Table 2
Multivariate assessment of the effect of sLR11 and other risk factors on OCS BMI, sLR11 and age were analyzed per 0.1 kg/m², 0.1 U, and 10 y increase, respectively. Models 1 and 2 are described in Methods. BMI: Body mass index.

	OR (95% CI)	*p* values
Model 1		
BMI, per 0.1 kg/m² increase	1.00 (0.99–1.02)	0.85
Diabetes	7.98 (2.29–27.77)	<0.01
Dyslipidemia	4.03 (1.61–10.08)	<0.01
Male	1.48 (0.54–4.07)	0.44
Hypertension	1.53 (0.67–3.48)	0.31
Smoking	2.14 (0.88–5.21)	0.09
sLR11, per 0.1 U increase	1.03 (1.00–1.05)	<0.01
Age, per 10 y increase	1.15 (0.72–1.82)	0.93
Model 2		
BMI, per 0.1 kg/m² increase	1.04 (0.91–1.15)	0.57
Diabetes	6.36 (1.97–20.51)	<0.01
Dyslipidemia	4.55 (1.86–1.61)	<0.01
sLR11, per 0.1 U increase	1.02 (1.00–1.04)	<0.05

The multivariate analysis of all variables (Model 1) for OCS showed that circulating sLR11 and the histories of diabetes or dyslipidemia were explanatory factor for OCS independent from other variables. The Model 2 analysis using the limited variables which have been shown to be significantly increased in OCS (see Table 1) showed that circulating sLR11 is still an independent factor for OCS.

culating sLR11 levels and age, sex, white blood cell, red blood cell, platelet, total cholesterol, LDL-cholesterol, non-HDL-cholesterol or fasting blood sugar.

5.4. Multiple regression analysis for sLR11 in all subjects

Table 4 shows multiple regression analysis for sLR11concentratin. Variables in simple liner regression analysis with $p<0.05$ were included into the multiple regression analysis model. The levels of sLR11 significantly correlated with HDL-cholesterol, triglyceride, HbA1c, BMI and HOMA-IR. Among these variables, only HbA1c concentration showed independent correlation with sLR11 levels (t-value = 3.02 $p<0.01$).

Table 3
Correlation of Serum Soluble Form of LR-11 with various parameters in all subjects.

	Pearsons correlation coefficient	*p* values
Age	0.121	0.14
Male	−0.150	0.07
Total cholesterol (mg/dl)	0.067	0.42
HDL-cholesterol (mg/dl)	−0.160	<0.05
Triglyceride(mg/dl)	0.161	<0.05
LDL-cholesterol(mg/dl)	0.101	0.11
Non-HDL-cholesterol (mg/dl)	0.144	0.08
Fasting blood sugar (mg/dl)	0.077	0.35
Insulin(μU/dl)	0.186	<0.05
HOMA-IR	0.242	<0.01
HbA1c (%)	0.272	<0.01
Body Mass Index (kg/m²)	0.182	<0.05

A negative correlation between sLR11 concentration and HDL-cholesterol ($r=-0.161$, $p<0.05$) and a positive correlation between sLR11 and triglyceride ($r=-0.161$, $p<0.05$) were found. Furthermore, BMI ($r=0.182$, $p<0.05$) and HbA1c ($r=0.272$, $p<0.01$) showed significant positive correlations with sLR11, respectively.

Table 4
Results of multiple regression analysis for soluble form of LR11 in all subjects.

	Partial regression coefficient (b)	t-value	*p* value
X			
HbA1c (%)	0.21	2.50	<0.01
HOMA-IR	0.11	1.23	0.22
Body Mass Index (kg/m²)	0.10	1.16	0.25
HDL-cholesterol (mg/dl)	−0.09	−1.05	0.29
Triglyceride (mg/dl)	0.05	0.59	0.56
Y Soluble form of LR 11			

X, explanatory factor; Y, subordinate variables; Correlation coefficient (R) = 0.35 F value = 4.1, $p=0.002$, ($n=150$); The levels of sLR11 significantly correlated with HDL-cholesterol, triglyceride, HbA1c, and BMI. Among these variables, only HbA1c concentration showed independent correlation with sLR11 levels (t-value = 3.02 $p<0.01$).

6. Discussion

Coronary organic stenosis is formed mainly with intimal thickness which is composed of proliferative intimal smooth muscle cells and matrix components accompanying with lipid pool [19–21]. In our cases, sLR11 was higher in OCS group than that of in NCA group. We have recently reported that LR11 is produced by the intimal SMCs, and considerable amounts of the shed sLR11 enhance SMC migration in vitro [13]. Therefore, high sLR11 concentration may reflect the pathophysiological condition of intimal SMCs. And we also reported that sLR11 is a circulating marker for IMT independent from the other classic risk factor for atherosclerosis in dyslipidemic subjects without CAD or diabetes [13]. Considering the facts that LR11 is highly expressed in intimal SMCs, macrophages, or lymphocytes [7–10], the above results strongly suggest that circulating sLR11 level reflect the amount of intimal SMC in coronary arteries. Next, the contributing risk factors for elevation of circulating sLR11 were studied. Although multiple regression analysis showed HbA1c was only significant factor correlated with sLR11, insulin resistance relating factors such as BMI, HOMA index, low HDL-cholesterol, high triglyceride were related with sLR11 in single regression analysis, indicating that sLR11 might be induced with the state of insulin resistance in addition to diabetes mellitus.

The reason why diabetic condition is tightly correlated with sLR11 levels in serum is not available yet, but it has been reported that arterial intimal thickening after balloon catheter injury was enhanced in diabetic animals than controls [6,14]. From these observations, diabetic condition may induce the expression of sLR11 directly, and also modify the phenotype of smooth muscle cell into so-called synthetic type in the arterial wall. Those possibilities are currently under investigation.

In summary, the results obtained from the patients with a suspicion of coronary artery diseases suggested that circulating sLR11 may relate to coronary organic stenosis, and that hyperglycemic condition is a promoting factor for expression of LR11 in vascular SMCs.

6.1. The limitation

The limitation of the present investigation is at first a lack of information about the serum sLR11 data at acute phases of coronary artery diseases. Second, the data may be influenced by the continuous medication. More of subjects have received the treatments against dyslipidemia with statins in OCS group than in NC group, and most of subjects have received against hypertension with angiotensin II receptor type 1 blockers (ARBs) in the present study (Table 1). Considering the facts that statins and ARBs inhibit the sLR11 expression in the cultured SMCs [10,13], the circulating sLR11 levels may be influenced by these treatments in addition to coronary artery diseases. Further studies to investigate the effects of these drugs on the circulating LR11 levels are in progress. Third, HOMA-IR was assessed as a marker of insulin resistance. If this is the case, glucose clamp method might be better than HOMA-IR; but they are problematic in daily practice, and HOMA-IR considered a reliable insulin resistance marker, in vivo, especially in subjects whose fasting blood glucose concentration were not so high. Therefore we used HOMA-IR as a marker of insulin resistance, considering the burden on patient and medical stuff.

Acknowledgment

These studies were supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology to Y.S. and H.B.

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Enhanced Circulating Soluble LR11 in Patients With Diabetic Retinopathy

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• PURPOSE: To investigate the relationship of circulating levels of soluble form of LR11 (sLR11; also called SorLA or SORL1), with the progression of proliferative diabetic retinopathy (PDR) in patients with type 2 diabetes mellitus.

• DESIGN: Cross-sectional study.

• METHODS: Fifty-four patients with type 2 diabetes mellitus were divided into 2 sex- and age-matched groups: one with PDR (n = 29) and the other with nonproliferative diabetic retinopathy (n = 25). The serum sLR11 levels were measured with an immunodetection system followed by chemifluorescence quantification.

• RESULTS: The serum sLR11 levels were higher in the PDR group than in the nonproliferative diabetic retinopathy group (5.8 ± 1.2 U vs 3.7 ± 1.3 U; $P < .01$). A multivariate regression analysis showed that circulating sLR11 is a factor contributing to the prediction of PDR independent of other classical risk factors, and an area under the receiver operating characteristic curve analysis revealed that the sensitivity and the specificity were equivalent to or more than those of other factors. Among the classical risk factors for PDR, glycosylated hemoglobin levels showed the highest correlation coefficient ($P < .01$) for the sLR11 concentrations.

• CONCLUSIONS: Serum sLR11 concentration may reflect the progression of PDR in patients with type 2 diabetes mellitus. sLR11, released from immature vascular cells and indicating the development of atherosclerosis, is expected to be a novel candidate biomarker indicating diabetic retinopathy in patients with type 2 diabetes mellitus. (Am J Ophthalmol 2012;154: 187–192. © 2012 by Elsevier Inc. All rights reserved.)

Accepted for publication Jan 27, 2012.

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SUSTAINED HYPERGLYCEMIA, EVEN IN THE ABSENCE of other risk factors, can increase the risk of microvascular complications.[1] Given the substantial quality-of-life burden that diabetic retinopathy can confer, the ability to detect early retinal vascular abnormalities sensitively in patients with diabetes mellitus is desirable. The detection of such markers of pathologic cell function in combination with treatment of hyperglycemia is needed.

LR11 (also called SorLA or SORL1), an low-density lipoprotein (LDL)-receptor family member, has been identified as a molecule expressed in intimal smooth muscle cells in the development of atherosclerosis and endothelial cells under the condition of dyslipidemia.[2,3] The released soluble form of LR11 (sLR11) promotes pathologic infiltration of macrophages into the damaged vessels.[2] We have shown that the circulating sLR11 levels were increased in patients with coronary artery disease[4] and dyslipidemic subjects with carotid atherosclerosis.[5] A multivariate analysis in these independent studies in patients with atherosclerosis indicated that the sLR11 levels were correlated distinctly with the glycemic level among the classical risk factors for atherosclerosis.[4,5]

Diabetic retinopathy mainly is caused by diffuse endothelial damage at the microvascular level. However, the interesting observations are that the retinopathy is tightly associated with increased cardiovascular mortality,[6–8] reduced coronary reactivity,[9] and poorer prognosis of coronary revascularization procedures.[10,11] Thus, high glucose levels may change the phenotype of endothelial cells as well as that of arterial smooth muscle cells; the pathologic cell phenotype in microvessels of the retina possibly is detected by the circulating sLR11 released from the damaged cells. In this analysis, we investigated the significance of circulating sLR11 with regard to proliferative diabetic retinopathy (PDR) in patients with type 2 diabetes mellitus. The factors contributing to the elevation of the serum sLR11 also were analyzed.

METHODS

• STUDY POPULATION: The subjects consisted of 56 consecutive Japanese patients with type 2 diabetes mellitus seeking treatment at the Department of Laboratory Vascular Function, Toho University Sakura Medical Center, who had already given blood samples. PDR was defined

0002-9394/$36.00
doi:10.1016/j.ajo.2012.01.035

TABLE 1. Comparison of Type 2 Diabetes Mellitus Patient Background Factors between Nonproliferative Diabetic Retinopathy and Proliferative Diabetic Retinopathy

	NPDR Group	PDR Group	*P* Value
No.	25	29	—
Male (%)	68.2	69	.95
Age (y)	66.0 ± 8.6	62.4 ± 9.7	.15
Duration of diabetes (y)	11.4 ± 7.8	11.9 ± 7.8	.71
Body mass index (kg/m^2)	23.8 ± 4.0	25.8 ± 3.7	.06
Hypertension (%)	63.6	58.6	.72
Dyslipidemia (%)	64.0	44.8	.16
eGFR (mL/minute per 1.73m^2)	60.2 ± 15.3	58.2 ± 28.0	.72
HbA1c (%)	6.5 ± 0.8	7.0 ± 1.4	.10
Fasting blood sugar (mg/dL)	124.6 ± 33.1	132.1 ± 38.2	.63
Total cholesterol (mg/dL)	183.8 ± 34.8	202.6 ± 40.7	.12
LDL cholesterol (mg/dL)	111.7 ± 30.6	124.1 ± 33.3	.26
HDL cholesterol (mg/dL)	47.7 ± 16.4	49.4 ± 10.6	.58
Triglyceride (mg/dL)	122.4 ± 43.1	122.7 ± 52.7	.93
Medications			
Insulin therapy (%)	13.6	65.5	< .0001
Administration of statin (%)	45.5	24.1	.11
Administration of ACE-I or ARB (%)	54.5	41.4	.43

ACE-I = angiotensin converting enzyme inhibitor; ARB = angiotensin receptor blocker; eGFR = estimated glomerular filtration rate; HbA1c = glycosylated hemoglobin; HDL = high-density lipoprotein; LDL = high-density lipoprotein; NPDR = nonproliferative diabetic retinopathy; PDR = proliferative diabetic retinopathy.

The data are presented as mean ± standard deviation or number of subjects (%). The unpaired *t* test was used for continuous variables, and the chi-square test was used for categorized variables.

according to the international clinical classification of diabetic retinopathy as neovascularization in the retina.[12] Vitreous surgeries had been performed to treat macular edema (n = 7), vitreous hemorrhage (n = 13), traction retinal detachment (n = 5), or neovascular glaucoma (n = 4). None of the nonproliferative diabetic retinopathy (NPDR) cases had retinal neovascularization. Patients with chronic heart disease with an ejection fraction of less than 50% or chronic renal failure with serum creatinine of more than 1.3 mg/dL were excluded from the study analysis.

• PATIENT DATA ANALYSIS: Blood samples were collected in the morning after an overnight fast. Lipid variables and fasting blood glucose were measured using standard laboratory techniques. The potential risk factors for atherosclerosis were analyzed, including age, sex, body mass index (BMI), smoking, and history of hypertension and dyslipidemia. Hypertension was defined as systolic pressure of more than 140 mm Hg or diastolic pressure of more than 90 mm Hg. Diabetes mellitus was defined as a fasting blood glucose level of more than 126 mg/dL, glycosylated hemoglobin (HbA1c) of more than 5.8%, or both. Dyslipidemia was defined as serum total cholesterol of more than 220 mg/dL and triglycerides of more than 150 mg/dL in the fasting state, or both, and high-density lipoprotein (HDL) cholesterol of less than 40 mg/dL, or a combination thereof. The serum creatinine level was assayed by an enzymatic method. The estimated glomerular flow rate was estimated using a modified traceable Modification of Diet in Renal equation, as proposed by the Working Group of Japan Chronic Kidney Disease Initiative[13]: estimated glomerular flow rate (mL/minute per 1.73 m^2) = $0.741 \times 175 \times \text{age}^{-0.203} \times \text{serum creatinine}^{-1.154}$ (if female × 0.742).

• MEASUREMENT OF SERUM SLR11: For the analysis of sLR11, fasting blood samples were collected and centrifuged immediately at 4000 g for 10 minutes, and the supernatant immediately was frozen in polypropylene tubes and stored at −80 C until use. Fifty microliters of serum was purified using 39-kDa receptor-associated protein-GST affinity beads (Cosmo Bio, Toyo city, Tokyo, Japan). For immunoblotting, equal amounts of protein extracted from pelleted beads were subjected to 10% sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) after heating to 95 C for 5 minutes, as described previously[5] under reducing conditions, and were transferred to a nylon membrane. Incubations were carried out with an antibody against LR11 (5-4-30-19-2 at 1:500 dilution),[5] followed by peroxidase-conjugated antimouse immunoglobulin G. The development was performed with the ECL detection reagents (Amersham Pharmacia, Piscataway, New Jersey, USA). The signals were quantified by densi-

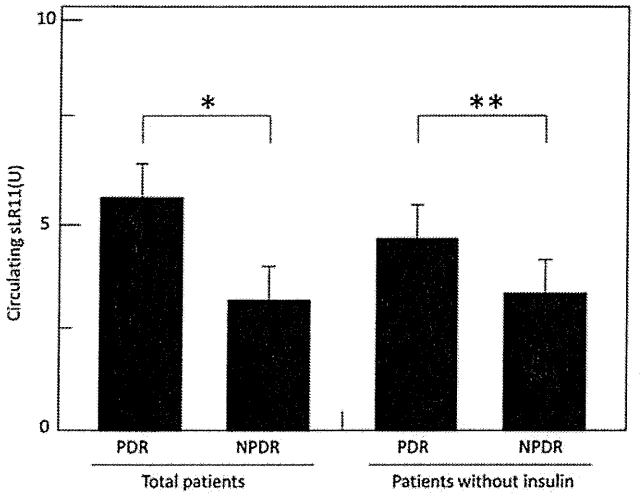

FIGURE 1. Bar graph showing circulating sLR11 levels in the patient groups with proliferative diabetic retinopathy (PDR) or without PDR (NPDR). The sLR11 levels of the total patients and those of the patients without insulin therapy were compared between the PDR and NPDR groups, respectively. Data are expressed as means ± standard deviation. For statistical analysis, the Student *t* tests was used. *P < .05, **P < .01.

tometric scanning using the NIH image software program (National Institutes of Health, Betheda, Maryland, USA). The sLR11 levels in each serum sample (50 μL) were determined as an averaged value of 3 quantified signal intensities resulting from independent assays using samples with blinded indications and were expressed as a ratio to that of standard serum. The immunologic estimation indicated that the signal of 1 U (in 50 μL serum) corresponded to approximately 50 ng/mL of recombinant sLR11.

• STATISTICAL ANALYSIS: The results are shown as means ± standard deviation or proportion (%) for each index. The statistical analyses were performed using the SPSS Statistical Package for Windows software program version 11.01.1. Comparisons between groups were performed using the Student t test. The data were subjected to a 1-way analysis of variance with the Dunnett multiple comparison of means. A Pearson correlation coefficient analysis was used to assess the associations between measured parameters. Subsequently, multiple linear regression analyses were used to calculate the odds ratio for PDR by controlling for all risk factors. These risk factors were scored as explanatory factors, and the subordinate variable was PDR = 1 and NPDR = 0. The sensitivity and specificity with respect to the presence of PDR were analyzed using a conventional receiver operating characteristic (ROC) curve. P values less than .05 were considered to be statistically significant.

TABLE 2. Results of Multivariate Analysis Investigating Risk Factors for Proliferative Diabetes Retinopathy in Subjects with Type 2 Diabetes Mellitus

	Odds Ratio (95% Confidence Interval)	*P* Values
Age, per 1-y increase	4.12 (0.78 to 0.996)	< .05
Male	0.01 (0.16 to 6.18)	.99
eGFR, per 1-U (mL/minute per 1.73 m^2) increase	0.80 (0.97 - 1.08)	.37
Total cholesterol, per 1-mg/dL increase	1.43 (0.99 to 1.04)	.34
HbA1c, per 1% increase	1.23 (0.24 to 2.04)	.51
sLR11, per 1-U increase	8.50 (1.63 to 12.25)	< .01

eGFR = estimated glomerular filtration rate; HbA1c = glycosylated hemoglobin; sLR11 = soluble form of LR11.

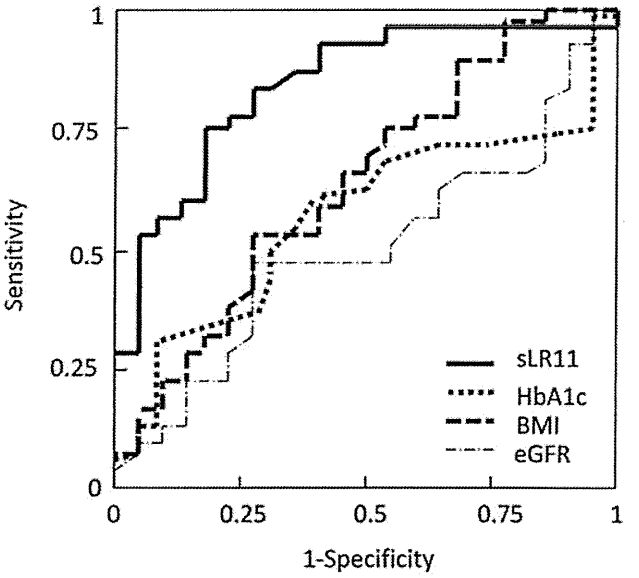

FIGURE 2. Receiver operating characteristic curve for discriminating the probability of type 2 diabetes mellitus patients developing proliferative diabetic retinopathy (PDR) from patients without PDR based on the levels of circulating soluble form of LR11 (sLR11), glycosylated hemoglobin (HbA1c), body mass index (BMI), or 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.

RESULTS

THE PATIENT CHARACTERISTICS ARE SHOWN IN TABLE 1. The age- and gender-matched NPDR and PDR groups comprised 25 and 29 subjects, respectively. There were no statistically significant differences in BMI, duration of diabetes, frequency of hyperlipidemia or dyslipidemia, or estimated glomerular flow rate between the NPDR and PDR subjects. There were also no statistically significant

TABLE 3. Area under the Receiver Operating Characteristic Curve Analysis Investigating Cutoff Values for Proliferative Diabetic Retinopathy

Marker	Cutoff	Sensitivity	Specificity	AUC %
sLR11(U)	4.2	0.78	0.77	85
HbA1c (%)	6.5	0.63	0.60	57
BMI (kg/m^2)	24.5	0.59	0.59	64
eGFR (mL/minute per 1.73 m^2)	120.5	0.5	0.46	50

AUC = area under the receiver operating characteristic curve; BMI = body mass index; eGFR = estimated glomerular filtration rate; HbA1c = glycosylated hemoglobin; sLR11 = soluble form of LR11.

TABLE 4. Correlation Analysis of Circulating sLR11 with Various Markers in All Subjects with Type 2 Diabetes Mellitus

	Pearson Correlation Coefficient	*P* Value
Age	−0.07	.63
Male	0.15	.29
Body mass index (kg/m^2)	0.21	.14
HbA1c (%)	0.32	< .01
Fasting blood sugar (mg/dL)	0.19	.17
eGFR (mL/minute per 1.73 m^2)	−0.19	.17
Total cholesterol (mg/dL)	0.27	.05
LDL cholesterol (mg/dL)	0.31	< .05
HDL cholesterol (mg/dL)	−0.10	.51
Triglyceride (mg/dL)	0.25	.07

eGFR, estimated glomerular filtration rate; HbA1c = glycosylated hemoglobin; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

differences in HbA1c, fasting blood sugar, or lipid concentrations between the NPDR and PDR subjects. Although there was no significant difference in the use frequency of statin, angiotensin converting enzyme inhibitor (ACE-I), or angiotensin II receptor type 1 blocker (ARB) between the 2 groups, the frequency of patients using insulin was significantly higher in the PDR subjects than that in the NPDR subjects.

The circulating sLR11 levels in the NPDR and PDR groups were 3.7 ± 1.8 U and 5.8 ± 2.7 U, respectively (Figure 1), indicating that the sLR11 levels in the PDR group were higher than those in the NPDR group ($P <$ .01). Note that we previously reported that the mean circulating sLR11 levels in 400 dyslipidemic subjects was 3.0 ± 1.0 U.[5] The sLR11 analysis restricted for the patients not treated with insulin showed that the sLR11 levels again were higher in the subjects with PDR (4.8 ± 1.2 U; n = 10) than in those with NPDR (3.7 ± 1.3; n = 12; $P <$ .05). Thus, circulating sLR11 levels were increased in type 2 diabetes mellitus patients with PDR regardless of medication with insulin therapy.

We analyzed the significance of the sLR11 concentration in comparison with other risk factors for PDR, including age, male gender, estimated glomerular flow rate, and the total cholesterol and HbA1c concentrations, in all subjects (Table 2). The multivariate analysis using all variables for PDR showed that the circulating sLR11 level, as well as younger age, strongly associated with PDR independent of other variables.

The ROC curves of the various factors were examined for discriminating the probability of the type 2 diabetes mellitus patients with PDR from the NPDR patients based on the levels of sLR11, the levels of HbA1c, the BMI, or the estimated glomerular filtration rate (Figure 2). The curves showed the fraction of true-positive results (sensitivity) and false-positive results (1-specificity) for various cutoff levels of each parameter. The cutoff level of sLR11 that gave the maximum sensitivity and specificity for PDR was 4.2 U. At the cutoff level, the sensitivity of sLR11 for PDR was 78%, and the specificity was 77%, equivalent to or more than the other classical risk factors, HbA1c, BMI, or estimated glomerular flow rate (Table 3).

Finally, to clarify the correlation between the sLR11 concentration and various clinical parameters in the studied patients, simple regression analyses were performed for the dependent variable (Table 4). The HbA1c levels and LDL cholesterol levels correlated positively with sLR11 ($r = 0.32$, $P <$ .01, and $r = .31$, $P <$ .05, respectively). No significant correlation was observed between the sLR11 and age, sex, BMI, fasting blood glucose, estimated glomerular flow rate, total cholesterol, HDL cholesterol, or triglyceride.

DISCUSSION

LR11 IS HIGHLY EXPRESSED IN THE ENDOTHELIAL CELLS under the condition of dyslipidemia as well as in the intimal smooth muscle cells migrated from media in the development of atherosclerosis.[2,3] Two recent independent studies for the subjects with dyslipidemia or coronary heart diseases have shown that the concentrations of soluble form, sLR11, were associated with the HbA1c levels in these subjects with different backgrounds.[4,5]

The key cytokines underlying the pathogenesis and development of PDR are similar to those leading to atherosclerosis. The barrier dysfunction of microvessels and retinal ischemia provokes an increase in the ocular levels of inflammatory cytokines and growth factors, including vascular endothelial growth factor, platelet-derived growth factor BB (PDGF-BB), and angiotensin II,[14–16] with increased expression of adhesion molecules,[17] all promoting retinal neovascularization. PDGF-BB and

angiotensin II trigger the increased expression of LR11 on vascular smooth muscle cells.[2,5] The LR11 expression in endothelial cells is induced under conditions of dyslipidemia, possibly through the activations of combination of cytokines and adhesion molecules.[2,3] Thus, considering that endothelial dysfunction is the first sign of microvascular injury at the organ level[18] and that the progression of diabetic microvascular complications is modulated by the severity of hyperglycemia through the gradual damages of the endothelium,[19] a high sLR11 concentration in the serum of diabetic patients with PDR may reflect the pathophysiologic endothelial dysfunction associated with diabetes, although the mechanism responsible for the release of sLR11 in circulation remains unresolved.

In the present study, the sLR11 levels in the PDR group were increased compared with the NPDR group, regardless of medication with insulin therapy (see Figure 1). The multivariate analysis of all variables showed that the circulating sLR11 level, as well as age, strongly associated with PDR, independent of other variables (see Table 2). The ROC analysis indicated that the sensitivity and specificity of sLR11 is the highest at a cutoff level of 4.2 as a marker of PDR (see Figure 2 and Table 3). Finally, the sLR11 concentration was correlated positively with the HbA1c level (see Table 4), which was consistent with previous observations with subjects with different profiles.[4,5]

Various studies on the pathogenesis of and risk factors for the development of PDR have been conducted, and hypertension and renal failure have been identified as important risk factors, along with poor blood glucose control.[20–23] ROC analyses using the present study subjects showed that the area under the ROC of sLR11 was equivalent to or more than those of the so-far established risk markers (see Table 3). Thus, LR11 may be an additional tool for discriminating patients with a high risk of developing diabetic retinopathy from the increasing population of patients with type 2 diabetes mellitus. Considering the lack of enough data for the role of LR11 in the basic mechanism of PDR, to clarify the clinical significance of LR11 in patients with PDR, further pathophysiologic studies to address the question that sLR11 is a marker or a triggering factor are required.

Thus, one limitation of the present investigation is the lack of information about the sLR11 data in the retina and proliferative membrane in patients with PDR. Second, the data may have been influenced by the continuous use of medication. The sLR11 levels of subjects with insulin therapy were not significantly different from those of subjects without insulin therapy (see Figure 1). In addition, most of the patients had received medication against hypertension with ARBs (see Table 1). Considering the fact that statins and ARBs inhibit the sLR11 expression in cultured cells,[2,5] the circulating sLR11 levels may be modified by these treatments. In this context, there was no significant difference in sLR11 levels between the subject groups with or without use of statin, or ACE-I, or ARB in the PDR subjects (data not shown). Finally, our results were obtained using relatively small sample sets. Clearly, further careful validation studies with larger sample sets to evaluate the effects of sLR11 on microvascular outcomes as primary end points will be required.

In summary, this study presented a novel and potentially clinically relevant new correlation of sLR11 with PDR, thus potentially providing a serum test to indicate patients at greater risk of developing PDR.

ALL AUTHORS HAVE COMPLETED AND SUBMITTED THE ICMJE FORM FOR DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST AND none were reported. Publication of this article was supported by a Health and Labor Sciences Research Grant for Translational Research, Chiyodaku, Tokyo, Japan (H.B.). Involved in Design of study (M.T., T.S.); Conduct of study (M.T., T.S.); Collection (M.T., T.S.), management (M.T., H.B., T.S., K.S.), analysis (M.T.), and interpretation (M.T., H.B., T.S., M.T., K.S.) of data; and Preparation (H.B., J.M.), review (H.B., T.S., J.M., M.T., K.S.), and approval (H.B., T.S., J.M., M.T., K.S.) of manuscript. The retrospective cross-sectional study protocol was approved by the Human Investigation Review Committee of Toho University Sakura Hospital, and informed consent was obtained from all of the patients. There was no statistical consultation or assistance.

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Clinica Chimica Acta 415 (2013) 191–194

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Increased circulating soluble LR11 in patients with acute coronary syndrome

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ARTICLE INFO

Article history:
Received 31 August 2012
Received in revised form 29 October 2012
Accepted 29 October 2012
Available online 2 November 2012

Keywords:
LR11
Smooth muscle cell
Biomarker
Acute coronary syndrome

ABSTRACT

Background: LR11 (also so called SorLA or SORL1) is a novel marker of intimal smooth muscle cell (SMC) proliferation. Vascular SMCs play important roles in the development of atherosclerosis interacting with macrophages in a vulnerable plaque of patients with acute coronary syndrome (ACS).The present study determines whether soluble LR11 (sLR11) is associated with ACS.
Methods: We studied 100 patients with coronary artery disease (CAD) comprising 50 consecutive patients with acute coronary syndrome (ACS; mean age 62.3 ± 13.0 years; male 78.0%) who were successfully treated with percutaneous coronary intervention and 50 age- and sex-matched stable angina pectoris (SAP) patients as control. Concentration of sLR11 was measured by sandwich enzyme-linked immunosorbent assay method.
Results: Circulating sLR11 was significantly increased in patients with ACS compared with SAP (9.88 ± 2.78 vs. 8.18 ± 1.11 ng/ml, $p<0.01$). Multivariate logistic regression analysis indicated that sLR11 was independently associated with ACS (odds ratio (OR), sLR11 quartile increment, 2.18, 95% confidence interval (CI) 1.21–4.19, $p<0.01$). Among various biomarkers of acute coronary syndrome, hsCRP were significantly correlated with LR11 ($r=0.480$, $p<0.01$).
Conclusions: There is a statistical significant association between LR11 and ACS and may be a useful biomarker for the development of acute coronary syndrome.

1. Introduction

Acute coronary syndrome (ACS) remains a major cause of mortality and morbidity despite advances in cardiovascular therapies [1–3] and it is mainly characterized by the rupture of lipid-rich vulnerable atherosclerotic plaque with subsequent thrombus formation [4,5]. Many studies have investigated the pathological mechanisms of ACS and inflammation is recognized as a key step in the pathogenesis of acute thrombotic events [6,7]. A novel diagnostic biomarker derived from ruptured plaque would be useful but remains to be established.

LR11 (also so called SorLA or SORL1) is a member of the LDL receptor family, and is highly expressed in atheromatous plaques of animal experimental model, especially in intimal smooth muscle cell (SMC) but not in medial SMC [8,9]. The overexpression of LR11 protein enhances SMC migration via the activation of the urokinase-type plasminogen activator receptor that regulates inflammatory monocyte adhesion [9,10]. We previously reported that circulating LR11 can be immunologically detected in serum using a novel sandwich enzyme-linked immunosorbent assay (ELISA) and specific monoclonal antibodies against human LR11 [11,12]. Circulating soluble LR11 levels positively correlate with the intima-media thickness in patients with dyslipidemia [11]. In addition, we also demonstrated increased levels of soluble LR11 in patients with stable coronary artery disease [13]. However, circulating LR11 levels in ACS have not been evaluated. The present study therefore evaluated the clinical significance of circulating LR11 in patients with ACS.

2. Materials and methods

2.1. Subjects

The present study is a cross-sectional case–control study. We enrolled 50 patients with ACS who were successfully treated with percutaneous coronary intervention (PCI) at Juntendo University Hospital between November 2008 and December 2009 and 50 age- and sex-matched patients with stable angina pectoris (SAP) as controls (mean age 62.4 ± 12.7 years; male, 78 %). Patients with previous coronary revascularization, malignant disease, inflammatory disease, and hemodialysis were excluded from the study. We defined ACS as acute myocardial

Abbreviations: ACS, acute coronary syndrome; CRP, C-reactive protein; PCI, percutaneous coronary intervention; SAP, stable angina pectoris; SMC, smooth muscle cell; STEMI, ST-segment elevation myocardial infarction; UAP, unstable angina pectoris; uPAR, urokinase-type plasminogen activator.

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http://dx.doi.org/10.1016/j.cca.2012.10.047

infarction (AMI) and unstable angina (UAP). AMI was defined as increased cardiac enzymes (troponin or MB fraction of creatine kinase) with ischemic symptoms and subclassified AMI as ST-segment elevation myocardial infarction (STEMI) and non ST-segment elevation myocardial infarction (NSTEMI) according to the presence or absence, respectively, of at least 0.1 mV ST-segment elevation in at least 2 contiguous leads. Unstable angina (UAP) was diagnosed based on the presence of ischemic symptoms with the ST–T change but without an increase in cardiac enzymes, or myocardial necrosis indicated as an increase in cardiac enzymes regardless of ST-segment change. The ACS group was divided into STEMI and UAP/NSTEMI subgroups. SAP was defined as effort angina with a stable profile of symptoms for at least 3 months before admission. Written informed consent was obtained from all patients to underego PCI using standard techniques. This study adhered to the Declaration of Helsinki and was approved by our institutional internal review board. The choice of stent type and device was left to the discretion of the operators at our cardiology center.

2.2. Blood samples

Arterial blood samples were collected from all patients before coronary angiography in the operating room. The samples were centrifuged at 1000×*g* for 10 min and serum samples were stored at −80 C. Soluble LR11 (sLR11) was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) method with our specific monoclonal antibodies directed against human LR11 that we previously established [12]. In brief, sLR11 in serum was immunologically identified as 250-kDa protein in serum fluid by SDS–PAGE separation, and was purified using a receptor-associated protein and monoclonal antibodies that bind to intact sLR11 without prior purification. The sLR11 immunoassay used a combination of anti-LR11 monoclonal antibodies (M3 and R14). Assay characteristics concerning sLR11 immunoassay, for example, the inter-, and intra-assay CVs, as well as the working range, and the mean backfit value for the lowest standard giving acceptable precision, and the lower limit of detection has been described in our previous article [12]. Concentrations of serum high-sensitive C-reactive protein (hs-CRP) were measured using a validated immunoassay and an autoanalyzer. Levels of circulating Troponin T (TnT) and CD40 ligand (CD40L) were measured in patients with ACS. Serum cardiac troponin T was measured using a chemiluminescent enzyme immunoassay kit (Determiner CL TnT, Kyowa Medex, Tokyo, Japan). CD40L was quantified using human CD40L ELISA kit (R&D systems, Minneapolis, MN). Other markers were determined by routine laboratory methods.

2.3. Statistical analysis

Results are expressed as means ± SD or as ratios (%) and numbers for categorical data. The distribution of continuous variables was visually assessed from frequency histograms and using the Kolmogorov–Smirnov test. The hs-CRP and blood glucose on admission (BG on Ad) were skewed and thus we used natural log-transformed hs-CRP and BG on Ad. Continuous variables were compared using an unpaired *t*-test or Mann–Whitney U-test. Categorical variables (presented as frequencies) were compared using either chi-square test or Fisher's exact probability test. LR11 values across the three groups were compared using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Associations between measured parameters were evaluated using Spearman's rank correlation coefficient analysis. Predictive variables for ACS adjusted for potential confounding factors were identified by multiple logistic regression analysis. The effects of biomarkers including hs-CRP, blood glucose on admission and sLR11 in this model were evaluated as quartile increments in the concentration of each. The univariate model included the variables of age, gender, diabetes, hypertension, dyslipidemia, current smoking, use of statins, angiotensin converting enzyme and/or angiotensin receptor blockers, hs-CRP, blood glucose on admission (BG on Ad) and sLR11. Statistically significant variables in the multivariable logistic regression analysis selected using stepwise forward selection were subsequently included in a new model. All data were statistically analyzed using JMP8.0 (SAS Institute Inc., Cary, NC) and SPSS v.18.0 (Chicago, IL). A $p<0.05$ was considered statistically significant.

3. Results

3.1. Characteristics of subjects

A comparison of the baseline characteristics between the ACS and SAP groups is shown in Table 1. Diabetes, metabolic syndrome, and hypertension were comparable between them, whereas current smoking and dyslipidemia were more frequent in the ACS and SAP group, respectively. The levels of total cholesterol, LDL-C, hs-CRP, WBC, and blood glucose on admission were significantly higher in the ACS group.

3.2. Levels of LR11

Levels of circulating soluble LR11 were significantly higher in patients with ACS than that with SAP (9.88 ± 2.78 vs. 8.18 ± 1.11 ng/ml, $p<0.01$). We also compared LR11 levels among 50 ACS patients subclassified as 32 patients represented STEMI and 18 diagnosed as NSTEMI/UAP, respectively. Levels of sLR11 were significantly higher in patients with STEMI compared with the other 2 groups (Fig. 1).

Table 1
Baseline Clinical Characteristics.

	SAP (n=50)	ACS (n=50)	P
Age	62.4±12.5	62.3±13.0	NS
Male Gender, n (%)	39 (78.0)	39 (78.0)	NS
Diabetes, n (%)	18 (36.0)	17 (34.0)	NS
Metabolic syndrome	30 (60.0)	23 (46.0)	NS
Hypertension, n (%)	38 (76.0)	33 (66.0)	NS
Dyslipidemia, n (%)	43 (86.0)	34 (68.0)	< 0.05
Current Smoker, n (%)	9 (18.5)	28 (56.0)	< 0.01
Family history, n (%)	15 (30.0)	13 (61.1)	NS
CKD, n (%)	9 (18.0)	11 (22.0)	NS
Angiographic degree of CAD			NS
1-vessel disease, n (%)	26 (52.0)	26 (52.0)	
2-vessel disease, n (%)	11 (22.0)	14 (28.0)	
3-vessel disease, n (%)	13 (26.0)	10 (20.0)	
LVEF, %	62.7±10.3	62.8.±9.9	NS
ACE-I/ARB	31 (62.0)	13 (26.0)	<0.01
Statin	37 (74.0)	13 (26.0)	<0.01
BMI, kg/m^2	24.2±2.9	25.2±3.5	NS
Waist, cm	88.2±8.2	90.7±8.2	NS
SBP, mmHg	146.3±24.4	139.3±24.0	NS
DBP, mmHg	77.5±13.2	80.2±17.9	NS
TC, mg/dl	171.1±37.4	204.3±38.5	<0.01
LDL-C, mg/dl	93.1±30.2	131.3±29.5	<0.01
HDL-C, mg/dl	49.3±13.1	45.1±11.1	0.09
TG, mg/dl	131.5±52.8	145.7±105.1	NS
FBG, mg/dl	103.5±21.9	109.9±28.2	NS
HbA1c, %	5.8±1.1	6.2±1.5	NS
BNP, ng/dl	56.3±83.1	105.2±209.4	NS
hs-CRP, mg/dl	0.25±0.68	1.33±2.67	<0.01
eGFR, ml/min/1.73 m^2	70.9±16.3	76.2±26.1	NS
WBC, /μl	5784±1562	9489±3125	<0.01
BG on admission	120.0±38.8	166.3±74.5	<0.01
sLR11, ng/ml	8.18±1.11	9.88±2.78	<0.01

ACE-I, angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blockers; BG, blood glucose; BNP, brain natriuretic peptide; CAD, coronary artery disease; CKD, chronic kidney disease; DBP, diastolic blood pressure; FBG, fasting blood glucose; LAD, left anterior descending artery; LCX, left circumflex coronary artery; LVEF, left ventricular ejection fraction; RCA, right coronary artery; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides.

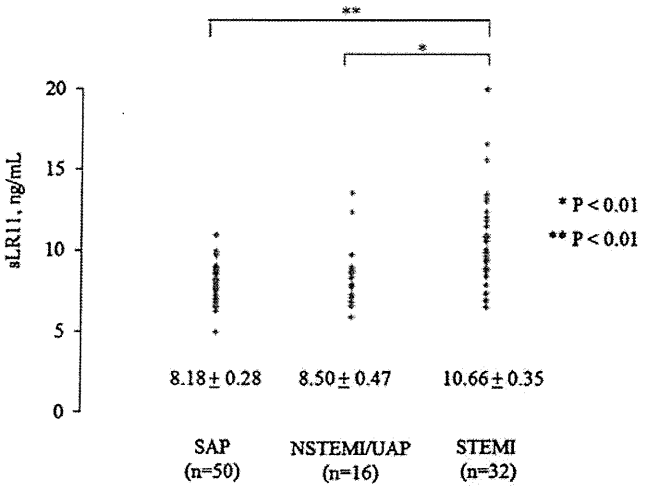

Fig. 1. Comparison of sLR11 among SAP, NSTEMI/UAP and STEMI. SAP, stable angina pectoris; NSTEMI/UAP, non ST-segment elevation myocardial infarction/unstable angina; STEMI, ST-segment elevation myocardial infarction.

3.3. Correlations between LR11 and clinical parameters

As shown in Table 2, serum LR11 levels positively correlated with BMI and LDL-C in all patients ($r=0.217$, $p<0.05$ and $r=0.304$, $p<0.01$, respectively). In ACS patients, LR11 positively correlated with hs-CRP ($r=0.480$, $p<0.01$, Fig.2A), WBC ($r=0.413$, $p<0.05$) and blood glucose on admission ($r=0.437$, $p<0.01$, Fig. 2B). LR11 tended to correlate swith TnT and CD40L but not significant ($r=0.231$, $p=0.11$ and $r=0.230$, $p=0.11$, respectively).

3.4. Multiple logistic regression analysis

We evaluated predictors of ACS for the entire study population using univariate and multivariate logistic regression analysis. The multivariate logistic regression analysis in Table 3 shows that sLR11 is independently associated with ACS after adjusting for confounding factors (odds ratio (OR), sLR11 quartile increment, 2.18, 95% CI 1.21–4.19, $p<0.01$).

4. Discussion

The present findings demonstrated that circulating soluble LR11 levels is significantly higher in patients with ACS than with SAP. We also showed that sLR11 is significantly and positively correlated with hs-CRP. Multivariate analysis indicated that increased sLR11 could be an independent variable for ACS after adjustment.

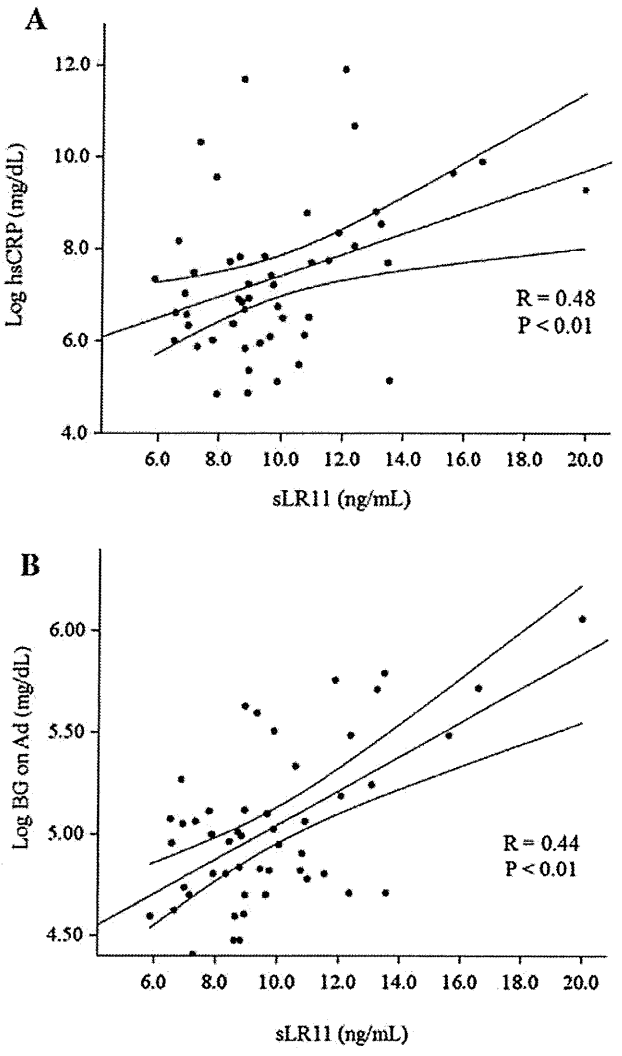

Fig. 2. Correlation between levels of sLR11 and various biomarkers in ACS. Fig. 2A, correlation between levels of sLR11 and hsCRP in ACS; Fig. 2B, correlation between levels of sLR11 and blood glucose on admission in ACS.

LR11, is a potential novel biomarker of vascular smooth muscle cells, that are abundantly expressed in the intimal SMC at the border between intima and media in plaque areas of apo E knockout mice [9]. Circulating LR11 positively correlates with intimal-media thickness in patients with dyslipidemia [11] and levels of soluble LR11 are higher in patients with coronary artery disease [13]. Our previous studies

Table 2
Correlations between LR11 and other parameters.

	r	P
Age	0.017	NS
BMI, kg/m^2	0.217	<0.05
SBP, mmHg	−0.029	NS
DBP, mmHg	0.130	NS
LDL-C, mg/dl	0.304	<0.01
HDL-C, mg/dl	−0.050	NS
TG, mg/dl	0.130	NS
BG, mg/dl	0.111	NS
eGFR, mL/min/1.73 m^2	−0.067	NS
HbA1c, %	0.020	NS
BNP, ng/mL	0.119	NS

BG on Ad, blood glucose on admission; DBP, diastolic blood pressure; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride.

Table 3
Univariate and multivariate logistic regression analysis model for prediction of ACS.

	Univariate			Multivaritate		
	OR	95%CI	P	OR	95%CI	P
Age, y	1.00	0.97–1.03	NS	1.04	0.99–1.10	NS
Gender, male	1.00	0.38–2.60	NS	Not selected		
Diabetes, yes	0.92	0.40–2.09	NS	0.24	0.04–0.91	<0.05
Hypertension, yes	0.61	0.25–1.46	NS	Not selected		
Dyslipidemia, yes	0.34	0.12–0.90	0.03	Not selected		
Current smoking, yes	5.80	2.40–15.1	<0.01	11.9	2.59–74.0	<0.01
Statin use, yes	0.13	0.05–0.30	<0.01	0.38	0.10–1.33	NS
ACEI/ARB use, yes	0.22	0.09–0.51	<0.01	0.36	0.09–1.31	NS
High-sLR11, QI	1.92	1.32–2.88	<0.01	2.18	1.21–4.19	<0.01
High-hsCRP, QI	2.48	1.65–3.90	<0.01	2.06	1.16–3.91	<0.05
High-BG on Ad, QI	2.18	1.47–3.32	<0.01	2.83	1.52–5.83	<0.01

ACE-I, angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blockers; BG on Ad, blood glucose on admission; QI, quartile increment.

implied that circulating LR11 reflects the pathological status of vascular smooth muscle cells in atherosclerotic lesions. Vascular smooth muscle cells play important roles in the development of atherosclerosis. Medial SMC migrate into the subendothelial space, proliferate, and produce extracellular matrix to form atheromatous plaques in response to inflammatory cytokines. The precursor of ruptured culprit lesions in patients with ACS is a thin cap fibroatheroma, characterized by a necrotic core and an overlying thin cap that lacks an extracellular collagen matrix [7,14]. The present study found significantly higher levels of soluble LR11 in patients with ACS. The pathological roles of LR11 in vulnerable plaque remain uncertain, but several possible explanations can be considered. Macrophages and vascular smooth muscle cells promote the local release of matrix metalloproteinases (MMP) that degrade supportive collagen, resulting in fibrous cap breakdown and enhanced plaque vulnerability [15–17]. The phenotypic modulation of smooth muscle cells influences the production of matrix-degenerating enzymes, monocyte recruitment and the expression of pro-inflammatory cytokines [18] and LR11 is expressed in synthetic intimal smooth muscle cells [19] that produce higher levels of matrix-degrading proteases resulting in thinning of the fibrous cap.

LR11 also significantly and positively correlated with hs-CRP, which plays an important role as an inflammatory mediator. Inflammation drives the formation and progression of atherosclerotic plaque and CRP is predominantly produced in the liver during the acute phase, but it is also expressed in smooth muscle cells within atherosclerotic lesions [20], where it is implicated in various aspects of atherogenesis and plaque vulnerability. We reported that soluble LR11 also regulates adhesion, migration and lipid accumulation in macrophages through urokinase-type plasminogen activator receptors [21]. We also demonstrated that sLR11 enhances scavenger receptor expression that contributes to foam cell formation in atherogenesis in vivo. These results imply that LR11 is a potential regulator of vulnerable plaque formation.

Soluble LR11 enhances the expression of urokinase-type plasminogen activator (uPAR) in macrophages and its expression is increased in the circulating monocytes of patients with AMI [22]. Urokinase-type plasminogen activator implicated in a broad spectrum of pathophysiological processes, including inflammation, fibrinolysis, proteolysis, atherogenesis and plaque destabilization, all of which contribute to the pathogenesis of myocardial infarction [23,24]. Cozen AE, et al. reported that macrophage-targeted overexpression of uPAR causes accelerated atherosclerosis, coronary artery occlusion, and premature death in apo E knockout mice [25]. Another study demonstrated that high levels of uPAR expression in vulnerable carotid plaque with high macrophage density and a ruptured fibrous cap [26]. These findings suggest that levels of soluble LR11 in ACS that are elevated via uPAR activation are associated with these pathophysiological conditions.

The present study has several limitations. Firstly, this is a single center study with a small patient cohort and thus unknown confounding factors might have affected the results regardless of the adjusted analysis. Further studies with a larger sample size are needed to confirm the results and to validate the clinical implication of LR11 as a diagnostic biomarker of ACS. Secondly, the limitations inherent in any cross-sectional study that a single sample at a specific time point might not reflect the natural course of a disease must be considered. The relationship between LR11 and other biomarkers at serial time points after patients with ACS are admitted to the hospital require further validation. Thirdly, several experimental studies have found that statin and angiotensin II type1 receptor blockers inhibit LR11 expression in SMCs [11,19]. More patients with the SAP group received statins and ARBs than the ACS group in the present study. This is because stable angina pectoris patients had been medically treated on the basis of current guideline, in contrast, most of patients with ACS admitted to our hospital for the first time without any medical background. These drugs might have attenuated circulating LR11 levels. In conclusion, there is a statistical significant association between LR11 and ACS. We believe that LR11 might serve as new biomarker reflecting different aspects of atherosclerosis, however the mechanism of how sLR11 plays a role in the pathophysiological conditions of ACS requires elucidation.

Acknowledgments

The authors thank the staff of the Department of Cardiovascular Medicine at Juntendo University and Chiba University Graduate School of Medicine. We also appreciate Yumi Nozawa for her secretarial assistance.

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Original Research Article

Dementia and Geriatric Cognitive Disorders

Dement Geriatr Cogn Disord 2010;30:28–32
DOI: 10.1159/000315539

Accepted: May 7, 2010
Published online: July 30, 2010

Increased Levels of Soluble LR11 in Cerebrospinal Fluid of Patients with Alzheimer Disease

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Key Words
LR11 · Lipoprotein receptor · Alzheimer disease · Biomarker · ApoE4

Abstract
Background: Recent genetic and pathological studies have suggested that a lipoprotein receptor, LR11, is intricately implicated in the pathogenesis of Alzheimer disease (AD). We have recently established a novel sandwich ELISA, which enabled the sensitive quantification of a soluble LR11 (sLR11). By this ELISA, we attempted to determine the difference in the levels of CSF sLR11 in AD patients. ***Methods:*** We examined CSF from 29 AD patients, 20 frontotemporal lobar degeneration patients and 27 age-matched control subjects. The CSF sLR11 level as well as the levels of tau and β-amyloid42 (Aβ42) were determined by sandwich ELISA. ***Results:*** The CSF tau level and tau/Aβ42 ratio were significantly increased ($p < 0.01$) in the AD patients. The CSF sLR11 level in the AD patients was significantly higher ($p < 0.01$) than that of the frontotemporal lobar degeneration patients and the controls. The *APOE*-ε4-positive AD patients have higher sLR11 levels than the *APOE*-ε4-negative patients ($p < 0.01$). ***Conclusions:*** These results suggest that the quantification of CSF sLR11 may serve as a biomarker of AD, although the diagnostic value for individual patients is limited. An elevated CSF sLR11 level in AD patients may be relevant to AD pathogenesis.

Introduction

Lipoprotein receptor LR11 is a member of the LDL receptor family and a type I membrane protein that is highly abundant in the brain. LR11 has a vacuolar protein sorting 10 protein domain that is involved in intracellular protein trafficking. Several lines of evidence suggest that LR11 is intricately implicated in the pathogenesis of Alzheimer disease (AD) [1]. First, variants of the LR11 gene were shown to be a genetic risk factor for sporadic AD [2]. Second, LR11 interacts with the amyloid precursor protein (APP) and alters APP trafficking to discrete compartments such that APP processing to generate amyloid-β (Aβ) is inhibited [3, 4]. Finally, LR11 expression is

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Table 1. Characteristics of patients and control subjects

	AD patients	FTLD patients	Control subjects
Number (male/female)	29 (13/16)	20 (12/8)	27 (12/15)
Age at examination, years	69.4 ± 11.1 (51–85)	69.3 ± 10.5 (52–89)	65.5 ± 12.2 (44–85)
Age at onset, years	64.2 ± 12.2 (47–78)	63.1 ± 10.2 (47–84)	n./a.
MMSE score	15.4 ± 9.3 (0–26)	13.8 ± 8.0 (0–26)	n./a.
CSF tau, pg/ml	520 ± 320 (160–1,150)**	223 ± 77 (118–343)	160 ± 99 (60–325)
CSF Aβ42, pg/ml	84.9 ± 36.1 (26.2–166.6)*	93.2 ± 41.2 (33.3–160.7)	104.2 ± 41.5 (23.0–206.3)
CSF tau/Aβ42	6.6 ± 1.1 (1.6–17.8)**	2.7 ± 1.3 (0.9–5.2)	1.8 ± 1.0 (0.3–3.6)

Values are shown as means ± SD (range). n./a.: data are not applicable.
* $p < 0.05$ compared with control subjects; ** $p < 0.01$ compared with FTLD patients and control subjects by ANOVA, followed by Tukey's post hoc test.

downregulated in the brains of AD patients [5, 6]. These findings prompted us to quantify the soluble LR11 (sLR11) levels in the cerebrospinal fluid (CSF) of patients with AD.

We have recently developed a novel sandwich enzyme-linked immunosorbent assay (ELISA) by producing specific monoclonal antibodies against human LR11, which enabled the sensitive quantification of sLR11 using CSF samples [7]. By this ELISA, we determined the difference in the levels of CSF sLR11 between patients with AD, patients with frontotemporal lobar degeneration (FTLD) and age-matched control subjects.

Materials and Methods

Participants

We studied 29 AD patients, 20 FTLD patients and 27 age-matched control subjects (table 1). The diagnosis of probable AD was made in accordance with the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer Disease and Related Disorders Association [8]. The clinical criteria for the diagnosis of FTLD were based on consensus criteria [9]. Each patient underwent neurological examination, neuroimaging (CT and/or MRI) and laboratory tests to exclude alternative causes of cognitive decline. The *APOE* genotype was determined by *HhaI* digestion of the PCR product in 21 AD patients. CSF samples were also collected from age-matched control subjects, who underwent lumbar puncture to exclude subarachnoid hemorrhage, for lumbar anesthesia or for diagnostic reasons in patients with leukemia in complete remission. The inclusion criteria of the control subjects were absence of memory complaints, preservation of general cognitive functioning and no active neurological and psychiatric diseases. All the samples showed values within the normal ranges including cell count, and glucose, protein and electrolyte levels. Samples were collected after informed consent had been obtained from the participants or their caregivers. The study protocol was approved by the Niigata University School of Medicine.

ELISA of CSF Samples

CSF was collected by lumbar puncture. Samples of CSF were centrifuged at 4,000 *g* for 10 min and the supernatant was immediately frozen in polypropylene tubes and stored at −80°C until use. The storage period of CSF in the freezer ranged from 0 to 3.5 years with an average of 2.1 years. The levels of CSF tau and Aβ42 were determined using commercially available ELISA kits (tau, Innogenetics, Belgium; Aβ42, High-Sensitive, Wako, Japan) as previously reported [10]. The sLR11 level was determined by sandwich ELISA as previously reported [7]. Briefly, a CSF sample (10 μl) diluted with sample buffer was reacted with the capture MAb M3 antibody and then incubated with the biotinylated reporter MAb R14 antibody. The LR11-Mab complex was reacted with horseradish-peroxidase-conjugated streptavidin. A standard curve was constructed using a purified LR11 protein.

Statistical Analysis

All statistical analyses were performed using commercially available software, SPSS 12.0J (SPSS Japan, Japan). The results are shown as means ± standard deviation (SD). Comparisons between groups of subjects were performed by analysis of variance (ANOVA), followed by Tukey's post hoc test. An exploratory correlation was examined using Pearson's correlation coefficient. Receiver operating characteristic curve (ROC) analysis was used to determine the best cutoff values for the measured biomarkers.

Results

There was no statistically significant difference in age at examination or onset among AD patients, FTLD patients and control subjects (table 1). We first quantified tau and Aβ42 levels in CSF, which are well-established CSF biomarkers of AD. As expected, the tau level and tau/Aβ42 of the AD patients were significantly higher than those of the FTLD patients ($p < 0.01$) and control subjects ($p < 0.01$). In addition, the Aβ42 level of the AD patients

Table 2. Diagnostic discrimination between Alzheimer patients and control subjects

CSF biomarker	Cutoff value	Sensitivity, %	Specificity, %	AUC %
sLR11, ng/ml	11.3	66	78	71
sLR11/Aβ42	1.4	71	88	79
Tau, pg/ml	287	82	95	88
Tau/Aβ42	2.4	89	81	85

AUC = Area under the ROC curve.

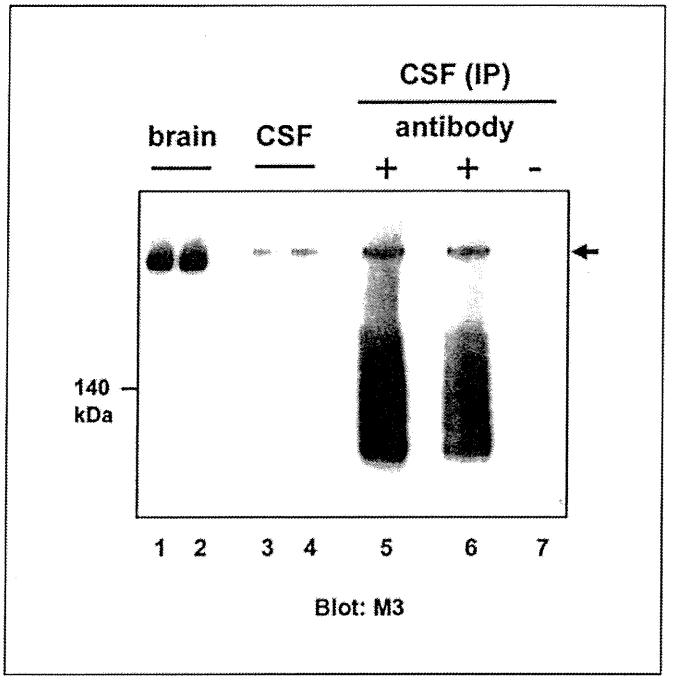

Fig. 1. Characterization of the antibodies against LR11. Human CSF samples (200 μl) from 2 individuals were immunoprecipitated with the R14 antibody (lanes 5 and 6) or without antibody (lane 7). The immune complexes were collected with protein-G-conjugated agarose beads, followed by separating by 6% Tris/glycine polyacrylamide gel electrophoresis. Fractioned proteins transferred to a PVDF membrane were immunoblotted with the M3 monoclonal antibody. Brain samples (lanes 1 and 2) and neat CSFs (10 μl; lanes 3 and 4) were also run on the same gel. A single band migrating at ~250 kDa corresponding to LR11 indicated by an arrow was detected in CSF. An antibody-derived smear band is present in the samples immunoprecipitated with the R14 antibody (lanes 5 and 6) but not in the sample without antibody (lane 7).

was significantly lower than that of the control subjects ($p < 0.05$).

We quantified the CSF sLR11 level of AD patients, FTLD patients and age-matched controls by sandwich ELISA [7]. First, the specificities of the monoclonal anti-LR11 antibodies, M3 and R14, used in ELISA, were examined by Western blot analysis. Immunoblot and immunoprecipitation assays using CSF samples detected a single band migrating at ~250 kDa corresponding to sLR11 (fig. 1). ELISA analysis revealed that the CSF sLR11 level in the AD patients was 13.1 ± 5.6 ng/ml (mean ± SD; median = 12.3; range = 4.6–31.7), which was significantly higher than those in the FTLD patients (8.3 ± 2.4 ng/ml; median = 8.5; range = 3.9–11.5) and controls (9.3 ± 4.5 ng/ml; median = 8.0; range = 3.0–25.9) ($p < 0.01$, fig. 2a). There was no significant correlation of CSF sLR11 level with age at examination or onset, or with Mini-Mental State Examination (MMSE) score. The CSF sLR11 level was comparable among 3 groups of AD patients divided by stage of mild (MMSE = 21–26; sLR11 = 13.2 ± 7.2 ng/ml), moderate (MMSE = 11–20; sLR11 = 12.2 ± 4.3 ng/ml) and severe (MMSE <10; sLR11 = 14.1 ± 3.5 ng/ml) dementia.

We determined the genotype of *APOE* of the AD patients. Sixty-two percent of our AD patients were *APOE*-ε4-positive. *APOE*-ε4-positive AD patients have higher sLR11 levels (13.1 ± 2.9 ng/ml, n = 13, range = 8.6–19.9) than *APOE*-ε4-negative patients (8.1 ± 3.3 ng/ml, n = 8, range = 4.6–15.0) ($p < 0.01$, fig. 2b). Comparable CSF sLR11 levels were found between AD patients heterozygous and homozygous for the *APOE*-ε4 allele.

The sensitivity and specificity of the CSF biomarkers including sLR11, sLR11/Aβ42, tau and tau/Aβ42 were determined by ROC analysis (table 2). ROC analysis of the CSF sLR11 levels in AD and control groups showed a sensitivity of 66%, a specificity of 78% and an area under the ROC curve (AUC) of 71% at a cutoff value of 11 ng/ml. The sLR11/Aβ42 ROC curve analysis increased the sensitivity to 71%, the specificity to 88% and the AUC to 79%. The tau level was the most effective discriminator with a sensitivity of 82%, a specificity of 95% and an AUC of 88% at a cutoff value of 287 pg/ml.

Discussion

Using the sensitive sandwich ELISA that we have recently developed [7], we demonstrated that the CSF sLR11 level is significantly higher in the AD patients than in the FTLD patients and age-matched control subjects. This result suggests that CSF sLR11 may serve as a potential bio-

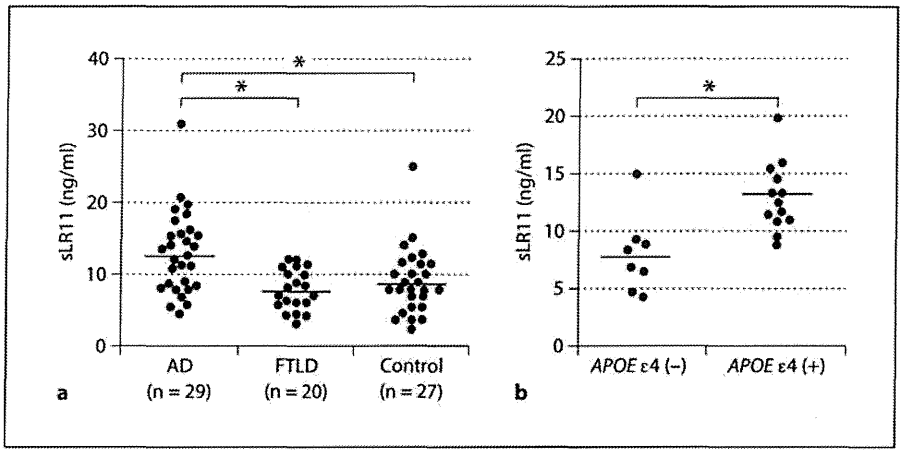

Fig. 2. Quantification of sLR11 in CSF. **a** sLR11 in CSF of AD patients, FTLD patients and age-matched control subjects was quantified by sandwich ELISA. Horizontal lines indicate median values. Statistical differences between groups were calculated by ANOVA, followed by Tukey's post hoc test. * $p < 0.01$. **b** Comparison of CSF sLR11 levels between *APOE*-ε4-positive and *APOE*-ε4-negative patients with AD. Horizontal lines indicate median values. Higher sLR11 levels were observed in *APOE*-ε4-positive than in *APOE*-ε4-negative AD patients (Mann-Whitney U test, * $p < 0.01$).

marker for AD. However, ROC analysis revealed that the measurement of sLR11 in CSF is not as sensitive for diagnostic status of AD as that of total tau; hence, the diagnostic significance of the CSF sLR11 level as a single biomarker might be limited.

In the present study, we showed that *APOE*-ε4-positive AD patients have higher levels of sLR11 than *APOE*-ε4-negative patients. This finding suggests that an elevated CSF sLR11 level in patients may be relevant to AD pathogenesis because the presence of *APOE*-ε4 constitutes a major risk factor for AD. LR11 binds multiple ligands including ApoE and plays a role in the endocytosis of ApoE-containing lipoproteins [11]. In CSF, ApoE is reported to form a complex with Aβ [12]. Interaction between ApoE and LR11 might interfere with the formation of the ApoE-Aβ complex, and this process may enhance Aβ deposition in the brain by increasing the amount of unbound Aβ species. Thus, it will be intriguing to examine the pathogenesis of AD through interaction of LR11 with the action of ApoE4.

In this study, we quantified a soluble form of LR11 in CSF. Full-length membrane-bound LR11 is first processed and activated by a furin-like activity, followed by ligand-induced ectodomain shedding by metalloprotease to release sLR11 [13–15]. A large fraction of the LR11 in the cell membrane is reported to release sLR11 into the culture medium upon proliferation of smooth-muscle cells [14]; however, it is largely unknown how the production of sLR11 is regulated in neuronal cells. In addition, the relationship between the sLR11 level in CSF and membrane-bound LR11 expression in brain parenchyma has not been determined yet. Thus, the mechanism underlying the elevation of CSF sLR11 levels in AD patients warrants further investigation.

In contrast to our results, Ma et al. [16] have recently reported that the CSF sLR11 levels were decreased in AD patients, although a relatively small number of AD patients (n = 19) were examined in their study. The reasons for the discrepancy between our findings and their results of CSF sLR11 level in AD patients have not been completely determined so far; however, a possible explanation may be the difference in the methodology used for the detection of CSF sLR11. In this regard, we took advantage of the availability of the sandwich ELISA using 2 antibodies with different recognition sites to quantify CSF sLR11, whereas Ma et al. [16, 17] performed semi-quantitative analysis by comparing the optical density of bands obtained by immunoblotting of sLR11. An alternative explanation is that Japanese AD patients may have different LR11 expression levels in the brain because an animal study has suggested that the LR11 level in the brain can be increased by a docosahexaenoic-acid-supplemented diet. To address this issue, we examined the LR11 level in the brains of Japanese AD patients (n = 5) in comparison with age-matched control subjects (n = 5). The analysis revealed that the levels of LR11 in the brains of Japanese AD patients were comparable to those in the brains of control subjects (unpublished data), and the results were in contrast to the previous reports [5, 6].

Although our study has potential clinical implications, it also has limitations. First, the diagnosis of the patients was based on clinical criteria, not on autopsy verification. It has been reported that global cognitive function correlated with LR11 expression in the brain, as determined by quantitative immunostaining of LR11 [6], whereas our study showed no correlation between CSF sLR11 level and MMSE score in AD patients. An important question is whether the change in CSF sLR11 levels

is related to LR11 expression levels in the AD brain parenchyma. Second, the difference in sLR11 levels between AD and control groups is relatively small; hence, the diagnostic value for individual patients appears to be limited. Future studies may establish the usefulness of the CSF sLR11 level as a longitudinal biomarker. Lastly, our results were obtained using relatively small sample sets. Clearly, further careful validation studies with larger sample sets will be required for the evaluation of CSF sLR11 as a biomarker of AD.

Acknowledgements

The authors wish to thank the participants for their cooperation. This study was supported by Grants-in-Aid from the Ministry of Health, Labour and Welfare, Japan, a Grant-in-Aid for Scientific Research (21200041) from the MEXT, Japan, the Takeda Science Foundation and the Suzuken Memorial Foundation.

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Journal of Alzheimer's Disease 28 (2012) 543–552
DOI 10.3233/JAD-2011-110983
IOS Press

Interrelations between CSF Soluble AβPPβ, Amyloid-β 1-42, SORL1, and Tau Levels in Alzheimer's Disease

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Handling Associate Editor: Thomas Leyhe

Accepted 22 September 2011

Abstract. Recently, light has been shed on possible interrelations between the two most important pathological hallmarks of Alzheimer's disease (AD): the amyloid cascade and axonal degeneration. In this study, we investigated associations between sβAPPβ, a product of the cleavage of the amyloid-β protein precursor (AβPP) by β-secretase, amyloid-β 1-42 ($A\beta_{42}$), soluble SORL1 (also called LR11 or SORLA), a receptor that is involved in AβPP processing, and the marker of axonal degeneration tau in the cerebrospinal fluid (CSF) of 76 patients with mild cognitive impairment (MCI), 61 patients with AD, and 17 patients with frontotemporal dementia, which neuropathologically is not related to the amyloid pathology. In the AD group, significant associations between sAβPPβ, tau ($p<0.001$), and soluble SORL1 ($p<0.001$) were detected according to linear regression models. In patients with MCI, sAβPPβ correlated significantly with tau ($p<0.001$) and soluble SORL1 ($p=0.003$). In the FTD group, only SORL1 ($p=0.011$) was associated with sAβPPβ and not tau. $A\beta_{42}$ was found to be significantly related to tau levels in CSF in the MCI group ($p<0.001$) and they tended to be associated in the AD group ($p=0.05$). Our results provide further evidence for a link between the two facets of AD pathology, which is likely to be mediated by the binding of Aβ oligomers to specifically targeted neurons, resulting in stimulating tau hyperphosphorylation and neurodegeneration.

Keywords: Alzheimer's disease, amyloid, amyloid-β 1-42, association, soluble AβPPβ, SORL1, tau

INTRODUCTION

The pathological hallmarks of Alzheimer's disease (AD) comprise extracellular fibrillar amyloid-β (Aβ) deposits and soluble Aβ oligomers (both products of the amyloid cascade), intracellular neurofibrillary tangles formed by abnormally phosphorylated tau protein, astrocytosis, and synaptic as well as neuronal loss [1]. It is an important and tempting research task to

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ISSN 1387-2877/12/$27.50

unveil how the multiple facets of AD pathology are interlinked.

The proteolytic breakdown of the amyloid-β protein precursor (AβPP) by β-secretase generates the β-secretase cleaved soluble AβPP (sAβPPβ) and the peptide C99. The subsequent proteolysis of C99 by γ-secretase results in the generation of several isoforms of Aβ. The fibrillar forms of Aβ, mainly consisting of the isoform $Aβ_{42}$ which is one of the main constituents of amyloid plaques, were initially considered to be the drivers of neuronal damage [2]. However, new observations provide evidence that small soluble Aβ oligomers which have no propensity for aggregation represent the most synaptotoxic species of the peptide [2]. Aβ oligomers are generated by the ability of β- and γ-secretase to execute proteolytic cleavage at different positions in AβPP, as well as by the probable involvement of other AβPP- and Aβ-degrading proteases. Interestingly, Aβ oligomers have been shown to be increased in the brain and in the cerebrospinal fluid (CSF) of patients with AD and to correlate with neurofibrillary tangle density [3-4]. According to findings of cell culture studies, they attach to synapses in the central nervous system and inhibit long-term potentiation, enhance long term depression, induce oxidative stress and abnormal phosphorylation of tau, and subsequently foster axonal degeneration [5–7]. It is known that Aβ oligomers activate glycogen synthase kinase-3β (GSK3β), Src family tyrosine kinases and phosphatidylinositol 3-kinase (PI3K), which are involved in the pathological hyperphosphorylation of tau [5, 8]. Intrahippocampal injection of an anti-oligomer antibody unexpectedly resulted in the clearing of both Aβ and tau pathology in a triple transgenic mouse model harboring mutant human AβPP, tau, and presenilin 1 [9]. Moreover antibodies against Aβ lead to a reduction of soluble Aβ oligomers, but not insoluble Aβ and lead to a decline of both GSK3β activation and tau phosphorylation [10]. However, the link between the amyloid cascade and tau pathology in AD still remains elusive, especially in the absence of data from patients suffering from AD.

In recent years, the sortilin-related receptor with A-type repeats (SORL1, also called LR11 or SORLA), a member of the apolipoprotein E and low-density lipoprotein receptor family, has captured scientific attention as a factor that is crucially implicated in the sorting of AβPP and in its interactions with secretases [11]. SORL1 is diffusely expressed throughout the brain and acts as an intracellular sorting receptor that engages in the Golgi apparatus-endosome transport [12]. SORL1 promotes the retention of AβPP in subcellular compartments which are less favorable for secretase processing and thereby reduces the extent of proteolytic breakdown into both amyloidogenic and non-amyloidogenic products [13]. The interaction between AβPP and SORL1 is not limited to the formation of complexes, but also comprises SORL1-dependent translocation of AβPP and a concomitant drastic decrease of AβPP cleavage [12]. Reduction of SORL1 levels in specific cell compartments leads to overproduction of Aβ [14], since the reduction of SORL1 switches AβPP away from the retromer recycling pathway and instead exposes AβPP to α- and β-secretase cleavage [12]. In line with these findings, the neuronal expression of SORL1 is dramatically decreased in AD brains [15–17]. However, SORL1 expression is not decreased in familial AD, suggesting that diminished SORL1 expression is not a consequence of amyloid accumulation [15]. Furthermore, SORL1 gene variants are assumed to be among the strongest genetic predisposition factors for AD [14, 18]. Nonetheless, no general consensus on the role of SORL1 genetic variants as risk factors for AD exists, since other investigations found only weak or no associations between SORL1 genetic variants and AD [19–24].

sAβPPβ is not prone to aggregation, and since it can be detected in the CSF, its levels in CSF do not reflect the generation only of $Aβ_{42}$ but of all Aβ peptides. The CSF is in direct contact with the central nervous system, therefore many alterations in the biochemical composition of brain parenchyma are reflected in the CSF, owing to the free exchange of molecules between the brain and the CSF [25]. The present study aimed to investigate possible associations between CSF levels of sAβPPβ, $Aβ_{42}$, and tau in patients with mild dementia in AD, patients with mild cognitive impairment (MCI), which in many cases represents a prodromal phase of AD [26], and patients with frontotemporal dementia (FTD) [27], a form of neurodegeneration which does not involve amyloid pathology. Since increased sAβPPβ may be associated with higher levels of Aβ oligomers, which might foster hyperphosphorylation of tau and subsequently axonal degeneration, a positive correlation between tau and sAβPPβ in patients with AD and possibly in the MCI group, but not in patients with FTD was expected. A further aim of the study was to elucidate possible relations between CSF SORL1 concentrations and sAβPPβ and $Aβ_{42}$, since according to the observations of cell culture studies SORL1 influences the cleavage of AβPP by secretases, resulting in the generation of sAβPPβ among further molecules. As a result, a negative correlation between

SORL1 and sAβPPβ and a positive between SORL1 and $A\beta_{42}$ in CSF possibly in all groups of participants was expected.

METHODS

The study protocol was approved by the ethics committee of the Faculty of Medicine at Technische Universität München. The study was conducted in accordance with the 1964 Declaration of Helsinki. All participants gave their written informed consent after an extensive description of the study aims and procedures.

Participants

The study encompassed 76 patients with MCI, 61 patients with mild dementia in AD, and 17 patients with FTD, who were recruited at the Department of Psychiatry and Psychotherapy at Technische Universität München. The examination of the patients included a history from the patient and from an informant, medical, neurological, and psychiatric examination, laboratory screening, structural brain imaging (MRI or CT), and a neuropsychological examination based on the German version of the Consortium to Establish a Registry for AD neuropsychological assessment battery (CERAD-NAB) [28]. The diagnosis of dementia was based on the criteria of the ICD-10 classification system [29]. To ensure that patients with dementia had not crossed the threshold to moderate dementia, patients with a score below 15 points on the MMSE were excluded from the study. This score has been found to discriminate mild from moderate dementia [30]. MMSE staging has been proven to be an effective clinical instrument for tracking the stages of dementia [30]. Patients with AD fulfilled the criteria of the National Institute of Neurological and Communicative Disorders and Stroke-AD and Related Disorders Association (NINCDS-ADRDA) for probable AD [31]. Patients with MCI met the revised consensus criteria of the International Working Group on MCI [32]. The diagnosis of FTD was established according to the revised Lund-Manchester criteria [33].

CSF sampling and analyses

CSF was collected in sterile polypropylene tubes, using atraumatic canulas placed in the L3/L4 or L4/L5 intervertebral space, and gently mixed. The CSF was centrifuged at 1800 g (4°C) for 10 min to remove cells and aliquots of the remaining CSF supernatants were stored in polypropylene tubes at −80°C.

Determination of tau, $A\beta_{42}$, and sAβPPβ levels

CSF $A\beta_{42}$, total tau (Innogenetics, Ghent, Belgium), and sAβPPβ (IBL, Gunma, Japan) in CSF were measured in duplicate with commercially available enzyme-linked immunosorbent assays (ELISA) according to the manufacturers' instructions as described previously in detail [34–36].

SORL1 concentrations

SORL1 concentrations in CSF were determined using ELISA by Sekisui Medical Co Ltd. (Ryugasaki, Japan) as described previously [37]. Briefly, 10 µl CSF was diluted with 100 µl sample buffer and added to the plate coated with mouse monoclonal antibody M3 [38]. Subsequently, after incubating with the biotinylated rat monoclonal antibody R14, the SORL-antibody complex reacted with horseradish peroxidase-conjugated streptavidin and substrate. A standard curve was constructed using a purified SORL1 protein. The final absorbance of each sample was measured at 450 nm. The intraassay and interassay coefficients of variation were 3.7% and 10.5% respectively [37]. SORL1 concentrations were determined in 57 patients with MCI, in 42 with AD, and in all patients with FTD.

Statistical analyses

Statistical analyses were implemented in IBM SPSS Statistics 19.0 for Windows. The normal distribution of data was checked using the Kolmogorov-Smirnov test. Differences between the groups with regard to age, sAβPPβ, $A\beta_{42}$, SORL1, and MMSE were tested by analysis of variance (ANOVA), and with regard to tau CSF concentrations with the Kruskal-Wallis test. Pairwise comparisons were performed using the Bonferroni's test or the *T*-test (normally distributed data) and the Mann-Whitney test (data not normally distributed). X^2 tests were employed for nominal (categorical) data. Possible associations between CSF tau on the one hand and sAβPPβ and $A\beta_{42}$ on the other hand in each of the three groups of the study sample were investigated with linear regression analysis models, into which tau concentrations were fed as dependent variable and sAβPPβ, $A\beta_{42}$, age, and gender as explanatory variables. The MCI group was dichotomized with regard to tau values, as markers of neurodegeneration, in order to investigate