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Levels of circulating soluble LR11, a regulator of smooth muscle cell migration, are highly associated with atherosclerotic plaques in patients with carotid artery stenosis

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ABSTRACT

Background: The levels of plasma sLR11, released from intimal SMCs, are positively associated with intima-media thickness (IMT) in asymptomatic subjects. We have evaluated the yet unknown pathological significance of sLR11 for plaque conditions in patients with carotid artery stenosis.

Methods: The presence of LR11 in carotid plaques was investigated using autopsy specimens. A clinical ultrasonography study for elucidating relationships between sLR11 and plaque condition was performed in 46 patients.

Results: Immunohistochemistry showed high levels of LR11 in SMCs within thickened intima and at the media-intima border of atherosclerotic carotid plaques. The levels of sLR11 in patients were clearly elevated compared to healthy controls. Univariate analysis of sLR11 revealed significant positive correlation with plaque score and a tendency to correlate with the stenotic fraction. Univariate and multiple regression analyses of plaque scores showed that sLR11, maximum IMT, and HDL-cholesterol independently determined plaque score. Finally, univariate analysis of initial sLR11 levels for changes in imaging markers after one-year follow-up showed that initial sLR11 levels significantly correlated with stenotic fraction progression.

Conclusions: The levels of sLR11, abundantly expressed in carotid atherosclerotic plaques, are highly associated with increased plaque score. sLR11 levels may be predictive of plaque conditions in patients with advanced carotid atherosclerosis.

1. Introduction

Atherosclerotic plaque progression in carotid arteries is a major cause of ischemic stroke through impaired cerebral blood flow, and also of thromboembolic stroke caused by plaque rupture with or without intra-plaque hemorrhage [1]. Surgical procedures such as carotid endarterectomy or carotid artery stenting are performed in patients with severe stenotic lesions, and, in fact, have been shown to significantly prevent the resultant cerebral infarction in such high-risk patients [2,3]. Nevertheless, appropriate subsequent management of their systemic atherosclerotic risks including diabetes, hypertension and dyslipidemia is indispensable for the improvement of prognosis regardless of the developing surgical procedures [4].

Carotid artery stenosis results from the atherosclerotic interaction between modified lipids, extracellular matrix, monocyte-derived macrophages and vascular smooth muscle cells (SMCs) that accumulate in the arterial wall [5]. Among them, SMCs play a regulatory role in the process of atherosclerosis through their migration, proliferation and release of extracellular matrices as the subsequent reactions against the accumulated lipids and/or cytokines secreted from other activated vascular cells. and lead to the formation of advanced and complicated atherosclerotic plaques [1,5,6]. Furthermore, in advanced lesions, SMCs are believed to constitute a fibrous cap covering the lipid core, one of main regulatory factors for plaque instability [5]. Thus,

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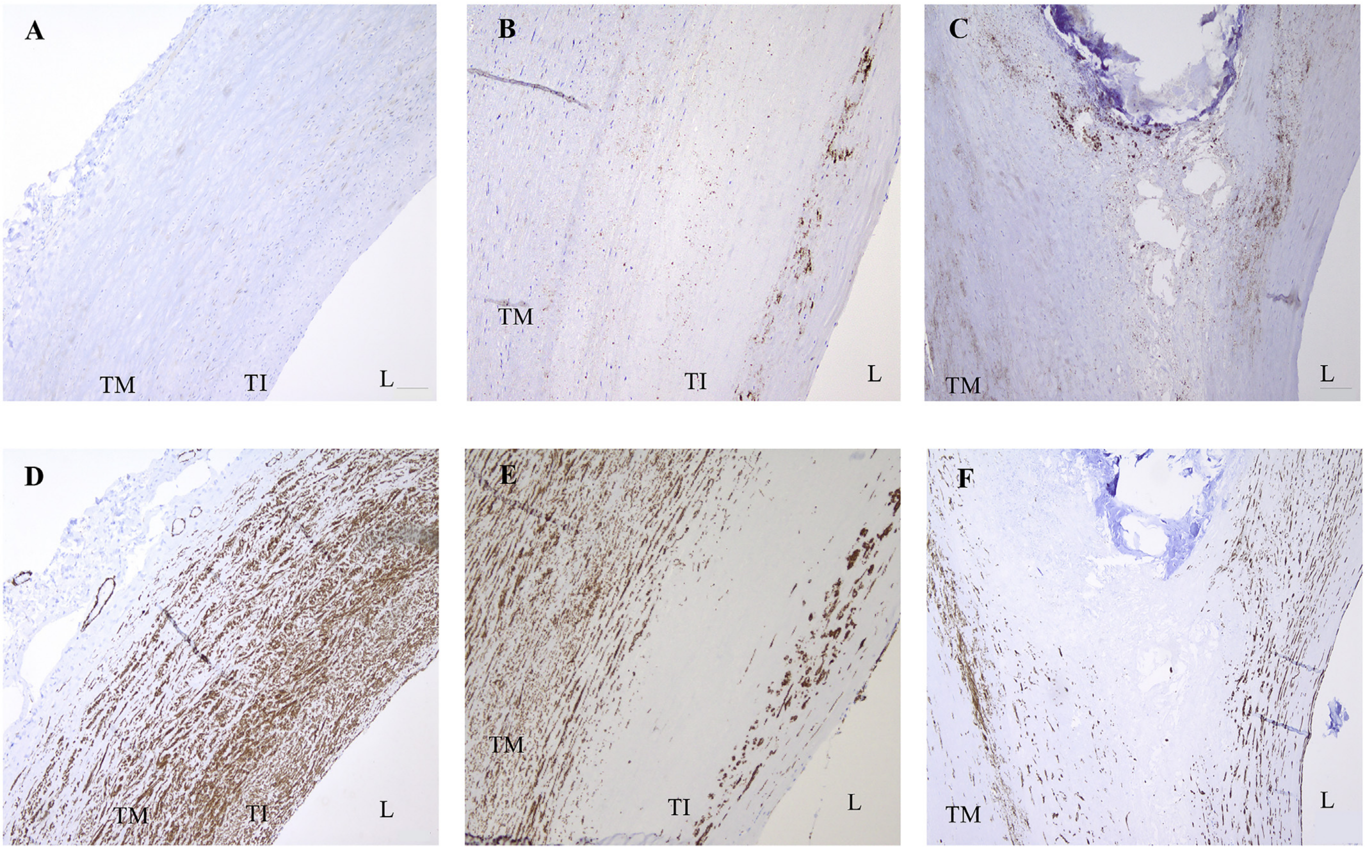

Fig. 1. Immunohistochemical analysis of LR11 in carotid artery specimens from autopsy patients with carotid atherosclerosis. Sections of tissue specimens were subjected to immunohistochemistry using an antibody against LR11 or smooth muscle actin (SMA). Non-atherosclerotic (A) and (D) and atherosclerotic (B), (C), (E) and (F) regions in specimens from the patients were stained with antibody against LR11 (A), (B) and (C) or SMA (D), (E) and (F). In SMCs located at intima, and also in those in media at the border of intima, LR11 was strongly immunostained. L, lumen; TI, tunica intima; TM, tunica media.

migration of vascular smooth muscle cells (SMCs) from the medial layer to the intimal layer is a key step in the development of advanced atherosclerosis [7,8]. Following migration, intimal SMCs, upon conversion in the media from a 'contractile' phenotype to a de-differentiated 'synthetic' phenotype, regulate various potential functions including matrix production, protease release, and cytokine secretion [7–9]. Migrated intimal SMCs are believed to play important roles in forming atherosclerotic plaques and determining their fragility upon interaction with other vascular and inflammatory cells. Using animal models and cultured SMCs, we have shown that disturbed interactions of SMCs with other vascular cells or phenotype conversion of SMCs in dyslipidemia lead to atherosclerosis progression and increased plaque fragility [10]. However, the clinical significance in atherosclerotic carotid stenosis of the intimal SMCs migrating under pathophysiological conditions has not yet been elucidated.

Low-density lipoprotein (LDL) receptor relative with 11 ligand-binding repeats (LR11, also called sorLA or SORL1), an unusually complex and highly conserved member of the family of LDL receptor relatives (LRs), has been discovered and molecularly characterized by us and others [11–13]. The receptor mediates the plasma membrane localization of urokinase type plasminogen activator receptor (uPAR), since the constitutively shed soluble form of the receptor (sLR11) binds to and colocalizes with uPAR on the cell surface [14]. LR11 is highly expressed in intimal SMCs at the intima-media border in the plaque areas of aorta from experimental models of atherosclerosis [15,16]. Furthermore, overexpression of LR11 in SMCs enhances their migration via increased surface localization of uPAR [16,17]. With a sandwich enzyme-linked immunosorbent assay (ELISA) for the exact quantitation of circulating sLR11 using specific monoclonal antibodies against human LR11 [18,19], it could be shown that the concentrations of sLR11 were increased in atherosclerotic patients with familial hypercholesterolemia [20–22] or coronary artery diseases [23–25]. Notably, several independent studies have shown that the sLR11 concentrations were highly associated with the mean intima-media thickness (IMT) of carotid arteries as a representative index indicative of systemic or coronary atherosclerosis using ultrasound imaging [17,26]. Thus, sLR11 levels have been suggested to reflect the conditions or degrees of migrated intimal SMCs in patients with atherosclerotic diseases involving the development of carotid atherosclerosis.

Based on the knowledge generated by the above observations for the potential involvement of sLR11 in carotid atherosclerosis, we have investigated the pathological significance of circulating sLR11 in patients with carotid artery stenosis by immunostaining of carotid artery plaques using autopsy specimens from two patients and by image analysis using ultrasonography in 46 patients with carotid artery stenosis.

2. Materials and methods

2.1. Subjects

For immunohistochemical analysis, two patients (a 84-yr-old male and a 81-yr-old female) who underwent autopsy were recruited, and the samples of atherosclerotic arteries were obtained from common carotid arteries. For ultrasound-imaging study, forty-six patients with carotid artery stenosis, who had ultrasonographically proven carotid artery stenosis, defined as stenosis of 50% or more, and treated by conventional pharmaceutical medications [27] between August 2013 and December 2014 at the Department of Neurosurgery, Toho University Medical Center Sakura Hospital, were enrolled. All patients were evaluated for carotid atherosclerosis by ultrasonography, and their

Table 1
Baseline and clinical characteristics of study subjects.

Variables	Mean ± SD
Age, years	72.2 ± 5.9
Male, n (%)	41 (89.1)
Current smoker, n (%)	28 (60.9)
BMI, kg/m^2	23.3 ± 2.6
Mean IMT, mm	1.0 ± 0.3
Maximum IMT, mm	5.7 ± 3.0
Plaque score	15.2 ± 4.5
Stenotic fraction, %	82.2 ± 7.7
TC, mg/dl	181.2 ± 34.9
LDL-C, mg/dl	106.1 ± 29.2
HDL-C, mg/dl	48.5 ± 15.7
TG, mg/dl	151.5 ± 77.8
HbA1c, %	6.5 ± 1.0
Cr, mg/dl	0.85 ± 0.23
eGFR, ml/min/1.73m^2	69.9 ± 18.6
CRP, mg/dl	0.4 ± 1.0
WBC, cells/μl	6820.2 ± 2181.5
History of CVD-no. (%)	9 (19.6)
History of CAD-no. (%)	6 (13)
History of hypertension-no. (%)	29 (63)
History of diabetes mellitus-no. (%)	16 (34.8)
History of dyslipidemia-no. (%)	29 (63)
History of renal dysfunction-no. (%)	2 (4.3)
Medication (at enrollment)	
Antiplatelet agents, n (%)	41 (89.1)
Statins, n (%)	24 (52.2)
Fibrates, n (%)	3 (6.5)
Hypoglycemic oral agents, n (%)	12 (26.1)
Insulin supplementation	3 (6.5)
Ca-blockers, n (%)	12 (26.1)
ARBs, n (%)	18 (39.1)
sLR11, ng/ml	12.2 ± 3.5

Study numbers of all variables are forty-six excluding stenosis area proportion (n = 38). BMI, body-mass index; IMT, intima-media thickness; TC, total cholesterol; TG, triglycerides; Cr, creatinine; eGFR, estimated glomerular filtration rate; CRP, C-reactive protein; WBC, white blood cell; CVD, cerebrovascular disease; CAD, coronary artery disease; ARBs, angiotensin II receptor blockers; sLR11, soluble form of LR11.

blood samples were collected on the same day. Follow-up ultrasonography was performed 6–14 months afterward. The following risk factors for atherosclerosis were evaluated: age, sex, smoking status, body mass index (BMI), plasma lipids, glycosylated hemoglobin (HbA1c), C-reactive protein (CRP), white blood cell counts (WBC), histories of coronary artery diseases (CAD), cerebrovascular diseases (CVD), hypertension, diabetes mellitus, dyslipidemia or renal dysfunction, and medications including antiplatelet agents, statins, fibrates, hypoglycemic agents, insulin supplementation, calcium blockers and angiotensin II receptor blockers. Smoking status was defined as current smoking. The diagnosis of CAD was based on a history of myocardial infarction or angina pectoris, and that of CVD was based on a history of cerebral bleeding or infarction. Hypertension was defined as systolic blood pressure ≧ 140 mmHg and/or diastolic blood pressure ≧ 90 mmHg or current use of antihypertensive medication [28]. Diabetes mellitus was defined as glycosylated hemoglobin (HbA1c) > 6.5% [29] or current use of hypoglycemic agents. Dyslipidemia was defined as low-density lipoprotein cholesterol (LDL-C) ≧ 140 mg/dL, high-density lipoprotein cholesterol (HDL-C) < 40 mg/dL, triglyceride (TG) ≧ 150 mg/dL [30] or current use of lipid-lowering agents. We defined renal dysfunction as an estimated glomerular filtration rate (eGFR) of < 60 ml/min/1.73m^2 [31] which was calculated using baseline serum creatinine. The exclusion criteria were patients who had vasculitis syndrome, aortic dissection, received surgical procedure with cardiovascular disease, received surgical procedure with carotid stenosis, and history of malignant disease. Patients with carotid atherosclerosis, defined as < 50% stenosis or without stenotic lesion, were also excluded. The study protocol was approved by The Ethics Committee of Toho University Sakura Medical Center (approval number 2014-066, S17105). Informed consent was obtained from the families of autopsy patients and all patients for data use in clinical study in accordance with the principles of the Declaration of Helsinki.

2.2. Immunohistochemistry

Three to four-micrometer-thick sections of paraffin-embedded specimens from the carotid arteries of autopsy samples were prepared. After deparaffinizion and hydration, for antigen retrieval the sections were subsequently treated with the Immunosaver (Nisshin EM, Tokyo, Japan) for staining of LR11 or treated with Envision™ Flex Target Retrieval Solution, High pH (Dako) for staining of smooth muscle actin (SMA), and finally treated at 95 °C for 45 min. Immunohistochemical reactions were performed with an autostainer (the EnVision™ FLEX, Dako, Glostrup, Denmark). Endogenous peroxidase activity was blocked by treatment with Envision™ Flex peroxidase-blocking solution (Dako). After washing with Envision™ Flex Wash Buffer (Dako), the slides were incubated at room temperature in a moist chamber for 30 min with 25 μg/ml mouse anti-human LR11 monoclonal antibody, A2–2-3 [32] or mouse anti-human SMA monoclonal antibody, 1A4 (IR611, DAKO). After washing, the slides were treated with Envision™ Flex HRP (Dako) for 20 min, followed by color development in Envision™ Flex DAB + Chromogen with Substrate Buffer (Dako). Finally, the slides were counterstained with hematoxylin.

2.3. Ultrasound imaging

High-resolution B-mode ultrasonography was performed with a 7.5 MHz linear-type probe (Hitachi, Tokyo) as previously described [17]. In accordance with the guidelines of the Japan Society of Ultrasonics [27], all scanning was conducted by experienced sonographers using the same ultrasound system and the same measuring method. Patients were investigated in the supine position with their body slightly turned from sonographer. The carotid arteries were carefully examined with regard to wall changes from different longitudinal (anterior oblique, lateral, and posterior oblique) and transverse views. Measurements of thickness were performed on the transverse image. The severity of carotid artery atherosclerosis was evaluated using the following four indices: mean IMT (mm), maximum IMT (mm), stenotic fraction (%), and plaque score [27,33–36]. To obtain each index, the extracranial carotid artery was divided into four segments of 15 mm in length each from the flow divider [33,34], and termed ICA (S1), carotid bulb (S2), distal CCA (S3) and proximal CCA (S4). The IMT was evaluated as the distance between the luminal-intimal interface and the leading edge of the medial-adventitial interface of the far wall. After localization of the common carotid artery, cross-sectional measurements were performed 10 mm proximal to the carotid bulb. Three points on the ipsilateral common carotid arteries without plaque were measured, and the mean IMT was defined as the average of them. Protruding lesions with an IMT ≥ 1.1 mm were defined as atheromatous plaques. Maximum IMT was defined as the maximal thickness of intima-media including plaques. To assess the severity of atherosclerosis, we used a "plaque score," which was calculated by summing up all plaque thicknesses in bilateral carotid arteries (S1-S4) (see Supplementary Fig. 1) [33,34]. The term "stenotic fraction" represents the stenotic area in % of the observed original lumen area.

2.4. Blood samples

Patients provided a blood sample for measurement of the study variables, including TC, HDL-C, LDL-C, TG, HbA1c, CRP, WBC, and sLR11. Blood samples were centrifuged and refrigerated at − 80 °C until processing. sLR11 was measured using a sandwich ELISA method (Sekisui Medical, Ryugasaki, Japan), using samples frozen at − 80 °C as reported previously [17]. Briefly, 12.5 μl of each plasma were used for

Table 2
Univariate analysis of sLR11 for all other variables.

Variables	*r* or Mean ± SD (yes/no)	95% CI Lower Upper	p values
Age	−0.037	−0.200 0.157	0.81
Male	12.06 ± 3.53/13.01 ± 1.44		NS
Current smoker	12.41 ± 3.55/12.77 ± 3.32		NS
BMI	−0.018	−0.433 0.387	0.91
Mean IMT	−0.067	−4.689 2.985	0.66
Maximum IMT	0.218	−0.161 1.051	0.15
Plaque score	0.450	0.138 0.554	0.002*
Stenotic fraction	0.315	−0.003 0.302	0.05*
TC	0.235	−0.006 0.053	0.12
LDL-C	0.271	−0.003 0.067	0.07
HDL-C	−0.015	−0.071 0.064	0.92
TG	0.089	−0.010 0.018	0.56
HbA1c	0.073	−0.841 1.367	0.63
Cr	0.175	−1.884 7.178	0.25
eGFR	−0.159	−0.086 0.026	0.29
CRP	0.204	−0.340 1.773	0.18
WBC	0.028	0.000 0.001	0.86
History of CVD	11.74 ± 2.93/12.25 ± 3.61		NS
History of CAD	12.09 ± 4.39/12.17 ± 3.39		NS
History of hypertension	11.92 ± 3.41/12.57 ± 3.66		NS
History of diabetes mellitus	11.84 ± 3.50/12.33 ± 3.52		NS
History of dyslipidemia	12.06 ± 3.49/12.32 ± 3.56		NS
History of renal dysfunction	9.83 ± 1.03/12.26 ± 3.52		NS
Medication			
Antiplatelet agents	11.91 ± 3.33/14.21 ± 4.43		NS
Statins	12.89 ± 4.01/11.36 ± 2.67		NS
Fibrates	9.41 ± 1.14/12.35 ± 3.51		NS
Hypoglycemic oral agents	11.93 ± 4.09/12.23 ± 3.33		NS
Insulin supplementation	13.15 ± 5.12/12.09 ± 3.41		NS
Ca-blockers	11.15 ± 2.88/12.51 ± 3.64		NS
ARBs	11.24 ± 3.29/12.75 ± 3.53		NS

Study numbers of all variables are forty-six excluding stenosis area proportion (n = 38). BMI, body-mass index; IMT, intima-media thickness; TC, total cholesterol; TG, triglycerides; Cr, creatinine; eGFR, estimated glomerular filtration rate; CRP, C-reactive protein; WBC, white blood cell; CVD, cerebrovascular disease; CAD, coronary artery disease; ARBs, angiotensin II receptor blockers; sLR11, soluble form of LR11.

the measurement of sLR11 by ELISA with specific monoclonal antibodies directed against human LR11. Other biochemical parameters were measured using routine laboratory methods at our institution. To evaluate the normal concentration range of sLR11, a group of healthy subjects without any underlying disease were recruited for comparison.

2.5. Statistical analysis

Statistical analysis was performed with SPSS version 17 (SPSS Japan, Inc., Tokyo). All descriptive data are expressed as means ± standard deviation or as proportions (%). The statistical significance of differences in sLR11 between patients with carotid artery stenosis and healthy subjects was assessed with independent *t*-test. Associations of sLR11 or plaque score levels with various risk factors were examined by Mann-Whitney's *U* test for categorical variables and by Pearson's correlation analysis for continuous variables; based on these results, which factors indicated or predicted the severity of carotid atherosclerosis were determined using stepwise multiple regression analysis. In multiple regression analysis, we employed plaque score as the dependent variable to quantitatively assess the severity of carotid lesions. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. LR11 is highly expressed in de-differentiated SMCs in carotid atherosclerotic plaques

We investigated the expression of LR11 using autopsy specimens from patients with carotid atherosclerosis by immunohistochemistry. Staining with a specific antibody against LR11, A2-2-3 [17], or SMA showed that the LR11 protein was barely or weakly detectable in SMCs of arterial walls without pathological intimal hyperplasia (Fig. 1, panels a and d). In contrast, the strong staining of LR11 protein was clearly visible in the SMCs located at intima, and also in those in media at the border of intima in the mild to severe stenotic areas (panels b, c, e and f). The intracellular LR11 protein was specifically visualized as granular immunostaining in the cytosol in larger magnifications (data not shown), as previously reported for SMCs and other cells [17,37]. These results suggest that LR11 is highly expressed in de-differentiated SMCs located in atherosclerotic plaques of carotid arteries, in agreement with previous observations in atherosclerotic plaques of aortas using animal models [15,16]; therefore, de-differentiated SMCs in carotid plaques potentially regulate circulating sLR11 levels by the release of the soluble fragment, sLR11, from the cell surface.

3.2. Circulating sLR11 levels are increased in patients with carotid artery stenosis

We analyzed the circulating sLR11 levels in 46 patients with carotid artery stenosis (72.2 ± 5.9 years, male 89.1%, and BMI 23.3 ± 2.6 kg/m^2) (Table 1). Using ultrasonography, mean IMT, maximum IMT, plaque score, and stenotic fraction were 1.0 ± 0.3 mm, 5.7 ± 3.0 mm, 15.2 ± 4.5, and 82.2 ± 7.7%, respectively. Considering the respective normal ranges observed in our institute, all indices were clearly increased, and particularly the increased levels of stenotic fraction were in agreement with advanced arterial stenosis in the carotid arteries of the patients under study. The biochemical characteristics showed that the plasma lipid, glucose and inflammatory marker levels were almost in the normal range. The proportions of patients with histories of cerebrovascular and coronary artery diseases were 19.6% and 13.0%, respectively. The proportions of patients with histories of hypertension, diabetes mellitus, dyslipidemia, and renal

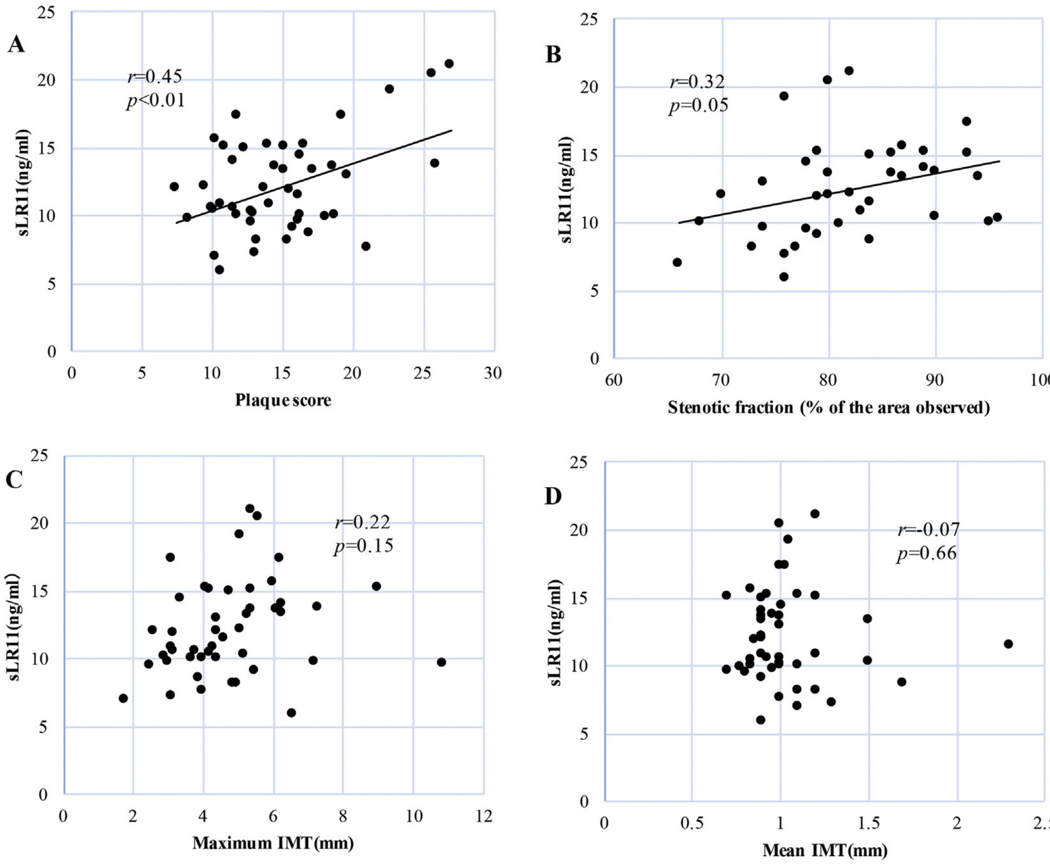

Fig. 2. Relationships between circulating sLR11 levels and four ultrasonographical parameters; plaque score (A, n = 46), stenotic fraction (in % of the area observed) (B, n = 38), maximum IMT (C, n = 46) and mean IMT (D, n = 46). IMT, intima-media thickness; sLR11, soluble form of LR11.

dysfunction were 63.0%, 34.8%, 63.0% and 4.3%, respectively. Among medications, the proportion of patients on antiplatelet agents was as high as 89.1%. Finally, the circulating sLR11 levels were 12.2 ± 3.5 ng/ml, which was clearly increased when compared with the levels of healthy volunteers (9.0 ± 2.6 ng/ml, n = 52, $p < 0.001$ by student *t*-test), and also considering the previously reported levels of healthy subjects, 8.7 ± 2.1 ng/ml (n = 87) [18] or 7.8 ± 1.6 ng/ml (n = 57) [19].

3.3. Circulating sLR11 levels are associated with plaque scores, together with a tendency for stenotic fractions, but not IMTs, in patients with carotid stenosis

We first analyzed the relationships of the increased circulating sLR11 levels in the patients with basal, imaging, and biochemical indices (Table 2). Univariate correlation analysis showed no significant correlation of sLR11 level with age, sex, biochemical indicators, or histories of diseases or medications. As for ultrasound-imaging indices, the sLR11 levels were significantly correlated with levels of plaque score, and tended to correlate with the levels of stenotic fraction, and not with mean or maximum IMT (Table 2 and Fig. 2). These results suggested that sLR11 is associated with the plaque and/or stenosis condition(s) of carotid arteries, rather than with the degree of intimal thickening, in patients with carotid stenosis.

3.4. Circulating sLR11 is an independent determinant for plaque score in patients with carotid stenosis

Based on the tight relationship between sLR11 and plaque score shown above, we subsequently performed univariate analysis of the association of plaque score with all indices in order to estimate the significance of sLR11 as a determinant for plaque score. The results showed that plaque score levels were significantly and positively correlated with levels of maximum IMT and sLR11, and negatively correlated with HDL-C levels (Table 3, left). Subsequent multiple regression analysis among these three factors revealed that sLR11 and maximum IMT, as well as HDL-C, were independent factors determining plaque score (Table 3, right). Thus, circulating sLR11 levels were associated with plaque scores as an independent determinant among all analyzed factors in patients with carotid stenosis.

3.5. After one-year follow-up, patients' circulating sLR11 levels were associated with increased stenotic fractions and showed a tendency for correlation with increased plaque score, but not IMTs

Finally, in order to further determine the clinical significance of circulating sLR11 levels for the prognosis of carotid atherosclerosis, we analyzed the relationships between the above-measured sLR11 levels and the changes in degrees of image indices one year later in those 27 of 46 patients who underwent follow-up ultrasonography. The changes of mean IMT, maximum IMT, plaque score, and stenotic fraction were −0.09 ± 0.38 mm, −0.30 ± 0.55 mm, 0.90 ± 1.99, and −0.44 ± 3.03%, respectively, in the observation periods. Univariate regression analysis showed that the initial sLR11 levels were significantly correlated with the progression of stenotic fraction ($r = 0.47$, p = 0.04), together with a tendency to correlate with those of plaque score ($r = 0.47$, p = 0.08) after one year, but not with levels of maximum IMT or mean IMT (Fig. 3). Thus, sLR11, as a circulating SMC migration regulator, is associated with the degrees of plaque conditions, as indicated by stenotic fraction and potentially by plaque score in patients with carotid artery stenosis in longitudinal observations.

Table 3
Univariate analysis and multiple regression analysis of plaque score with other variables.

Variables	Univariate analysis			Multiple regression analysis		
	r or Mean ± SD (yes/no)	95% CI Lower Upper	p values	β	95% CI Lower Upper	p values
Age	−0.126	−0.327 0.135	0.41			
Male	15.26 ± 4.68/12.44 ± 1.90		NS			
Current smoker	15.36 ± 4.68/14.31 ± 4.35		NS			
BMI	0.199	−0.191 0.852	0.21			
Mean IMT	0.182	−1.933 7.917	0.23			
Maximum IMT	0.36	0.205 1.713	0.014*	0.24	0.013 1.265	0.045*
Stenotic fraction	−0.035	−0.230 0.186	0.83			
TC	−0.179	−0.062 0.016	0.23			
LDL-C	0.092	−0.033 0.061	0.55			
HDL-C	−0.457	−0.210 -0.054	0.001*	−0.43	−0.191 -0.058	< 0.001*
TG	0.039	−0.015 0.020	0.80			
HbA1c	−0.042	−1.664 1.260	0.78			
Cr	0.288	−0.055 11.424	0.052			
eGFR	−0.144	−0.108 0.038	0.34			
CRP	0.063	−1.115 1.688	0.682			
WBC	0.061	−0.001 0.001	0.687			
History of CVD	16.76 ± 5.08/14.57 ± 4.39		NS			
History of CAD	14.48 ± 3.05/15.02 ± 4.74		NS			
History of hypertension	14.73 ± 4.79/15.33 ± 4.18		NS			
History of diabetes mellitus	14.62 ± 3.92/15.13 ± 4.88		NS			
History of dyslipidemia	15.17 ± 4.73/14.59 ± 4.29		NS			
History of renal dysfunction	13.65 ± 3.04/15.01 ± 4.61		NS			
Medication						
Antiplatelet agents	14.94 ± 4.38/15.02 ± 6.26		NS			
Statins	16.05 ± 5.24/13.75 ± 3.34		NS			
Fibrates	13.90 ± 3.32/15.02 ± 4.63		NS			
Hypoglycemic oral agents	15.44 ± 5.39/14.80 ± 4.31		NS			
Insulin supplementation	15.77 ± 6.03/14.89 ± 4.50		NS			
Ca-blockers	14.30 ± 4.46/15.18 ± 4.60		NS			
ARBs	14.44 ± 4.47/15.28 ± 4.62		NS			
sLR11	0.45	0.233 0.940	0.002*	0.39		0.002*

Study numbers of all variables are forty-six excluding stenosis area proportion (n = 38). BMI, body-mass index; IMT, intima-media thickness; TC, total cholesterol; TG, triglycerides; Cr, creatinine; eGFR, estimated glomerular filtration rate; CRP, C-reactive protein; WBC, white blood cell; CVD, cerebrovascular disease; CAD, coronary artery disease; ARBs, angiotensin II receptor blockers; sLR11, soluble form of LR11.

4. Discussion

We have investigated the pathological and clinical significance of sLR11, a migration regulator of intimal SMCs, as an index for atherosclerotic plaque conditions in patients with carotid artery stenosis using immunohistochemistry and ultrasonography for imaging carotid plaques. Previous studies have suggested that circulating sLR11 concentrations are associated with carotid atherosclerosis using mean IMT as a systemic atherosclerosis marker in patients with diabetes mellitus [26] or asymptomatic healthy volunteers [17], as well as with coronary atherosclerosis in patients with coronary artery diseases [23–25], or with systemic atherosclerosis in those with familial hypercholesterolemia [20–22]. Based on the consecutive observations in these independently performed studies, we hypothesized that circulating sLR11 concentrations may potentially reflect the pathological conditions of migrated intimal SMCs in atherosclerotic plaques themselves, in addition to the patients' systemic atherosclerotic conditions. The properties of intimal SMCs are known to likely determine the formation of fragile plaques, subsequently leading to ischemic stroke through impaired cerebral blood flow and/or thromboembolic stroke through plaque rupture [1,10]. The subjects in this study were elderly, non-obese patients who with or without treatment had close to normal values for lipids and glucose (Table 1). Most of the patients had not suffered from vascular diseases, although the proportions of patients on medications for hypertension or dyslipidemia were over 60%. Plasma sLR11 concentrations were indeed elevated in comparison to the values of healthy subjects in the present as well as previous studies [18,19], and in fact seem to be equivalent or higher than those in patients with advanced atherosclerosis; acute coronary syndrome, 9.9 ± 2.8 ng/ml (n = 50) [24]; familial hypercholesterolemia, 9.9 ± 2.6 ng/ml (n = 123) [22], and close to the CAD patient groups (Q4) with the highest long-term adverse cardiac events > 14.36 ng/ml (n = 109) [38].

The results of statistical analyses presented in Tables 2 and 3, and Figs. 2 and 3, strongly suggested that in patients with carotid artery stenosis, sLR11 was strongly associated with plaque score in combination with stenotic fraction, and less so with mean or maximum IMTs. Finally, a one-year follow-up study revealed that initial sLR11 levels were associated with an increase in stenotic fraction together with a tendency to correlate with progressed plaque scores. Thus, circulating sLR11 levels were associated with the ultrasound imaging indices for conditions of atherosclerotic plaques in carotid arteries; moreover, sLR11 levels appeared indicative of the subsequent progression of the imaging indices in patients with carotid artery stenosis.

Another novel finding of sLR11 in relation to the imaged carotid atherosclerosis was immunohistochemistry of carotid plaques using autopsy specimens. The staining firstly uncovered the characteristic expression of LR11 in human atheromatous lesions (see Fig. 1). Abundant protein was principally located in SMCs in the intima and in the media at the border with the intima, as previously observed in the aorta of experimental animal models [15,16], together with the staining of granular pattern in the cells, again as previously observed in other cells [17,37].

Limitations of the present study are the unfortunate restrictions to the availability of samples both for immunohistochemistry and the clinical study. The autopsy samples were restricted because of few lesions of advanced carotid atherosclerosis. We need, and plan, to further investigate the staining in atherosclerotic plaques from patients with heterogeneous backgrounds. As for clinical analysis, subjects were meticulously collected from a single institute; however, the patient number may not be fully sufficient for extensive analysis using

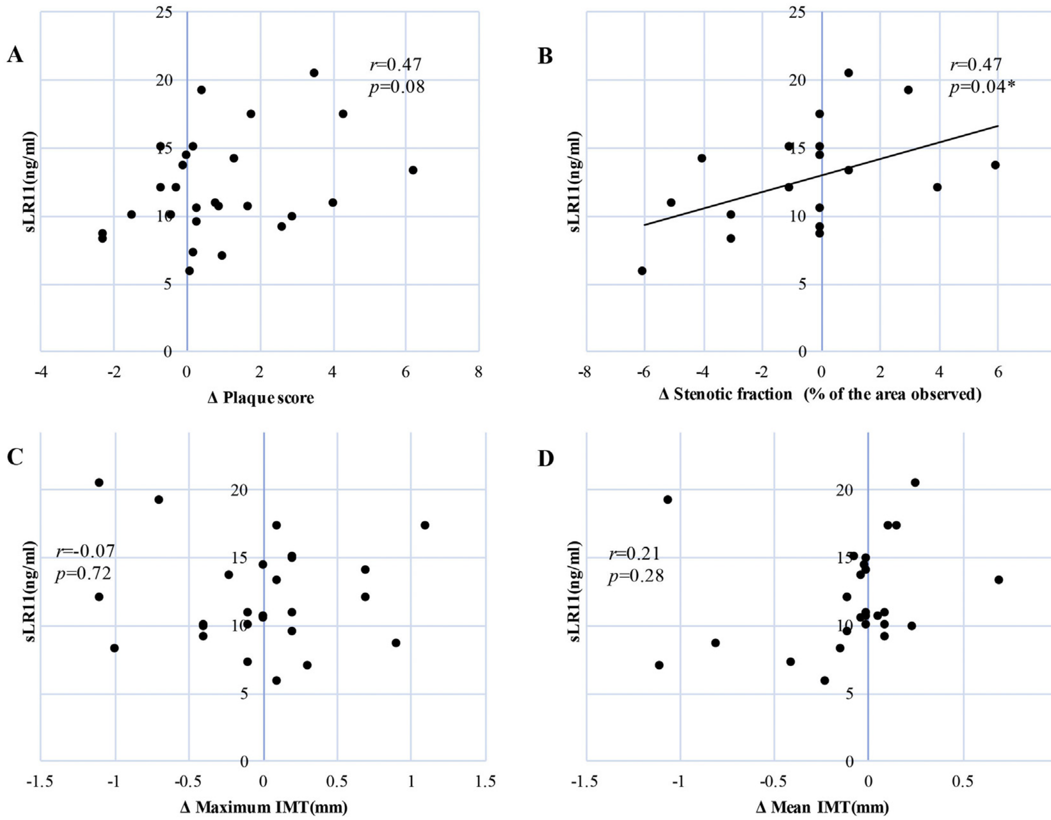

Fig. 3. Relationships between sLR11 and four ultrasonographical parameters after one year follow-up; plaque score (A, n = 27), stenotic fraction (% of the area observed) (B, n = 18), maximum IMT (C, n = 27) and mean IMT (D, n = 27). IMT, intima-media thickness; sLR11, soluble form of LR11.

complete multivariate regression methods. Therefore, the current study is principally conducted in a cross-sectional research design, limiting the possibilities to identify causal relationships among sLR11, ultrasound indices, and possibly other risk factors. Clearly, further studies using subjects with different characteristics (sex, age, BMI, and treatment conditions) should be helpful for the evaluation of pathological mechanisms involving sLR11 in carotid atherosclerosis. Because the changes in ultrasound imaging indices used in this study are the sum of changes in intimal thickness, medial thickness, and hypertrophy of SMCs, which can largely result from, e.g. hypertension, the influence of medical treatment(s) may require consideration as important factors in the patients analyzed. Based on the current results, a future prospective larger-scale study for the comparison of patients with or without carotid artery stenosis using cerebrovascular events as endpoints is indicated.

In conclusion, the present study demonstrates that sLR11 was abundantly expressed in likely de-differentiated SMCs of carotid atherosclerotic plaques, and that the circulating levels were associated with plaque scores and size of stenotic fractions in cross-sectional and one-year observation analyses in patients with carotid artery stenosis. Considering the insights gained from the current study, circulating sLR11 may be a marker reflecting the pathological conditions of intimal SMCs in patients with carotid artery stenosis.

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Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict to interest with respect to this manuscript.

Author contributions

MH, MJ, KT, NH and HB are responsible for the work described in this paper. MH, MJ, NH, TN and HB were involved in the conception, design, or planning of the study. MH, MJ, TN and HB were involved in the analysis of data. MH, MJ, HE, NH, WS, NS, TN and HB were involved in the interpretation of results. HM, KT, WS and HB substantially contributed to the drafting of the manuscript.

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