenzie JAG, et al. LRG1 promotes angiogenesis by modulating endothelial TGF-beta signalling. Nature 2013;499:306-11.
8. Chen C, Chen X, Huang H, et al. Elevated plasma and vitreous levels of leucine-rich-alpha2-glycoprotein are associated with diabetic retinopathy progression. Acta Ophthalmol 2019;97:260-4.
9. Pek SL, Tavintharan S, Wang X, et al. Elevation of a novel angiogenic factor, leucine-rich-alpha2-glycoprotein (LRG1), is associated with arterial stiffness, endothelial dysfunction, and peripheral arterial disease in patients with type 2 diabetes. J Clin Endocrinol Metab 2015;100:1586-93.
10. Rodriguez-Poncelas A, Mundet-Tuduri X, Miravet-Jimenez S, et al. Chronic kidney disease and diabetic retinopathy in patients with type 2 diabetes. PLoS One 2016;11, e0149448.
11. Chen YW, Wang YY, Zhao D, et al. High prevalence of lower extremity peripheral artery disease in type 2 diabetes patients with proliferative diabetic retinopathy. PLoS One 2015;10, e0122022.
12. Liu JJ, Pek SLT, Ang K, Tavintharan S, Lim SC. Study SD. Plasma leucine-rich alpha-2-glycoprotein 1 predicts rapid eGFR decline and albuminuria progression in type 2 diabetes mellitus. J Clin Endocrinol Metab 2017;102:3683-91.
13. Liu JJ, Tavintharan S, Yeoh LY, et al. High normal albuminuria is independently associated with aortic stiffness in patients with type 2 diabetes. *Diabetic medicine: a journal of the*. British Diabetic Association 2014;31:1199-204.
14. Wilkinson CP, Ferris 3rd FL, Klein RE, et al. Proposed international clinical diabetic retinopathy and diabetic macular edema disease severity scales. Ophthalmology 2003;110:1677-82.
15. Rognant N, Lemoine S, Laville M, Hadj-Aissa A, Dubourg L. Performance of the chronic kidney disease epidemiology collaboration equation to estimate glomerular filtration rate in diabetic patients. Diabetes Care 2011;34:1320-2.
16. Kidney Disease KIDGO. Improving global outcomes (KDIGO) CKD work group. KDIGO 2012 clinical practice guideline for the evaluation and management of chronic kidney disease. Kidney Int Suppl 2013;3:1-150.
17. Baron RM, Kenny DA. The moderator-mediator variable distinction in social psychological research: conceptual, strategic, and statistical considerations. J Pers Soc Psychol 1986;51:1173-82.
18. MacKinnon DP, Fairchild AJ, Fritz MS. Mediation analysis. Annu Rev Psychol 2007;58:593-614.
19. Zhang X, Neelam K, Low S, et al. Central arterial stiffness is associated with the presence and severity of diabetic retinopathy among Asians with type 2 diabetes mellitus. Singapore Med J 2016;57:S50.
20. Burgansky-Eliash Z, Nelson DA, Bar-Tal OP, Lowenstein A, Grinvald A, Barak A. Reduced retinal blood flow velocity in diabetic retinopathy. Retina 2010;30:765-73.
21. Abu El-Asrar AM, Nawaz MI, Kangave D, Siddiquei MM, Ola MS, Opdenakker G. Angiogenesis regulatory factors in the vitreous from patients with proliferative diabetic retinopathy. Acta Diabetol 2013;50:545-51.
22. Abu El-Asrar AM, Imtiaz Nawaz M, Kangave D, Siddiquei MM, Geboes K. Osteopontin and other regulators of angiogenesis and fibrogenesis in the vitreous from patients with proliferative vitreoretinal disorders. Mediators Inflamm 2012;2012, 493043.
23. Yoshida Y, Yamagishi S, Matsui T, et al. Protective role of pigment epithelium-derived factor (PEDF) in early phase of experimental diabetic retinopathy. Diabetes Metab Res Rev 2009;25:678-86.
24. Capitao M, Soares R. Angiogenesis and inflammation crosstalk in diabetic retinopathy. J Cell Biochem 2016;117:2443-53.

25. Fujimoto M, Serada S, Suzuki K, et al. Leucine-rich alpha2 -glycoprotein as a potential biomarker for joint inflammation during anti-interleukin-6 biologic therapy in rheumatoid arthritis. Arthritis Rheumatol 2015;67:2056-60.
26. Ha YJ, Kang EJ, Lee SW, et al. Usefulness of serum leucine-rich alpha-2 glycoprotein as a disease activity biomarker in patients with rheumatoid arthritis. J Korean Med Sci 2014;29:1199-204.
27. Cai XL, Wang F, Ji LN. Risk factors of diabetic retinopathy in type 2 diabetic patients. Chin Med J (Engl) 2006;119:822-6.
28. Xie XW, Xu L, Wang YX, Jonas JB. Prevalence and associated factors of diabetic retinopathy. The Beijing Eye Study 2006 Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie 2008;246:1519-26.
29. Zhong ZL, Han M, Chen S. Risk factors associated with retinal neovascularization of diabetic retinopathy in type 2 diabetes mellitus. Int J Ophthalmol 2011;4:182-5.
30. Nathan DM, Buse JB, Davidson MB, et al. Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: a consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes. Diabetes Care 2009;32:193-203.
31. Huang OS, Tay WT, Ong PG, et al. Prevalence and determinants of undiagnosed diabetic retinopathy and vision-threatening retinopathy in a multiethnic Asian cohort: the Singapore Epidemiology of Eye Diseases (SEED) study. Br J Ophthalmol 2015;99:1614-21.
32. Lin CC, Lai MS, Syu CY, Chang SC, Tseng FY. Accuracy of diabetes diagnosis in health insurance claims data in Taiwan. J Formos Med Assoc 2005;104:157-63.