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Author contributions

Y.K. planned and designed the study, performed the experiments, and wrote the paper. K.I., Y.U., S.T., K.N., M.K., A.S., T.T., K.K., and S.M. carried out the experiments and analysed data. K.N. gave conceptual advice.

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Short Communication

Peripheral leukocyte anomaly detected with routine automated hematology analyzer sensitive to adipose triglyceride lipase deficiency manifesting neutral lipid storage disease with myopathy/triglyceride deposit cardiomyovasculopathy

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ABSTRACT

Adipose triglyceride lipase (ATGL) deficiency manifesting neutral lipid storage disease with myopathy/triglyceride deposit cardiomyovasculopathy presents distinct fat-containing vacuoles known as Jordans' anomaly in peripheral leucocytes. To develop an automatic notification system for Jordans' anomaly in ATGL-deficient

Abbreviations: ATGL, adipose triglyceride lipase; NLSD-M, Neutral lipid storage disease with myopathy (NLSD-M); TGCV, triglyceride deposit cardiomyovasculopathy.

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patients, we analyzed circulatory leukocyte scattergrams on automated hematology analyzer XE-5000. The BASO-WX and BASO-WY values were found to be significantly higher in patients than those in non-affected subjects. The two parameters measured by automated hematology analyzer may be expected to provide an important diagnostic clue for homozygous ATGL deficiency.

1. Introduction

Adipose triglyceride lipase (ATGL, EC 3.1.1.3) deficiency is caused by mutations in ATGL gene, also called *PNPLA2* [1–3]. It presents profound lipid accumulation mainly in skeletal and cardiac muscles, manifesting neutral lipid storage disease with myopathy (NLSD-M)/triglyceride deposit cardiomyovasculopathy (TGCV) [3–7]. Only up to 40 patients have been reported globally [3–13]. Most of the reported cases were diagnosed in adulthood except for one case each in childhood and adolescence [12,13]. In adulthood, the myopathy and cardiomyopathy can be severe and rapidly progressive, and refractory to various therapies. Affected patients with ATGL deficiency exclusively exhibit persistent lipid droplets in the cytoplasm of circulatory neutrophils known as Jordans' anomaly (Fig. 1A) [3–13]. In earlier life, clinical symptoms seem to be absent or minimal in most cases, however Jordans' anomaly has been documented in subclinical or preclinical adolescents with ATGL deficiency [12,13]. Blood smear examination with May-Giemsa staining has been used for the detection of vacuoles in leucocytes. This report concerns a simple, easy and feasible laboratory test using a routine automated hematological analyzer that detects leukocyte abnormalities in patients with myopathy or cardiomyovasculopathy and possibly leads to a diagnosis of homozygous ATGL deficiency.

2. Methods

2.1. Subjects and specimens

Four homozygous ATGL-deficient patients (3 males and 1 female, 45–60 years of age) (Table 1) and nine heterozygous family members (4 males and 5 females, 17–83 years), lacking ATGL deficiency-associated symptoms, were enrolled. The diagnosis of ATGL deficiency was based on gene analyses together with clinical manifestations of myopathy, including easy fatigability, reduced exercise capability and limb weakness, and cardiomyopathy. Forty-three healthy subjects (14 males and 29 females, 32–84 years) lacking the mutations in ATGL gene and having no abnormality under the physical examination were also enrolled as controls. The peripheral blood specimens were collected with EDTA. Written informed consent was obtained from the enrolled subjects before study initiation.

2.2. Sample analysis

Blood specimens were analyzed by the XE-5000 automated hematology analyzer (Sysmex, Kobe, Japan) and investigated all the parameters including WBC/BASO channel of the XE-5000 to screen for Jordans' anomaly. In the WBC/BASO channel, its hemolyzing reagent, Stromatolyzer FB (Sysmex), lyses plasma membranes of cells other than basophils in the specimen and, as a result, the cytosolic components of non-basophils are released from the cells. Lipid droplets released from ATGL-deficient leukocytes which retain their shape in aqueous environment due to their own lipid monolayer membranes can be detected as smaller particles [14]. Thus, in the WBC/BASO scattergram, almost intact basophils, nucleus of

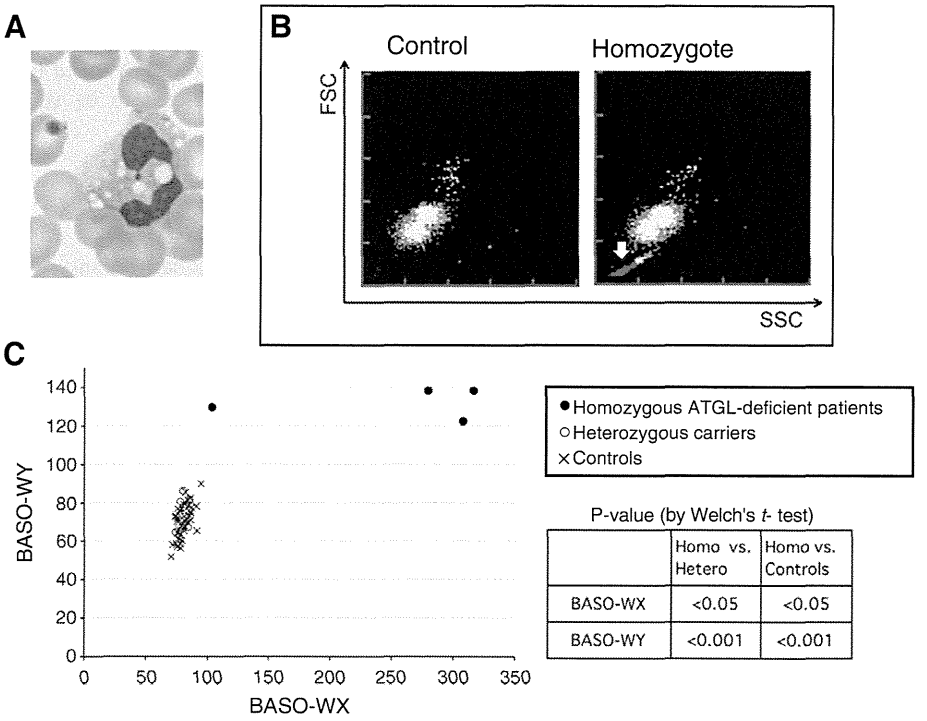

Fig. 1. (A) A representative image of May-Giemsa staining of blood smears from ATGL-deficient patients. Lipid droplets in peripheral leucocytes known as Jordans' anomaly were found in the neutrophils from all the ATGL-deficient patients. (B) Typical BASO scattergrams of control's blood (left) or homozygote's blood (right). Horizontal (X) and vertical (Y) axes indicate side and forward scattered light intensity, respectively. Colors indicate basophils (white dots), degenerated white blood cells (pale blue dots), and the other small particles (blue dots, white arrow). In these samples, BASO-WX and BASO-WY values are 79.5 and 60.3 (left, healthy), or 362.9 and 139.5 (right, patients), respectively. (C) Scatter plot of BASO-WX and BASO-WY. BASO-WX/BASO-WY values for ATGL-deficient patients (●), heterozygous ATGL carriers (○), controls (X). BASO-WX/BASO-WY values for ATGL-deficient patients were significantly higher than in other groups in Welch's *t*-test.

non-basophil leukocytes, and relatively large cytosolic components including lipid droplets or debris are detected as particles (Fig. 1B). Parameters named BASO-WX and BASO-WY, which stand for the spread of particle distribution in side and forward scattered light, respectively, are calculated in the WBC/BASO channel.

Table 1
Backgrounds of four adipose triglyceride lipase-deficient patients.

Case	Sex	Gene mutations	Reference	Present age	Cardiac function	Skeletal myopathy	Age at diagnosis[a] (Jordan's anomaly)
1	M	c.865C>T	6	50	NYHA4	Mild	41
2	M	c.696+1G>C	8	47	NYHA4	Mild	33
3	F	477_478dupCTCC	4	45	NYHA1	Severe	31
4	M	c.576delC	11	60	NYHA3	Mild	58

[a] All patients showed Jordans' anomaly in their blood smears at the diagnoses.

2.3. Statistical analysis

An analysis of Welch's *t*-test was performed to compare differences in BASO-WX and BASO-WY values between homozygous and heterozygous or controls. $p < 0.05$ was set to be statistically significant.

3. Results and discussion

After confirming that all the specimens from the four ATGL-deficient patients had Jordans' anomaly by examining their blood smears stained with May-Giemsa (Fig. 1A), we investigated all the parameters of the XE-5000 automated hematology analyzer to find any change corresponding to Jordans' anomaly. The WBC/BASO scattergram revealed an increased number of small particles in the homozygous patients, typically shown as the blue dots in Fig. 1B (white arrow in the right panel). Such change was not observed in the controls (Fig. 1B left) and in the heterozygote carriers (data not shown). The observed small particles are supposed to be the lipid droplets released from the patients' leukocytes, because lipid droplets can be expected to retain their spherical forms with neutral lipid core and phospholipid monolayer surface in this aqueous environment [14]. The BASO-WX and BASO-WY values obtained from the WBC/BASO channel of XE-5000 were significantly higher in the ATGL-deficient patients than those in the non-affected heterozygotes and the controls (Fig. 1C): 251.8 ± 100/132.0 ± 7.7 (BASO-WX/BASO-WY, mean ± SD) for the ATGL-deficient patients, 80.2 ± 3.8/74.8 ± 8.2 for the heterozygous carriers, and 80.4 ± 5.5/70.3 ± 8.2 for the controls.

We, therefore, anticipate that detection of the leucocyte abnormality in a routine automated hematological analysis may be a first step toward the diagnosis of homozygous ATGL deficiency. We further suggest that detected positive subjects should undergo more detailed analyses, including ATGL gene analyses, to establish the diagnosis in the clinical practice.

In homozygous ATGL deficiency, once the clinical presentations of myopathy and cardiomyopathy occur, it has been difficult so far to regress or resolve symptoms through any conventional treatments [3–12]. We reported two patients with severe cardiomyovasculopathy and heart failure requiring cardiac transplantation [6,8]. We recently provided data indicating that up-regulation of peroxisome proliferated activated receptor-γ and the related genes may promote triglyceride accumulation in the skeletal and cardiac muscles in ATGL deficiency [8]. We believe that the development of an easy method to detect cellular triglyceride accumulation is desired. It is quite likely that the change in the leukocyte emerges before the development of myopathy or cardiomyovasculopathy, as it does in other congenital lipid storage diseases such as Gaucher's and Niemann–Pick disease, which show distinct lipid storage in circulatory and bone marrow macrophages [15].

In our settings, heterozygous carriers could not be differentiated from control subjects, even though Jordans' anomaly has been reported in some heterozygous ATGL deficiency [16]. We speculate that one of the reasons for this may be that heterozygous leukocytes may have enough ATGL enzymatic activity to reduce the number and/or size of intracellular lipid droplets, so that BASO-WX and BASO-WY parameters were not different between heterozygous and control subjects.

It has been known that Jordans' anomaly in leukocytes is present not only in ATGL deficiency but also in Chanarin–Dorfman syndrome also called NLSD with ichthyosis, which is caused by deficiency of the protein CGI-58, an activator of the ATGL enzyme [17,18]. Further, in carnitine palmitoyltransferase deficiency type 1, a fatty acid beta-oxidation disorder engendering hypoglycemia and acidosis, this anomaly can sometimes be found in blood smears [19]. It would be of interest to know whether the present system can detect leucocyte abnormalities in these disorders, even we did not have the chance to test the possibility because of the disease rarity.

The sensitivity and specificity of BASO-WX and BASO-WY for ATGL deficiency remains to be investigated, however we believe that this automatic detection of changes in leukocytes with an automated hematology analyzer may provide an earlier diagnostic clue for ATGL deficiency to clinicians, who encounter patients with neuromuscular and cardiovascular disorders, whose causes are unknown.

In order to increase information on the natural history and pathophysiology in NLSD/TGCV patients, we have started an international registry system on the web (http://www.tgcv.org/r/home.html).

4. Conclusions

The BASO-WX and BASO-WY values obtained from automated hematology analyzer XE-5000 could help to detect Jordans' anomaly. A notification system using an automated hematology analyzer may prompt the earlier and easier diagnosis of homozygous ATGL deficiency.

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Short Communication

Disease-associated marked hyperalphalipoproteinemia

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ABSTRACT

Marked hyperalphalipoproteinemia (HAL) is a heterogeneous syndrome. To clarify the pathophysiological significance of HAL, we compared clinical profiles between marked HAL subjects with and without cholesteryl ester transfer protein (CETP) deficiency. CETP deficiency was associated with cardiovascular diseases and strokes in the HAL population, particularly in female. HAL women without CETP deficiency tended to have higher prevalence with cancer history. HAL may not always be a longevity marker, but be sometimes accompanied with pathological conditions.

1. Introduction

Hyperalphalipoproteinemia (HAL) had been regarded as a longevity syndrome. Matsuzawa et al. reported that a man with HAL unexpectedly had a corneal opacity which is a clinical sign for high density lipoprotein (HDL) deficiency [1]. Following studies revealed that genetic deficiency of cholesteryl ester transfer protein (CETP) is a major cause for HAL in Japan [2,3]. CETP is a plasma glycoprotein which facilitates the transfer of cholesteryl ester from HDL to apolipoprotein

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B-containing lipoproteins, then determine the plasma levels of HDL-cholesterol and low-density lipoprotein (LDL)-cholesterol levels [4]. This protein also regulates the lipid composition and particle size of lipoproteins.

CETP deficiency presents marked HAL and relative decrease in LDL-cholesterol level [5]. Such lipid profiles are generally believed protective for cardiovascular diseases (CVDs) and strokes, however, there has been a controversy whether this genetic deficiency is overall anti- or pro-atherogenic [6–8]. In addition, it is noteworthy that some clinical trials with CETP inhibitors recently failed and terminated [9], suggesting that further understanding pathophysiological significance for HAL is obviously required.

Here, we examined the prevalence of CVDs and strokes in HAL subjects with and without CETP deficiency along with their respective lipid profiles in a specific community, Akita Prefecture, Japan, where we reported that genetic CETP deficiency accumulates [10].

2. Subjects and methods

2.1. Subjects

The surveyed population comprised residents aged over 20-years-old in a community in Daisen City, Akita Prefecture, Japan (http://www.city.daisen.akita.jp/content/docs/english/), which includes Omagari area where genetic CETP deficiency accumulates [9,10].

After the opt-out in the community journal, we directly sent a request letter to 343 people with marked HAL (HDL-C > 100 mg/dL) based upon the annual health examination for the last three years. Unrelated 181 individuals (53%) agreed to participate in this study. Physical examination, blood test, and interview for medical histories and records of CVDs and strokes were performed. Based upon the analyses of the CETP gene and the protein levels, the subjects with HAL were divided into CETP-deficient and non-CETP-deficient groups.

This study was approved by the ethical committee in Osaka University.

2.2. Medical interview

We performed interviews on smoking, alcohol consumption, and medical histories for CVDs, stroke, diabetes mellitus, hypertension, hyperlipidemia, and cancer.

Diagnoses of hypertension and diabetes mellitus were made according to the criteria of Japanese Society of Hypertension and Japan Diabetes Society. CVDs include non-fatal myocardial infarction, angina pectoris, congestive heart failure, and arteriosclerosis obliterans. Strokes include cerebral infarction and cerebral hemorrhage, but exclude subarachnoid hemorrhage and strokes associated with atrial fibrillation. Cancers included any malignant tumors treated previously and currently.

2.3. CETP gene analyses

We performed direct sequencing of the DNA fragments amplified by polymerase chain reaction to detect two common CETP gene mutations [11,12]: intron 14 splicing defect (c.1321 + 1G>A, rs5742907) and missense mutation in exon 15 (c.1376A>G, rs2303790).

2.4. CETP protein mass

CETP protein mass was measured by the commercial available ELISA kit according to the manufacturer's protocol [13,14].

2.5. Criteria for CETP deficiency

Criteria of CETP deficiency was one of the following: 1) either of the common genetic mutations with c.1321 + 1G>A or c.1376A>G. We previously reported that these two CETP gene mutations contributed to approximately 90% of the genetic CETP deficiency in Japan (13, 14); 2) CETP mass was below 2.0 μg/mL.

We decided to use this cut-off value because the mean CETP mass level of the heterozygote for the missense mutation in exon 15 was 1.65 ± 0.31 μg/mL, as reported by Goto et al. [14].

2.6. Lipoproteins analyses

Serum lipoproteins were analyzed by analytical HPLC service system (LipoSEARCH®) at Skylight Biotech Inc. (Akita, Japan), as previously described [15].

2.7. Statistical methods

Data are presented as means (SD). All pair-wise comparisons between CETP- and non-CETP deficient groups were performed with the two-sided Student's *t*-test, and differences in percent values between these two groups were examined by Fisher's exact test. p Values < 0.05 were considered significant.

3. Results

Among the 181 participants with marked HAL, the numbers of CETP-deficient and non-CETP-deficient subjects were 71 and 110, respectively. There were no statistical significance of age and listed coronary risk factors, including hypertension, diabetes mellitus, and cigarette smoking (Table 1).

Among 71 CETP-deficient subjects, 2 were revealed to be homozygous. Prevalence of CVDs history was significantly higher in CETP-deficient group than in non-CETP-deficient group ($p = 0.016$). Particularly in female subgroups, the prevalence of CVDs and strokes was significantly higher in CETP-deficient female ($p = 0.02$ for CVD, $p = 0.028$ for ischemic stroke) (Table 1). Furthermore, the prevalence of cancer history tended to be higher in non-CETP-deficient females than in CETP-deficient ones, although not significant statistically (Table 1). Among HAL women without CETP deficiency, the histories for gastric and uterine/breast cancers seem to be higher.

The particle sizes of HDL and LDL were not different significantly between CETP-deficient and non-CETP-deficient groups. HDL-TG/HDL-cholesterol ratio was significantly decreased in CETP-deficient group than non-CETP-deficient group ($p = 0.002$), whereas LDL-TG/LDL-cholesterol ratio was significantly increased in CETP-deficient group ($p = 0.01$) (Table 1), which is compatible with our previous reports [16,17].

4. Discussion

In the previous cross-sectional study in Omagari area, Japan, where CETP deficiency accumulates, we found that there was a U-shaped relationship between plasma HDL-cholesterol and ischemic electrocardiographic changes for the first time [10]. Zhong et al. reported that heterozygous CETP deficiency may be associated with CVDs in Japanese-American population in Hawaii [18], consistent with results of our previous study. Further, recent reports have drawn U-shaped relationship between plasma HDL-C levels and prevalence of CVDs in the other subjects and population [19,20]. The results of this study, together with those of previous studies, provide evidence that HAL is not always promising for the preventions of CVDs and strokes.

We and others reported that CETP deficiency results in qualitative and quantitative abnormalities in both HDL and LDL [16,17], as shown in Table 1. Triglyceride-rich LDL had lower affinity for LDL receptor [17] and may be susceptible for oxidation in plasma. There seems to be controversial whether large and cholesterol-rich HDL from CETP deficiency had reduced or improved ability for cholesterol efflux from lipid-laden macrophages, depending on their experimental settings [16,21,22].

Unexpectedly, we noticed that the cancer history tended to be more frequent in HAL without CETP deficiency than with CETP deficiency (Table 1). It is known that the Akita Prefecture has one of the highest cancer mortalities among all prefectures in Japan last couple of decades. Further study would be of significance to know the association between HAL and cancer for public health as well as medical science.

The present study has the following limitations: 1) we focused on subjects with marked HAL who voluntarily participated. Therefore, residents with some clinical problems might be more motivated to participate compared with those without any clinical problems, which might raise a possibility that the

Table 1
Clinical profiles in subjects with marked hyperalphalipoproteinemia with and without CETP deficiency.

	CETP deficiency	Non-CETP deficiency	P
Total number	71	110	
Age (y)	67 ± 12	64 ± 13	0.263
CETP mass (mg/mL)	1.7 ± 0.5	2.8 ± 0.5	0.0009
Coronary risk factors			
Hypertension	22 (31%)	36 (33%)	0.878
LDL-cholesterol (mg/dL)	98 ± 24	103 ± 29	0.284
Diabetes mellitus	4 (6%)	10 (9%)	0.572
Smoking habit	18 (26%)	29 (26%)	1.00
Triglycerides:cholesterol ratio in HDL	0.15 ± 0.03	0.21 ± 0.03	0.002
Triglycerides:cholesterol ratio in LDL	0.28 ± 0.04	0.22 ± 0.04	0.01
Cardiovascular disease	10 (14%)	3 (3%)	0.016
Stroke	5 (7%)	4 (4%)	0.487
Ischemic	5 (7%)	3 (3%)	0.271
Hemorrhagic	0 (0%	1 (1%)	1.00
Cancers	8 (11%)	19 (17%)	0.399
Gastric cancer	5 (7%)	10 (9%)	0.786
Male (n)	28	44	
Cardiovascular disease	3 (11%)	2 (5%)	0.386
Stroke	1 (4%)	4 (9%)	0.645
Ischemic	1 (4%)	3 (7%)	1.00
Hemorrhagic	0 (0%)	1 (2%)	1.00
Cancers	5 (18%)	6 (14%)	0.747
Gastric cancer	4 (14%)	5 (14%)	0.734
Others	1 (4%)	1 (4%)	1.00
Female (n)	43	66	
Cardiovascular disease	7 (16%)	1 (2%)	0.02
Stroke	4 (9%)	0 (0%)	0.028
Ischemic	4 (9%)	0 (0%)	0.028
Hemorrhagic	0 (0%)	0 (0%)	1.00
Cancers	3 (7%)	13 (20%)	0.165
Gastric cancer	1 (2%)	5 (8%)	0.404
Uterine, breast cancers	2 (5%)	7 (11%)	0.48
Others	0 (0%)	1 (2%)	1.00

Data are presented as mean ± SD (p value assessed by use of Student's *t*-test) and percentages by Fisher's exact test.
Diagnoses of hypertension and diabetes mellitus were made according to the criteria of the Japanese Society of Hypertension and the Japan Diabetes Society.
Cardiovascular diseases include non-fatal myocardial infarction, angina pectoris, congestive heart failure, and arteriosclerosis obliterans.
Stroke includes cerebral infarction and cerebral hemorrhage, and excludes subarachnoid hemorrhage and strokes associated with atrial fibrillation. Cancers include any malignant tumors treated previously and currently.

disease prevalence might be overestimated in both CETP- and non-CETP deficient HAL groups. It would be of importance to compare the disease prevalence in subjects with marked HAL with that in normolipidemic subjects in the same community; 2) we did not know the molecular basis for HAL without CETP deficiency, although molecules such as hepatic triglyceride lipase [7,22] were reported as responsible for some types of HAL.

In conclusion, marked HAL is not always beneficial for the prevention of CVDs and strokes. Rather, marked HAL may be occasionally associated with the developments of these life-threatening diseases, depending on their sexes and genetic backgrounds.

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Association of the ASCO Classification with the Executive Function Subscores of the Montreal Cognitive Assessment in Patients with Postischemic Stroke

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Background: The ASCO classification can evaluate the etiology and mechanisms of ischemic stroke more comprehensively and systematically than conventional stroke classification systems such as Trial of Org 10172 in Acute Stroke Treatment (TOAST). Simultaneously, risk factors for cognitive impairment such as arterial sclerosis, leukoaraiosis, and atrial fibrillation can also be gathered and graded using the ASCO classification. *Methods:* Sixty patients with postischemic stroke underwent cognitive testing, including testing by the Japanese version of the Montreal cognitive assessment (MoCA-J) and the mini-mental state examination (MMSE). Ischemic strokes were categorized and graded by the ASCO classification. In this phenotype-based classification, every patient is characterized by the A-S-C-O system (A for Atherosclerosis, S for Small vessel disease, C for Cardiac source, and O for Other cause). Each of the 4 phenotypes is graded 0, 1, 2, or 3, according to severity. The conventional TOAST classification was also applied. Correlations between individual MoCA-J/MMSE scores and the ASCO scores were assessed. *Results:* The total score of the ASCO classification significantly correlated with the total scores of MoCA-J and MMSE. This correlation was more apparent in MoCA-J than in MMSE, because MoCA-J scores were normally distributed, whereas MMSE scores were skewed toward the higher end of the range (ceiling effect). Results for individual subtests of MoCA-J and MMSE indicated that cognitive function for visuoexecutive, calculation, abstraction, and remote recall significantly correlated with ASCO score. *Conclusions:* These results suggest that the ASCO phenotypic classification of stroke is useful not only for assessing the etiology of ischemic stroke but also for predicting cognitive decline after ischemic stroke. **Key Words:** ASCO phenotypic classification—poststroke cognitive impairment—Montreal cognitive assessment—mini-mental state examination.

The burden of stroke stemming from its effect on cognition has been underestimated for a long time. Ischemic stroke is a major cause of adult chronic disability and represents an important cause of cognitive decline and dementia. Poststroke cognitive impairment (PSCI) appears in about one third of patients with stroke.[1] The prevalence of cognitive impairment among patients with a history of stroke is similar to that of subjects

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10 years older without a history of stroke.[2] In addition, a recent study demonstrated that poststroke mild cognitive impairment (MCI) is progressive and develops into dementia in 24.4% of patients within 3 years, giving a mean conversion rate of approximately 8% per year.[3] Patients with PSCI showed a much poorer prognosis. Thus, the concept of PSCI, including poststroke MCI, should be emphasized for early detection and treatment, even if the impairment is not severe enough to meet the criteria for dementia.

The ASCO phenotypic classification is proposed as a new classification of stroke.[4] The ASCO classification can evaluate the etiology and mechanisms of ischemic stroke more comprehensively and systematically than conventional stroke classification systems such as the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification. Increasing knowledge about ischemic stroke mechanisms and the introduction of new diagnostic techniques have supported the promotion of the new ASCO phenotypic classification, which aims to characterize patients using different grades of evidence for ischemic stroke subtypes. With this new way of classifying patients, no information is neglected when the diagnosis is made, and treatment can be adapted to the observed phenotypes and the most likely etiology. Furthermore, risk factors for dementia such as atherosclerosis, small vessel disease, and atrial fibrillation can be summarized and graded using the ASCO classification.

For the detection of cognitive impairment after ischemic stroke, the Montreal cognitive assessment (MoCA) is more sensitive than the mini-mental state examination (MMSE).[5] The MoCA is a simple, standalone, cognitive screening test with a good sensitivity and specificity in detecting MCI and, unlike the MMSE, includes executive tasks.[6] However, additional predictive methods for early detection of PSCI are urgently needed. For example, medial temporal atrophy has predictive value for cognitive decline after stroke but it seems to be less informative than the MoCA or MMSE. If the ASCO classification has a predictive value for PSCI, its clinical value will be enhanced.

The main aim of this study was to evaluate the additive value of the ASCO phenotypic classification in assessing cognitive impairment of patients with postischemic stroke over the use of the Japanese version of the MoCA (MoCA-J) and MMSE. We found additional evidence that the ASCO classification can not only assess the etiology of ischemic stroke but also predict cognitive decline after ischemic stroke.

Materials and Methods

Subjects

Sixty patients with first-ever ischemic stroke were enrolled in this study more than 3 months after admission to the Kobe University Neurology Clinic between 2012 and 2013. Patients taking part in the study gave informed consent as approved by the Committee of Medical Ethics within our faculty. All procedures were performed in accordance with the guidelines for clinical study from the Ethical Committee of Kobe University. Patients with infratentorial infarction or strategic single-infarct dementia were excluded. Patients with other dementing illnesses including Alzheimer disease and Lewy body dementia were not enrolled in this study to exclude the possibility of nonvascular causes of neurodegenerative cognitive impairment in the subjects.

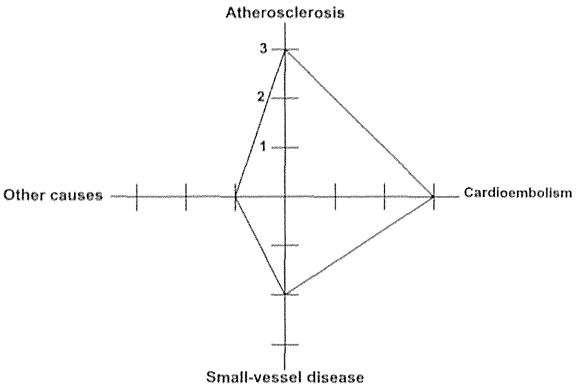

Figure 1. *Example of the grading system (phenotyping) in a patient with an ipsilateral carotid stenosis >50% (atherosclerosis, grade 3), a single, deep branch artery stroke (small vessel disease, grade 2), atrial fibrillation (cardioembolism, grade 3), and a platelet count of 700,000/mm^3 (other causes, grade 1). Stroke subtype is A3-S2-C3-O1.*

Ischemic Stroke Assessment by the ASCO and TOAST Classifications

Sixty consecutive patients with postischemic stroke were assessed by the ASCO classification of stroke. All ischemic strokes were confirmed by imaging studies. The ischemic stroke was categorized and graded by the ASCO phenotypic classification. In this phenotype-based classification, every patient is characterized by the A-S-C-O system (A for Atherosclerosis, S for Small vessel disease, C for Cardiac source, O for Other cause). Each of the 4 phenotypes is graded 0, 1, 2, or 3, according

Table 1. *The ASCO phenotypic classification of stroke*

A	Atherosclerosis
S	Small vessel disease
C	Cardiac disease
O	Other causes
3	Definitely a potential cause of the index stroke
2	Causality uncertain
1	Unlikely a direct cause of the index stroke (but disease is present)
0	Disease not present
9	Insufficient workup

Table 2. *Clinical features and demographics of patients*

Variable	Value
Mean age, y (range)	68 ± 13 (39-92)
Gender (M:F)	40:20
Modified Rankin Scale, mean ± SD (range)	1.65 ± .7 (0-4)
NIHSS ± SD (range)	1.82 ± .6 (0-7)
Hypertension, n (%)	45 (75)
Diabetes mellitus, n (%)	23 (38.3)
Dyslipidemia, n (%)	28 (46.7)
Cigarette smoking, n (%)	25 (41.7)
MoCA-J ± SD (range)	19.6 ± 4.8 (3-28)
MMSE ± SD (range)	24.3 ± 4.6 (5-30)

Abbreviations: M:F, male:female; MMSE, mini-mental state examination; MoCA-J, Montreal cognitive assessment-Japanese version; NIHSS, National Institute of Health Stroke Scale; SD, standard deviation.

to severity. The ASCO classification methods are illustrated in Figure 1 and Table 1. In this study, the original ASCO classification grading was modified as follows for quantitative evaluation: the original grade 1 was grade 3 in the revised version, and the original grade 3 was grade 1 in the revised version. The total ASCO score was 12 (A, 3; S, 3; C, 3; O, 3). The stroke subtype with the highest ASCO grade was regarded as the most likely etiology of the stroke (A, Atherosclerosis; S, Small vessel disease; C, Cardioembolism; O, Other causes). The ischemic strokes of the patients were also categorized by the conventional TOAST method.

Neuropsychologic Examination

All subjects underwent a general physical and neurologic examination, and a neuropsychologic assessment, including MoCA-J and MMSE more than 3 months after ischemic stroke. MoCA-J less than 24 and MMSE less than 24 were regarded as indicating cognitive decline in this study. Two neurologists were involved in the neuropsychologic assessment; if their assessments did not agree, patients were re-examined.

Statistical Analysis

Correlations of individual cognitive activity with the scores of MoCA-J and MMSE were assessed using the Pearson correlation analysis. Subtests of the MoCA-J and MMSE were evaluated by dividing the mean subtest score by its standard deviation. A lower Z-score indicates greater discrimination between subjects.

Results

Patient Demographic Data

Clinical features and demographic data of patients are summarized in Table 2. All patients had at least 1 risk factor for ischemic cerebrovascular disease, such as hypertension, diabetes mellitus, dyslipidemia, or cigarette smoking. The mean modified Rankin Scale score was 1.65 (range, 0-4) and the mean National Institute of Health Stroke Scale (NIHSS) score was 1.82 (range, 0-7). These patients' clinical histories and radiologic features excluded the possibility of coexisting strategic single-infarct dementia.

Stroke Subtyping Classification by ASCO and TOAST

Ischemic strokes were categorized and graded by the ASCO phenotypic classification. The corresponding categorizations by the ASCO and TOAST classifications are

Table 3. *ASCO and TOAST classifications in the present study*

	ASCO, %	TOAST, %
A	23.33	21.67
S	30.00	35.00
C	30.00	26.67
O	6.67	6.67
Undeterminded	–	10.00

	TOAST				
ASCO	A, N = 13	S, N = 21	C, N = 16	O, N = 4	Undetermined, N = 6
A	**13**	18	13	2	5
S	11	**21**	15	3	6
C	3	4	**16**	0	4
O	0	0	0	**4**	0

Abbreviations: ASCO, A for Atherosclerosis, S for Small vessel disease, C for Cardiac source, O for Other cause; TOAST, Trial of Org 10172 in Acute Stroke Treatment.

Comparison of ASCO results at evidence grade 3 with TOAST results, giving percentages of etiologies found with both classification systems. TOAST results (column) and the corresponding ASCO score distribution (rows) for evidence grades 1-3.

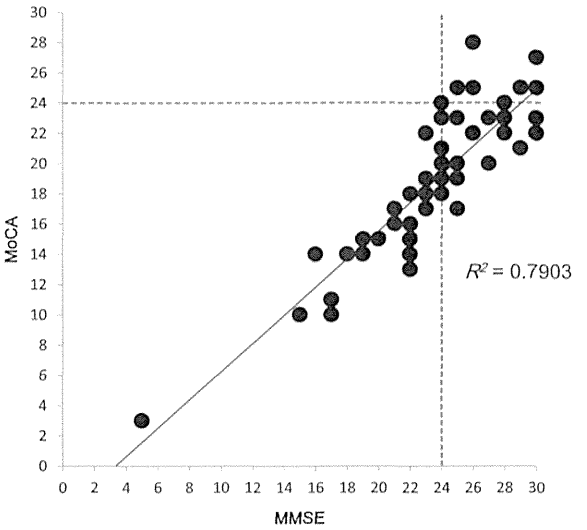

Figure 2. *Distribution of MoCA-J and MMSE scores of the patients after ischemic stroke. A significant relationship exists between MoCA-J and MMSE (R^2 = .79; P < .05; Pearson correlation analysis). MoCA-J scores are normally distributed, whereas MMSE scores are skewed toward the higher end of the range. Abbreviations: MMSE, mini-mental state examination; MoCA-J, Montreal cognitive assessment-Japanese version.*

summarized in Table 3. Grade 3 ASCO evidence was found in 23% of the subjects for Atherosclerosis (A), 30% of the subjects for Small vessel disease (S), 30% of the subjects for Cardiac source (C), and 6.7% of the subjects for Other cause (O). Eighty-eight percent of the patients were classified into more than 1 category. All cases could be determined by the ASCO classification. On the other hand, using the TOAST classification, the distribution was 22% (Atherosclerosis), 35% (Lacunae), 27% (Cardioembolism), and 6.7% (Others). Specifically mentioned, 10% cases were diagnosed as undetermined because more than 2 causes existed. There was a high concordance for grade 3 etiologic classification between the ASCO and TOAST systems.

Relationship between MoCA-J and MMSE

There was a significant relationship between MoCA-J and MMSE (R^2 = .79; P < .05; Fig 2). MoCA-J scores were normally distributed, whereas MMSE scores were skewed toward the higher end of the range. Of the 37 patients with an impaired MoCA-J score (<24), only 20 (54%) had an impaired MMSE score (<24), whereas all 20 patients with an impaired MMSE score had an impaired MoCA-J score. Results for individual subtests of the MoCA-J and the MMSE are summarized in Table 4. Coefficients of variation >5 were found in 4 MMSE subtests (orientation, registration, naming, and language) but in no MoCA-J subtests.

Relationship between the ASCO Classification and Tests of Cognitive Function

In all patients with postischemic stroke, there was a significant correlation between the ASCO score and MoCA-J score (R^2 = .47; P < .05; Fig 3A). There was also a significant correlation between the ASCO score and MMSE score (R^2 = .41; P < .05; Fig 3B). Compared with MoCA-J scores, MMSE scores were skewed toward the higher end of the range (ceiling effect) and MoCA-J was more sensitive to cognitive decline in patients with postischemic stroke than MMSE. Results for individual subtests of MoCA-J indicated that cognitive function tests for visuoexecutive, abstraction, and remote recall were correlated closely with the ASCO score. Results for individual subtests of MMSE indicated that cognitive function tests for calculation and remote recall were correlated closely with the ASCO score.

Discussion

This study shows that the total score of the ASCO classification significantly correlates with the total scores of MoCA-J and MMSE. This correlation was more apparent in MoCA-J than in MMSE, because MoCA-J scores were normally distributed, whereas MMSE scores were skewed toward the higher end of the range (ceiling effect). Results for individual subtests of MoCA-J and MMSE indicated that cognitive function for visuoexecutive, calculation, abstraction, and remote recall function significantly correlated with the ASCO score in patients with PSCI.

Table 4. *Cognitive test results: average subtest scores*

MoCA-J	Visuoexecutive/5	Naming/3	Attention/6	Language/3	Abstraction/2	Recall/5	Orientation/6
Average (SD)	3.4 (1.4)	2.6 (.8)	4.6 (1.3)	1.4 (.7)	1.1 (.8)	1.2 (1.0)	5.2 (1.4)
Z-score	2.4	3.4	3.6	1.9	1.3	1.1	3.8

MMSE	Orientation/10	Registration/3	Attention/ calculation/5	Recall/5	Naming/2	Language/6	Drawing/1
Average (SD)	8.9 (1.8)	2.9 (.2)	2.8 (1.7)	1.1 (1.1)	2.0 (.3)	5.6 (.7)	.9 (.3)
Z-score	5.0	12.5	1.6	1.0	7.4	8.6	3.0

Abbreviations: MMSE, mini-mental state examination; MoCA-J, Montreal cognitive assessment-Japanese version; SD, standard deviation.

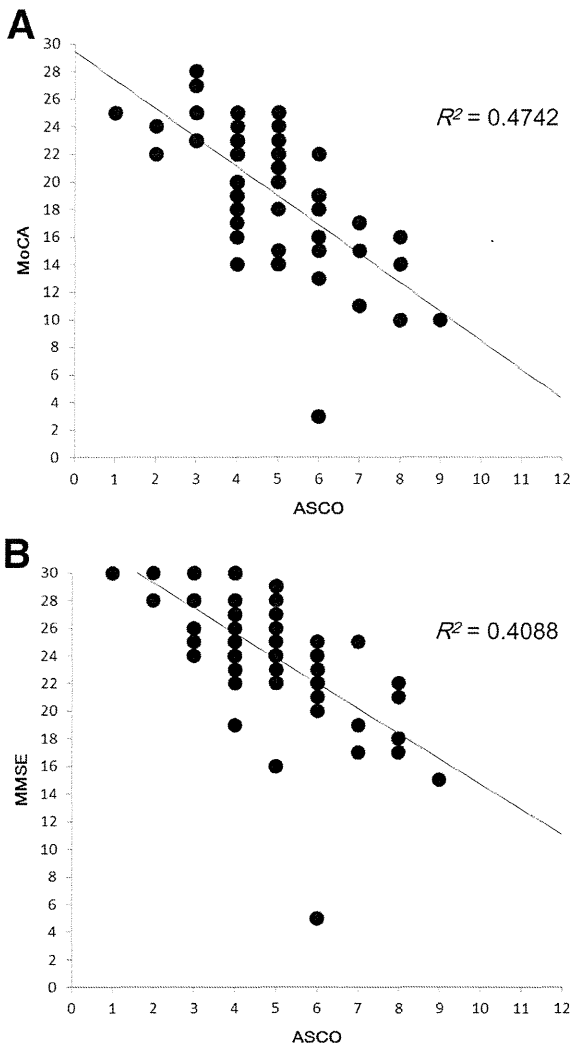

Figure 3. *Distribution of MoCA-J, MMSE, and ASCO scores of patients after ischemic stroke. A significant correlation exists between the ASCO phenotypic classification total score and MoCA-J (R^2 = .47; P < .05) (A). There is also a significant correlation between the ASCO phenotypic classification total score and the MMSE score (R^2 = .41; P < .05; Pearson correlation analysis) (B). Compared with MoCA-J scores, MMSE scores are skewed toward the higher end of the range (ceiling effect). Abbreviations: ASCO, A for Atherosclerosis, S for Small vessel disease, C for Cardiac source, O for Other cause; MMSE, mini-mental state examination; MoCA-J, Montreal cognitive assessment-Japanese version.*

Stroke and cognitive impairment pose major threats to elderly people. PSCI includes all forms of cognitive decline that develop after stroke, even if not severe enough to fit the criteria for dementia after stroke.[7,8] The true cognitive burden of stroke is probably underestimated because cognitive impairment of stroke is 3 times more common than in stroke-free subjects.[9] PSCI significantly worsens the survival rate of patients even compared with patients with Alzheimer disease.[10] Nevertheless, little attention is usually paid to cognitive impairment after stroke. In the present situation, guidelines for care and rehabilitation of patients with stroke neither clearly define the optimal timing nor the most appropriate instruments for cognitive evaluation after ischemic stroke. Recently, several effective treatments for PSCI have been reported. Administration of donepezil in the acute phase of ischemic stroke improved the MMSE score and prognosis of the patients.[11] Early poststroke rehabilitation is associated with a reduction in dementia risk among patients with ischemic stroke.[12] Furthermore, early treatment of poststroke depression by antidepressant drugs may improve cognitive ability and survival rate.[13] In addition, the diagnostic concept "Cognitive Impairment No Dementia (CIND)", namely vascular mild cognitive impairment, predicts further cerebrovascular events in addition to cognitive decline.[14] Thus, early detection and treatment of PSCI are essential not only to improve cognitive decline but also to prevent secondary ischemic stroke. Appropriate instruments to detect PSCI are urgently needed.

Several batteries of neuropsychologic tests are available for predicting PSCI. The MoCA has been recommended for vascular cognitive impairment. For example, the MoCA score at acute phase after stroke has predictive value for PSCI.[5] Several clinical studies have demonstrated that patients with PSCI often have impaired executive function and that MoCA is more sensitive than the MMSE for detection of cognitive decline after stroke.[5] In this study, MMSE tended to overlook cognitive decline after stroke and MoCA-J could detect PSCI more sharply than MMSE. Compared with MoCA-J, MMSE scores were skewed toward the higher end of the range because of the easiness of the tasks (ceiling effect). To detect PSCI, MoCA-J should be used to assess the cognitive ability of patients with postischemic stroke.

The ASCO classification of stroke is proposed by Amarenco et al.[4] The concept is to introduce a complete "stroke phenotyping" classification as distinguished from past classifications that subtype strokes by characterizing only the most likely cause of stroke. In clinical practice, many ischemic strokes have complex etiologies and cannot be diagnosed as belonging to only 1 category by a conventional classification system such as TOAST.[15] Simultaneously, the ASCO score reflects risk factors not only for ischemic stroke but also for cognitive impairment such as arterial stenosis,[16] leukoaraiosis,[17] and atrial fibrillation.[18] Patients with arterial stenosis, leukoaraiosis, or atrial fibrillation tend to show more serious cognitive impairment. That was probably why the ASCO score was associated with PSCI as assessed by MoCA-J and MMSE in this study. In addition, subtest analyses demonstrated that MoCA-J and MMSE revealed cognitive impairment for visuoexecutive, calculation, abstraction, and remote recall function, and the executive functions are often impaired in vascular cognitive impairment syndromes including PSCI.

In this study, the cognitive scores of patients with ASCO scores 4-6 seemed to have a large variance, and

the ASCO score could not reveal subtle cognitive declines. We speculate that the grading (0, 1, 2, or 3) criteria of the ASCO classification are still inaccurate. For example, grade for small vessel disease (S) does not define the degree of leukoaraiosis or microbleeds.[4] A strict and appropriate indication of the degree of leukoaraiosis should be given. In addition, under other causes (O), frequent causes of ischemic stroke such as vasculitis and paraneoplastic syndrome are not included in the present ASCO classification. Several rare, but important, causes of stroke should be added.

There are several limitations of this study. For example, we did not follow the 60 patients long enough. The temporal profiles of cognitive function of patients with post-ischemic stroke should be explored. In addition, a recent study has demonstrated that there is substantial heritability for ischemic stroke and PSCI and this varies markedly for different stroke subtypes.[19,20] In future, the cognitive ability of patients with PSCI should be explored in a perspective of genetic heritability.

In conclusion, these results suggest that the ASCO phenotypic classification of stroke is useful not only for assessing the etiology of ischemic stroke but also for predicting cognitive decline after ischemic stroke.

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Overexpression of LARGE suppresses muscle regeneration via down-regulation of insulin-like growth factor 1 and aggravates muscular dystrophy in mice

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Several types of muscular dystrophy are caused by defective linkage between α-dystroglycan (α-DG) and laminin. Among these, dystroglycanopathy, including Fukuyama-type congenital muscular dystrophy (FCMD), results from abnormal glycosylation of α-DG. Recent studies have shown that like-acetylglucosaminyltransferase (LARGE) strongly enhances the laminin-binding activity of α-DG. Therefore, restoration of the α-DG–laminin linkage by LARGE is considered one of the most promising possible therapies for muscular dystrophy. In this study, we generated transgenic mice that overexpress LARGE (LARGE Tg) and crossed them with *dy*^{2J} mice and fukutin conditional knockout mice, a model for laminin α2-deficient congenital muscular dystrophy (MDC1A) and FCMD, respectively. Remarkably, in both the strains, the transgenic overexpression of LARGE resulted in an aggravation of muscular dystrophy. Using morphometric analyses, we found that the deterioration of muscle pathology was caused by suppression of muscle regeneration. Overexpression of LARGE in C2C12 cells further demonstrated defects in myotube formation. Interestingly, a decreased expression of insulin-like growth factor 1 (IGF-1) was identified in both LARGE Tg mice and LARGE-overexpressing C2C12 myotubes. Supplementing the C2C12 cells with IGF-1 restored the defective myotube formation. Taken together, our findings indicate that the overexpression of LARGE aggravates muscular dystrophy by suppressing the muscle regeneration and this adverse effect is mediated via reduced expression of IGF-1.

INTRODUCTION

Dystroglycan (DG) is encoded by a single gene, *Dag1*, located on human chromosome 3p21 and cleaved into two proteins, α- and β-DG, by post-translational processing (1). α-DG is an extracellular peripheral membrane protein and although its molecular mass is predicted to be 72 kDa from its amino acid sequence, its apparent molecular mass in skeletal muscle is 156 kDa owing to extensive glycosylation. α-DG binds to several extracellular matrix proteins including laminin, agrin and perlecan (1) and synaptic proteins such as neurexin and pikachurin (2,3). Transmembrane protein β-DG anchors α-DG at the extracellular surface of the plasma membrane (4). The cytoplasmic domain of β-DG interacts with dystrophin, a large cytoplasmic protein that binds to F-actin (5). Thus, DG plays a central role in stabilizing

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the plasma membrane by acting as an axis that links the extracellular matrix to the cytoskeleton.

α-DG is composed of distinct three domains: the N-terminal domain, the mucin-like domain and the C-terminal domain. The N-terminal domain is cleaved by the proprotein convertase furin and secreted outside cells (6,7). The mucin-like domain is highly glycosylated by *O*-linked oligosaccharides. Chiba *et al.* identified the *O*-mannosyl glycan, Siaα2-3Galβ1-4GlcNAcβ1-2Man, which is attached to the mucin-like domain of α-DG and is necessary for binding activity with laminin (8). The *O*-mannose is phosphorylated at the 6th position and further extended on the distal side of the phosphate by a repeating disaccharide -3Xylα1-3GlcUAβ1-. This extension is catalyzed by like-acetylglucosaminyltransferase (LARGE) and regulates the binding activity of α-DG (9,10). Most recently, it was demonstrated that SGK196 phosphorylates the 6th position of *O*-mannose of GalNAcβ1-3GlcNAcβ1-4Man on α-DG after the mannose had been modified by glycosyltransferase-like domain containing 2 (GTDC2) and β3-*N*-acetylgalactosaminyltransferase 2 (B3GALNT2) (11).

The linkage between α-DG and laminin is crucial to stabilize the sarcolemma, and disruption of this linkage caused by aberrant glycosylation of α-DG underlies the pathogenesis of several types of muscular dystrophy (12). These disorders are collectively called dystroglycanopathy. Their phenotypes range from severe congenital muscular dystrophy (CMD) to milder adult-onset limb-girdle muscular dystrophy (13). The most severe end of this spectrum includes Walker–Warburg syndrome (OMIM236670), muscle–eye–brain disease (OMIM253280) and Fukuyama-type congenital muscular dystrophy (FCMD) (OMIM253800). These severe forms of muscular dystrophy are often associated with brain anomalies and ocular defects (13). FCMD is the second most common childhood muscular dystrophy in Japan. The founder mutation, a SINE-VNTR-*Alu* retrotransposal insertion to the 3′ non-coding region, as well as several point mutations of *fukutin* has been reported in Japan and other countries (14,15). Thus far, mutations in 17 genes encoding known or putative glycosyltransferases have been identified as causative of the dystroglycanopathy. Among their gene products, enzymatic activities of protein *O*-mannosyltransferase 1 (POMT1) (16,17), protein *O*-mannosyltransferase 2 (POMT2) (17,18), protein *O*-linked mannose β-1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) (19), LARGE (10,20), SGK196 (11,21), GTDC2 (19,22) and B3GALNT2 (11,23) have been shown to be involved in the formation of *O*-mannosyl glycan on α-DG. Defects in dolichol kinase (DOLK) (24), GDP-mannose pyrophosphorylase B (GMPPB) (25), dolichol-P-mannose synthase polypeptide 1 (DMP1) (26), dolichol-P-mannose synthase polypeptide 2 (DMP2) (27) and dolichol-P-mannose synthase polypeptide 3 (DMP3) (28) lead to dystroglycanopathy by inhibiting the biosynthesis of dolichol-P-mannose, which is necessary for the initial step of *O*-mannosylation. Although the functions of fukutin (14), fukutin-related protein (FKRP) (29), β-1,3-*N*-acetylglucosaminyltransferase 1 (B3GNT1) (30), isoprenoid synthase domain containing (ISPD) (31,32) and transmembrane protein 5 (TMEM5) (33) have not been elucidated, they are thought to be involved in the formation of *O*-mannosyl glycan. In addition to these secondary defects in glycosylation, a primary mutation was identified in the N-terminal domain of α-DG in a patient with dystroglycanopathy (34).

Apart from dystroglycanopathy, defects in the ligand of α-DG, laminin-211, also lead to muscular dystrophy. Laminin α2 chain-deficient congenital muscular dystrophy (MDC1A) (OMIM607855) presents as severe CMD associated with dysmyelination of peripheral nerves and white matter abnormalities in the brain. MDC1A is one of the most common forms of CMD, and it accounts for 30–40% of all patients with CMD (35). The causative mutations of MDC1A were identified in the gene encoding laminin α2 chain, *LAMA2* (36,37). These facts imply that the axis composed of α-DG and laminin is crucial to protect muscle cells from dystrophic changes.

Barresi *et al.* reported, using fibroblasts from patients with dystroglycanopathy such as FCMD and muscle–eye–brain disease, that overexpression of LARGE facilitated the glycosylation of α-DG and restored the reduced laminin-binding activity of α-DG irrespective of the gene involved (38). Therefore, the up-regulation of laminin-binding activity of α-DG by the overexpression of LARGE could provide a novel therapeutic strategy for muscular dystrophy, regardless of whether the primary mutation resides in LARGE or not. To test this hypothesis, we generated transgenic mice overexpressing LARGE (LARGE Tg mice) and crossed them with dy^{2J} mice (39) and MCK-fukutin conditional knockout mice (*FKTN* cKO mice) (40), a model of MDC1A and FCMD, respectively. Remarkably, the muscular dystrophy of both dy^{2J} and *FKTN* cKO mice was aggravated by the overexpression of LARGE. Morphometric analyses demonstrated that the regeneration of muscle fiber was suppressed in these mice. Furthermore, the transfection of C2C12 cells with LARGE led to deficits in proliferation and fusion of myoblasts. Finally, we found that the expression of insulin-like growth factor (IGF-1) was reduced in both LARGE Tg mice and LARGE-transfected C2C12 cells, and supplementation of IGF-1 rescued the suppressed myotube formation of C2C12 cells. These data indicate that the overexpression of LARGE suppresses the regeneration of skeletal muscle, at least partially, via down-regulation of IGF-1 and worsens the muscular dystrophy of dy^{2J} and *FKTN* cKO mice.

RESULTS

Generation and characterization of LARGE transgenic mice

First, to test the effect of overexpression of LARGE, we generated transgenic mice that overexpress LARGE (LARGE Tg mice) using CAG promoter (41). Large Tg mice were born, grew normally and exhibited no obvious motor or behavioral abnormalities. The overexpression of LARGE was demonstrated in each tissue, including skeletal muscle, cardiac muscle, brain, peripheral nerve, kidney and liver by western blotting (Fig. 1A). Consistently, the immunoreactivity of IIH6, which recognizes glycosylated form of α-DG, and the laminin-binding activity of α-DG were markedly increased in each tissue of LARGE Tg mice, as compared with the wild type (Fig. 1A and B). In the brain of LARGE Tg mice, the enhanced signal of IIH6 was localized to the brain surface and capillaries (Fig. 1B). Further, *in situ* overlay assay showed increased binding activity of skeletal and cardiac muscles for both laminin and agrin (Fig. 1C). The expression of laminin α2, β1 and γ1 chains was not significantly changed as confirmed by immunofluorescent analysis (Supplementary Material, Fig. S1A).

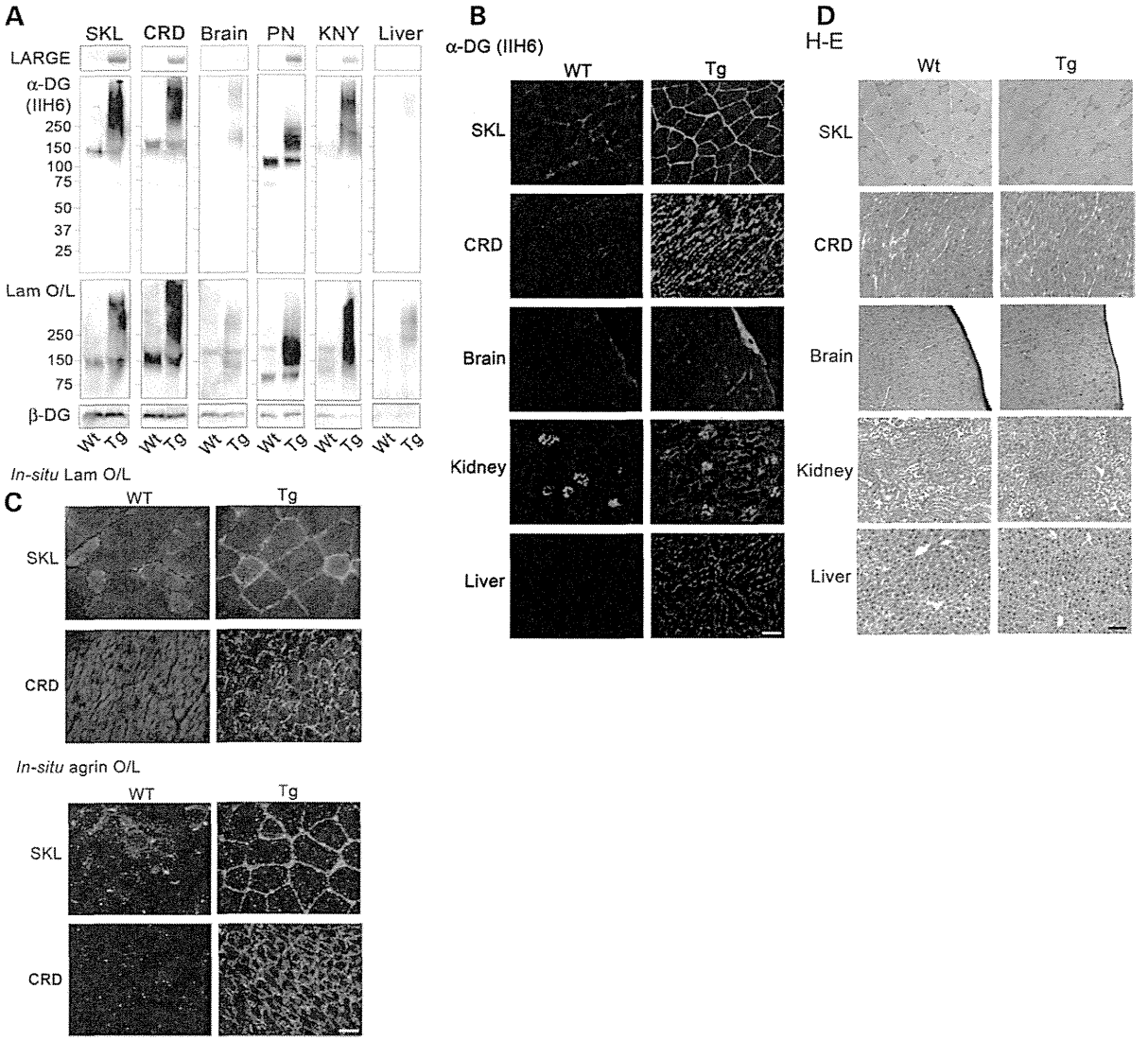

Figure 1. Overexpression of LARGE and hyperglycosylation of α-DG in LARGE Tg mice. (**A**) Western blot analysis showed that LARGE was expressed in greater quantities in all tissues tested including skeletal muscle (SKL), cardiac muscle (CRD), brain, peripheral nerve (PN), kidney (KNY) and liver of LARGE Tg mice (Tg) in comparison with wild type (WT). Immunoreactivity of IIH6, an antibody against the glycan structure of α-DG and laminin-binding activity demonstrated by laminin blot overlay assay (Lam O/L) strongly increased in these tissues. Expression of β-DG was unchanged. (**B**) Immunofluorescent analysis demonstrated that immunoreactivity of IIH6 markedly increased in each tissue of LARGE Tg mice. (**C**) *In situ* laminin and agrin overlay assay showed increased binding activity of skeletal (SKL) and cardiac (CRD) muscle for both laminin and agrin in LARGE Tg mice. (**D**) H-E staining of each tissue of LARGE Tg mice was indistinguishable from control. Scale bar represents 50 μm.

On western blotting, the expression of each laminin chain was not altered, except for the finding that laminin α1 and β1/γ1 chains were up-regulated in the cardiac muscle and liver (Supplementary Material, Fig. S1B). Hematoxylin and eosin (H-E) staining revealed no morphological changes in each tissue observed (Fig. 1D).

Overexpression of LARGE leads to aggravation of muscular dystrophy in dy^{2J} mice

In dy^{2J} mice, a model for MDC1A, a 57-amino acid deletion and a 1-amino acid substitution in the N-terminal domain of the laminin α2 chain lead to the expression of an N-terminally truncated protein (39). Although the mutant protein exhibits defective polymerization and reduced heparin binding (42), it has not been determined whether its binding activity to α-DG is still preserved. Thus, we first conducted a pH 12 extract overlay assay, in which α-DG on blots were overlaid with pH 12 extracts from the skeletal muscle of dy^{2J} mice that contain native laminin-211. When α-DG from control mouse was overlaid with the pH 12 extract from dy^{2J} mice, the laminin-211 bound to α-DG, which was also detected by IIH6 and anti-α-DG core protein, to the same degree as that from the control extract (Fig. 2A, left). The laminin-211 from dy^{2J}