knowledge about the key players regulating the mobilization of leukemia cells is still severely limited [1,9,10]. Considering the progress in cell biological and genetic classification of leukemia cells, one approach to address the unsolved issues is the identification of an appropriate circulating molecule(s) released directly from the leukemic cells.

LR11 (also called SorLA or SORL1) is a type I membrane protein, from which a large soluble extracellular part, sLR11, is released by proteolytic shedding [11–14]. Substantial studies have shown that LR11 plays important roles both in intimal thickening and macrophage-foam cell formation in the process of atherosclerosis and in amyloidogenesis of Alzheimer disease [15,16]. The smooth muscle cell (SMC)-derived sLR11 causes macrophage infiltration in injured arteries by binding to urokinase-type plasminogen activator receptor (uPAR; CD87) on the cell surface [17,18]. Membrane-bound LR11 regulates the intracellular trafficking and processing of amyloid precursor protein (APP) by impairing the cleavage of APP through secretases in a way that leads to reduced levels of amyloid beta protein, the major component of amyloid plaques [19]. Thus, the levels of the membrane-bound form in neurons and of the soluble form in cerebrospinal fluid are highly associated with the pathogenesis of Alzheimer disease [20–22], and the circulating levels of the soluble form reflect the progression of atherosclerosis [23,24].

In addition, Zhang et al. reported high levels of LR11 mRNA in human $CD34^+CD38^-$ immature hematopoietic precursors [25]. However, little is known about the expression and the role(s) of LR11 and/or sLR11 in human hematopoietic cells. CD87 binds to and facilitates CD11b/CD18-mediated adhesion of human monocytes and induces proinflammatory signaling in human polymorphonuclear neutrophils [26]. Furthermore, CD87 is highly expressed in acute myeloid leukemia (AML) cells, especially myelo-monocytic subtypes, and patients with higher proportions of $CD87^+$ cells show a significantly lower remission rate and higher relapse risk [27,28]. Here, we investigated whether circulating sLR11 may represent the pathological conditions of undifferentiated leukemic cells.

2. Materials and methods

2.1. Antibodies and cells

Monoclonal antibodies (A2-2-3, M3) against LR11 were described previously [29]. M3 was used for flow cytometric analysis, and A2-2-3 for immunoblotting. Human leukemia cell lines, Daudi, MOLT-4, TALL-1, CCRF-SB, HL-60, ML-2, NB-4, RPMI8226, U937, and K562 were purchased from ATCC. The neuroblastoma cell line IMR32 was used as control cells expressing LR11 mRNA and protein, and releasing the soluble form as described [18,29]. All cultured cells were maintained in the media recommended by ATCC with 10% fetal bovine serum (FBS, Terumo). For mRNA and protein experiments, cells were used after incubation with serum-free media (1×10^6 cells/ml) for 30 h. For immunoblot analysis of sLR11 in the culture media, following serum starvation for 24 h, the cells were cultured with fresh serum-free media, and after 6 h the conditioned media were collected. The collected media were used for immunoblot analysis of sLR11 after concentration using Centricon-100 (Millipore). Before each experiments, cell viability was tested by trypan blue dye exclusion and confirmed the viability >95%.

2.2. Flow cytometry

Samples were analyzed on a JSAN desktop cell sorter (Bay Bioscience) with FlowJo software (Tree Star) as described [30]. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-LR11 monoclonal antibody M3 [29] in the presence or absence of antibodies against CD4, CD8, CD14, CD19, CD34, CD38, or CD11b (Becton Dickinson, San Jose, CA). For comparison of surface expression, mean fluorescence intensity (MFI) was evaluated. Surface expression of LR11 on peripheral leukocytes was analyzed in three healthy volunteers.

2.3. sLR11 ELISA

sLR11 levels were determined by the sandwich ELISA previously reported [29]. Briefly, samples (10 μl) diluted with sample buffer were reacted with the capture monoclonal antibody M3, and then incubated with the biotinylated rat monoclonal reporter antibody R14. The LR11-antibody complex was quantitated with horseradish peroxidase-conjugated streptavidin using purified LR11 protein as standard.

2.4. Immunoblotting

Cultured cells were washed three times with PBS, and collected in solubilization buffer (200 mmol/l Tris-maleate, pH 6.5, 2 mmol/l $CaCl_2$, 0.5 mmol/l PMSF, 2.5 μmol/l leupeptin and 1% Triton X-100) as described [24]. For immunoblotting, equal amounts of membrane protein or collected media were subjected to 10% SDS-PAGE after heating to 95 °C for 5 min as described under reducing conditions, and transferred to a nitrocellulose membrane. For immunodetection, A2-2-3 (1:500 dilution) was used, followed by peroxidase-conjugated anti-mouse IgG. Development was performed with the ECL detection reagents (Amersham Pharmacia, Piscataway, NJ). The signals were quantified by densitometric scanning using NIH image™ software.

2.5. Real-time quantitative PCR

Total RNA was prepared from cultured cells using an RNeasy kit (Qiagen, Valencia, CA) as described [24]. The methods for real-time quantitative PCR (qPCR) have been described [24]. For quantification of transcript levels, qPCR was performed using SYBR green PCR master mix and the PCR primers for LR11 mRNA (Hs00300475_s1, Applied Biosystems, Foster City, CA). mRNA amounts were normalized to levels of 18S ribosomal mRNA (Hs99999901_s1), which served as internal standard.

2.6. Patients

Flow cytometric analysis of LR11 and CD87 expression on leukemic cells was performed and MFI was evaluated in AML and acute lymphoblastic leukemia (ALL) patients between 2009 and 2011 in Chiba University Hospital. For sLR11 measurement, 139 subjects with various hematological diseases including 43 AML and 23 ALL patients diagnosed from 1999 to 2010 in Chiba University Hospital or affiliated hospitals and last followed-up in June 2010 were enrolled in this study, which was approved by the Human Investigation Review Committee of the Chiba University Graduate School of Medicine or affiliated hospitals. All acute leukemia patients were treated by chemotherapy according to the protocols established by the Japan Adult Leukemia Study Group (JALSG, http://www.jalsg.jp/english/01/e_index.html). Serum samples of patients with AML or ALL were collected at diagnosis and at remission, and subjected to analysis of sLR11 by ELISA. All clinical data were subjected to statistical analysis. One hundred serum samples from healthy volunteers were used for control.

2.7. Statistical analysis

Comparisons of serum sLR11 levels between patient and control samples, and between tertiles of peripheral blast proportions were evaluated by Dunnett's test. Serum sLR11 levels at diagnosis and at remission were compared using Wilcoxon's signed-rank test. As for variable selection, the stepwise procedure was set to

a threshold of 0.15 for inclusion or exclusion in linear regression analyses. All comparisons were planned and the tests were two-sided. A P value of <0.05 was considered to be statistically significant. All statistical analyses were performed using the JMP (ver 7.0.2, SAS Institute Inc., Cary, NC) and SAS (ver 9.2, SAS Institute Inc.) programs.

3. Results

3.1. Surface expression of LR11 on leukocytes and leukemic cells

First, we examined the expression of LR11 on leukocytes and leukemic cells by flow cytometry. Most $CD14^+$ peripheral monocytes express

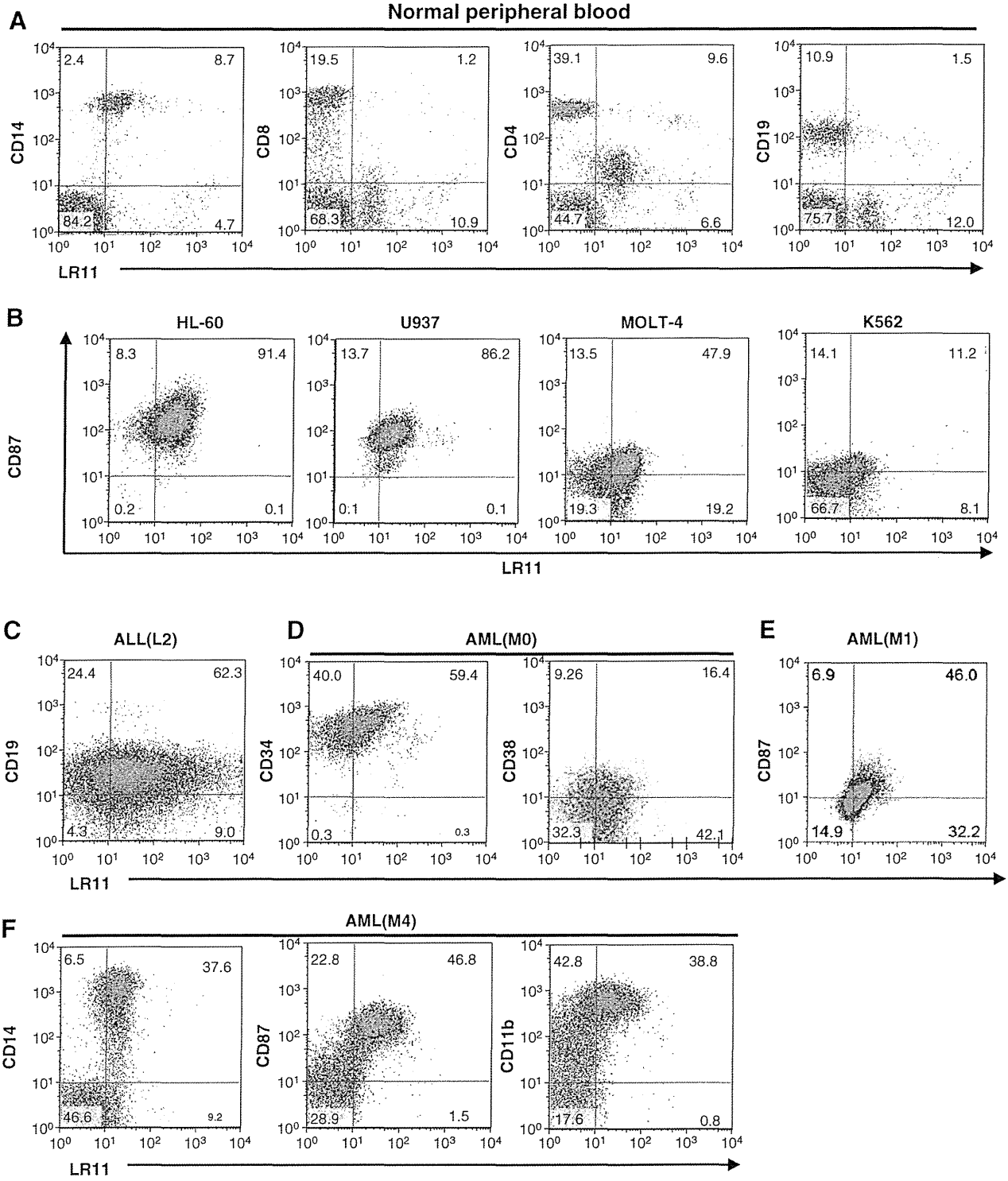

Fig. 1. Identification of LR11-expressing cells in peripheral blood leukocytes and leukemia cells by flow cytometry. (A) Peripheral blood mononuclear cells obtained from a normal subject were stained with the indicated antibodies. (B) Co-expression of LR11 and CD87 on leukemic cell lines. Representative data were shown. (C–F) Co-expression of LR11 and CD87 on leukemic cells. Bone marrow or peripheral blood mononuclear cells obtained from leukemia patients were stained with the indicated antibodies. The percentage of cells in each quadrant is indicated.

Table 1
Surface expression of LR11 and uPAR (CD87) on patients' leukemic cells.

Patient	FAB	LR11		uPAR (CD87)		Double positive	
	Classification	(%)	Positivity[a]	(%)	Positivity[a]	(%)	Positivity[a]
AML							
1#	M0	59.4	+	16.0	+	12.9	+
2	M1	30.9	+	24.2	+	14.3	+
3[b]	M1	78.2	+	52.9	+	46.0	+
4	M1	64.9	+	34.2	+	24.7	+
5	M2	33.7	+	8.2	−	2.5	−
6	M2	71.0	+	34.3	+	28.7	+
7	M2	18.7	+	8.0	−	4.1	−
8	M2	5.8	−	3.9	−	1.4	−
9	M2	73.7	+	42.1	+	33.0	+
10	M2	24.9	+	1.0	−	0.6	−
11	M2	22.7	+	38.9	+	17.6	+
12	M2	51.1	+	20.0	+	13.1	+
13	M2	8.7	−	2.8	−	1.9	−
14	M2	62.2	+	25.2	+	20.5	+
15	M3	66.5	+	37.0	+	30.6	+
16	M3	72.6	+	9.0	−	7.8	−
17	M3	14.4	+	1.0	−	0.4	−
18[b]	M4	48.6	+	69.6	+	46.8	+
19	M4	25.0	+	13.0	+	10.9	+
20	M4	89.3	+	61.8	+	56.1	+
21	M4	34.9	+	10.1	+	6.4	−
22	M4	25.7	+	33.1	+	13.1	+
23	M4	15.5	+	12.0	+	4.6	−
24	M6	92.1	+	31.5	+	29.1	+
Mean ± SD		45.1 ± 25.7	22/24 (91.7%)	24.5 ± 19.1	17/24 (70.8%)	16.0 ± 33.8	15/24 (62.5%)
ALL							
1[b]	L2	76.3	+	20.6	+	14.5	+
2	L2	9.5	−	6.6	−	1.1	−
3	L2	47.3	+	26.3	+	11.7	+
4	L2	63.0	+	1.6	−	0.8	−
5	L2	60.2	+	22.5	+	17.6	+
6	L2	12.7	+	5.2	−	1.0	−
7	L2	90.8	+	5.4	−	5.4	−
8	L2	93.3	+	59.0	+	56.7	+
Mean ± SD		56.6 ± 32.1	7/8 (87.5%)	18.4 ± 18.9	4/8 (50%)	13.6 ± 18.6	4/8 (50%)

[a] Data cut-off level is 10%.
[b] Patients with AML Nos. 1, 3, and 18 and ALL No. 1 are corresponding to Fig. 1D, E, F, and C, respectively.

LR11 (Fig. 1A), whereas the expression is much lower in most T cells ($CD4^+$, $CD8^+$), B cells ($CD19^+$), or granulocytes (data not shown). The leukemia cell lines HL-60 (promyelocytic), NB-4 (promyelocytic), U937 (monocytic), ML-2 (myeloblastic), MOLT-4 (lymphoblastic), and CCRF-SB (lymphoblastic) express LR11 on their cell surface; the expression on K562 (chronic myelogenous) and TALL-1 (lymphoblastic) was lower (Fig. 1B). In addition, most of LR11 positive cells express CD87 on their cell surface. Thus, LR11 is significantly co-expressed with CD87 on the surface of leukemic cells.

Next, we explored the expression of LR11 on the surface of leukemia cells in 24 AML patients and 8 ALL patients. Representative data were shown in Fig. 1C–F. The $CD19^+$ cells in ALL (FAB L2) showed the highest levels of LR11, within a wide range (Fig. 1C). Over 50% of $CD34^+$ cells in AML (FAB M0) were LR11-positive, whereas LR11-positive blasts predominated in the $CD38^-$ fraction (Fig. 1D). Some leukemic cells co-expressed both LR11 and CD87 (Fig. 1E). Among AML patients, in FAB M4 the majority of mononuclear cells with high CD14-expression were LR11-positive with a narrow LR11 expression range and 97% and 98% of the LR11-expressing mononuclear cells were also positive for CD87 and CD11b, respectively (Fig. 1F). In total, 22 out of 24 AML cases (91.7%) and 7 out of 8 ALL cases (87.5%) were positive for LR11 on their leukemic cells, whereas 70.8% of AML cases and 50% of ALL cases were positive for CD87 (Table 1). In these patients, a significant portion of CD87-positive cells also co-expressed LR11 on their cell surface.

3.2. Correlation of LR11 levels and released sLR11 in leukemic cell lines

Next, we examined the significance of sLR11 from immature leukemia cells. Quantitative PCR and ELISA revealed differences in LR11 expression in mRNA and released sLR11 of 10 leukemia cell lines of different origins, respectively (Table 2). There was a clear-cut correlation between the levels of mRNA and released sLR11 protein (Fig. 2A).

Table 2
The relationship of mRNA expression, surface LR11 and CD87, and levels of cell-released sLR11 in various kinds of leukemia-derived cell lines.

Cell lines	LR11 mRNA (qPCR)[a]	sLR11 in culture media (ELISA)[a]	Mean fluorescence intensity	
			LR11	CD87
Daudi	3.7 ± 0.3	2.8 ± 0.6	7.8	10.5
MOLT-4	2.2 ± 0.1	2.9 ± 0.2	16.6	13.3
TALL1	0.4 ± 0.2	0.4 ± 0.1	5.4	5.7
CCRF-SB	7.9 ± 0.8	5.1 ± 0.7	14.5	10.6
HL-60	3.1 ± 0.5	3.6 ± 0.4	28.2	205.0
ML-2	3.8 ± 0.4	3.0 ± 0.8	46.5	126.0
NB-4	2.7 ± 0.2	1.8 ± 0.1	30.3	32.8
RPMI8226	1.0 ± 0.1	0.4 ± 0.2	10.3	16.9
U937	4.1 ± 0.7	3.2 ± 0.6	26.5	118.0
K562	0.1 ± 0.0	0.0 ± 0.0	10.6	8.9

[a] LR11 mRNA levels and sLR11 levels in the culture media are expressed relative to that in IMR32. Data are presented as mean ± SD (n = 3).

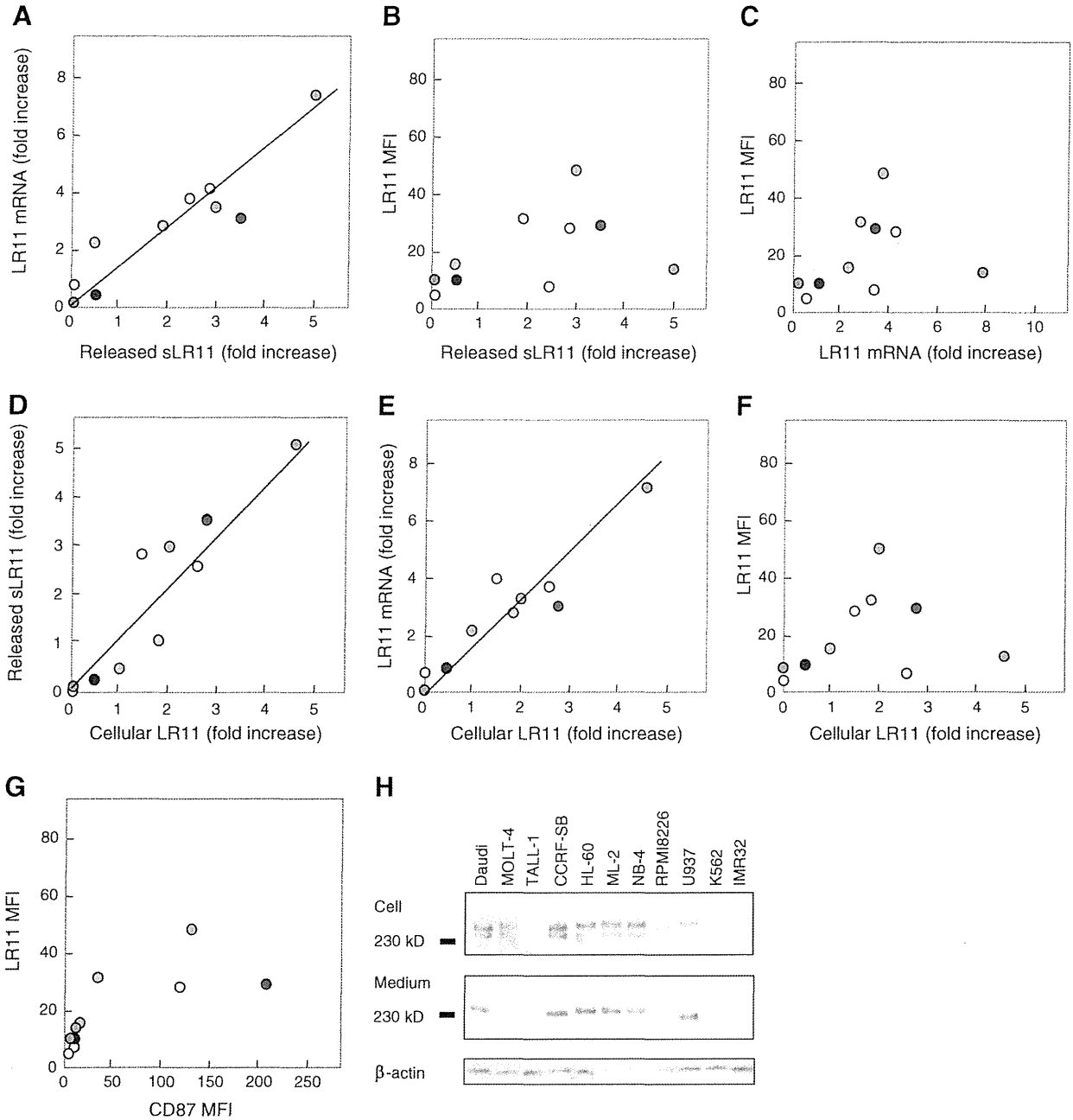

Fig. 2. The relationship between mRNA, cell protein, surface LR11, and levels of cell-released sLR11 in various kinds of leukemia-derived cell lines. (A–G) The relationships between the levels of LR11 mRNA and sLR11 protein (A), between the levels of surface LR11 (MFI) and sLR11 protein (B), between the levels of surface LR11(MFI) and LR11 mRNA (C), between the levels of sLR11 and cellular LR11 protein (D), between the levels of LR11 mRNA and cellular LR11 protein (E), between the levels of surface LR11 (MFI) and cellular LR11 protein (F), and between the levels of surface LR11 (MFI) and CD87 (MFI) (F) were analyzed using the results shown in Table 2 and (H). The signals for cellular LR11 in (H) were quantified by densitometric scanning using NIH software. The Spearman's rank correlation coefficients and P values are $r=0.94$ and $P<0.001$ in (A), $r=0.95$ and $P<0.001$ in (D), and $r=0.93$ and $P<0.001$ in (E), respectively. There were no significant correlations in (B), (C), (F) and (G). (H) LR11 protein in cells (1×10^6 cells/ml) after serum starvation for 30 h, and sLR11 in the serum-free media collected for 6 h. LR11 was detected as signals migrating at around 250 kDa by immunoblot analysis with antibody A2-2-3 directed against LR11. The deduced molecular weights of the membrane-bound form and the soluble form are approximately 250 and 230 kDa, respectively (17–19). Data are representative of 3 independent experiments.

On the other hand, surface expression levels of LR11 (expressed as MFI) were not significantly correlated with the levels of released sLR11 protein or LR11 mRNA (Fig. 2B and C, respectively). LR11 has been shown to localize in intracellular vesicles and to be released through shedding by proteinases from the membrane of neurons and smooth muscle cells [14–16,22,24]. We therefore analyzed the expression levels of cell-associated LR11 in the above cultured cells and the released protein by immunoblotting (Fig. 2H). There were significant correlations between cellular LR11 levels and the levels of released sLR11 or LR11 mRNA (Fig. 2D and E). On the other hand, there was no clear correlation between the cellular LR11 levels and surface LR11 expression levels (Fig. 2F), mainly because a few cell lines showed extremely high or low MFI of LR11 on the cell surface (e.g., CCRF-SB and Daudi), although the released sLR11 levels were dependent on the cellular LR11 levels in most of cell lines (Fig. 2H). The surface LR11 levels were not strictly restricted by the surface CD87 expression levels (Table 2, Fig. 2G). Thus, the release of LR11 by leukemia cell lines of different origins appeared to be the result of a dual regulation by transcriptional activity and proteinase shedding.

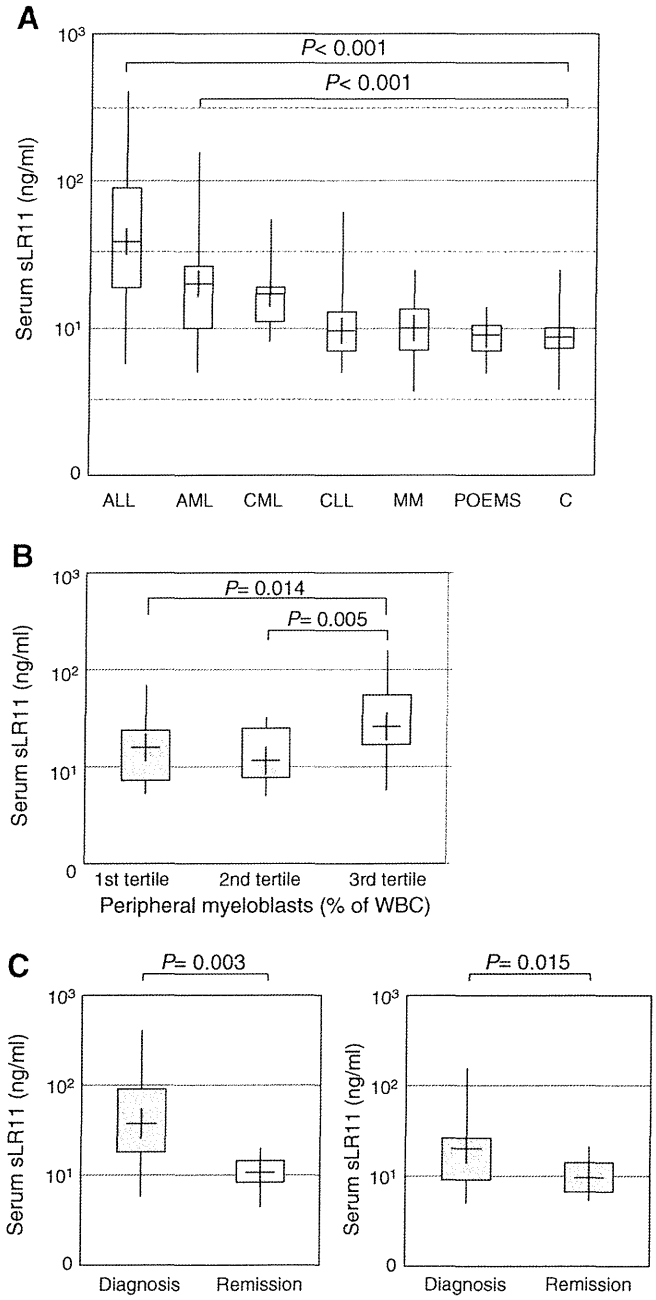

Fig. 3. Circulating sLR11 levels in patients with acute leukemias. (A) Upper and lower edges of boxes indicate upper and lower quartiles, respectively; upper and lower bars indicate maximum and minimum, respectively; short horizontal lines represent median values. MM indicates multiple myeloma; POEMS, POEMS syndrome; C, control subjects without diagnosed hematological diseases. (B) Serum sLR11 levels at diagnosis in patients with AML, grouped by tertiles (n = 14 each) of peripheral myeloblasts (% of WBC). Peripheral myeloblast ranges for 1st, 2nd, and 3rd tertiles were <20.0%, 23.0% to 64.0%, and >67.5%, respectively. P values were determined by Dunnett's test. (C) The paired data at diagnosis and at remission are shown for all patients with ALL (left) and AML (right).

3.3. Serum sLR11 in acute leukemia

Next, serum sLR11 levels were determined in 139 patients with diagnosed acute leukemias and other hematological diseases (Supporting Table S1, Fig. 3). sLR11 levels of acute leukemia patients at diagnosis were significantly increased over those in subjects without hematological disease (n = 100; median, 7.7 ng/ml; range, 4.7–17.2, S.D., 3.3). In ALL, we determined a median of 37.1 ng/ml (range, 5.7–407.0, S.D., 93.5; P<0.001) and in AML, a median of 20.0 ng/ml (range, 5.0–157.5, S.D., 29.1; P<0.001) (Fig. 3A). sLR11 levels in patients with chronic myeloid leukemia (CML; median, 17.1 ng/ml; range, 8.1–55.0, S.D., 11.1), chronic lymphocytic leukemia (CLL; median, 9.6 ng/ml; range, 5.0–62.0, S.D., 11.6), multiple myeloma (MM, median, 10.0 ng/ml; range, 3.7–25.0, S.D., 4.8), and POEMS syndrome (median, 9.0 ng/ml; range, 4.9–14.0, S.D., 2.7) were not significantly different from controls. To identify factors influencing serum sLR11 levels at diagnosis, we used Stepwise regression models including age, peripheral WBC count, peripheral blast population, peripheral blast count, bone marrow blast population, and serum LDH level. The correlation between peripheral blast proportion in AML and ALL and the sLR11 level was independent of other prognostic risk factors at diagnosis (Table 3). Among 42 AML patients, sLR11 levels of subjects in the highest tertile of peripheral blast proportion (>67.5% of WBC) were 2.44- and 3.05-fold higher than those in the middle (23.0–64.0% of WBC) and lowest tertiles (<20.0% of WBC), respectively (Fig. 3B). Paired sample analysis of patients with AML and ALL who achieved complete remission (CR) showed significantly decreased sLR11 levels compared to those at diagnosis, and most notably, in CR the levels were all below 20 ng/ml (Fig. 3C). These data suggest that sLR11 levels indeed are representative of the pathological conditions of patients with acute leukemias.

Table 3
Stepwise regression analysis for factors influencing serum sLR11 levels at diagnosis.

Variable	Estimate	Standard error	Adjusted β-coefficients	R^2	P value
AML					
Peripheral blast proportion (% of WBC)	0.38	0.12	0.45	0.21	0.003
ALL					
Peripheral blast proportion (% of WBC)	1.97	0.61	0.72	0.34	0.004

Stepwise regression models included age, peripheral WBC count, peripheral blast population, peripheral blast count, bone marrow blast population, and serum LDH level. The stepwise procedure was set to a threshold of 0.15 for inclusion and exclusion.

4. Discussion

This study has revealed that LR11 is a novel surface molecule for monocytes. Furthermore, LR11 as well as CD87, a partner of sLR11, were expressed on the surface of leukemic cells in AML and ALL for formation of a complex mediating functional intracellular signal activation. Since the patient data indicate a tight association of sLR11 levels with peripheral blast proportions independently of other prognostic risk factors, the level of sLR11 at diagnosis may serve as novel candidate information reflecting the status and efficacy of treatment of acute leukemias.

Zhang et al. reported that the high level of LR11 mRNA expression was found in peripheral white blood cells, and among white blood cells, LR11 was highly expressed in neutrophils and lower in lymphocytes, but not in monocytes. In contrast to these data, we found by flow cytometry that the surface expression of LR11 was higher in monocytes than in neutrophils and lymphocytes. LR11 is mostly localized in endosome vesicles in many kinds of cells [15,16], in which LR11 is believed to be important for the transport of intracellular proteins, such as APP in neuron [16]. The granular staining pattern in the cells has suggested that the soluble form of LR11 exists in addition to membrane-bound form in the vesicles. Thus, we have shown that the soluble form of LR11 is active for the complex formation with CD87 on the membrane as membrane bound form [12,17,18]. These data suggested that the levels of the released form of LR11 and of the surface form are possibly regulated by the combined activities of mRNA transcription and post-translational protein transport with subsequent proteinase-mediated shedding from the surface. In this context, LR11 has been shown to be released from neurons and

SMCs by the potential actions of proteinases including ADAM17/TACE [11–14].

Questions remain about the mechanisms underlying enhanced levels of surface LR11 and circulating sLR11 in leukemia cells of many different, but not all, origins. We have previously shown that sLR11 accelerates the co-localization of uPAR (CD87) and integrin $\alpha_v\beta_3$ in the membrane of SMCs [24]. Moreover, uPAR can regulate the adhesion of HSPCs to the bone marrow microenvironment and the homing and engraftment of HSPCs, in part via integrin $\alpha_4\beta_1$ [31]. In this study, most of leukemic cells and leukemic cell lines co-express LR11 and uPAR on their cell surface, although there were also leukemic cells without the harmonized expressions of both molecules (see Tables 2 and 3, and Fig. 2). Thus, sLR11 might interact with uPAR in association with cell-specific integrin isoforms to regulate mobility-related functions in leukemia cells of some origins.

Since sLR11 levels are associated with the peripheral blast proportion in acute leukemias, but are not elevated in chronically proliferative diseases such as CML, CLL, and MM, circulating sLR11 levels are unlikely to be simply associated with proliferating cell numbers. Rather, sLR11 levels appear to be predictive for pathogenic properties of immature blasts, including their migration and attachment activities. Recombinant sLR11 induces adhesion and migration of HL-60 and U937 cells, and these actions are involved in G-CSF-mediated leukocyte mobilization (Shimizu et al., unpublished observations). The fact that sLR11 is not increased in POEMS syndrome, in which extremely high levels of circulating VEGF cause vessel injury [32], suggests that increased sLR11 levels in acute leukemias are not a result of vascular injury, but rather a consequence of the enhanced release from leukemic cells.

Currently, the limitation of this study is the small number of patients available for studies, particularly for that of ALL. Further extensive flow-cytometric analysis of leukemic cells in AML patients with each FAB subtype as well as in ALL patients, in relation to sLR11 levels in the circulation, is expected to reveal the pathophysiological significance of sLR11 as a leukemia marker.

In summary, we have identified sLR11 as a novel circulating molecule directly released from leukemic cells in acute leukemias. Considering the lack of circulating molecules shed from the surface of leukemia cells, a prospective clinical trial testing the efficacy of sLR11 as a candidate biomarker for clinical characteristics and chemotherapeutic outcome in acute leukemia patients is now in progress.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2012.06.025.

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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.)

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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

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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-

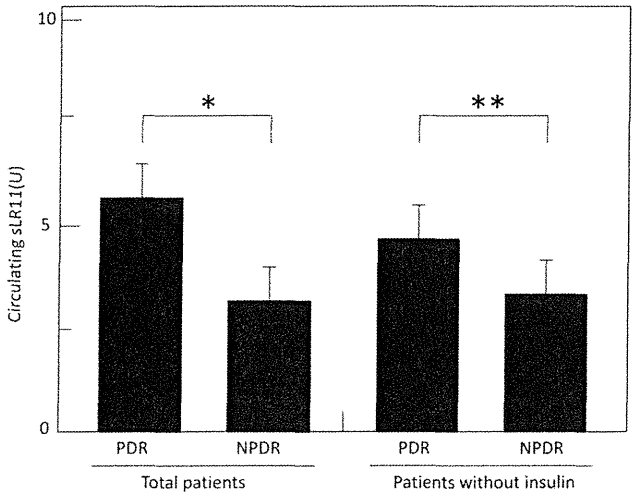

FIGURE 1. Bar graph showing circulating sLR11 levels in the patient groups with proliferative diabetic retinopathy (PDR) or without PDR (NPDR). The sLR11 levels of the total patients and those of the patients without insulin therapy were compared between the PDR and NPDR groups, respectively. Data are 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.

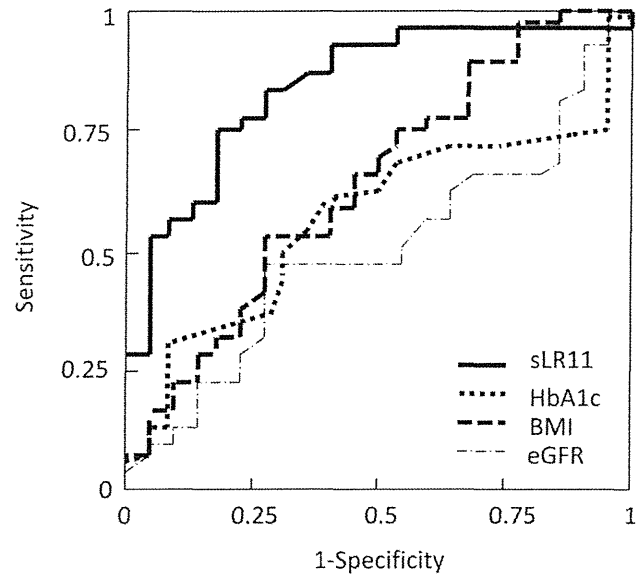

FIGURE 2. Receiver operating characteristic curve for discriminating the probability of type 2 diabetes mellitus patients developing proliferative diabetic retinopathy (PDR) from patients without PDR based on the levels of circulating soluble form of LR11 (sLR11), glycosylated hemoglobin (HbA1c), body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

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ORIGINAL PAPER

SORL1 genetic variants and cerebrospinal fluid biomarkers of Alzheimer's disease

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Abstract The neuronal sortilin-related receptor with A-type repeats (SORL1, also called LR11 or sorLA) is involved in amyloidogenesis, and the *SORL1* gene is a major risk factor for Alzheimer's disease (AD). We investigated AD-related CSF biomarkers for associations with *SORL1* genetic variants in 105 German patients with mild cognitive impairment (MCI) and AD. The homozygous CC-allele of single nucleotide polymorphism (SNP) 4 was associated with increased Tau concentrations in AD, and the minor alleles of SNP8, SNP9, and SNP10 and the haplotype CGT of these SNPs were associated with increased SORL1 concentrations in MCI. SNP22 and SNP23, and the haplotypes TCT of SNP19-21-23, and TTC of SNP22-23-24 were correlated with decreased Aβ42 levels in AD. These results strengthen the functional role of *SORL1* in AD.

Keywords Amyloid cascade · Biomarker · Mild cognitive impairment · Dementia · Genetic risk

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Introduction

The neuronal sortilin-related receptor with A-type repeats (SORL1, also called LR11 or sorLA) has been linked to protective effects against amyloidogenesis in Alzheimer's disease (AD) [1]. SORL1 seems to be capable of regulating the intracellular trafficking and processing of amyloid precursor protein (APP) by impairing the cleavage of APP through α-secretase, β-secretase (β-site APP-cleaving-enzyme-1, BACE1), and γ-secretase in a way that leads to reduced levels of soluble APP (sAPP) and amyloid beta protein (Aβ), the major component of amyloid plaques [2]. In line with this theory, reduced SORL1 expression has been demonstrated in human brains with amyloid pathology [3]. *SORL1* gene variants can reduce SORL1 expression or function and thereby increase Aβ production as well as AD risk [4]. Recently, multiple single nucleotide polymorphisms (SNP) within the *SORL1* gene have emerged as risk factors for sporadic AD in a variety of populations. Although replications are inconsistent, implicating influences of multi-ethnicity and allelic heterogeneity [4, 5], several independent studies have observed that significant associations were located in 2 distinct regions: the 5′ end and the 3′ end of the *SORL1* gene [4]. So far, only few studies have reported associations of *SORL1* variants with cerebrospinal fluid (CSF) endophenotypes in

AD [6–8]. In the present study, we have investigated eleven AD risk SNPs in a German sample to evaluate the effect of *SORL1* variants on the CSF levels of Aβ42, total TAU, sAPPα, sAPPβ, and SORL1 protein as well as on the CSF activity of BACE1.

Methods

The study population consisted of 44 Caucasian patients with probable AD according to NINCDS-ADRDA criteria and 61 patients with mild cognitive impairment (MCI) according to the revised International Working Group on MCI consensus criteria recruited from a university-based memory clinic in compliance with standardized guidelines [9, 10]. Written informed consent was obtained according to the 1975 Helsinki Declaration and the study protocol was approved by the ethics committee of the medical faculty at Technische Universität München.

The CSF concentrations of Aβ42, Tau (Innogenetics, Zwijndrecht, Belgium) as well as sAPPα and sAPPβ (Immuno-Biological Laboratories Co. Ltd., Gunma, Japan) were measured by enzyme-linked immunosorbent assay (ELISA) as described previously [11]. BACE1 activity in CSF was determined as the fluorescence signal of europium, which is proportional to the activity of BACE1, by a commercial BACE1 assay kit (Perkin Elmer Inc., Turku, Finland) according to a standard protocol [12, 13]. SORL1 concentration in CSF was quantified by ELISA in the laboratories of Sekisui Medical Co Ltd. (Ryugasaki, Japan) according to published procedures [14]. Genomic DNA was extracted from whole blood, and the apolipoprotein E *(APOE)* genotype was determined by a polymerase chain reaction and restriction enzyme digestion, simultaneously utilizing two distinct restriction enzymes, according to standard procedures.

Five marker SNPs at the 5′ end of the *SORL1* gene, rs661057 (SNP4), rs11600875, rs668387 (SNP8), rs689021 (SNP9), and rs641120 (SNP10), as well as 6 markers at the 3′ end, rs2070045 (SNP19), 21rs18ex26 (SNP21), rs1699102 (SNP22), rs3824968 (SNP23), rs2282649 (SNP24), and rs1010159 (SNP25), were selected from the published data based on their significant association with AD risk in Caucasian populations [4, 5, 7]. The genotypes were determined using TaqMan assays (SNP assays-on-demand) on a StepOne analyzer with StepOne software v2.1 (all assays, machine, and software from Applied Biosystems, Carlsbad, CA, USA).

Deviations from the Hardy–Weinberg equilibrium to exclude population stratification were tested for all 11 *SORL1* SNPs (http://www.oege.org/software/hwe-mr-calc.shtml) [15]. The sample size required to detect a significant difference between carriers and non-carriers with 90% power and a type I error rate of 0.05 was estimated in G-Power v3.1.3 [16] at $N = 14$ per group according to previous results [7] (mean Aβ42 concentration difference between carriers and non-carriers of the *SORL1* SNP23 T-allele of 56.60 ng/L with a shared standard deviation of 41.59 ng/L).

Patient characteristics were compared between the AD and the MCI groups using parametric tests for normally distributed data in the Predictive Analytics Software package (PASW) v18 (The SPSS Inc., Chicago, IL, USA). Analysis of covariance (ANCOVA) in PASW was used to test for the genotypic or allelic effect of all 11 SNPs of interest on CSF biomarker concentrations, adjusting for age, gender, and *APOE*, which was coded as a dichotomous variable for carriers and non-carriers of the ε4 allele. In addition, three-marker haplotypes of SNP8/SNP9/SNP10, SNP19-21-23, SNP22-23-24, and SNP23-24-25, again selected from the literature according to their linkage disequilibrium (LD) and the significant association with AD risk, were reconstructed and assessed with the Haplo.stats package in R software v2.1 (http://www.r-project.org/). The associations between *SORL1* haplotypic variants and CSF biomarker concentrations were examined in multivariate linear models after adjustment for age, gender, and *APOE* ε4 carrier status. Only genetic frequency higher than 5% was considered. Significance was set at $p < 0.05$. The study was driven by a priori hypotheses; therefore, no correction for multiple comparisons was applied [17] in accordance with similar previous studies [18].

Results

The demographic and clinical characteristics are summarized in Table 1; genotype and allele frequencies are provided in the Supplementary Tables 1 and 2. None of the 11 SNPs showed significant deviation from the Hardy–Weinberg equilibrium in the AD group; in the MCI group, deviation was only observed for SNP21 (Supplementary Table 1). The APOE ε4 allele was associated with lower Aβ42 levels in the MCI group ($p < 0.001$, $N = 61$). The single-marker analysis revealed significant associations between Aβ42 concentrations and the synonymous coding SNP22 and SNP23 at the 5′ end of the gene in the AD group. Carriers of the SNP22 C-allele ($p = 0.04$, $N = 22$) and SNP23 A-allele ($p = 0.04$, $N = 23$) had lower levels of Aβ42 than non-carriers (Supplementary Table 3). In the haplotype analyses, we observed associations of haplotype TCT (frequency 36.9%) of SNP19-21-23 ($p = 0.04$, $N = 38$) and TTC (frequency 24.7%) of SNP22-23-24 ($p = 0.04$, $N = 38$) with decreased CSF Aβ42 levels in the AD group (Fig. 1a). At the 3′ end of the gene, a significant association between the homozygous minor allele CC of

Table 1 Characteristics of the study sample

	AD (N = 44)	MCI (N = 61)	p value
Age at lumbar puncture*	66 (9.6)	65 (8.7)	0.44
Age at onset of symptoms*	6 (8.8)	63 (8.8)	0.62
Men:women	23:21	35:26	0.81
Schooling, years*	13 (2.9)	1 (2.7)	0.91
MMSE score*	23 (3.1)	27 (1.9)	<0.001**
ApoE4 carrier, n (%)	26 (59.1%)	27 (44.3%)	0.55
Aβ42 (ng/L)*	551.8 (233.52)	771.1 (350.84)	<0.001**
TAU (ng/L)*	627.8 (384.24)	383.9 (255.87)	<0.001**
sAPPα (ng/mL)*	287.1 (159.21)	332.2 (166.75)	0.17
sAPPβ (ng/mL)*	897.0 (402.65)	1047.2 (493.75)	0.10
BACE1 (FU/µL)*	8333.06 (2585.76)	9381.67 (3239.94)	0.08
SORL1 (µg/L)*	11.9 (4.69)	11.9 (4.28)	0.95

SNP single nucleotide polymorphism, *CSF* cerebrospinal fluid, *Aβ42* amyloid beta 42, *sAPPα, sAPPβ* alpha- and beta-soluble amyloid precursor protein, *BACE1* β-site APP-cleaving-enzyme-1, *SORL1* sortilin-related receptor with A-type repeats, *AD* Alzheimer's disease, *MCI* mild cognitive impairment, *FU* fluorescence units

* Mean (SD), ** significant at $p < 0.05$

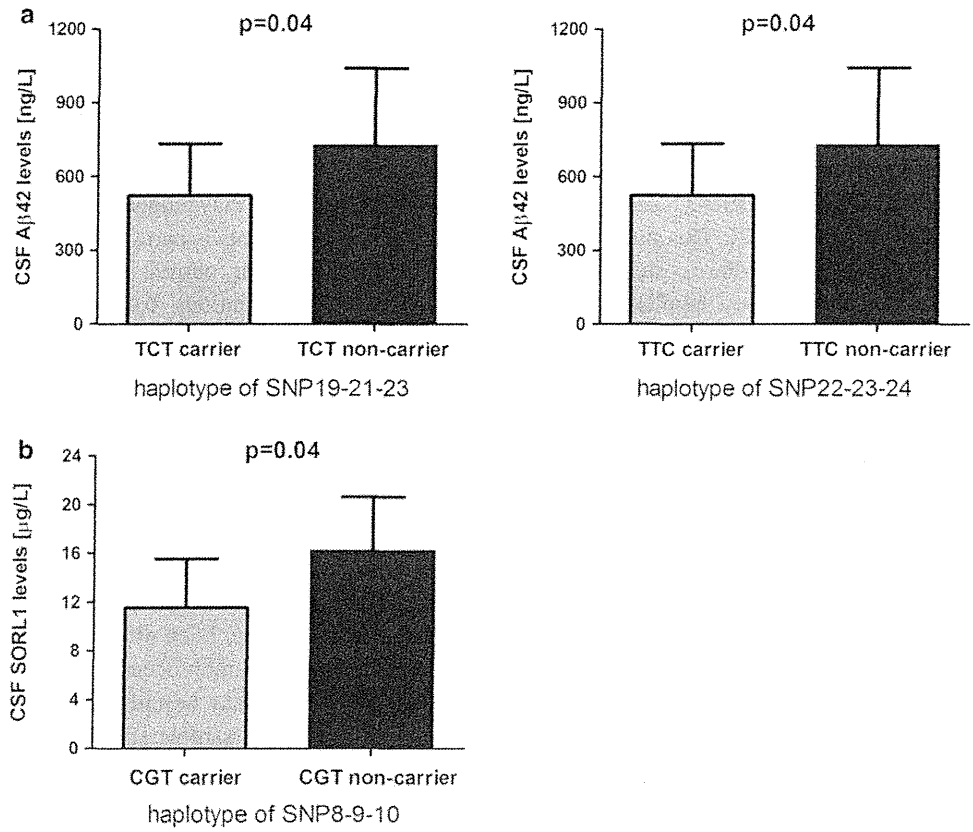

Fig. 1 **a** Effects of SORL1 haplotypes on CSF Aβ42 levels in the AD group; and **b** effects of SORL1 haplotypes on CSF SORL1 levels in the MCI group

SNP4 and increased Tau levels was observed (p = 0.03, N = 7) in the AD group. No association was found in heterozygous carriers, which points to a strong gene dosage effect (Supplementary Table 4). In the MCI group, at the 3′ end of the gene, SNP8, SNP9, and SNP10 showed significant associations with CSF SORL1 levels in a way that minor allele carriers had increased SORL1 concentrations (SNP8 TT: p = 0.04; SNP9 AA: p = 0.04; SNP10 CC: p = 0.04) (Supplementary Table 5). Again, these associations were driven by the homozygous carriers of the minor alleles of each of the three SNPs. In the haplotype analyses, a significant association between reduced CSF SORL1

levels was found with haplotype CGT (frequency 22.5%) of SNP8-9-10 in the MCI group ($p = 0.04$, $N = 55$) (Fig. 1b). There were no associations between sAPP levels and BACE1 activity with any of the SNPs or haplotypes.

Discussion

SORL1 regulates the intracellular sorting of APP and hinders APP cleavage and thereby Aβ production [1, 2]. The *SORL1* gene has been identified as a major risk factor for sporadic AD [4]. In the present study, associations between *SORL1* genetic variants and CSF levels of Aβ42, Tau, and SORL1 were observed at two distinct gene regions in patients with MCI and probable AD. Associations between *SORL1* genetic variants and CSF sAPPα and sAPPβ concentrations as well as BACE1 activity were not observed.

In the AD group, lower CSF Aβ42 levels were found in carriers of the exonic SNP22 (C-allele) and SNP23 (A-allele), and haplotypes TCT of SNP19-21-23 and TTC of SNP22-23-24 at the 3′ gene end. It has been demonstrated that SNP19 is in strong linkage disequilibrium with SNP22 and SNP23 in various Caucasian cohorts [19]. SNP21, on the other hand, has been reported as AD-related *SORL1* polymorphism in a German cohort [3] and the haplotype TGA of SNP19-21-22 correlated with lower CSF Aβ42 in AD before [7]. This finding was not replicated in our work, probably due to the low frequency of these markers in our sample (Supplemental Tables 1 and 2). In the initial genetic association study [4], the SNP22 C-allele, SNP23 T-allele, and haplotype CTT of SNP22-23-24 were associated with an increased risk for AD. In contrast, in our study, reduced Aβ42 levels were correlated with genotypes and haplotypes consisting of the alternative alleles. This inconsistency suggests that *SORL1* allelic heterogeneity and ethnic variants may also play a role [20]. Since exonic SNPs of the *SORL1* gene are present in the mature mRNA, they could directly alter translation and thus protein levels [21]. Therefore, the 3′ end SNPs, in particular the synonymous coding SNPs, might directly influence the function of the SORL1 protein and thereby alter the CSF levels of Aβ42.

We also found that Tau levels were associated with CC homozygotes of SNP4 in the AD group. The C-allele of SNP4 has been associated with AD among Caucasian populations in multiple independent cohorts and genome-wide association studies before [4, 5, 20, 22–25]. Although the present work is a case-only study that precludes a statement on the association of *SORL1* SNPs with AD risk per se, our data still confirm that the SNP4 C-allele is significantly associated with upregulated CSF Tau levels, which in turn are correlated to neurodegenerative pathology.

SORL1 protein is considered an important regulator of amyloidogenesis since reduced SORL1 levels may lead to dysfunctional retromer trafficking and upregulated cerebral Aβ production [1]. It remains inconclusive how reduced SORL1 protein expression in AD brain is related to alterations of SORL1 in CSF. It has been reported that the expression of SORL1 protein is reduced in brain tissue from patients with sporadic AD [3]. The two published CSF studies are inconsistent in this regard, reporting both decreased [26] and increased [27] SORL1 levels in AD compared with healthy controls. We identified associations between CSF SORL1 concentrations and three AD risk marker SNPs in the MCI group; the homozygous minor allele carriers of the intronic SNP8 (T-allele), SNP9 (A-allele), and SNP10 (A-allele) had increased SORL1 concentrations in CSF. Moreover, the haplotype analysis confirmed that a three-marker haplotype CGT (a combination of the major alleles) of SNP8/SNP9/SNP10 was associated with reduced CSF SORL1 levels in the MCI group. These three SNPs have been confirmed as the most significant AD risk markers within the *SORL1* gene in Caucasian samples in a recent meta-analysis including 11,592 cases and 17,048 controls [28]. The association of three 5′ end SNPs in our study with CSF SORL1 concentrations is consistent with the allelic disease association in this meta-analysis. Since MCI often represents pre-dementia AD, our data may suggest that the influence of *SORL1* genetic variants is particularly relevant in early clinical AD stages.

Our current study extends the existing literature on associations between SORL1 genetic variants and AD biomarkers, thereby supporting the role of SORL1 as an important influence factor on AD pathogenesis. Limitations include the rather small study sample and the lack of longitudinal data as well as neuropathological verification of the diagnoses. Therefore, replication studies with independent larger samples are warranted. We did not aim to replicate the results from previous genetic association studies; neither did we aim to identify new risk SNPs, and no control group was included because of this study design choice. Lack of consistent replication of genetic findings is a common occurrence in the study of complex phenotypes and may be indicative of inadequate power resulting from small sample size and genetic or environmental heterogeneity. The use of CSF biomarkers for genetic studies of AD may provide increased statistical power and important insight into the biological mechanisms by which these variants modulate disease risk. In any study attempting to associate genetic information with pathology, the exact effect of genetic variants on phenotypic variation often remains unclear. On the one hand, the genetic variants may have a direct effect on markers of pathology; on the other hand, neighboring SNPs in LD with the variant tested or other downstream factors may also have an influence.

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Conflict of interest The authors declare that they have no conflict of interest.

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

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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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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