Soluble form of LR11 is highly increased in the vitreous fluids of patients with idiopathic epiretinal membrane

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Abstract

Purpose LR11 (also called SorLA or SORL1) is a migration regulator of adherent cells with the immature proliferative phenotype. The present study investigated the clinical and pathological involvements of the soluble form of LR11 (sLR11) in idiopathic epiretinal membrane (iERM).

Methods The subjects were 51 patients with iERM (24 cellophane macular reflex (CMR) and 27 preretinal macular fibrosis (PMF)) and 45 patients with macular hole as age and sex-matched controls. Vitreous sLR11 and TGFβ2 levels were measured by ELISA.

Results The sLR11 levels in the vitreous fluids of patients with iERM (20.2 ± 8.1 ng/mL) were significantly higher than those in controls (11.4 ± 4.7 ng/mL). Among the patients with iERM, the vitreous sLR11 levels were significantly higher in PMF (23.6 ± 8.2 ng/mL), than those in CMR (16.5 ± 5.9 ng/mL). Multivariate regression analysis of the studied factors showed that sLR11 was a unique factor independently contributing to the discrimination of the iERM patients against the control subjects (odds ratio [OR] 1.35 per 1-ng/mL increase, 95% CI 1.09-1.67; P = 0.004). ROC analysis showed that the sensitivity and the specificity of sLR11, but not of other studied factors, categorized into the rank of moderate accuracy. Finally, there was a positive correlation (R = 0.588; P = 0.003) between the vitreous levels of sLR11 and TGFβ2 using the available samples.

Conclusions sLR11 levels in vitreous fluids were specifically increased in patients with iERM, suggesting the involvement in the pathology of proliferative and migrating cells for the development of iERM.

Keywords Soluble LR11; Idiopathic epiretinal membrane; Vitreous fluids; Transforming growth factorβ2; Vitreous surgery

Introduction

Epiretinal membrane (ERM), a disease with proliferative ectopic tissues of non-fibrovascular origins, is caused idiopathically or subsequently with diabetic retinopathy, retinal detachment, uveitis or retinal vein occlusion [1]. Idiopathic ERM (iERM) is often observed with posterior vitreous detachment (PVD) from the internal limiting membrane, in which the proliferative and migrating cells of heterogeneous origins, such as glial cells, retinal pigment epithelial cells, fibroblasts, myofibroblasts, retinal endothelial cells and hyalocytes, have been immunohistochemically identified with the extracellular matrices (ECMs) secreted from the cells [1-4]. The enhanced proliferation and migration of the above pathological cells have been reported to be activated by various cytokines and physiological factors [3,5,6]. Although several studies have identified transforming growth factor (TGF) β2 as a key cytokine for the formation of the pathological membranes in iERM [7-9], the molecular mechanism underlying the excessive activation of heterogeneous cells remains not fully understood.

LDL receptor-relative with 11 ligand-binding repeats (LR11; also known as SorLA or SORL1) is a type I membrane protein that plays a key role in the migration of immature vascular smooth muscle cells [10]. Significantly, a large soluble extracellular part of LR11, sLR11, is released from cell surface by proteolytic shedding, and the concentrations in body fluids can be quantitated by ELISA [11]. The measurements of sLR11 in various diseases revealed that the circulating sLR11 levels were increased in atherosclerotic diseases, and thus the levels reflected the pathological states of migrating and proliferating immature adherent cells in atheroma [12-15].

In ophthalmologic proliferating diseases, the sLR11 levels in vitreous fluids, as well as in the sera, were increased in the patients with proliferative diabetic retinopathy (PDR), and also those with non-PDR (NPDR) [16]. Thus, the vitreous sLR11 levels may reflect the pathological conditions of undifferentiated immature adherent cells migrated into vitreous space from unknown ocular origins.

Based on the above background, we constructed a hypothesis that vitreous sLR11 levels may be involved in the pathologically migrating and proliferating cells important for abnormal membrane formations in patients with iERM. In this study, we first evaluated the vitreous sLR11 levels in patients with iERM, in comparison to those in patients with macular hole (MH) as age- and gender- matched controls. Subsequently, the characteristics of vitreous sLR11 in the patients with iERM were analyzed by uni-variable and multi-regression association analyses with other clinical factors among all patients.

Methods

Sample Collection

Fifty-one patients with iERM (24 cellophane macular reflex (CMR) and 27 preretinal macular fibrosis (PMF)), who have been treated by vitreous surgery at Toho University Sakura Medical Center in the period between June 2009 and August 2013, were recruited in this study. CMR and PMF were diagnosed by the fundus camera view, and graded using the same protocols and graders as previously reported [17,18]. Exclusion criteria included a history of glaucoma, diabetic retinopathy, uveitis, or retinal vein

occlusion. Forty-five patients with macular hole (MH), who have been treated by vitreous surgery, were recruited as age- and gender- matched controls. Vitreous fluids were obtained from patients at the vitreous surgery, and immediately frozen at -80□ until use. All procedures were in full compliance with the guidelines of the Declaration on Helsinki, and were approved by the institutional review Board/Ethics Committee of Toho University Sakura Medical Center.

Measurement of sLR11 and TGFβ2 Levels in Vitreous Fluids

sLR11 levels in the vitreous fluids were determined by sandwich ELISA (Sekisui Medical, Ryugasaki, Japan) [11]. Briefly, samples of vitreous fluid (10 μL) diluted with sample buffer were reacted with the capture monoclonal antibody (mAb) M3, and then incubated with the biotinylated reporter mAb R14. The LR11-mAb complex was reacted with horseradish-peroxidase-conjugated streptavidin. A standard curve was constructed using a purified sLR11 protein (Sekisui Medical, Ryugasaki, Japan). The vitreous sLR11 levels were compared between patients with iERM and controls, and between patients with CMR and those with PMF. The TGFβ2 levels in the vitreous fluid were determined by sandwich ELISA (R&D Systems, Minneapolis, MN).

Statistics

The results are shown as means ± standard deviation or proportion (%) for each index. The statistical analyses were performed using SPSS (SPSS Inc, Chicago, IL). The following factors were analyzed as clinical and laboratory characteristics of patients

with iERM and MH: male (%), age (y), body mass index (kg/m^2), estimated glomerular filtration rate (eGFR, mL/minute per 1.73m^2), fasting plasma glucose (mg/dL), total cholesterol (mg/dL), creatinine (mg/dL), albumin (mg/dL), TGFβ2 (pg/mL), sLR11 (ng/mL), and proportions (%) of diabetes mellitus, hypertension or hyperlipidemia. Hypertension was defined as systolic pressure of more than 140 mmHg or diastolic pressure of more than 90 mmHg, or use of antihypertensive agents. Diabetes mellitus was defined as a fasting plasma glucose level of more than 126 mg/dL, glycosylated hemoglobin (HbA1c) of more than 6.5 %, or both. Hyperlipidemia was defined as a total cholesterol level of more than 220 mg/dL or case of hyperlipidemia treatment. Comparisons between groups were performed using the unpaired t-test, or Yates 2×2 chi square test between groups. Logistic regression analysis was used to determine independent factors for iERM. Sensitivity and specificity with respect to the presence of iERM was analyzed using conventional receiver operating characteristics (ROS) curve. Pearson's correlation coefficient analysis was used to assess the associations between measured parameters. Multiple regression analysis was performed to assess the influence of independent variables on the vitreous levels of sLR11. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Clinical and Laboratory Characteristics of Patients with iERM

The clinical and laboratory characteristics of fifty-one patients with idiopathic iERM （PMF or CMR） were compared with those of forty-five patients with MH as age- and

gender-matched controls (Table 1). There were no significant differences in body mass index, proportions of diabetes mellitus, hypertension and hyperlipidemia, eGFR, fasting plasma glucose, total cholesterol, creatinine, and albumin between the patients with iERM and the controls. The levels of TGFβ2 in the available twenty-three vitreous fluids were significantly higher in patients with iERM (n=16), than in controls (n=7). Among the patients with iERM, there were also no differences between twenty-seven patients with PMF and twenty-four patients with CMR in gender, age, PVD, body mass index, proportions of diabetes mellitus, hypertension and hyperlipidemia, eGFR, fasting plasma glucose, total cholesterol, creatinine, and albumin (Table 2). The levels of central macular thickness and TGFβ2 in the available sixteen vitreous fluids were both significantly higher in the patients with PMF than those with CMR.

Vitreous sLR11 Levels were Increased in Patients with iERM, and Particularly in Those with PMF

The sLR11 levels in the vitreous fluids of the patients with iERM were 20.2 ± 8.1 ng/ml, and significantly higher than those in controls (11.4 ± 4.7 ng/mL) (Table 1). Furthermore, among the patients with iERM, the vitreous fluid sLR11 levels in patients with PMF (23.6 ± 8.2 ng/mL) were significantly higher than those in patients with CMR (16.5 ± 5.9 ng/mL) (Table 2). Thus, sLR11 levels in vitreous fluids were increased in patients with iERM, particularly in the severely proliferating subtype, PMF.

Logistic Regression and Roc Analyses Identified sLR11 as an Independent Risk

Factor Discriminating iERM

We next performed a logistic regression analysis to know factors independently contributing to the discrimination of patients with iERM against controls (Table 3). Among all characteristic factors (see Table 1) as explanatory variables, increase in sLR11, but not other factors, was significantly correlated with the probability of iERM. ROC analysis for discriminating the probability of patients with iERM against controls showed that the cutoff level of sLR11 that gave the maximum sensitivity (0.82) and specificity (0.83) for iERM was 12.7 (Table 4 and Figure 1). As a result, the area under the curve (AUC) of sLR11 (82%) was ranked in the range of moderate accuracy (1.09 to 1.67). On the other hand, the AUCs of age (64%), eGFR (43%), total cholesterol (45%) or fasting plasma glucose (48%) at the cutoff levels that gave the maximum sensitivity and specificity for iERM were not high enough for the moderate accuracy.

Vitreous sLR11 Levels were Independently Correlated with Ages, and not with Other Characteristics, among All Subjects

The above results indicated that the vitreous sLR11 level was indicative of the pathological conditions of iERM, independent from other clinical and laboratory factors. We therefore analyzed the relationships of sLR11 with other factors among all subjects. Previous studies have reported that serum sLR11 levels were associated with the levels of low density lipoprotein (LDL)-cholesterol, HbA1c and albumin in patients with diabetic retinopathy [12,16]. Pearson's correlation analysis of dependent variables showed that the vitreous sLR11 levels were positively correlated with ages, and the

levels in subjects with hyperlipidemia were significantly higher than those without hyperlipidemia (Table 5). In addition, the vitreous sLR11 levels also showed positive and negative correlation tendencies with the values of serum albumin and eGFR, respectively. Subsequent multiple regression analysis among the above four factors showed that age was only an independent variable to determine vitreous sLR11 levels in the subjects (Table 6), as shown in previous studies among atherosclerotic patients [14, 19]. Thus, sLR11, an independent risk factor for iERM, was not associated with other clinical or biochemical factors without age in the study subjects.

Vitreous sLR11 Levels were Associated with Vitreous TGFβ2 Levels

Finally, in order to know the pathological significance of the increased sLR11 levels in the vitreous fluids of patients with iERM, we analyzed the relationships between the vitreous levels of sLR11 and TGFβ2, in twenty-three samples available from all subjects (Figure 2). There was a significant and positive correlation between the levels of sLR11 and TGFβ2 (R = 0.588; P = 0.003) among the samples. These results suggested that sLR11, a molecule released from the proliferating immature vascular cells [12-15] may be also involved in the mechanism underlying TGFβ2-mediated formation of the pathological membranes in iERM.

Discussion

The present study showed several statistical findings concerning the role of sLR11 in the vitreous fluids in the pathogenesis of iERM. Firstly, sLR11 levels in the vitreous

fluid of patients with iERM, particularly with the proliferative subtype, PMF, were significantly higher than those of the age- and gender-matched controls. Second, multivariate analysis revealed that sLR11 was a risk factor correlated with the probability of iERM, independent from other clinical characteristics including age, diabetes mellitus and hypertension. ROC analysis for discriminating the probability of patients with iERM against the controls showed that the sensitivity and the specificity of sLR11, but not those of other studied factors, were high enough to be categorized into the rank of moderate accuracy. Finally, there was a positive correlation between the levels of sLR11 and TGFβ2 in the vitreous fluids using the available samples among all subjects.

The risk factors for iERM have been extensively studied in heterogeneous populations. Among them, age, hyperlipidemia and diabetes mellitus have been shown to be potent risks in a multi-ethnic United States population [18]. The Hisayama study showed that age and serum cholesterol levels were the relevant risk factors for ERM in the Japanese population [20]. Kawasaki, et al. has reported that age and diabetes mellitus were the two representative risk factors for the development of ERM in another Japanese population [21]. In our study, multivariate analysis revealed that the probability of iERM was not correlated with age, hyperlipidemia, diabetes mellitus, fasting plasma glucose or total cholesterol, among the investigated risk factors including sLR11 (see Table 3). ROC analysis showed that the AUC values for age, total cholesterol and fasting plasma glucose were obviously low in comparison to those of sLR11 in the studied subjects (see Table 4 and Figure 1). Interestingly, subsequent Pearson's

correlation analysis with the followed multiple regression analysis showed that sLR11 was correlated with age and dyslipidemia. These results suggested that sLR11 was a novel factor indictable of the possibility of iERM, and may be involved in the associations of the previously established risks (ex. age, hyperlipidemia or diabetes mellitus) with the development of iERM. In this context, several studies have shown that the circulating sLR11 levels were increased in patients with hyperlipidemia [19] and diabetes mellitus [12,16]. There were also previous studies showing the positive correlation of circulating sLR11 with age among atherosclerotic patients [14,19,22].

PVD is one of triggers for the development of iERM [2], and the detachment causes micro-injury in the internal limiting membrane (ILM) and, in response, glial and other cells start migration through the retinal surface to the injured regions in ILM [1,2,23].

In PVD case, residual vitreous cortex that is the posterior wall of the premacular pocket serves as a framework for cellular proliferation [24]. Even in case of no PVD, posterior wall of the premacular pocket serve as a collagen sheet for iERM. The proliferated cell may modify the nature of the iERM [24]. In the process of formation of iERM, several cytokines and growth factors including TGFβ, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and nerve growth factor (NGF), have been identified as promoting factors for the increased migration and/or proliferation of heterogeneous cells [7-9,25,30]. Among them, TGFβ is believed to play an important role in the intraocular fibrosis [7,26,27] and regulates the proliferation, migration and, furthermore, trans-differentiation of retinal muller cells [28]. Recent studies have shown that a complex ECM network including several types

of collagens promotes the migration and the proliferation of epiretinal cells in the pathological membranes [29-31]. TGFβ has been again suspected to be involved in the role of pathological ECM network in the development of iERM [32]. The levels of TGFβ have been shown to be indeed increased in the vitreous fluids of patients with iERM [7, 33]. In this context, sLR11 has been recently reported to inhibit the activation of smad 1, 3, 5 and 8, as the downstream signals mediated by the BMP receptor [22], a member of the TGFβ receptor superfamily. Increased sLR11 levels in the vitreous fluids may be involved in the pathogenesis of fibrous membrane formation in association with the TGFβ2 signals through the regulation of common smad signals.

Limitation of the present study was at first the lack of histocytochemical analysis for the identification of LR11 in the membrane pathology for patients with iERM. Additional pathological experiments are needed to clarify the involvement of sLR11 in the pathogenesis of iERM. Second, the patient number was not enough for the extensive statistical analysis using the clinical stages or complications in patients with iERM. Finally, the association analysis between sLR11 and TGFβ2 was performed in the restricted samples available from all study subjects. Further analyses are essentially needed to investigate the causal relationship of sLR11 with other previously reported cytokines and growth factors important for the pathology of iERM, in addition to TGFβ2, in future.

In summary, the present study has shown that an increase in vitreous sLR11 level was a novel and specific discriminating factor for iERM, thereby sLR11 may play a pathological role in the abnormal membrane formation through the migration and

proliferation of heterogeneous cells in patients with iERM.

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Compliance with ethical standards All procedures performed in studies involving human participants were in accordance with the institutional review board of Toho University Sakura Medical Center (No.S16036) and with the 1964 Helsinki declaration. Informed consent was obtained from all individual participants included in the study.

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Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speaker’s bureaus; membership, employment,

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Figure legends

Figure 1.

Receiver operating characteristic curve for discriminating the probability of patients with iERM from controls based on vitreous levels of soluble form of LR11 (sLR11), age, fasting plasma glucose (FPG), total cholesterol (TC) and estimated glomerular filtration rate (eGFR). The curves show the fraction of true-positive results (sensitivity) and false-positive results (1-specificity) for various cutoff levels of each parameter.

Figure 2.

Correlation between vitreous levels of sLR11 and TGFβ2 in twenty-three samples available from all subjects. Statistical differences for the correlation between groups were calculated by Pearson's correlation analysis.

Table 1 Clinical and Laboratory Characteristics of Patients with iERM and Controls

	iERM	Control	*p*
No.	51	45	-
Male (%)	68.2	69.0	0.53
Age (y)	67.6 ± 8.9	65.6 ± 5.4	0.19
Body mass index (kg/m^2)	23.1 ± 3.3	21.9 ± 2.4	0.42
Diabetes mellitus (%)	9.8	6.67	0.72
Hypertension (%)	45.1	33.3	0.30
Hyperlipidemia (%)	21.6	21.6	1.00
eGFR (mL/minute per 1.73m^2)	68.2 ± 19.3	72.3 ± 14.7	0.44
Fasting plasma glucose (mg/dL)	114.8 ± 36.2	108.9 ± 15.3	0.30
Total cholesterol (mg/dL)	209.2 ± 29.9	220.6 ± 39.7	0.12
Creatinine (mg/dL)	0.76 ± 0.19	0.73 ± 0.22	0.60
Albumin (mg/dL)	4.60 ± 0.49	4.52 ± 0.26	0.15
TGFβ2（pg/mL）	339.1 ± 41.9	297.1 ± 24.9	0.03
sLR11 (ng/mL)	20.2 ± 8.1	11.4 ± 4.7	< 0.001

iERM idiopathic epiretinal membrane, *MH* macular hole, *eGFR* estimated glomerular filtration rate, *TGFβ2* transforming growth factor β2, *sLR11* soluble form of LR11.

The values of age, body mass index, eGFR, fasting plasma glucose, total cholesterol, creatinine, albumin and TGFβ2 between patients with iERM and controls were statistically analyzed by unpaired t-test. The proportions of male, diabetes mellitus, hypertension, hyperlipidemia between patients with iERM and controls were analyzed by 2×2 chi square test. The levels of TGFβ2 in vitreous fluids were measured in the available sixteen patients with iERM and those with seven controls.

Table 2 Clinical and Laboratory Characteristics of Patients with PMF and Those with CMR

	PMF Group	CMR Group	*p*
No.	27	24	-
Male (%)	70.1	69.0	0.78
Age (y)	67.4 ± 10.7	67.9 ± 6.6	0.84
Central macular thickness（μm）	505.6 ± 69.0	399.5 ± 64.1	< 0.001
Posterior vitreous detachment (%)	91.7	85.1	
Body mass index (kg/m^2)	22.4 ± 2.97	23.8 ± 3.9	0.43
Diabetes mellitus (%)	11.1	8.6	1.00
Hypertension (%)	37	54.1	0.26
Dyslipidemia (%)	18.5	22.2	0.73
eGFR (mL/minute per 1.73m^2)	71.8 ± 15.3	64.1 ± 23.4	0.26
Fasting plasma glucose (mg/dL)	110.7 ± 25.9	119.7 ± 46.3	0.39
Total cholesterol (mg/dL)	214.5 ± 28.9	202.9 ± 31.2	0.18
Creatine (mg/dL)	0.76 ± 0.2	0.75 ± 0.2	0.84
Albumin (mg/dL)	4.72 ± 0.6	4.56 ± 0.2	0.25
TGFβ2（pg/mL）	364.8 ± 30.8	312.1 ± 34.5	0.009
sLR11 (ng/mL)	23.6 ± 8.2	16.5 ± 5.9	0.001

PMF preretinal macular fibrosis, *CMR* cellophane macular reflex, *eGFR* estimated glomerular filtration rate, *TGFβ 2* transforming growth factorβ 2, *sLR11* soluble form of LR11.

The values of age, body mass index, eGFR, fasting plasma glucose, total cholesterol, creatinine, albumin, TGFβ2, central macular thickness between patients with PMF and those with CMR were statistically analyzed by unpaired t-test. The proportions of male, diabetes mellitus, hypertension, hyperlipidemia between patients with PMF and those with CMR were analyzed by 2×2 chi square test. The levels of TGFβ2 in vitreous fluids were measured in the available eight patients with PMF and those with eight CMR.

Table 3 Results of Multivariate Analysis Investigating Risk Factors for iERM (n = 96)

	Odds Ratio (95% Confidence Interval)	*P*
sLR11, per 1-ng/ml increase	1.35 (1.09 to 1.67)	0.004
Male	0.84 (0.08 to 8.3)	0.88
Age, per 1-y increase	1.12 (0.93 to 1.35)	0.25
Body mass index, per 1-kg/m^2 increase	1.39 (0.75 to 2.59)	0.31
Diabetes mellitus, per 1% increase	0.79 (0.09 to 67.4)	0.80
Hypertension, per 1% increase	9.56 (0.94 to 96.9)	0.06
Hyperlipidemia, per 1% increase	0.22 (0.03 to 1.51)	0.12
eGFR per-1U (mL/minute per 1.73m^2) increase	1.02 (0.97 to 1.06)	0.43
Fasting plasma glucose, per-1U (mg/dL) increase	1.02 (0.99 to 1.01)	0.26
Total cholesterol, per 1- mg/dL increase	0.99 (0.96 to 1.01)	0.25
Creatinine, per 1- mg/dL increase	5.48 (0.04 to 68.8)	0.61
Albumin, per 1- mg/dL increase	0.56 (0.03 to 8.6)	0.56

iERM idiopathic epiretinal membrane, *sLR11* soluble form of LR11, *eGFR* estimated glomerular filtration rate.

Table 4 ROC Analysis Investigating Cutoff Values for Discriminating the Probability of Patients with iERM against Controls

	Cutoff	Sensitivity	Specificity	AUC (%)
sLR11 (ng/mL)	12.7	0.82	0.83	82
Age (y)	66.5	0.73	0.50	64
eGFR (mL/minute per 1.73m^2)	79.5	0.32	0.67	43
TC (mg/dL)	202.0	0.56	0.50	45
FPG (mg/dL)	99.5	0.71	0.61	48

ROC receiving operating characteristic curve, *AUC* area under the receiver operating characteristic curve, *iERM* idiopathic epiretinal membrane, *sLR11* soluble form of LR11, *eGFR* estimated glomerular filtration rate, *TC* total cholesterol, *FPG* fasting plasma glucose.

Table 5 Pearson's Correlation Analysis of Vitreous sLR11 with Other Parameters in the Study Subjects

	r or Mean ± SD	*P*
Male	16.81±8.3/15.66±7.9	0.53
Age (y)	0.220	0.03
Body mass index (kg/m^2)	-0.028	0.90
Diabetes mellitus, yes/no	18.28 ± 9.2 / 20.45 ± 7.8	0.51
Hypertension, yes/no	18.72 ± 7.9 / 21.58 ± 7.8	0.62
Hyperlipidemia, yes/no	23.72 ± 6.8 / 19.27 ± 8.0	0.04
eGFR (mL/minute per 1.73m^2)	-0.233	0.09
Fasting plasma glucose (mg/dL)	0.006	0.95
Total cholesterol (mg/dL)	-0.05	0.63
Creatinine (mg/dL)	0.133	0.20
Albumin (mg/dL)	0.177	0.09

sLR11 soluble form of LR11, *eGFR* estimated glomerular filtration rate.

Table 6 Multiple Regression Analysis of Vitreous sLR11 with Other Parameters in the Study Subjects

	β	*P*
Age (y)	0.400	0.008
Hyperlipidemia, yes/no	-0.070	0.63
eGFR (mL/minute per 1.73m^2)	-0.147	0.30
Albumin (mg/dL)	0.003	0.98

sLR11 soluble form of LR11, *eGFR* estimated glomerular filtration rate.

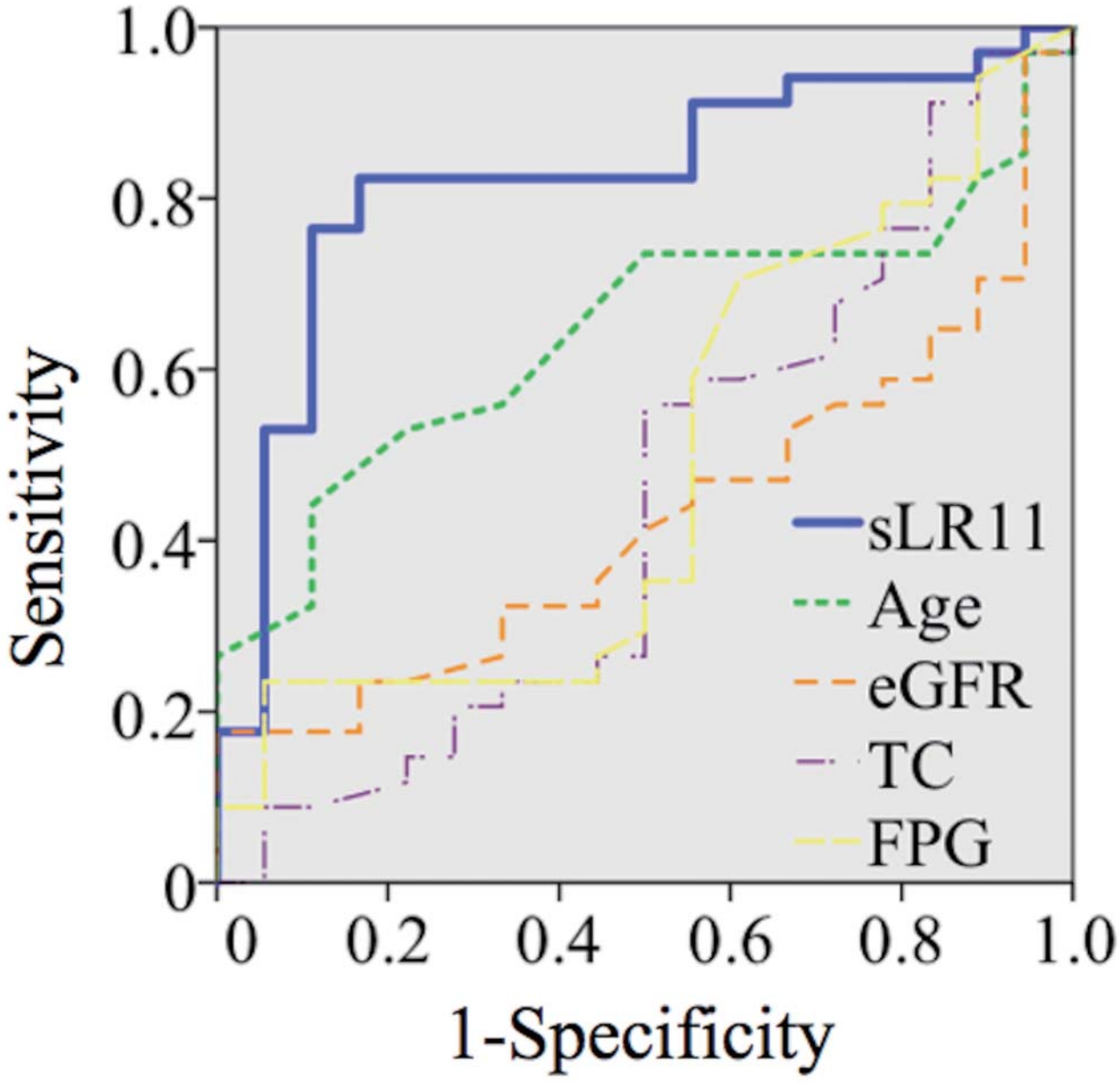
1.0
0.8
0.6
0.4
0.2
0
Sensitivity
0
0.2
0.4
0.6
0.8
1.0
1-Specificity
sLR11
Age
eGFR
TC
FPG

Figure 1

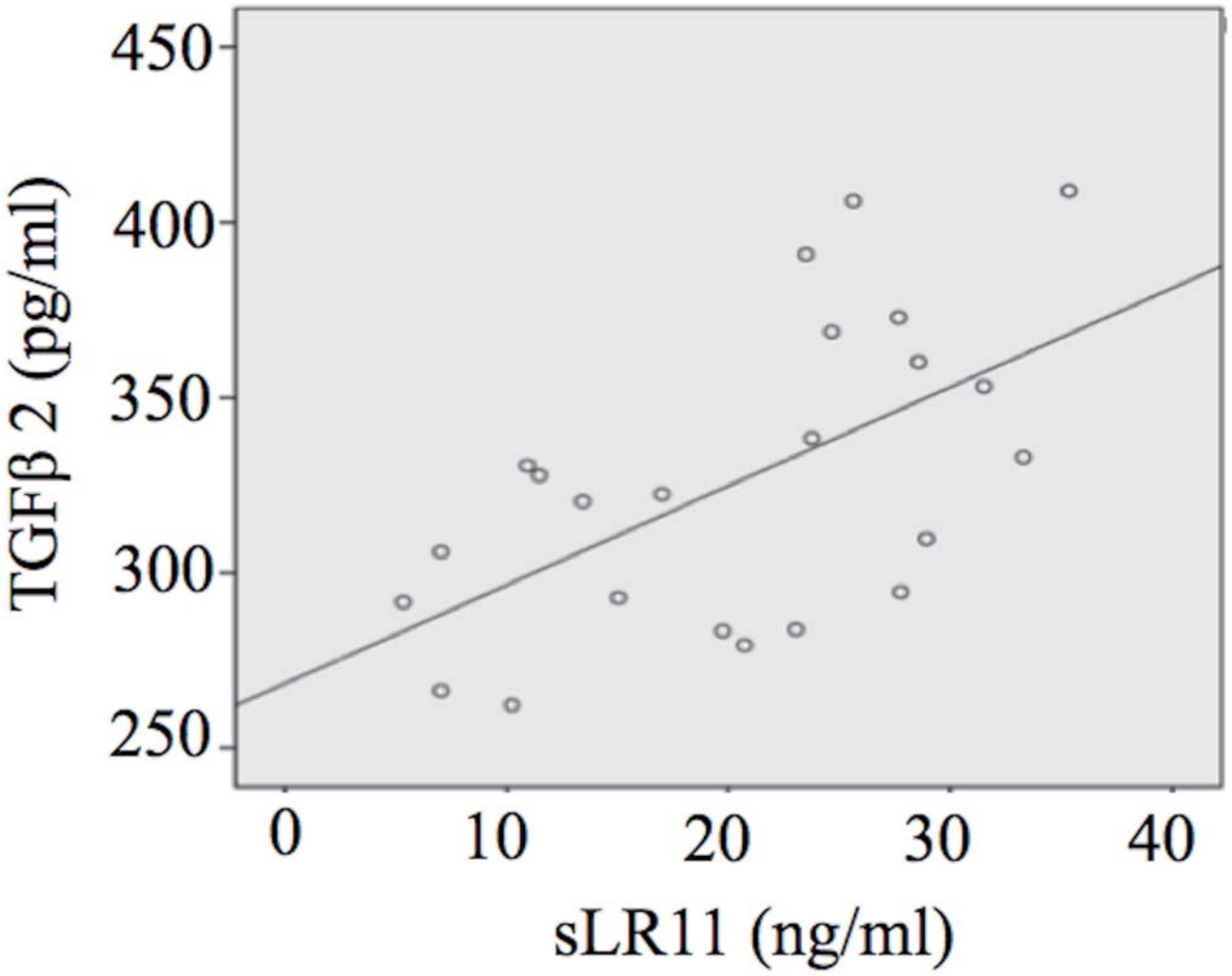

Figure 2