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順天堂醫事雜誌

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(949th issue)

JUNTENDO MEDICAL JOURNAL

順天堂醫事雑誌

The History of *Juntendo Medical Journal*

This *Juntendo Medical Journal* has been published under the Japanese name *Juntendo Igaku* (順天堂医学) from 1964 to 2012. However, the origin of *Juntendo Medical Journal* dates back to the oldest medical journal in Japan, *Juntendo Iji Zasshi* (順天堂醫事雑誌), which had been published between 1875 and 1877 (total of 8 issues). Between 1885 and 1886, Juntendo issued a limited release of a research journal titled *Houkoku* [*Juntendo Iji Kenkyukai*] (報告) for a total of 39 issues.

In 1887, *Juntendo Iji Kenkyukai Houkoku* (順天堂醫事研究會報告) was published with the government's approval and we used to regard this as the first issue of *Juntendo Medical Journal*. Since then, *Juntendo Medical Journal* has undergone a series of name changes: *Juntendo Iji Kenkyukai Zasshi* (順天堂醫事研究会雑誌), *Juntendo Igaku Zasshi* (順天堂医学雑誌), and *Juntendo Igaku* (順天堂医学).

Now in commemoration of the 175th anniversary of Juntendo University, starting with the first volume issued in 2013 (Volume 59 Number 1), we return to *Juntendo Medical Journal*'s original Japanese title in 1875-*Juntendo Iji Zasshi* (順天堂醫事雑誌). We also reconsidered the numbering of the journal and set the first issue in 1875 as the initial publication of *Juntendo Medical Journal*. The Volume-Number counting system and the English name *Juntendo Medical Journal* started in 1955 from the January 10 issue. Although this is not our intension, we will retain the Volume-Number counting system to avoid confusion. However, Volume 59 Number 1 will be the 882nd issue, reflecting the sum of all issues to date: 8 issues of *Juntendo Iji Zasshi* (順天堂醫事雑誌), 39 issues of *Houkoku* [*Juntendo Iji Kenkyukai*] (報告) (47 issues combined), and 834 issues from *Juntendo Iji Kenkyukai Houkoku* (順天堂醫事研究會報告) in 1887 to the present.

出典：小川秀興（OGAWA Hideoki, M.D., Ph.D.）：順天堂醫事雑誌（Juntendo Medical Journal）2013；59：6-10.

本誌は昭和39年（1964年）から平成24年（2012年）末まで『順天堂医学』として刊行されてきた．しかし，その起源は明治8年（1875年）から10年（1877年）にかけて発刊された日本最古の医学誌『順天堂醫事雑誌』（計8巻）にある．さらに明治18年（1885年）から19年（1886年）まで，会員限定配本として順天堂醫事研究會の雑誌『報告』（計39集）が発行されている．

その後『順天堂醫事研究會報告』が明治20年（1887年）に官許を受けて公刊されたので，順天堂ではこれを通刊1号としてきた．以来，『順天堂醫事研究会雑誌』，『順天堂医学雑誌』，『順天堂医学』と名称を変更して刊行されてきた．

今般，順天堂が創立175周年を迎える平成25年（2013年）の59巻1号を期して，本来の名称である『順天堂醫事雑誌』と復刻し，その起源である明治8年（1875年）第1巻をもって創刊号（通刊第1号）とすることとした．従来の巻号と欧文誌名は，昭和30年（1955年）1月10日発行のものを1巻1号としており，欧文誌名もこれより付け始めたもので不本意であるが，混乱を避けるためにこれらを継承する．ただし，通刊数は明治8年（1875年）から19年（1886年）にかけて刊行された『順天堂醫事雑誌』8巻分と順天堂醫事研究會の雑誌『報告』39集，計47巻分を通巻834号に加え，59巻1号を通刊882号とした．

出典：小川鼎三，酒井シヅ：順天堂医学　1980；26：414-418.
小川秀興：順天堂醫事雑誌　2013；59：6-10.

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Call for feature article proposals

To introduce the latest medical findings, Juntendo Medical Journal features a specific focus area for each issue. We would like to request all our readers to address any suggestions or proposals for suitable focus areas to our editorial office.

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From the illustrator: I go to Naritasan Shinsho-ji Temple for New Year's visit every year, and found something interesting at one of souvenir shops lined on the sidewalk leading to the temple. I bought "a fantastic moon swing with two frogs on it" and right away displayed it as a welcome sign in my art class.

Perspectives

Juntendo Medical Journal
2024. 70(2), 104–111

Long-term Prognosis of Pediatric Ocular Disease

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Several problems differentiate the treatment of children, especially those with congenital ocular disease, from adults, including the absence of complaints and the complication of systemic diseases. However, the most challenging is the continuing developing anatomical and functional development and immaturity in children. Consequently, the timing of disease onset and treatment can greatly affect the prognosis, and the prognosis cannot be confirmed without long-term follow-up periods.

The prognosis for unilateral congenital cataract is very poor. However, some cases achieved good vision with successful refractive correction and amblyopia therapy, suggesting that long-term parental enthusiasm and adherence are important for the visual prognosis.

Penetrating keratoplasty is rarely performed in children, and outcomes at our hospital have been extremely poor for congenital corneal opacity over the past 28 years. The visual prognosis is also poor for large limbal dermoids approaching the center of the cornea, which did not respond to preoperative amblyopia therapy. Consequently, early excision, lamellar keratoplasty, wearing of hard contact lenses, and amblyopia therapy were considered necessary.

Treatment of pediatric ocular disease should consider the pros and cons, methods, and timing, especially the development of the pediatric eye and the time of onset of the disease.

Key words: pediatric ocular disease, congenital cataract, congenital corneal opacity, Peter's anomaly, limbal dermoid

Introduction

Pediatric ocular diseases, especially congenital ocular diseases, pose some distinctive problems that do not occur in the adult disease, including the absence of complaints, and complications by systemic abnormalities which hinder examination, diagnosis, and treatment. The biggest challenge is that children are still developing anatomically and functionally, and are immature. Consequently, the timing of disease onset and treatment is critical to the prognosis. Furthermore, the prognosis, including the development of complications, cannot be determined without long-term follow up.

Development of the pediatric eye

The anatomical changes in the eye include the lengthening of the ocular axis from 16 mm at birth to 22 mm at one year and 24 mm in the adult, and nearly tripling of the volume of the eye associated with this increase. Therefore, the length of the ocular axis increases rapidly during the first year of life, after which the anterior part of the eye does not change much, but the posterior part of the eye continues to grow even after the age of 10 years. As the ocular axis elongates, the cornea is rapidly flattened at 6 months[1].

Visual acuity in children is about 0.02 at birth and reaches 1.0 after the age of 3 years[2]. Visual

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acuity only develops during a limited period in childhood. Visual sensitivity is not very high in the first 2 months of life, is high from 3 months to 2 years, and then decreases until about 8 years[3]. However, the prognosis for vision with unilateral congenital cataract is poor even if surgery is performed within a few months of birth, whereas amblyopia therapy is effective even in older children.

Congenital cataract

The prognosis for unilateral congenital cataract is very poor. Surgery is needed within a few months to achieve good vision, but specialist consultation is not always possible at that time[4]. Cataract surgery is a procedure to remove the cloudy lens, so the surgery results in strong hyperopia. Intraocular lenses (IOLs) are inserted in adults, but the power of the IOL is calculated based on the axial length and the corneal curvature, which change significantly during childhood growth, and so correct selection of the lens power is difficult.

Refractive correction can be achieved with eyeglasses or contact lenses in the absence of IOLs. However, such eyeglasses require lenses with very high power resulting in aniseikonia, in which the object seen appears larger than the object seen in the other eye. For this reason, correction with contact lens is necessary. However, these lenses also have special power and are difficult to care for. Furthermore, strict occlusion therapy is needed after correcting hyperopia as treatment for amblyopia. However, occlusion treatment is also quite difficult and the duration of occlusion is less than half of the recommended time[5]. Considering these factors, IOLs are thought to be more advantageous than contact lenses because the hyperopia is always corrected to some extent although selection of the power remains a problem.

A large prospective study by a North American group, the Infant Aphakia Treatment Study group, has largely concluded the prognostic impacts, and advantages and disadvantages of IOL implantation. The prognosis of 114 patients with unilateral congenital cataracts treated surgically before 6 months of age, half with contact lens and half with IOL implants, was prospectively studied. At one year of age, no difference in visual acuity was found between the two groups. However, there were 2.5 times more intraoperative complications, 6 times more additional surgeries, and 3 times more adverse events with IOLs[6].

Contact lenses seem to be the better choice by far, but the absence of any difference in visual acuity despite these various problems suggests that a longer study may detect differences. Comparison of the groups at age 5 years again found no difference in visual acuity. Half of the participants had visual acuities of less than 0.1, and most had strabismus and high frequency of glaucoma[7]. Visual acuity did not differ between the contact lens and IOL groups as expected at age 10 years, when visual development was almost complete. Forty-four percent of patients had visual acuity of 0.1 or less. The final conclusion was that IOL implantation was neither beneficial nor detrimental to visual acuity[8].

Three patients with unilateral congenital cataracts were treated surgically early in life, before 6 months, and followed up for more than 7 years, consistent with the previous study, in Juntendo University Nerima Hospital. The preoperative severity of the cataracts was similar in all three patients, as shown in Figure 1. However, the final visual acuity was 0.07 in Case 1, 0.7 in Case 2, and 1.2 in Case 3 (Table 1).

Case 1 was first diagnosed after 3 months. The mother had earlier noticed leukocoria in her right eye and complained at the one-month checkup, but no close examination was done. The surgery was performed within 10 days of the first visit as an associate emergency. She had a maximum visual acuity of 0.4 at age 3 years. However, the patient could not tolerate wear contact lenses or occlusion well, and she developed strabismus within 2 years, so we abandoned binocular vision and instructed her to wear glasses, but she was not able to do that very well either.

Case 2 could not or did not wear contact lenses, but exotropia appeared at 2 years after surgery, so the patient wore both glasses and contact lenses, and did her best to occlude her healthy eye, which enabled her to achieve visual acuity up to 0.7.

Case 3 was able to continuously wear contact lenses and strictly occlude her healthy eye. It is very rare for a child to achieve 1.2 vision. The reason is that the mother was a nurse who still works at our hospital and had previously worked in the ophthalmology department. Consequently,

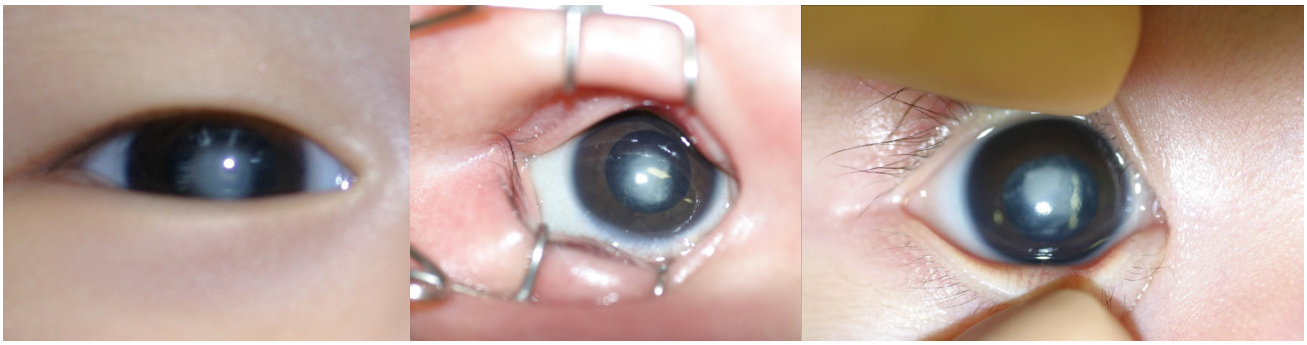

Figure 1 Preoperative photographs of the anterior segment of the eyes showing cataracts with dense central opacities. *Left*: Case 1, *center*: Case 2, *right*: Case 3.

Table 1 Summary of three cases of unilateral congenital cataract

Case No.	Age at first visit (days)	Age at surgery (days)	Compliance of occlusion	Compliance of wearing glasses or contact lenses	Eye position	Final VA (age)	Best VA (age)	Postoperative complication
1	108	116	Not good	Not good	Esotropia	0.07 (10 years 5 months)	0.4 (3 years 1 month)	None
2	40	40	Good	Not good	Exotropia	0.7 (7 years 7 months)	0.7 (5 years 7 months)	Temporal choroidal detachment
3	39	39	Good	Good	Esotropia	1.2 (9 years)	1.2 (6 years 9 months)	Synechia iridis posterior

VA: visual acuity.

her mother understood the importance of refractive correction and occlusion therapy to achieve good vision.

Congenital corneal opacity

Congenital corneal opacity is a rare disease, found in only two to three of every 100,000 live births[9, 10], of which 40% are Peter's anomaly, followed by dermoid and sclerocornea, and includes corneal dystrophy, which is rarely seen, metabolic disorders such as Hurler's syndrome, which is also rarely seen, congenital glaucoma, and forceps delivery trauma.

The main treatment for corneal opacity is keratoplasty. A review of all keratoplasties performed at our hospital over a 28-year period from 1981 to 2008 revealed that only 25 children underwent penetrating keratoplasty, equivalent to about 1% of the adult population. Transparent grafting is reasonably successful for keratoconus and herpetic keratitis, but very poor for congenital conditions such as Peter's anomaly and congenital corneal staphyloma, as shown in Table 2. Furthermore, visual acuity is very poor in patients with Peter's anomaly even if keratoplasty was successful, compared to acquired conditions such as keratoconus or herpes keratitis (Table 3). Poor postoperative results are widely reported with long-term Peter's anomaly[11–13].

Table 2 Penetrating keratoplasty under age 15 years and rates of clear grafts

	Number of eyes	Clear grafts	Rate of clear graft (%)
Peter's anomaly	9	2	22.2
Keratoconus	9	8	88.9
Herpetic keratitis	6	4	66.7
Corneal staphyloma	1	0	0
Total	25	14	56.0

Table 3 Visual acuity with clear graft

	Preoperative	Postoperative
Peter's anomaly	1: ?	0.01
	2: ?	0.01
Keratoconus	0.01–0.1	≥0.8 except one with 0.1 vision
Herpetic keratitis	1: 0.02	0.7
	2: 0.01	0.1
	3: 0.02	0.8
	4: 0.01	0.4

However, good outcomes are known[14, 15], various poor prognostic factors such as vascular invasion, glaucoma, and lens abnormalities have been identified, and new methods of treatment using Descemet's stripping automated endothelial keratoplasty have been reported[16]. In any case, early surgery and thorough low vision treatment are necessary.

I present a case of bilateral Peter's anomaly with early onset glaucoma. The patient was one month old when she was first seen at our hospital, and had already been treated for glaucoma, which is a complication of Peter's anomaly, at another hospital. She had high intraocular pressure after coming to our hospital, so she underwent a trabeculotomy to control the intraocular pressure and penetrating keratoplasty in her left eye at age 6 months and in her right eye at age 1 year 3 months. However, the graft in the left eye became cloudy at 8 months and the right graft became cloudy at 1 month after keratoplasty. Subsequently, she underwent 3 transplants, cataract extraction, 2 trabeculotomies, and 3 trabeculectomies in her left eye, and 2 trabeculotomies and 2 trabeculectomies in her right eye. Figure 2 shows the anterior segment of the eyes at the time of initial examination, after unsuccessful keratoplasty, and at age 22 years. Her visual acuity was finger counting in both eyes at her last visit, which was considered a better outcome than no

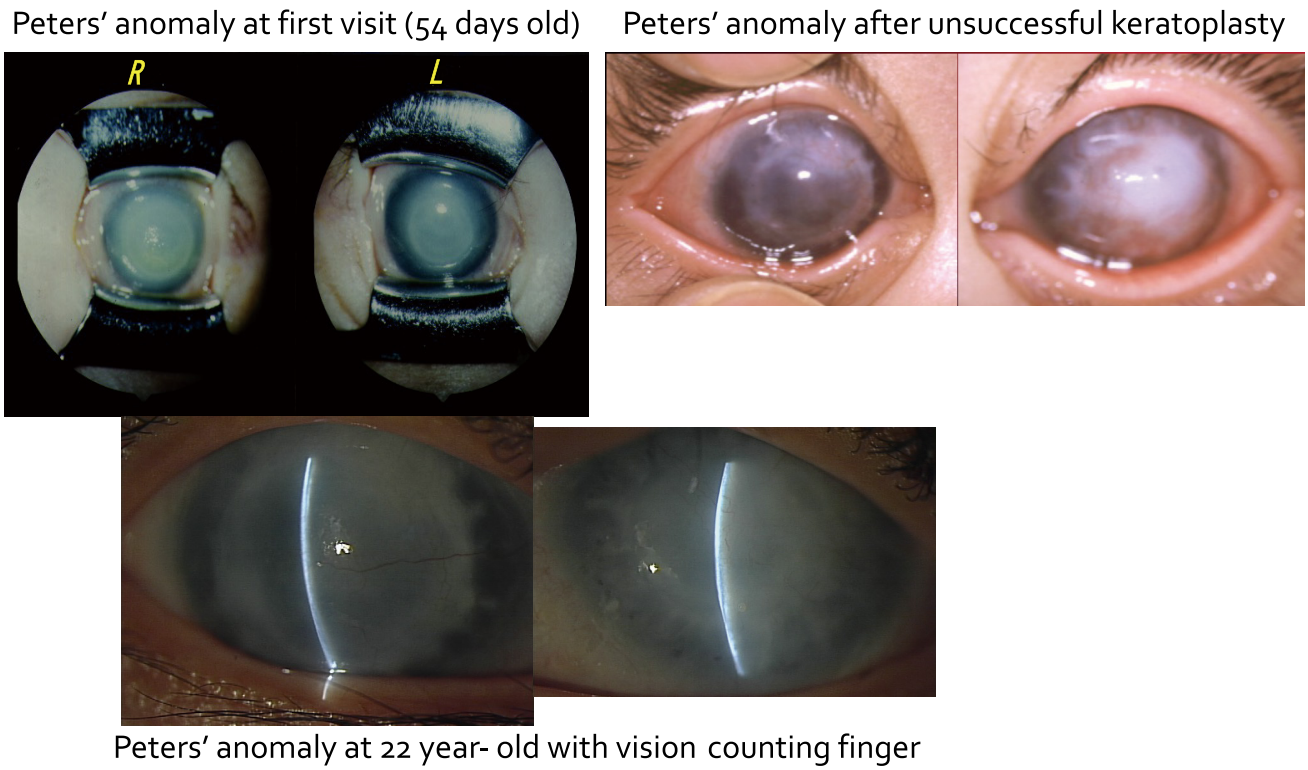

Figure 2 Photographs of the anterior segment of the eyes at the time of initial examination at age 54 days (*upper left*), after unsuccessful keratoplasty (*upper right*), and at age 22 years (*lower*) in a patient with Peter's anomaly with clouded grafts.

surgery although the visual prognosis was poor.

We have experienced 60 cases of pediatric lamellar keratoplasty in 18 years. Most cases were limbal dermoid and the prognosis was reasonable (Table 4). Limbal dermoids are congenital choristomatous lesions consisting of ectodermal and mesodermal elements and appear as yellowish-white, dome-shaped masses, usually at the inferotemporal limbus of the eye[17]. Dermoids can affect the visual acuity by inducing regular or irregular astigmatism and hyperopic anisometropia[18]. However, if the dermoid or associated lipid infiltration encroach on the visual axis, the visual prognosis is very poor[18, 19]. Therefore, we investigated whether the degree of encroachment or encroachment index (EI) of the dermoid tumor can be used as a prognostic factor of visual acuity after lamellar keratoplasty.

The medical records of eight boys and nine girls with limbal dermoids were reviewed (Table 5). Age at surgery, amblyopia therapy, preoperative cylindrical power, tumor size (largest diameter), and visual acuity at the final visit were recorded. The EI of the limbal dermoids was calculated as the ratio of the distance from the estimated (because the limbus is covered by the tumor) limbus to the papillary edge of the tumor divided by the distance from the estimated limbus to the center of the pupil (Figure 3 *upper left*). A value of 1.0 indicated that the tumor was at the center of the pupil, and <1.0 that the tumor had not reached the center. Values >1.0 indicated that the tumor had spread past the center of the pupil. The dermoid tumor was unilat-

Table 4 Lamellar keratoplasty under age 15 years

	Number of eyes	Number of clouded grafts
Limbal dermoid	45	2
Herpetic keratitis	2	0
Corneal ulcer	2	0
Gelatinous drop-like corneal dystrophy	2	2
Others	9	5
Total	60	9

Table 5 Summary of 17 cases

Case No.	Amblyopic therapy	Preoperative cylindrical power (D)	Age at surgery (years)	Size of the tumor (mm)	ECRs	Visual acuity
1	−	0.5	6	7	0.26	1.5
2	+	2.25	8	5.5	0.26	1.2
3	−	1	5	8	0.44	1.2
4	+	1.5	5	6	0.47	1.2
5	−	1.25	12	8.5	0.50	1.2
6	+	4.25	10	8	0.50	1.2
7	+	3.25	9	8.5	0.53	1.2
8	+	4	9	4.5	0.56	1
9	+	6.25	13	8	0.65	1.2
10	+	4	10	8	0.67	1
11	+	7	6	9.5	0.67	0.8
12	+	8.5	7	10	0.70	1
13	+	8	6	10	0.80	0.2
14	+	15.5	4	10	0.81	0.04
15	+	12	3	11	0.94	0.15
16	+	9	1	10	1.06	0.4
17	+	0.25	3	8	1.07	0.01

D: diopters.

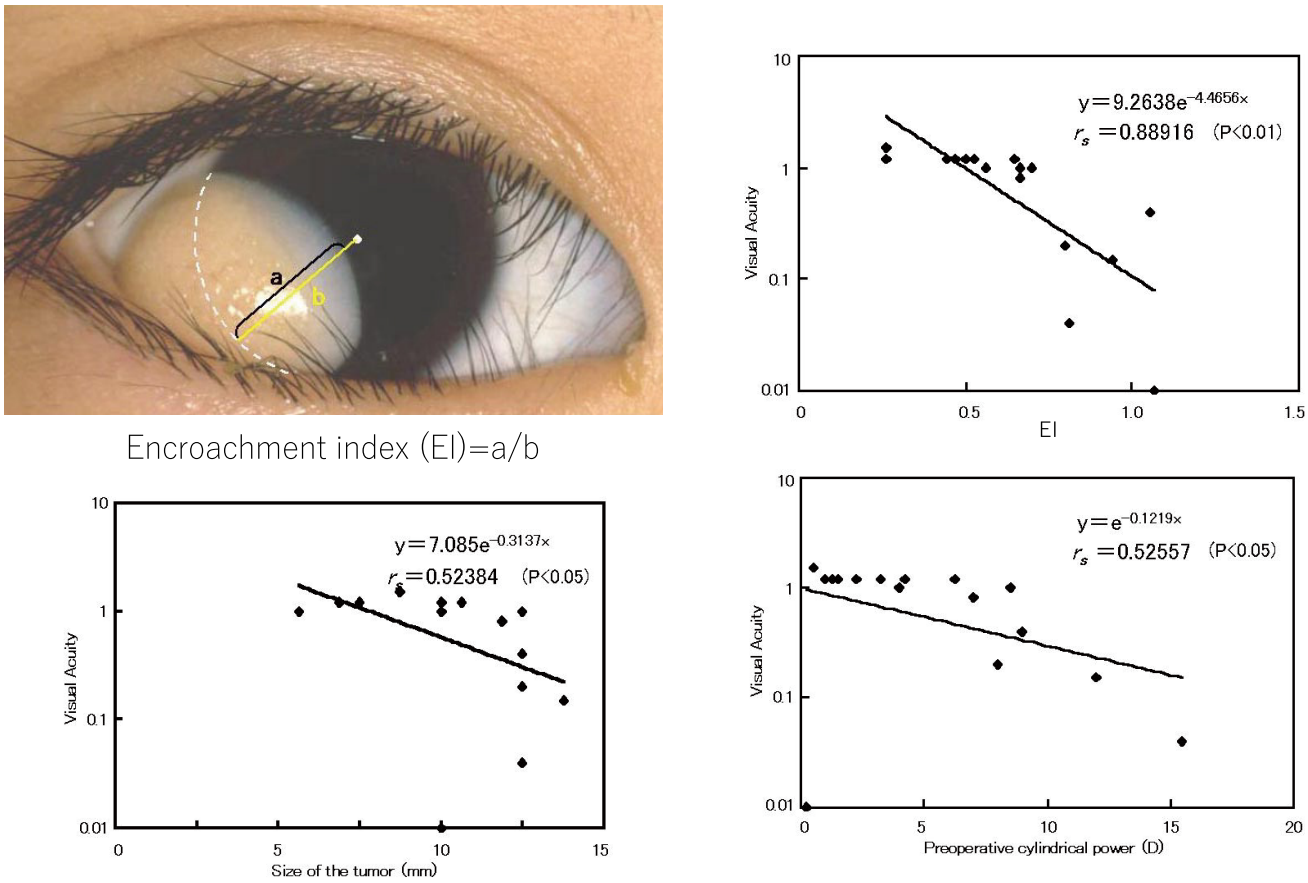

Figure 3 *Upper left*: Photograph of a relatively large limbal dermoid tumor to demonstrate calculation of the encroachment index (EI). The EI is the ratio of the distance from the estimated limbus to the papillary edge of the tumor (a) divided by the distance from the estimated limbus to the center of the pupil (b) along the axis of the tumor. *Upper right*: Correlation between visual acuity and EI. *Lower left*: Correlation between visual acuity and tumor size. *Lower right*: Correlation between visual acuity and preoperative cylindrical power.

eral in all cases, and the follow-up time ranged from 6 months to 244 months. Patients with multiple tumors, other ocular disease or mental retardation were excluded.

Fifteen patients received single lamellar keratoplasty with good cosmetic results. One patient (17) had 3 operations because of graft melting and another patient (15) had two operations because the graft was too small. Six of the 17 patients had auricular appendages and were diagnosed with Goldenhar's syndrome.

Correlation coefficient of the final visual acuity was 0.889 with the EI ($P = 1.87 \times 10^{-6}$; Figure 3 *upper right*), 0.524 with the tumor size ($P = 0.031$; Figure 3 *lower left*), and 0.526 with the preoperative cylindrical power ($P = 0.03$; Figure 3 *lower right*; Spearman's rank correlation coefficient). Five patients had final visual acuity of less than 0.7, and four had had surgery after age 3 years. Tumor larger than 10 mm, cylindrical power >7.0 diopters, and EI >0.8 were risk factors for poor visual acuity. However, Case 12 with a large 10 mm tumor, 8.5 diopters cylindrical power, and EI of 0.7 had good final visual acuity, and Case 17 with a relatively small tumor, low cylindrical power, and EI of 1.07 had poor visual acuity. Most importantly, all patients with EI >0.8 had poor visual acuities. The EI is easy to calculate and the high correlation with the final visual acuity after lamellar keratoplasty indicates that corneal extension is the most important factor for the visual prognosis and EI can used as a prognostic factor for patients indicated for lamellar keratoplasty for limbal dermoid.

These large dermoids would not respond to amblyopia therapy, so we decided to treat as soon as possible and then correct amblyopia with hard contact lenses and occlusion therapy. The representative case shown in Figure 4 had a limbal dermoid extending to the center of the cornea. In general, surgery is performed after amblyopia treat-

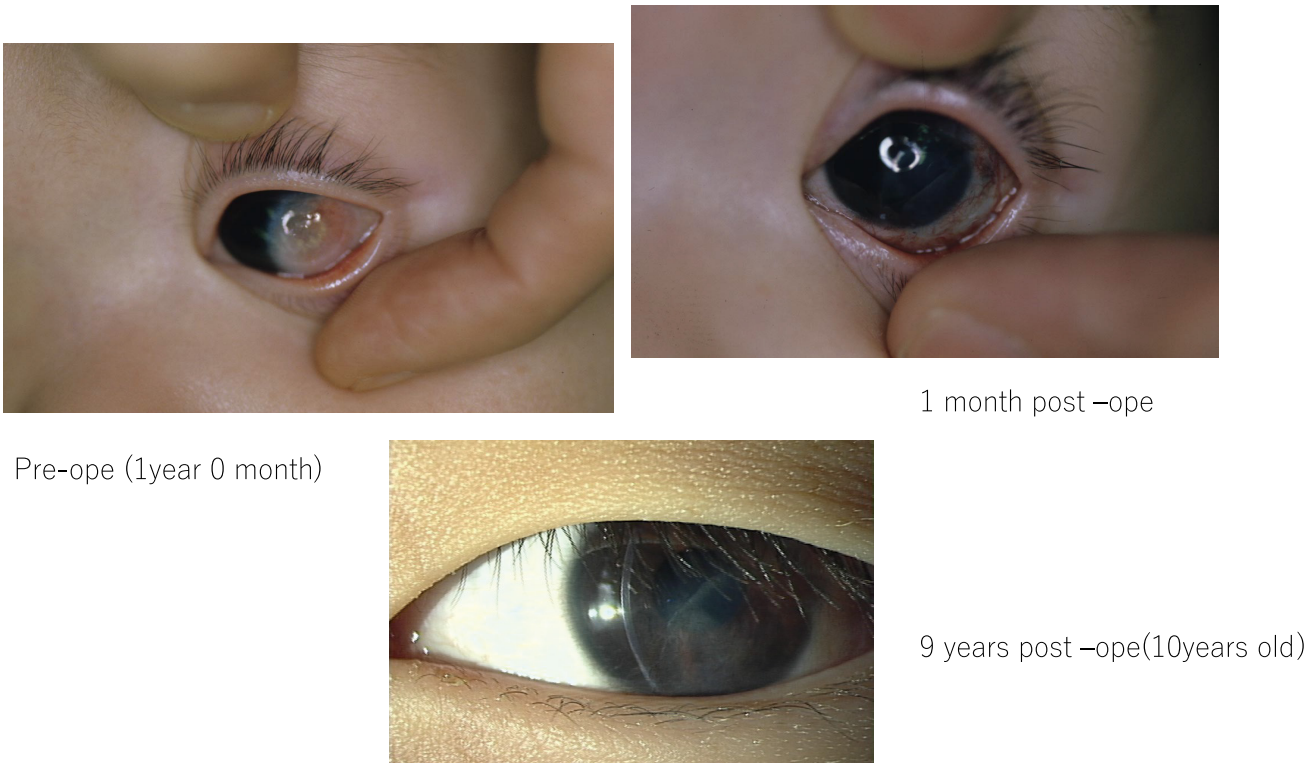

Figure 4 A representative patient with large limbal dermoid treated by early surgery and correction with hard contact lenses and occlusion therapy. *Upper left*: Preoperative photograph (age 1 year 0 month). *Upper right*: Postoperative photograph after 1 month. *Lower*: postoperative photograph after 9 years (age 10 years).

ment, mostly at age 5 years or later, but this patient was treated at age 1 year, and after wearing hard contact lenses with occlusion therapy, she was able to achieve 0.4 vision.

In conclusion, treatment should consider that the child patient is still developing, and the prognosis will not be confirmed until after a longer follow-up period.

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Author contributions

The author read and approved the final manuscript.

Conflicts of interest statement

The author declares that there are no conflicts of interest.

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Abstract

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Identification of IgE Cross-reactive Allergens Causing Food Allergies Using Murine Models

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Key words: food allergy, IgE cross-reactivity, house dust mite, salmon, murine model

Background

Pollen food allergy syndrome (PFAS)/oral allergy syndrome (OAS) is caused by IgE cross-reactive allergens. Generally, PFAS has been diagnosed by taking a dietary history, measuring serum levels of allergen-specific IgE, and performing a skin prick test. However, the molecular mechanisms by which aeroallergens cross-react with food allergens are elusive. In this study, we aimed to develop methods for comprehensive identification of unknown IgE cross-reactive allergens, that may cause food allergies, using murine models.

Methods

Mice were sensitized by intraperitoneal administration of either alum alone as a control or alum plus pollen (e.g., ragweed, birch) or Dermatophagoides pteronyssinus (Der p) extract. Allergenic protein microarray analysis was conducted using mouse serum to identify food extract highly bound to serum IgE from the sensitized mice. IgE cross-reactivity was evaluated by ELISA and murine models of local anaphylaxis. IgE cross-reactive food proteins were identified by mass spectrometry after protein separation. Recombinant proteins of interest were generated for further analysis.

Results

Ragweed pollen showed strong IgE cross-reactivity with fennel and black pepper among edible plants both in vitro and in vivo[1]. IgE cross-reactivity was also observed between coho salmon and Der p. In addition, mass spectrometry analysis identified tropomyosin as the IgE cross-reactive protein contained in coho salmon and Der p extracts in our models[2].

Conclusions

We developed a new screening method using allergenic protein microarray technology and murine model sensitized with environmental allergen. This method will be useful to identify the unknown IgE cross-reactive allergens that may be responsible for food allergies (Figure 1).

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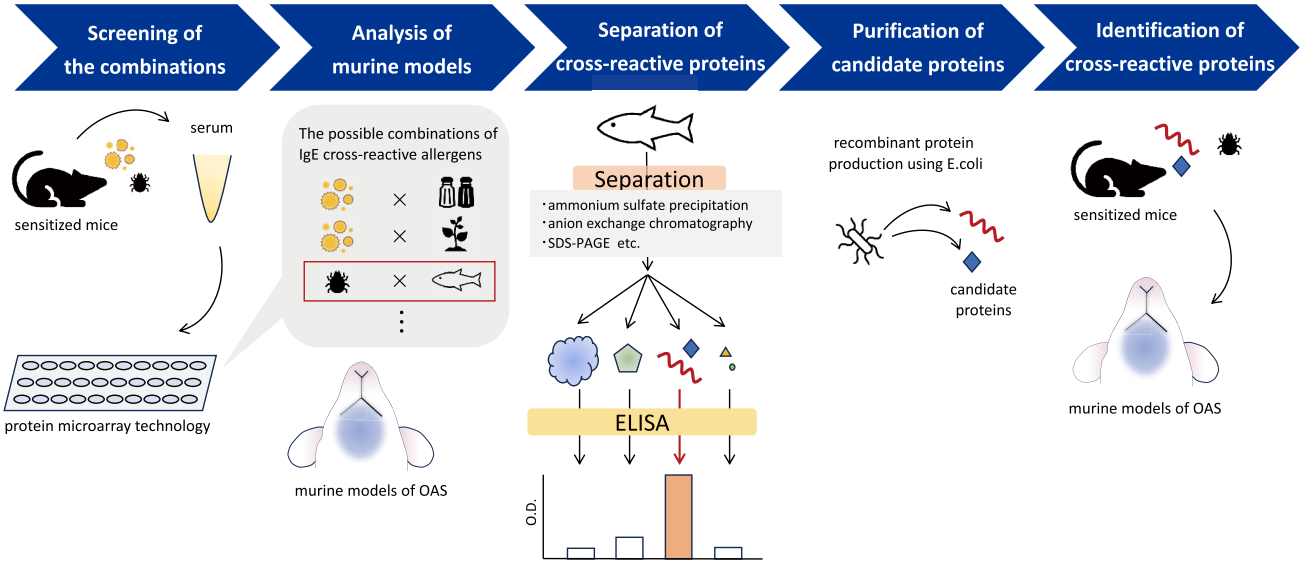

Figure 1 A new method to comprehensively identify IgE cross-reactive allergens

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Author contributions

RY performed all the experiments and participated in writing the manuscript. KI assisted with the analysis of murine model and the in vitro experiments, analyzed the data, and actively participated in manuscript writing. TA assisted with the in vitro experiments and statistical analysis and analyzed the data. AM assisted with the in vitro experiments. AK and NN assisted with the in vivo experiments. HO and KO analyzed the data. JK conceived the project, analyzed the data, and actively participated in manuscript writing. All authors contributed to the article. All authors read and approved the final manuscript.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Perspectives

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The Detection of Neutrophil Activation by Automated Blood Cell Counter in Sepsis

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Neutrophils serve as the frontline defenders in the host's response to infections. However, the available methods for assessing the activated status of neutrophils are still limited. The immature cells that appear during sepsis are large with complex cytoplasmic components and rich nucleic acids, making them diagnosable by cell population data analysis using the automated cell counter. The changes are expressed as increased forward scattered light, side fluorescence light, and side fluorescence distribution width. Additionally, changes in side fluorescence light may indicate the neutrophil extracellular trap formation and can be useful for the diagnosis of sepsis-associated disseminated intravascular coagulation.

Key words: sepsis, neutrophil, cell count, cell death, neutrophil extracellular traps

Introduction

Neutrophil is the most abundant and fast-reacting leukocytes in sepsis. Recent research elucidated the critical roles of neutrophils in the frontline of the host defense against infection. They demonstrate bacteriocidal activity even after cell death. Other than apoptotic cell death, proinflammatory cell death such as necrosis, pyroptosis, and ferroptosis increased during sepsis, and the blood smear findings revealed disrupted nuclear membranes with the dispersion of nuclear contents and the presence of burst neutrophils[1] (Figure 1). In addition, turnover of the cell cycle is increased, and an accelerated turnover is partially detected by the increased cell counts and the presence of immature neutrophils namely, band neutrophils. However, other parameters are not easily assessable.

NE-WY and NE-SFL, markers of bacterial sepsis?

Park et al.[2] reported the usefulness of the specific automated blood cell counter for dividing the neutrophils into subtypes depending on their phenotypes. Popular cell counters (cell analyzer), such as Sysmex XN20® analyzer (Sysmex Corporation, Kobe, Japan) and DxH800® (Beckman Coulter Inc., Miami, FL, USA) are able to analyze the morphological characteristics of cells and provide information about various cell population data (CPD) that reflect the detailed status of neutrophil activation. The neutrophil parameters obtained by representative cell analyzer Sysmex XN-20 were as follows: forward scattered light (NE-FSC), side scattered light

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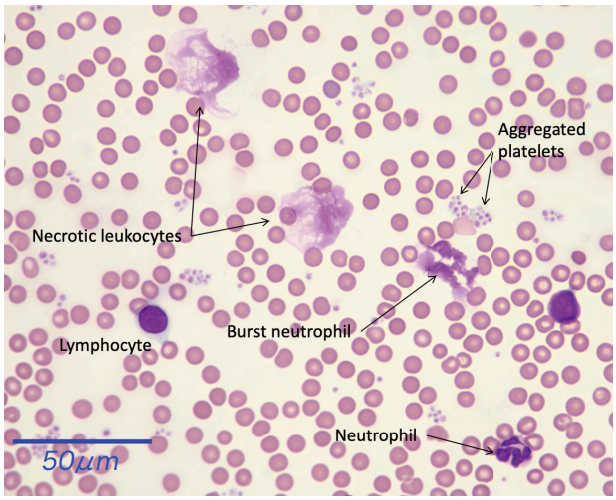

Figure 1 Peripheral blood smear findings in sepsis
Sepsis was induced by *E.coli* injection to the rats. The blood sample was collected, and the blood smear was fixed with methanol and stained with May-Grunwald Giemsa. Some neutrophils were enlarged, and the nuclear contents were expelled outside the cells.

(NE-SSC), side fluorescence light (NE-SFL), and the variances of the above indicators. Those are side scattering light distribution width (NE-WX), side fluorescence distribution width (NE-WY), and forward scattering light distribution width (NE-WZ) (Figure 2). Among them, variations in RNA/DNA contents represented by NE-WY and NE-SFL were reported to be significantly higher in patients with sepsis compared to the healthy controls[2-4]. In a population of patients at the onset of fever, NE-SFL, NE-WY, NE-WZ, and Monocytes-WZ parameters reached the highest AUC scores for predicting sepsis[5]. Furthermore, unsupervised K-means clustering in the sepsis group separated patients with high procalcitonin from the others. Since immature neutrophils are rich in nucleic acids, the increase in immature cells can explain the high levels of NE-WY and NE-SFL. Narumi et al.[6] reported that there were no significant differences in the NE-WY or NE-SFL among patients with sepsis, bacteremia, and focal infection. However, they showed that NE-WY and NE-SFL showed a very high differentiation ability for sepsis, and NE-WY, NE-SFL, and NE-FSC were independent predictors of sepsis[7]. These findings reflected the blood smear findings of increased immature cells, complex internal structure, and specific morphology, such as the presence of toxic granules and vacuolization. Notably, NE-FSC was significantly lower in patients with sepsis in their study. Low NE-FSC indicates the decrease in mean cell size which was ambivalent to the increase of imma-

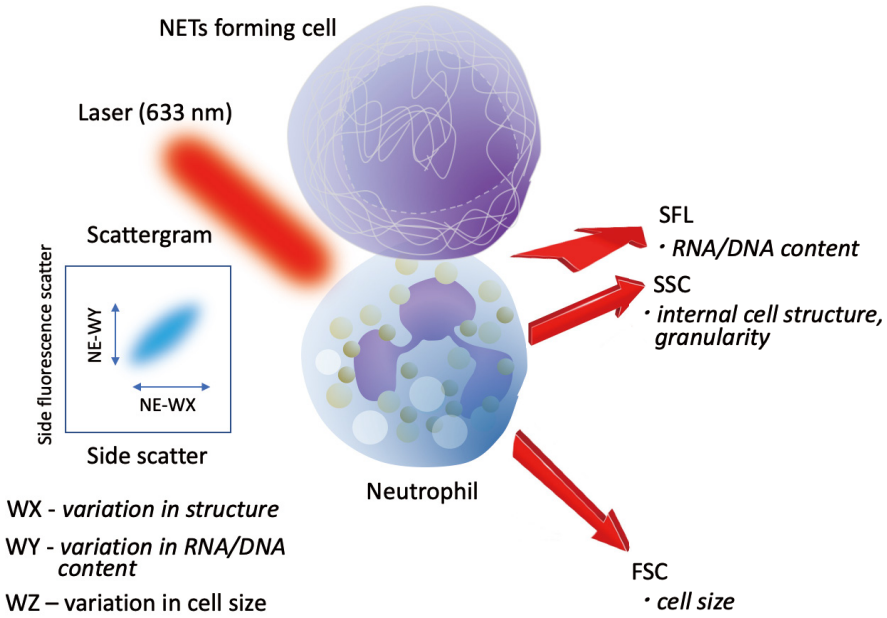

Figure 2 The mechanism of cell population data analysis
Cell population data were obtained with an automated hematological analyzer. The analyzer allows the measurement of neutrophil fluorescence by impedance and by fluorescence flow cytometry at 633 nm. The analysis included the labeling of neutrophils with fluorescent dyes. Signal fluorescence intensity was used to measure the RNA/DNA content.

ture neutrophils. Since apoptotic neutrophils are increased in sepsis, decreased NE-FSC with increased NE-WZ reflect the mixture of apoptotic and immature neutrophils. Although the usefulness of CPD analysis for the diagnosis of sepsis warranted further study, it is noteworthy that the above parameters are easily and quickly calculated from the data of a complete blood count without additional cost.

NE-SFL, a marker of sepsis-induced disseminated intravascular coagulation?

Disseminated intravascular coagulation (DIC) is a critical complication of sepsis, and disease severity is known to increase considerably when patients are complicated by DIC. A multicenter study revealed the prevalence of DIC was 45.7% in sepsis due to acute respiratory distress syndrome, with a mortality rate reaching 40.7%[8]. These data were confirmed in two prospective multicenter cohorts of septic shock patients, in which 43 and 36% of the patients – respectively – developed DIC[9, 10]. DIC was strongly associated with septic shock severity, sequential organ failure assessment (SOFA), and mortality (45.2% in DIC group versus 28.3% in non-DIC, $p<0.001$). Neutrophil activation plays a pivotal role in the development of DIC by upregulating thromboinflammation in the vasculature[11]. NETosis is a type of cell death with releasing neutrophil extracellular traps (NETs) and is involved in the development of immunothrombus and DIC[12].

In 100 septic shock patients – including 35 DIC – Stiel et al.[13] reported that NE-SFL was significantly higher in patients with DIC compared to non-DIC patients: 66.6 (59.3-80.4) versus 50.0 (46.6-56.2) ($p<0.01$). With a cut-off at 57.3 arbitrary units, the area under the ROC curve was 0.882 ($p<0.0001$) for early DIC diagnosis, with a sensibility is 90.91% and a specificity of 80.60%. Interestingly, NE-SFL values were increased *in vitro* in a range identical to that observed in septic patients in ionomycin-induced NETosis in blood samples from healthy subjects, while NE-FSC and NE-SSC were not significantly different.

Delabranche et al.[14] confirmed the potential link between NETosis and DNA decompaction expressed by NE-SFL, by showing that indirect markers of NETosis (nucleosomes and DNA-myeloperoxidase) were significantly increased in DIC patients and that NE-SFL, NETs, and elevated nucleosome concentrations were all correlated to DIC ($p<0.05$).

Finally, Stiel et al.[15] detected circulating NETs forming cells using an immunofluorescent staining technique in the peripheral blood obtained from patients with sepsis and DIC. At the same time, they reported that the chromatin decompaction during the pathway of NETosis could be detected by CPD analysis.

The appearance of NETosis is characterized by the loss of intracellular membranes before the integrity of the plasma membrane is compromised. As a result, decondensed chromatin spreads in the cytoplasm, and the cells expand with the damage to the cellular membrane[16]. Therefore, automated cell analyzers can capture the increased variation in cell size expressed by large NE-WZ and increased cell size expressed by large NE-FSC[2].

In summary, CPD using an automated cell analyzer is a low-cost, routinely available, and rapid measure to evaluate neutrophil activation. Neutrophil activation is represented by the presence of large and chromatin-rich immature neutrophils and reactive neutrophils with toxic granules and vacuolization, which can be detected by CPD analysis. Although it is unknown whether NETosis or other types of cell death can be accurately detected by this measure, the CPD assessment is promising for early detection of sepsis and sepsis-associated DIC.

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JH and TI wrote and reviewed the manuscript. FM and LM revised the manuscript. All authors read and approved the final manuscript.

Conflicts of interest statement

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Perspectives

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The Effect of Antiplatelet Therapy on COVID-19

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Platelets are one of the major targets of SARS-CoV-2. Activated platelets release prothrombotic substances, express adhesion molecules, and activate coagulation, thereby contributing to the thrombotic tendency in COVID-19. However, the antiplatelet therapy is not recommended in the current international guidelines. We think that the initiation timing and the target severity are the causes of the failure in clinical trials. As shown in the clinical studies that examined the effects of anticoagulants, early initiation in moderate severity is necessary for the success of antithrombotic therapy. Future trials are warranted to study the effects of antiplatelets in such conditions.

Key words: COVID-19, platelet, thrombosis, aspirin, P2Y12 inhibitor

The critical roles of platelets in the pathogenesis of coronavirus disease 2019 (COVID-19) have been widely accepted. Activated platelets significantly facilitate prothrombotic effects by releasing microvesicles, platelet factor 4 (PF4), von Willebrand factor (VWF), and other prothrombotic proteins. At the same time, platelets increase the expression of adhesion molecules such as P-selectin and C-type lectin-like receptor 2 (CLEC-2) on the surface[1]. Postmortem histopathological examination has noted microvascular thrombi with megakaryocyte and platelet-fibrin deposition in the damaged organs[2]. Predominant roles of platelets contributing to the development of vaccine-induced immune thrombotic thrombocytopenia (VITT) are also recognized in conjunction with the polyanion from the vaccine component[3] (Figure 1). Moreover, there is speculation about the potential involvement of activated platelets in the pathogenesis of the condition known as 'Long COVID'[4].

The intriguing observation is that while anticoagulation with heparin or low-molecular-weight heparin is the established therapy for COVID-19-associated coagulopathy, the use of additional antiplatelet agents does not reduce the incidence of thrombosis and does not enhance the outcome of COVID-19[5]. As a result, international guidelines for antithrombotic treatment in COVID-19 recommend against the supplementary use of antiplatelets alongside anticoagulant therapy. There have been two important studies that give a hint to solve the question of why antiplatelets could not show a favorable effect. REMAP-CAP is a randomized controlled trial (RCT) that examined the effect of P2Y12 inhibitors[6], and the other is a large-scale cohort study by Chow et al.[7] that evaluated the effect of aspirin. Although Chow's cohort study showed the association between early aspirin use and lower odds of 28-day mortality, the REMAP-CAP failed to show an increase in organ support-free days. The essential differences between these two studies are the disease severity and treatment

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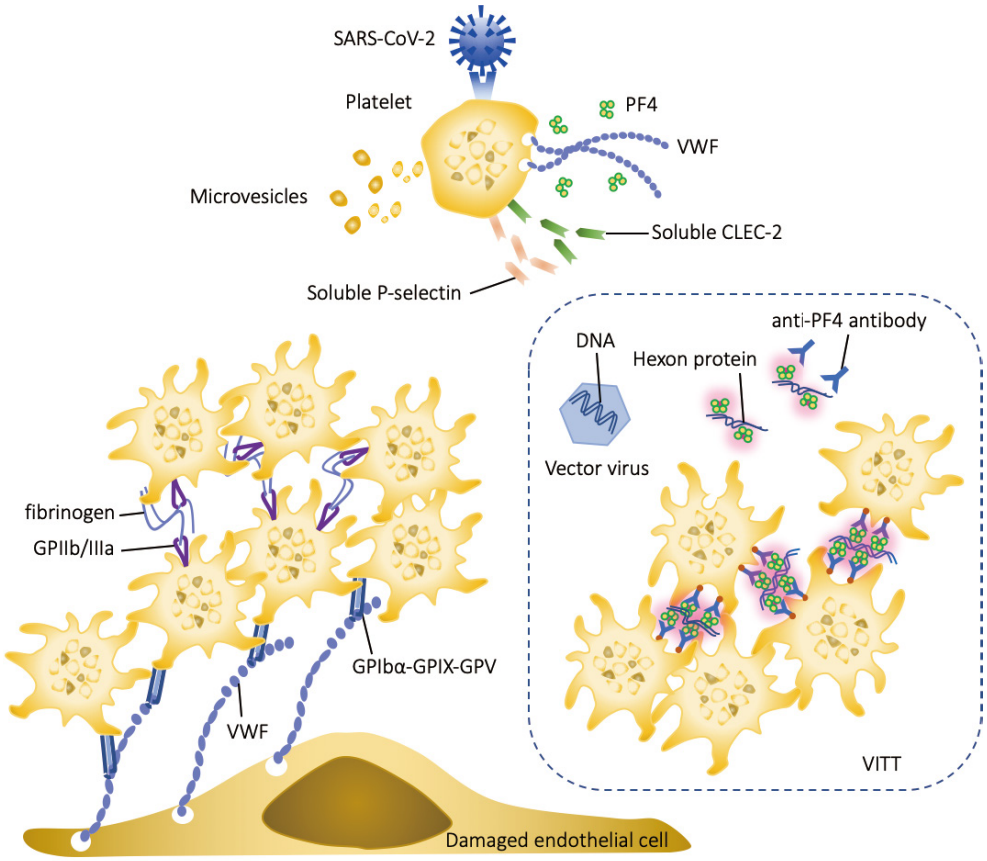

Figure 1 The role of platelets in COVID-19 and vaccine-induced immune thrombotic thrombocytopenia SARS-CoV-2 binds to angiotensin-converting enzyme-2 (ACE2) on platelets and stimulates the release of contents from α-granules, such as von Willebrand factor (VWF) and platelet factor 4 (PF4). Simultaneously, the expression of P-selectin and C-type lectin-like receptor 2 (CLEC-2) is upregulated, leading to increased levels of circulating soluble P-selectin and soluble CLEC-2. Activated platelets aggregate on the vascular endothelium by binding to the VWF released from endothelial cells, forming a thrombus. In vaccine-induced immune thrombotic thrombocytopenia (VITT), positively charged PF4 binds to DNA or other polyanions (hexon-protein) and exhibits antigenicity after a conformational change, stimulating the production of anti-PF4/polyanion antibodies that induce platelet aggregation.

timing. REMAP-CAP evaluated critically ill ICU patients requiring organ support, while Chow studied moderately ill hospitalized patients with aspirin. From a similar perspective, the dose-escalation study of heparin in COVID-19 in the multiplatform RCT reported increased organ support-free days in therapeutic dosing of noncritically ill COVID-19 patients, but not in critically ill patients but rather a risk for increased bleeding[8]. Based on these results, the disease severity and treatment timing are suggested to be vital factors determining the efficacy of antithrombotic therapy. Once thrombosis has occurred in critically ill patients, increased anticoagulation and additional antiplatelet therapy are unlikely to be effective. A previous RCT (RECOVERY) also failed to show a reduction in mortality, further supporting the importance of early timing for antiplatelet therapy[9]. However, another earlier RCT (ACTIV-4B) designed to evaluate aspirin in non-hospitalized outpatient was terminated early due to low event rates and small increases in minor and clinically relevant non-major bleeding in the aspirin arm[10], serving as a reminder of the critical importance of careful patient selection in such studies. The failure of these studies does not deny the critical role of platelets in COVID-19, and it is noteworthy to mention that the proportion of surviving hospital discharge was 71.5% in the antiplatelet group and larger than that in the control group (67.9%) (adjusted odds ratio, 1.27 [95% credible interval, 0.99-1.62]) in REMAP-CAP. Based on these considerations, we believe that additional studies should be considered to determine the proper timing and optimal patient group for anti-

platelet therapy in COVID-19.

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Author contributions

ES and TI wrote and reviewed the manuscript. Both authors read and approved the final manuscript.

Conflicts of interest statement

The authors declare that they have no conflict of interest.

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Perspectives

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Why is DIC a Rare Diagnosis in the 21st Century?

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Disseminated Intravascular Coagulation (DIC) has been a common diagnosis made by health care givers since the dawn of the 20th century. However, currently, this diagnosis is entertained rarely in clinical settings that can predispose to this complication. The incidence of four common clinical scenarios traditionally associated with DIC, sepsis, trauma, obstetrical disorders, and cancers, are on the increase due to better diagnostics and management strategies, but DIC is rarely diagnosed in these disease categories currently. The authors suggest the rarity of a DIC diagnosis is due to varied understanding of the pathophysiology of this condition. In this perspectives, we would like to present reasons for this change in consideration and encourage caregivers to consider a DIC diagnosis at an early stage based on new criteria to help patients benefit from available treatments.

Key words: disseminated intravascular coagulation, sepsis, diagnostic criteria, bleeding

Definition of DIC

Disseminated Intravascular Coagulation (DIC) is defined by the International Society on Thrombosis and Haemostasis (ISTH) as an acquired syndrome characterized by the intravascular activation of coagulation without a specific localization and arising from different causes. It can originate from and cause damage to the microvasculature; if the damage is sufficiently severe, organ dysfunction can result[1]. The different subcomponents of this definition have to be satisfied for the diagnosis of DIC, and it is useful to examine them in detail.

Firstly, DIC is an acquired syndrome that arises from different causes, and as such, the diagnosis should ONLY be entertained in patients with an underlying trigger; and for the same reason, the diagnosis SHOULD be entertained when a predisposing clinical situation exists in a patient who may have clinical and laboratory evidence of dysregulated coagulation activation. Four common causes were described earlier, while other clinical situations with dysregulated coagulation activation can also lead to DIC[2].

Loss of localization and intravascular coagulation activation are the other two crucial components of DIC pathogenesis. The physiological process of clot formation at the site of endothelial injury, which is always limited to the vessel wall, becomes pathological in DIC, wherein thrombus formation is not localized and starts to develop and propagate intravascularly. This "intravascular dissemination" is detected by laboratory tests which can point to uncontrolled thrombin generation and form the

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basis of DIC diagnostic criteria[3].

The third and often overlooked component of the definition is the important role of the microvasculature. Although studied in elaborate detail by several basic science researchers, hemostasis perturbation occurring at the microvascular endothelium is not easily translatable to clinical practice[4]. In this context, the last part of the definition may, however, assist. The pathological process that originates from the microvasculature in DIC can result in organ dysfunction if sufficiently severe. In other words, impairment of organ (or multiorgan) function can be the "clinical" manifestation of DIC. In routine clinical practice, the onset of organ impairment is seen either clinically for acute lung injury or for neurological manifestations such as confusion- as examples or based on laboratory parameters as in the case of abnormal renal function. However, in patients who have a well-known trigger, organ dysfunction may be an early sign of DIC in the absence of clear alternate explanations. Unfortunately, a DIC diagnosis is often not entertained early in a disease course but rather later when there is multisystem thrombosis, a stage where therapeutic interventions are unlikely to be of benefit[5].

Laboratory criteria for DIC

Diagnostic criteria for DIC were developed by the ISTH two decades ago that included platelet count, prothrombin time, plasma fibrinogen, and a fibrinolytic biomarker like D-dimer[1]. The British Society of Haematology guidelines recommended repeating these tests since one set of tests would only provide a snapshot of the pathological process, while serial testing helps in understanding the worsening or improvement of the DIC[6]. One of the prominent issues in the underdiagnosis of DIC is the lack of dependence on these easily available tests in patients with likely triggers for DIC. This is evident in the huge difference in the number of patients diagnosed with DIC in Japan, where reliance on diagnostic criteria like the Japan Ministry of Health and Welfare (JMHW) criteria (developed much before the ISTH) and the Japanese Association for Acute Medicine (JAAM) criteria guide the physicians to diagnose more patients with DIC[7]. A recent cohort study performed in Japan reported the prevalence of DIC was 50.9% in septic patients, and the patients with DIC showed a higher incidence of multiple organ dysfunction (32.0% vs. 13.1%) and worse mortality (24.8% vs. 17.5%)[8]. Several other reasons may be considered for the low rate of DIC diagnosis outside Japan

- DIC is usually a complication of sepsis, trauma, cancer, or obstetrical pathologies. This means a good understanding of the DIC pathophysiology should be present among infectious disease doctors, intensive care physicians, trauma experts, oncologists, and obstetricians.
- Infrequent use of DIC diagnostic criteria by these specialists
- Attribution of abnormal laboratory results to "other" reasons. For example, thrombocytopenia is a very common presentation in critical care units and oncology patients, prolonged prothrombin time may be due to vitamin K deficiency or liver disease, increased fibrinogen and fibrinolytic biomarkers occur in many conditions requiring hospital admission, including the DIC triggers
- Entertaining the diagnosis of DIC only when the patient has multi-system thrombosis or uncontrollable bleeding when therapeutic measures are futile.

An answer to the above conundrum is considering DIC when i) the different laboratory tests are taken in conjunction; rather than in isolation which can overcome the issue of attributing the results to other causes, and ii) repeating the tests after a time interval that shows worsening test results, sometimes along with organ impairment. In this context, the development of the simple diagnostic criteria, i.e., sepsis-induced coagulopathy (SIC) criteria by the authors, has been extremely beneficial[9] (Figure 1). Importance is given to the sequential organ failure assessment (SOFA) score AND the simple laboratory markers, platelet count, and prothrombin time in this multiply validated criteria for patients with sepsis[10]. Another big advantage of the SIC criteria is the ability to use it in low-resource settings since the components are easily performed. Other than sepsis, a pregnancy-specific score for diagnosis of DIC in obstetrics has also been validated in multiple studies[11].

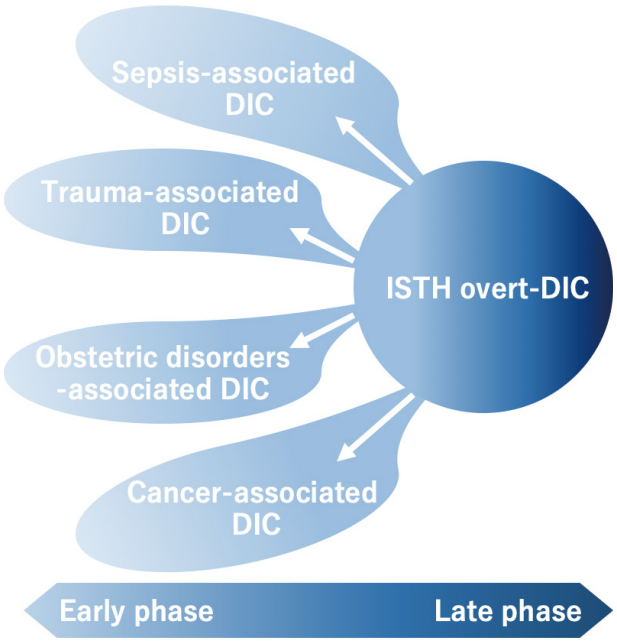

Figure 1 The trend toward the early and specific DIC diagnostic criteria
Sepsis, trauma, obstetrical disorders, and cancers frequently serve as common underlying diseases for disseminated intravascular coagulation (DIC). While the International Society on Thrombosis and Haemostasis (ISTH) DIC criteria have been widely accepted as the standard, there is a growing need for simple and easily applicable DIC criteria that allow for early diagnosis specific to each underlying disease, given the advocated delay in diagnosis.

Looking to the future

So far, endothelium and its biomarkers of injury have been overlooked in the clinical and diagnostic work-up of patients with DIC. The inclusion of the SOFA score in the SIC criteria is a significant step forward in this regard to include disease severity scores. However, despite several studies showing the crucial role of the vascular endothelium in the pathophysiology of DIC, the biomarkers included in these studies have not yet become mainstream and as such, cannot be widely recommended at the current stage for DIC diagnosis without prospective trials and validation studies. What can be done until then is to go back to the basics, i.e., using the ISTH definition and more usage of diagnostic criteria (e.g., SIC) among specialists who deal with the conditions that lead to DIC. Based on this consideration, this will allow earlier diagnosis of more patients with DIC at a stage where therapeutic measures can potentially reverse the uncontrolled intravascular coagulation activation and limit its dissemination.

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Author contributions

JT wrote, and TI, ES, and JHL reviewed and revised the manuscript. All authors read and approved the final manuscript.

Conflicts of interest statement

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Perspectives

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Designing Future Clinical Trials for Sepsis-associated Disseminated Intravascular Coagulation

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Defining success in a clinical trial is not necessarily a straightforward task, especially when the target population is critically ill patients where few agents have demonstrated effectiveness. This has been the case for trials of anticoagulation in patients with sepsis-associated disseminated intravascular coagulation (DIC), which have generally examined patients with severe sepsis but not specifically DIC. Limitations of existing studies include inadequate anticoagulant doses and delayed initiation of treatment. Furthermore, 28-day mortality has been adopted as the primary endpoint but is affected by a panoply of factors other than anticoagulant therapies and may not be the most relevant measure. Future trials must address several current limitations in order to improve our understanding of the role of anticoagulation in patients with sepsis-associated DIC.

Key words: sepsis, disseminated intravascular coagulation, clinical trial, anticoagulants, composite endpoint

Patient screening

Patient screening is an essential component of clinical trial design yet remains a major challenge for clinical trials of sepsis-associated DIC. This may, in large part, reflect the fact that a priori screening for disseminated intravascular coagulation (DIC) in sepsis is not routinely performed outside of Japan. In addition, most diagnostic criteria for DIC consist of multiple laboratory parameters, including platelet count, prothrombin time, fibrin/fibrinogen degradation products (i.e., D-dimers), and fibrinogen, which may not be routinely followed in critically ill patients. The sepsis-induced coagulopathy (SIC) criteria, composed of only platelet count and prothrombin time-international normalized time (PT-INR) as laboratory parameters alongside the sequential organ failure assessment (SOFA) score[1)], offer a more feasible method for patient identification. Notably, the use of the SIC criteria has been shown to capture almost all cases that progress to overt DIC, and its scoring is suitable for screening[2)]. Therefore, we recommend patient screening for clinical trial inclusion using the SIC criteria to ensure appropriate candidates are not overlooked.

Patient selection

Defining the proper patient population is critical for success in clinical trials. Most studies examining the effects of anticoagulant therapies have been conducted in patients with severe sepsis; however, none of them have shown benefit in this population. Nevertheless, anticoagulant therapy has been found to be effective in patients with sepsis-associated DIC[3)], and the effect of anticoagulant therapy has been more prominent in patients with greater disease severity[4)]. These reports examined studies that adopted 28-day mortality as a primary endpoint. When all-cause mortality is the endpoint, it is

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reasonable to think that the study protocol should specify a target mortality. We recently proposed a stepwise classification strategy using a decision tree with variables including baseline SOFA score, antithrombin activity, underlying disease, sex, and age to identify patients with an estimated mortality rate ranging from 20 to 40%[5]. This type of approach is recommended in the design of future clinical trials.

Anticoagulants

Antithrombin concentrate and recombinant thrombomodulin are frequently used in Japan. Nevertheless, the effectiveness of either agent has not been proven in a randomized controlled trial (RCT). Antithrombin is the most abundant and arguably the most important physiological anticoagulant, inhibiting several key enzymes of the coagulation system. Antithrombin activity is significantly decreased in sepsis-associated DIC, both from physiologic consumption and permeability-related extravasation; thus, supplementation is considered to be a rational approach. Surprisingly, and despite suggested benefit in smaller studies, the largest RCT of antithrombin in sepsis-associated DIC failed to demonstrate efficacy[6,7]. Multiple causes were suspected regarding the failure of the RCT, highlighting the need for additional consideration in the development of future trials.

Similar findings have been reported for recombinant thrombomodulin in sepsis-associated DIC. Thrombomodulin is a transmembrane anticoagulant expressed on the surface of the vascular endothelium. Thrombomodulin activates protein C by binding with thrombin, and activated protein C inhibits coagulation factors Va and VIIIa. Although a positive effect of recombinant thrombomodulin was reported in patients with DIC[8], the SCARLET trial, the first RCT that targeted sepsis-associated coagulopathy, failed to demonstrate efficacy[9].

Dose and timing

The dose and timing of anticoagulant administration are anticipated to be critical in mitigating the disease progression of sepsis-associated DIC. Unfortunately, the optimal dose may not yet be determined, as is the case for antithrombin concentrate. We previously examined the relationship between antithrombin dose and patient survival, finding that 3,000 IU/day of antithrombin administration for three days was associated with superior survival compared to 1,500 IU/day for three days[10]. In addition, Akahoshi et al.[11] have examined any relationship between antithrombin activity post-supplementation and patient outcomes, reporting significantly higher survival rates in patients who achieved antithrombin activity ≥ 80%. Since the post-treatment antithrombin activity did not reach this level in many of the patients in the aforementioned RCT of antithrombin supplementation, we hypothesize that a standard dosage in Japan (1,500 IU/day, and lower than the other countries) might have been insufficient to see a benefit, and a higher dose was likely required.

Inadequate timing in the initiation of anticoagulant therapy is another important issue to consider. In the SCARLET trial, 28-day mortality improved by only 2.6% in the intention-to-treat population allocated to recombinant thrombomodulin. However, more than 20% of the patients recovered before treatment initiation, and subgroup analysis in patients who fulfilled entry criteria at baseline revealed a reduction in 28-day all-cause mortality by 5.4%[9]. While it is understandable that obtaining informed consent takes time, especially when patients are critically ill, it is necessary to interpret RCT results in light of relevant limitations.

Endpoint setting

Traditionally the gold standard for endpoint analysis in sepsis trials has been 28-day mortality. Since mortality is influenced by numerous factors beyond the target therapies, a recent sepsis trial investigating the impact of Vitamin C instead used the change in organ failure assessed by SOFA score as the primary outcome. Interestingly, despite no significant differences in that primary endpoint, patients infused with Vitamin C did demonstrate a significant reduction in 28-day all-cause mortality[12]. The use of a composite endpoint as a primary outcome has been applied in recent clinical trials[13]. In this method various events observed in the disease are amalgamated as outcomes. For example, in the case of DIC, in addition to death, improvements in organ function and DIC resolution can be considered for the single study outcome. The composite endpoint offers several advantages, such as improving the ability to detect differences, allowing

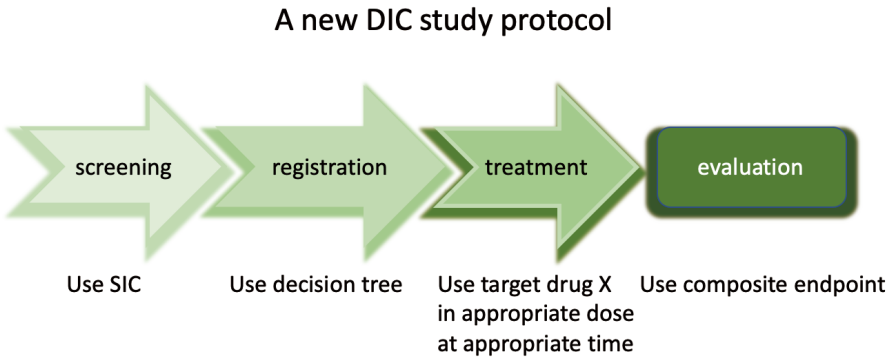

Figure 1 Proposal of a new study protocol examining the effects of anticoagulants for sepsis-associated DIC
It is important to look back at the factors that caused the failure of clinical trials and refine our protocols going forward. Major contributing factors have included the lack of patient screening, absence of mortality estimation, inappropriate use of the target agents, and inadequate evaluation.
DIC: disseminated intravascular coagulation, SIC: sepsis-induced coagulopathy

smaller sample sizes, and shortening study completion times[14]. Moreover, its usefulness has already been demonstrated in trials targeting COVID-19-associated coagulopathy[15].

Conclusion

There are multiple reasons for the failure of clinical trials to define therapeutic efficacy despite mechanistic rationale for why a drug may be beneficial. Was the patient's eligibility screened appropriately? Were the inclusion criteria properly set? Was the target agent properly used? Was the endpoint adequate? Given the nuance related to each of these questions and the fact that any potential benefit from a single agent is likely not as measurable as hoped, it is important to define and refine our clinical trial designs continuously (Figure 1).

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Author contributions

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Perspectives

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Stereotaxic Coordinates of Human Hypothalamic Nuclei Used for Region of Interest Analyses in Functional Magnetic Resonance Imaging

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The hypothalamus maintains homeostasis by controlling various organs and the central nervous system, but analyzing the human hypothalamic nuclei is challenging. Our previous studies applied areal parcellation to high-resolution functional magnetic resonance imaging (fMRI) data to delineate hypothalamic nuclei boundaries. This article presents stereotaxic coordinates of these nuclei for fMRI analyses, offering guidance on defining regions of interest and appropriate spatial smoothing kernel sizes. The provided coordinates aid future research in nuclear level hypothalamus analyses.

Key words: functional magnetic resonance imaging, human, hypothalamus

The hypothalamus can be considered as a functional center of the human body that maintains homeostasis by controlling various organs and the central nervous system. The hypothalamus contains lots of nuclei that play distinct functional roles, so it is essential to analyze these nuclei separately. However, separating these nuclei embedded within a small structure is challenging using noninvasive neuroimaging in humans. To resolve this issue, in our previous studies[1–6], we have applied areal parcellation[7–9] to high-resolution functional magnetic resonance imaging (fMRI) data to delineate the boundaries between these hypothalamic nuclei. Since we have covered most of the hypothalamic nuclei in our studies, it seems now worthwhile to summarize the published data and propose practical tools for fMRI analyses of the nuclei. Specifically, in this article, we present stereotaxic coordinates of these nuclei that can be used to define the regions of interest (ROIs) for the nuclei in spatial resolutions of 1.25 mm and 2 mm. We also propose an appropriate kernel size of spatial smoothing that maximizes the signal to noise ratio with minimal unwanted signal contamination.

Table 1 shows the stereotaxic coordinates of the human hypothalamic nuclei, namely the medial preoptic nucleus (MPO), suprachiasmatic nucleus (SCN), supraoptic nucleus (SO), paraventricular nucleus of the hypothalamus (PVH), anterior nucleus of the hypothalamus (AH), ventromedial nucleus of the hypothalamus (VMH), dorsomedial nucleus of the hypothalamus (DMH), arcuate nucleus (ARC), posterior nucleus of the hypothalamus (PH). The mammillary body is not included here since it is structurally evident. The lateral hypothalamic area (LHA) is not included either as

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Table 1 List of MNI coordinates of the hypothalamic nuclei

Resolution	1.25 mm isotropic			2.0 mm isotropic		
Nucleus	*x*	*y*	*z*	*x*	*y*	*z*
L MPO	−1.5	+2.3	−12.8	−2.0	+2.0	−12.0
R MPO	+1.5	+2.3	−12.8	+2.0	+2.0	−12.0
L SCN	−1.8	+1.5	−15.8	−2.0	+2.0	−16.0
R SCN	+1.8	+1.5	−15.8	+2.0	+2.0	−16.0
L SO	−5.5	+1.8	−12.8	−6.0	+2.0	−12.0
R SO	+4.3	+1.0	−12.5	+4.0	+2.0	−12.0
L PVH	−1.8	−0.5	−10.5	−2.0	±0.0	−10.0
R PVH	+1.5	+0.3	−10.8	+2.0	±0.0	−10.0
L AH	−2.0	−1.5	−18.0	−2.0	−2.0	−18.0
R AH	+1.8	−1.5	−18.0	+2.0	−2.0	−18.0
L VMH	−1.5	−4.1	−14.3	−2.0	−4.0	−14.0
R VMH	+1.3	−4.3	−14.2	+2.0	−4.0	−14.0
L DMH	−1.5	−4.3	−11.2	−2.0	−4.0	−12.0
R DMH	+1.5	−4.5	−10.8	+2.0	−4.0	−10.0
L ARC	−2.3	−4.8	−17.0	−2.0	−4.0	−18.0
R ARC	+2.3	−4.8	−17.0	+2.0	−4.0	−18.0
L PH	−1.5	−8.3	−8.3	−2.0	−8.0	−8.0
R PH	+1.5	−8.3	−8.3	+2.0	−8.0	−8.0

L = left, R = right, MPO = medial preoptic nucleus, SCN = suprachiasmatic nucleus, SO = supraoptic nucleus, PVH = paraventricular nucleus of the hypothalamus, AH = anterior nucleus of the hypothalamus, VMH = ventromedial nucleus of the hypothalamus, DMH = dorsomedial nucleus of the hypothalamus, ARC = arcuate nucleus, PH = posterior nucleus of the hypothalamus.

it is relatively large. Thus, the listed nuclei are located in the medial part of the hypothalamus and can be approximately defined as $X < 3$ or $X > -3$, excluding the middle line ventricular voxels, of the hypothalamus.

In the 1.25 mm resolution, the coordinates are the centroids of multi-voxel ROIs for the hypothalamic nuclei calculated based on the areal parcellation method. Although it is possible to use the coordinates for your ROI analyses, it would be more appropriate to use the multi-voxel ROI. The ROI files can be found at the dryad website (https://doi.org/10.5061/dryad.6q573n620) or please contact us for details. The Gaussian smoothing kernel could be 4 mm in full width at half maximum, in either single-voxel or multi-voxel ROIs (see Discussion in Ogawa et al., 2022[4] for validation). However, the SCN is one exception in the list, and the smoothing kernel should be 2 mm because of its smaller size (see Discussion in Oka et al., 2024[6] for validation). In the 2 mm resolution, as the size of each voxel is four times larger, it is appropriate to use the single voxels listed in the table as ROIs, and the smoothing kernel size should be set the same as previously mentioned at 4 mm in all nuclei except for the SCN (2 mm). When scanning original image data, the image resolution should be at 1.25 mm to make use of the 1.25 mm analysis. However, if scanning at this resolution is not possible or a large database taken in 2 mm resolution is utilized, the 2 mm analysis may be used. We hope this guidance is helpful to future followers of the nuclear level analyses of the hypothalamus.

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Author contributions

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The authors declare that there are no conflicts of

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Original Articles

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An Association Study Between Educational Attainment-related Genes and Cognitive Functions in Japanese Patients with Schizophrenia Based on Full Pleiotropy

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Objectives: This study presents the multifaceted effects of candidate loci identified by genome-wide association studies on parameters such as educational background and the clinical symptoms of Japanese patients with schizophrenia along with detailed psychological measurements. This study aimed to investigate whether gene mutations that affect cognitive dysfunction are (1) related to the onset of schizophrenia and (2) also affect cognitive dysfunction in patients with schizophrenia.
Design: Case-control study.
Methods: This study evaluated 12 single-nucleotide polymorphisms (SNPs) (rs10189857, rs2175263, rs9398171, rs12670234, rs6466056, rs11156875, rs2018916, rs11663602, rs11885093, rs9404453, rs2473938, and rs4275659) that are common in Japanese individuals and demonstrated a relationship with schizophrenia and educational attainment in a previous genome-wide study. We included 640 Japanese patients (schizophrenia group) and 640 healthy participants (control group). Both groups were investigated for the relationship between the SNPs and educational attainment as well as psychometric evaluations of cognitive function.
Results: The 12 SNPs were not identified as genetic risk factors for schizophrenia. However, rs9404453 was associated with a decline in educational achievement, educational performance, Japanese Adult Reading Test (JART100) score, and Wechsler Adult Intelligence Scale-Revised (WAIS-R) (full-scale intelligence quotient [FSIQ]) score in patients with schizophrenia, SNP rs6466056 was associated with a decline in the WAIS-R (FSIQ) score, and SNP rs11663602 was associated with a decline in the JART100 score.
Conclusion: The SNPs rs9404453, rs6466056, and rs11663602 may be associated with academic performance or cognitive decline in patients with schizophrenia, although the overall findings from psychological tests did not show the expected consistency.

Key words: schizophrenia, pleiotropy, educational achievement, JART, WAIS-R

Introduction

Schizophrenia (SCZ) is a typical psychiatric disorder with a prevalence of approximately 1%. SCZ exhibits several genetic features, which often overlap for long periods of time because the disorder presents with positive symptoms, such as hallucinations and delusions, as well as negative symptoms, such as autism, withdrawal, and social dysfunction[1, 2)]. The etiology and pathophysiology of SCZ may include multiple genetic and environmental factors; however, the underlying mechanisms remain unclear. The fact that the incidence of SCZ in identical twins is 50% and that in children born to the

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same parents is tenfold higher strongly suggests the involvement of heredity.

The advancements in genetic analysis technology since 2000 have allowed genome-wide association studies (GWASs) based on gene polymorphisms, and recent GWASs have suggested that certain candidate gene regions are associated with SCZ. Moreover, some neuropsychological tests, including the Wechsler Adult Intelligence Scale-Revised (WAIS-R)[3,4], the Japanese Adult Reading Test (JART; Japanese version of the National Adult Intelligence Test for Estimating Premorbid Intelligence)[5,6], and the Frontal Lobe Cognitive Function Test[7-9], have identified intellectual and cognitive dysfunction in patients with SCZ.

Several reports have evaluated the relationship between SCZ and educational achievement[10,11], and a GWAS of correlation with educational achievement has been performed on a large sample[12]. Leveraging GWASs to identify genetic correlations between complex traits and diseases can help elucidate the pathophysiology of diseases[13]. In this regard, one study demonstrated that three independent single-nucleotide polymorphisms (SNPs; rs9320913, rs11584700, and rs4851266) are important throughout the genome[12]. Similarly, in our previous study, the genetic region of 2q32.3 was suggested to affect educational achievement and cognitive decline in SCZ[14]. Another GWAS identified 10 loci that were shared between SCZ and college level, and 29 loci that were shared between SCZ and years of education[15], while a GWAS meta-analysis revealed new loci and genetic correlations for general cognitive function, providing new insights into the genetics of neurocognitive function[16].

While previous studies have demonstrated that selected loci are associated with cognitive dysfunction and SCZ, we hoped to determine whether similar results could be obtained in Japanese patients with SCZ. Therefore, this study investigated whether such gene mutations that affect cognitive dysfunction are (1) related to the onset of SCZ and (2) also affect cognitive dysfunction in SCZ.

Methods

Participants

This case-control genetic association study included 640 unrelated Japanese patients with SCZ (302 men, 328 women; mean age ± SD = 38.1 ± 11.4 years). The diagnosis of SCZ was confirmed according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders-V (DSM-V) after a structured clinical interview with the patient. Patients with schizoaffective disorders or mood disorders were excluded. In addition, 640 healthy individuals (322 men, 318 women; age, mean ±SD = 43.3± 11.9 years) were recruited from Saitama and Tokyo and included in the study as a control group. Healthy individuals were defined as those who did not meet the current or past criteria for any Axis I disorder (DSM-V). Overall, patients in both groups met the following criteria: (1) absence of systemic or neurological diseases, (2) absence of head trauma complicated by loss of consciousness, and (3) no medical history of dependency on alcohol or other substances. The mean age was significantly lower in the SCZ group than in the control group ($p < 0.001$), whereas the sex distribution was comparable between the two groups ($p = 0.14$). Notably, the number of subsamples that underwent assessments for both SCZ and cognitive functioning was 252 in both cases.

SNP selection and genotyping

Peripheral white blood cells were used to extract genomic DNA with help of the QIAamp® DNA Blood Maxi Kit (Qiagen, Courtaboeuf, France). Twelve SNPs (rs10189857, rs2175263, rs9398171, rs12670234, rs6466056, rs11156875, rs2018916, rs11663602, rs11885093, rs9404453, rs2473938, and rs4275659) that are common in Japanese individuals and had demonstrated relationships with SCZ and educational attainment in a previous GWAS[15] were investigated in the present study. We limited our analysis to the 12 loci whose expression frequency in Japanese individuals was estimated to be more than 20% as per the Thermo Fisher website (https://www.thermofisher.com/jp/ja/home/brands/thermo-scientific.html). We excluded loci with low expression frequencies in the Japanese population because we considered the number of samples in our cohort to be extremely small. The SNP analyses were performed using TaqMan® technology (Assay-by-Design™) on an ABI7500 system (Applied Biosystems, Foster City, CA, USA). However, the probes and primers were developed by the Assay-by-Design™ service (Applied Biosystems). Polymerase chain reaction

(PCR) was performed with a standard PCR MasterMix reagent kit in a 4-μL volume. The SNP analysis results were validated by using a direct DNA sequencing method (TaqMan® method) in a few randomly selected participants to check for errors. The results from direct sequencing were similar to those obtained using the TaqMan® method for all investigated SNPs. The general and common information (e.g., gene name and position) of the selected SNPs is provided in Supplementary table 1.

Clinical and cognitive assessments

Experienced psychiatrists interviewed patients and their family members to evaluate their clinical symptoms. These interviews were conducted at the beginning of the study period and if the patients showed any new acute symptoms during the study period. Psychiatrists also examined the patients using a cognitive test battery. Age at disease onset was defined as the age at the first presentation of any SCZ symptom reported in the DSM-V, and was determined on the basis of interviews with the patients and their family members, in addition to the relevant medical records. Daily antipsychotic doses were converted to chlorpromazine (CP)-equivalent doses[17]. Clinical symptoms were evaluated using the Brief Psychiatric Rating Scale (BPRS), in which each item was rated on a 7-point scale, and the total rating was compared between the groups[18].

Social adjustment and cognitive function assessments were performed after the patients' severe symptoms improved from the time of admission. Comprehensive Assessment of Symptoms and History (CASH)[19] and the Modified Premorbid Adjustment Scale (MPAS)[20] were used to evaluate social status. For the "current occupation" and "previous occupation" items in CASH, we simply used the classifications of "employed" or "unemployed" because this item was difficult to analyze. Cognitive assessments were performed using WAIS-R[21] to assess present intelligence, JART[22] to evaluate premorbid intelligence, and verbal fluency tests[23,24] and the Stroop test[25,26] for assessment of the prefrontal cortex and cognitive functions[27].

Statistical analysis

A two-tailed Student's t-test was used to compare the mean values of continuous variables, while categorical variables were compared using Chi-square (χ^2) test. All statistical analyses were performed using SPSS Statistics software version 21 (IBM, Chicago, IL, USA). For the case-control association study, Hardy-Weinberg equilibrium (HWE) tests for the SNPs were performed using SNPAlyze software version 7.0 Pro (Dynacom, Yokohama, Japan). The HWE tests were performed for all loci in both groups. All statistical significance values were two-tailed and were analyzed using Bonferroni correction (probability level of $p < 0.05/3$ SNPs = 0.0167 in each analysis). Power was calculated using a prevalence rate of <0.01 with an additive or multiplicative model, assuming various degrees of allelic frequencies and odds ratios for the SNPs.

The Kruskal-Wallis test was performed to highlight potential differences in clinical characteristics (three genotyped patient groups for each SNP). Accordingly, a post-hoc analysis was conducted using the two-tailed Mann-Whitney U test. As a method to analyze the relationship between SNP and each parameter, we selected the multiple linear regression analysis, following previous our research[14]. A correlation test was performed to detect the factors related to differences in clinical characteristics among the genotypes based on their correlations with altered clinical characteristics. As a result, only potentially significant factors (independent variables) were included in the multiple linear regression analysis for potentially significantly different clinical characteristics among the genotypes (dependent variables) using genotypes as dummy variables (0/1; e.g., G/G = 1, A/G, and A/A = 0).

Results

Genetic case-control analyses

Twelve SNPs were genotyped in both the study groups, and the completeness of genotyping was between 99.0% and 99.6%. No deviations from the HWE were observed in either group (all $p > 0.05$; Table 1). None of the SNPs showed significant associations between their allelic or genotypic frequencies and SCZ.

Table 1 Distribution and statistical analysis of educational attainment SNPs in Japanese patients with schizophrenia

	Genotype frequency (%)			*p*-value	HWE c/s	Allele frequency (%)		χ^2	*p*-value	Odds ratio (95%CI)
rs2175263	A/A	A/T	T/T			A	T			
Schizophrenia	188 (26.3)	362 (48.5)	196 (26.3)	0.6882	0.942/0.421	738 (49.5)	754 (50.5)	0.3433	0.5579	1.0439 (0.904–1.205)
Controls	174 (26.5)	374 (50.1)	198 (26.5)			722 (48.4)	770 (51.6)			
rs2473938	C/C	C/G	G/G			C	G			
Schizophrenia	62 (8.21)	336 (44.5)	357 (47.3)	0.0958	0.005/0.197	460 (30.5)	1050 (69.5)	3.4864	0.0619	0.8639 (0.741–1.007)
Controls	67 (9.01)	366 (49.3)	310 (41.7)			500 (33.6)	986 (66.4)			
rs4275659	C/C	C/T	T/T			C	T			
Schizophrenia	379 (50.5)	317 (42.2)	55 (7.32)	0.0221	0.069/0.325	1075 (71.6)	427 (28.4)	6.9734	8.27E−03	0.8113 (0.695–0.948)
Controls	328 (43.6)	355 (47.1)	70 (9.30)			1011 (67.1)	495 (32.9)			
rs6466056	C/C	C/T	T/T			C	T			
Schizophrenia	420 (55.6)	292 (38.8)	40 (5.32)	0.8285	0.060/0.281	1132 (75.3)	372 (24.7)	0.0541	0.8161	1.0199 (0.8639–1.2041)
Controls	421 (55.9)	297 (39.4)	35 (4.65)			1139 (75.6)	367 (24.4)			
rs9398171	C/C	C/T	T/T			C	T			
Schizophrenia	45 (6.00)	303 (40.4)	402 (53.6)	0.2037	0.4288/0.257	393 (26.2)	1107 (73.8)	1.1008	0.2941	0.9156 (0.7765–1.0796)
Controls	49 (6.57)	268 (35.9)	429 (57.5)			366 (24.5)	1126 (75.5)			
rs9404453	A/A	A/G	G/G			A	G			
Schizophrenia	313 (42.6)	336 (45.7)	86 (11.7)	0.9192	1/0.807	962 (65.4)	508 (34.6)	0.1213	0.7276	1.0273 (0.8827–1.1956)
Controls	309 (42.0)	335 (45.6)	91 (12.4)			953 (64.8)	517 (35.2)			
rs11156875	A/A	A/G	G/G			A	G			
Schizophrenia	301 (40.7)	332 (44.9)	107 (14.5)	0.4601	0.398/0.343	934 (63.1)	546 (36.9)	1.5964	0.2064	0.9089 (0.7837–1.0541)
Controls	282 (37.8)	344 (46.1)	120 (16.1)			908 (60.9)	584 (39.1)			
rs11663602	A/A	A/C	C/C			A	C			
Schizophrenia	172 (22.3)	369 (49.3)	207 (27.7)	0.5352	0.108/0.770	713 (47.7)	783 (52.3)	0.4309	0.5115	0.9531 (0.8256–1.1002)
Controls	189 (25.3)	351 (47.1)	206 (27.6)			729 (48.9)	763 (51.1)			
rs11885093	C/C	C/T	T/T			C	T			
Schizophrenia	333 (44.3)	340 (45.3)	78 (10.4)	0.0646	0.011/0.564	1006 (67.0)	496 (33.0)	0.3119	0.5765	1.0443 (0.8969–1.2161)
Controls	337 (45.7)	299 (40.6)	101 (13.7)			973 (66.0)	501 (34.0)			

HWE, Hardy–Weinberg equilibrium; CI, confidence interval; c/s, controls/schizophrenia

Genotype effect on clinical characteristics

In the SCZ group, 252/640 patients were admitted to Juntendo Koshigaya (Saitama) or Juntendo Hospital (Tokyo) due to acute symptom exacerbation. The inpatients underwent both clinical and cognitive assessments. However, not all of these 252 patients could be examined using all cognitive assessment tests. For instance, although the BPRS scores could be estimated in all 252 patients, some patients could not be examined using complicated assessments such as the WAIS-R. Furthermore, some clinical information could not be easily evaluated on the basis of the information obtained from patients and their family members. For instance, the evaluation of parental educational achievements in CASH could not be easily performed for patients with deceased parents. Thus, the number of patients included in the analysis of each clinical variable was different, and the detailed case numbers for each test in the battery are shown in Table 2.

Social status

Among the eight subscales of CASH, only "Educational Achievement of Subject" and "Educational Performance" scores showed statistically significant differences correlated with the rs9404453 genotype, with p-values of 0.035 and 0.01, respec-

Table 2 Clinical characteristics and test scores in each genotype from study participants

Variables	Patients	rs6466056			rs9404453			rs11663602		
		C/C	C/T	T/T	A/A	A/G	G/G	A/A	A/C	C/C
	*n = 252	n = 150	n = 93	n = 9	n = 108	n = 109	n = 35	n = 71	n = 119	n = 62
Clinical variables		Mean ± SD (min-max)								
Sex, M/F		77/73	41/52	6/3	51/57	56/53	17/18	40/31	53/66	31/31
Age, mean ± SD, (years)		37.0 ± 12.9 (15-76)	36.5 ± 14.2 (14-76)	30.1 ± 7.6 (19-43)	38.1 ± 13.3 (14-76)	35.5 ± 13.2 (16-76)	35.3 ± 13.2 (17-63)	36.6 ± 11.2 (17-63)	36.3 ± 14.1 (14-76)	37.0 ± 13.9 (15-76)
Onset (years)		25.2 ± 8.8** (14-76)	23.1 ± 8.8** (13-53)	20.0 ± 5.8** (13-30)	24.7 ± 8.6 (13-52)	23.6 ± 8.6 (11-53)	24.9 ± 10.1 (12-54)	23.7 ± 7.2 (13-53)	24.2 ± 9.3 (13-54)	24.9 ± 9.5 (11-53)
Post hoc analysis (Mann-Whitney U tests)		C/C vs T/T; $\chi^2 = -1.748$, $p = 0.081$ C/C vs C/T; $\chi^2 = -2.201$, $p = 0.028$ T/T vs C/T; $\chi^2 = -0.826$, $p = 0.409$								
Duration of disease (years)		12.6 ± 10.1 (0-48)	13.0 ± 10.3 (0-39)	9.6 ± 4.8 (1-19)	14.1 ± 10.2 (0-41)	11.9 ± 10.0 (0-48)	10.5 ± 9.3 (0-35)	14.0 ± 11.1 (1-48)	11.7 ± 9.3 (0-41)	12.9 ± 10.1 (0-41)
DUP (months)		17.7 ± 22.6 (0-96)	14.0 ± 20.4 (0-96)	23.3 ± 33.0 (0-96)	16.8 ± 22.8 (0-96)	15.2 ± 21.2 (0-96)	19.9 ± 24.1 (0-96)	18.7 ± 24.1 (0-96)	14.7 ± 21.4 (0-96)	17.5 ± 21.9 (0-96)
CED (mg/day)		1021.2 ± 544.1 (2-2760)	1057.6 ± 641.9 (150-4400)	766.1 ± 210.3 (350-1065)	1070.7 ± 661.1 (120-4400)	1015.3 ± 491.8 (200-2300)	919.3 ± 533.2 (2.7-1975)	986.3 ± 478.5 (3-2050)	1027.9 ± 628.4 (200-4400)	1066.2 ± 577.6 (150-2460)
Clinical symptoms										
BPRS scores (total)	*n = 252	n = 150	n = 93	n = 9	n = 108	n = 109	n = 35	n = 71	n = 119	n = 62
		35.8 ± 11.3 (0-82)	36.1 ± 12.7 (0-84)	40.4 ± 10.4 (31-63)	35.0 ± 12.7 (0-63)	37.2 ± 10.4 (10-84)	35.7 ± 12.9 (20-82)	35.1 ± 11.3 (0-82)	35.8 ± 11.7 (0-84)	37.6 ± 12.4 (0-63)
Social status										
CASH	*n = 190	n = 116	n = 66	n = 8	n = 79	n = 81	n = 30	n = 51	n = 90	n = 49
Current employed/ unemployed		107/10	62/6	6/2	74/7	71/11	30/0	46/5	89/5	42/8
Previous employed/ unemployed		46/67	29/36	2/5	34/45	33/43	12/18	18/30	41/52	20/26
Educational achievement of subject		12.4 ± 2.3 (9-21)	12.5 ± 2.5 (8-20)	11.6 ± 1.2 (9-13)	**12.7 ± 2.3**** (**8-20**)	**12.0 ± 2.4**** (**9-18**)	**12.7 ± 2.3**** (**9-21**)	12.0 ± 2.3 (9-20)	12.4 ± 2.5 (8-21)	12.9 ± 2.3 (9-18)
		Post hoc analysis (Mann-Whitney U-tests)			A/A vs G/G; $\chi^2 = -0.507$, $p = 0.612$ **A/A vs A/G; $\chi^2 = -2.444$, $p = 0.015$** G/G vs A/G; $\chi^2 = -1.574$, $p = 0.115$					
Educational performance		3.1 ± 0.9 (1-6)	2.8 ± 0.9 (1-5)	3.8 ± 1.7 (2-7)	**3.0 ± 0.8**** (**1-5**)	**3.2 ± 1.0**** (**1-7**)	**2.7 ± 1.0**** (**1-6**)	3.0 ± 1.0 (1-5)	3.0 ± 1.0 (1-7)	3.2 ± 0.8 (1-5)
		Post hoc analysis (Mann-Whitney U-tests)			A/A vs G/G; $\chi^2 = -1.851$, $p = 0.064$ A/A vs A/G; $\chi^2 = -1.627$, $p = 0.104$ **G/G vs A/G; $\chi^2 = -2.879$, $p = 0.004$**					
Educational achievement of parents		11.6 ± 3.7 (2-21)	13.2 ± 3.2 (6-21)	13.0 ± 3.1 (9-16)	12.0 ± 3.8 (2-21)	12.1 ± 3.1 (2-16)	13.4 ± 3.7 (9-21)	12.1 ± 2.9 (6-16)	12.5 ± 3.7 (2-21)	12.0 ± 3.8 (2-18)
		rs6466056			rs9404453			rs11663602		
		C/C	C/T	T/T	A/A	A/G	G/G	A/A	A/C	C/C
Psychometrics										
Frontal lobe function	*n = 113	n = 62	n = 45	n = 6	n = 46	n = 49	n = 18	n = 30	n = 55	n = 28
Verbal fluency test		24.8 ± 11.6 (5-67)	24.7 ± 9.8 (7-49)	24.9 ± 10.1 (9-39)	26.1 ± 12.0 (6-67)	22.9 ± 9.5 (5-49)	27.1 ± 11.2 (9-45)	23.3 ± 12.9 (6-67)	24.9 ± 10.1 (5-54)	26.1 ± 9.9 (8-50)

Stroop test (time)		105.4 ± 51.6 (10–271)	102.5 ± 60.6 (−6–335)	126.5 ± 75.2 (59–231)	108.1 ± 51.8 (15–220)	105.3 ± 66.5 (−6–335)	98.5 ± 35.4 (37–208)	94.6 ± 48.1 (−6–195)	101.9 ± 51.9 (10–271)	123.7 ± 69.3 (24–335)
Intelligence scales	*n = 64	n = 34	n = 25	n = 5	n = 20	n = 31	n = 13	n = 19	n = 31	n = 14
JART 100		87.1 ± 19.1 (55–121)	89.2 ± 18.4 (57–122)	92.6 ± 11.4 (81–107)	**89.6 ± 18.0** (57–114)**	**84.1 ± 17.8** (55–118)**	**97.3 ± 18.5** (68–122)**	**81.1 ± 16.6** (57–114)**	**92.1 ± 18.0** (55–121)**	**88.4 ± 19.3** (55–122)**
Post hoc analysis (Mann–Whitney U-tests)					A/A vs G/G; $\chi^2 = -1.482$, $p = 0.138$			A/A vs C/C; $\chi^2 = -1.601$, $p = 0.109$		
					A/A vs A/G; $\chi^2 = -1.526$, $p = 0.127$			**A/A vs A/C; $\chi^2 = -2.81$, $p = 0.005$**		
					G/G vs A/G; $\chi^2 = -2.656$, $p = 0.008$			C/C vs A/C; $\chi^2 = 0.36$, $p = 0.717$		
WAIS-R (FSIQ)		**71.3 ± 13.6** (48–114)**	**79.2 ± 13.3** (58–110)**	**72.7 ± 23.8** (44–110)**	**79.4 ± 12.7** (60–110)**	**69.8 ± 13.7** (44–110)**	**78.4 ± 17.9** (52–114)**	76.1 ± 14.2 (52–110)	76.3 ± 16.0 (48–114)	68.6 ± 12.3 (44–89)
Post hoc analysis (Mann–Whitney U tests)		C/C vs T/T; $\chi^2 = -0.133$, $p = 0.894$			A/A vs G/G; $\chi^2 = -0.221$, $p = 0.825$					
		C/C vs C/T; $\chi^2 = -2.533$, $p = 0.011$			**A/A vs A/G; $\chi^2 = -2.720$, $p = 0.007$**					
		T/T vs C/T; $\chi^2 = -0.826$, $p = 0.409$			G/G vs A/G; $\chi^2 = -1.654$, $p = 0.098$					
(VIQ)		77.6 ± 13.7 (53–119)	83.9 ± 17.4 (48–121)	78.2 ± 20.0 (55–113)	84.5 ± 16.6 (53–121)	75.8 ± 13.0 (55–113)	83.7 ± 19.4 (48–119)	81.5 ± 14.9 (53–119)	81.9 ± 17.1 (55–121)	74.6 ± 14.1 (48–97)
(PIQ)		70.1 ± 14.6 (49–103)	74.2 ± 16.1 (29–97)	78.8 ± 20.1 (54–103)	77.0 ± 11.8 (61–97)	69.5 ± 14.6 (49–103)	72.0 ± 21.6 (29–103)	75.0 ± 14.3 (54–97)	73.9 ± 15.2 (49–103)	65.4 ± 17.2 (29–97)

BPRS, Brief Psychiatric Rating Scale; CED, chlorpromazine-equivalent dose; DUP, duration of untreated psychosis; JART, Japanese version of the National Adult Reading Test; FSIQ, full score intelligence quotient

*Because some patients were difficult to be examined by complicated assessments, such as WAIS-R, and some correct clinical information was difficult to obtain from patients and their family (e.g.,,, Educational Achievement of Parents of CASH because of the death of parents). Thus, the numbers of patients in each clinical variable were different.

**p-values with statistical significance among the genotypes are presented in bold; then, post hoc analysis was performed between two genotype combinations.

tively. The A/A genotype demonstrated significantly higher "Educational Achievement of Subject" scores than the A/G genotype ($\chi^2 = -2.444$, p = 0.015) (Table 2). Moreover, the A/G genotype also showed a significantly higher "Educational Performance" score than the G/G genotype ($\chi^2 = -2.879$, p = 0.004). However, none of the subscale scores differed significantly in relation to the rs6466056 and rs11663602 genotypes. The MPAS, total BPRS, and Frontal Lobe Cognitive Function test scores were comparable among the genotypes of each of the three SNPs (Table 2).

Psychometrics

The SNP rs6466056 showed a significant difference in relation to the full-scale intelligence quotient (FSIQ) values ($\chi^2 = 6.109$, p = 0.047), and post-hoc tests with Bonferroni correction showed that the FSIQ values of individuals with the C/C genotype were significantly lower than those of individuals with the C/T genotype ($\chi^2 = -2.533$, p = 0.011). The SNP rs9404453 showed a significant difference in relation to the JART score ($\chi^2 = 7.548$, p = 0.023) and FSIQ values ($\chi^2 = 7.826$, p = 0.020). Post-hoc tests showed that the JART score and FSIQ values of individuals with the A/G genotype were significantly lower than those of individuals with the G/G genotype, with p-values of 0.008 and 0.007, respectively. The SNP rs11663602 showed a significant difference in relation to the JART score ($\chi^2 = 7.897$, p = 0.019), and post-hoc tests showed that the JART score of individuals with the A/A genotype was significantly lower than that of individuals with the A/C genotype ($\chi^2 = -2.81$, p = 0.005). Performance IQ (PIQ) and verbal IQ (VIQ) values were comparable among the three genotypes (Table 2).

Multiple linear regression analysis

To identify possible confounders affecting cognitive function, multiple regression analyses were performed with the BPRS score, educational history, and CP-equivalent dose at discharge for each SNP. The multiple regression analysis was performed on the basis of a previous study[14]. Specifically, for rs6466056, the independent variables were age, BPRS score, education history, CP-equivalent dose, and each SNP, and the dependent variable was the total IQ value. For rs9404453, the independent variables were age, BPRS score, education history,

CP-equivalent dose, and each SNP, and the dependent variable was the total IQ value. For rs11663602, the independent variables were age, BPRS score, education history, CP-equivalent dose, and each SNP, and the dependent variable was the JART100 estimated IQ value.

rs6466056 showed a significance probability < 0.05 for the dependent variable total IQ value. The significance probability was highly relevant, being 0.000 for the BPRS score and 0.013 for rs6466056. The standardized coefficients were −0.428 for the BPRS score and 0.287 for rs6466056, with the former having a slightly higher impact. The multiple regression equation was as follows: total IQ = BPRS score × (−0.685) + rs6466056 × 8.689 + 95.445. The R value was 0.541, while the adjusted R^2 value was 0.267, and the goodness-of-fit of the multiple regression equation was low. We hypothesized that these findings indicated an association. In the stepwise method, the CP-equivalent dose at hospital discharge did not vary in relation to the JART100 score or the total IQ value.

rs9404453 also showed a significance probability < 0.05 in relation to the dependent variable total IQ value. The significance probability was highly relevant, being 0.001 for the BPRS score and 0.011 for rs9404453. The standardized coefficients were −0.388 for BPRS and −0.302 for rs9404453, with the former having a slightly greater impact. The multiple regression equation was BPRS × (−0.621) − rs9404453 × 9.041 + 101.013. The R value was 0.545; the adjusted R2 value was 0.272, and the goodness-of-fit of the multiple regression equation was low.

No multicollinearity was observed between rs11663602 and educational history for the dependent variable JART100 estimated IQ, with an R value of 0.435. The significance probability was <0.05. Educational background and rs11663602 were highly relevant, with significance probabilities of 0.000 and 0.005, respectively. The standardized coefficients were 0.341 for educational background and −0.236 for rs11663602, with the former having a slightly higher impact. The multiple regression equation was as follows: JART100 estimated IQ = Education × 2.46 − 9.731 × rs11663602, which was +61.24. However, the goodness-of-fit of the multiple regression equation was low, with an R value of 0.435 and an R2 value of 0.176.

Discussion

In this study, we examined 12 SNPs (rs10189857, rs2175263, rs9398171, rs12670234, rs6466056, rs11156875, rs2018916, rs11663602, rs11885093, rs9404453, rs2473938, and rs4275659) that had been previously associated with educational attainment in a GWAS. We also determined the education-related clinical characteristics of Japanese patients with SCZ.

Our results demonstrate that these SNPs were not risk factors for the development of SCZ in Japanese patients. This finding is consistent with most previous GWASs, since the gene region where these three SNPs are located has not been identified as a genetic risk factor for SCZ.

Three of the 12 SNPs investigated in this study-rs6466056, rs9404453, and rs11663602-showed some degree of difference in the clinical features of SCZ related to education or cognitive functioning. rs6466056 produced significant differences in WAIS-R scores; rs9404453 in years of education, academic achievement, and JART100 and WAIS-R scores; and rs11663602 in JART100 scores. Of these, rs9404453 does not encode a gene, and its clinical significance is unknown. However, rs6466056 and rs11663602 act as introns of *SRPK2* and *KCNG2*, respectively, although their clinical significance is unknown.

SRPK2 is a serine/arginine-rich splicing factor (SRSF, a protein encoded by the *SRSF* gene and an essential factor in pre-mRNA splicing) and functions as a protein kinase involved in neuronal apoptosis[28] and the pathogenesis of Alzheimer's disease[29]. The *KCNG2* gene encodes a subunit of the voltage-gated potassium channel Kv6.2, is involved in neurotransmitter release and neuronal excitability, and has been implicated in opioid dependence[30].

For rs6466056, a significant difference was observed in all IQ test results but no significant difference was observed in JART scores, suggesting an association between disease status and IQ decline. For rs9404453, a significant difference was observed in all IQ test and JART100 scores. rs11663602 was also associated with significant differences in JART100 scores, suggesting that, similar to rs9404453, it is associated with lower pre-disease IQ.

The results of the stepwise multiple regression analysis suggested that the presence of these SNPs was related to IQ and JART scores, independent of CP-equivalent doses and BPRS scores. The WAIS-R and JART100 scores represent the IQ after and before the onset of SCZ[31], respectively, suggesting that the presence or absence of these SNPs affects IQ. Since the CASH contains many categorical variables, its findings were categorized by employment status for convenience. We then examined each SNP, but found no statistically significant differences due to the small number of samples. Moreover, none of the patients with the G/G phenotype for rs9404453 were employed at present.

This study had several limitations. First, patients using drugs other than antipsychotics were excluded. Second, although the WAIS-R was used to measure IQ, the clinical features of SCZ include impairments in verbal comprehension, working memory, perceptual organization, and processing speed. However, these variables are also measured using the WAIS-IV and may be correlated with the PIQ. Currently, we are collecting data on these four variables using the WAIS-IV to investigate their relationships with SNPs in a future study. Finally, we selected 12 candidate SNPs from the largest recent GWAS to show the relationship between these SNPs and both SCZ and educational background. Additional investigations are needed to validate the role of SNPs in cognitive dysfunction in patients with SCZ. The association between SNPs and intelligence in healthy controls was not investigated in this study, and the influence of other factors on the association between SNPs and intelligence cannot be ruled out. In addition, the number of samples for each psychological test was different because all patients who underwent psychological tests were inpatients, and a considerable number of these patients could not complete the tests because of their complexity or the severity of their psychiatric symptoms. In addition, the Bonferroni correction was only applied within each SNP, and no correction for the multiplicity of the 12 SNPs overall was indicated. For the rs9404453 gene polymorphism, the A/A genotype was associated with a higher educational background than the A/G genotype, whereas the A/G genotype was associated with higher educational performance than the G/G genotype. However, both the JART100 and WAIS-R (FSIQ) results were poor for the A/G genotype. Although the results of multiple psychological tests were inconsistent, the Educational Performance values were not prioritized because of the possibility of chance results because of poor accuracy. This is because Educational Performance is a self-reported numerical value by patients and their families and is a relative evaluation score that does not represent absolute academic ability and cannot be said to have high validity.

In conclusion, 12 SNPs (rs10189857, rs2175263, rs9398171, rs12670234, rs6466056, rs11156875, rs2018916, rs11663602, rs11885093, rs9404453, rs2473938, rs4275659) that affect cognitive dysfunction as identified by GWASs were not related to the onset of SCZ. However, three of these SNPs may be associated with a decline in educational performance and cognitive function in patients with SCZ. Although some items showed significant differences in relation to some SNPs, the expected consistency was not obtained when evaluating the psychological tests as a whole.

Ethics approval statement

All participants provided written informed consent before participation. This study was conducted in compliance with the Declaration of Helsinki of the World Medical Association and was approved by the Research Ethics Committee of Juntendo University (2015014).

Data availability statement

The research data are not shared. The raw data used in the present study cannot be made publicly available because disclosure of personal data was not included in the research protocol of the present study. The data are not publicly available due to privacy and ethical restrictions.

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Author contributions

All the authors contributed to the conceptualization, design, and writing of this manuscript. All the authors have read and approved the final version of the manuscript.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Supplementary table 1 General and common explanation about selected SNPs

No.	SNPs	locus	Most severe consequence	apped gene(s)	Minor allele	MAF
1	rs10189857	2p16.1	Intron	BCL11A	A	0.4894
2	rs2175263	Chr.3: 16	Intragenic	*OTOL1*	T	0.44
3	rs9398171	6q21	Intron	FOXO3	C	0.4984
4	rs12670234	7q11.22	Intron	CALN1	A	0.4922
5	rs6466056	Chr.7: 10	Intron	SRPK2	T	0.38
6	rs11156875	14q13.2	Intron	PRORP	G	0.2384
7	rs2018916	16q21	Intergenic	LINC02165	C	0.4884
8	rs11663602	18q23	Intergenic	CTDP1,KCNG2	A	0.4169
9	rs11885093	Chr.2: 57	Intragenic	*VRK2, FANCL*	T	0.32
10	rs9404453	Chr.6: 103	Intragenic	ADGB	G	0.39
11	rs2473938	Chr.6: 113	Intergenic	LOC	G	0.32
12	rs4275659	12q24.31	Intron	ABCB9	C	0.4884

Original Articles

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Maternal Protein Restriction Inhibits Insulin Signaling and Insulin Resistance in the Skeletal Muscle of Young Adult Rats

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Objectives: Infants with fetal growth restriction (FGR) are at a risk of developing metabolic syndromes in adulthood. We hypothesized that skeletal muscle degeneration by nutrition-restricted FGR results in abnormal insulin signaling and epigenetic changes.

Material and Methods: To develop a protein-restricted FGR model, rats were fed a low-protein diet (7% protein) during the gestational period; rats fed a normal diet (20% protein) were used as controls. At 8 and 12 weeks of age, the pups were subjected to oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) to evaluate insulin resistance. At 12 weeks, the mRNA and protein levels of insulin signaling pathway molecules in the skeletal muscles were examined. DNA methylation of promoters was detected. DNA extracted from skeletal muscles was used as a template for methylation-specific PCR analysis of *GLUT4*.

Results: The body weight of FGR rats from birth to 8 weeks was significantly lower than that of the controls; no significant difference was observed between the groups at 12 weeks. In the OGTT and ITT, the incremental area under the curve (iAUC) was significantly higher in FGR rats than in the controls at 12 weeks. The mRNA and protein levels of Akt2 and GLUT4 in the plantar muscles were significantly lower in FGR rats than in the controls. *GLUT4* methylation was comparable between the groups.

Conclusions: Protein-restricted FGR rats showed insulin resistance and altered insulin signaling in skeletal muscles after 12 weeks. However, we could not demonstrate the involvement of DNA methylation in this model.

Key words: fetal growth restriction, insulin resistance, skeletal muscle, insulin signaling, DNA methylation

Introduction

Fetal growth restriction (FGR) is known to increase predisposition to a variety of chronic diseases such as hypertension, cardiovascular diseases, obesity, insulin resistance/type 2 diabetes mellitus (T2DM), and other metabolic syndromes in adulthood[1, 2)]. Fetal growth is dependent on the continuous transfer of nutrients and oxygen from the mother via the placenta. Maternal undernutrition represents a global problem as it is associated with the incidence of chronic diseases in newborns; it also affects the development of newborns[3)]. Barker and Hales first proposed the "thrifty phenotype" hypothesis, according to which fetal undernutrition is strongly associated with numerous chronic conditions later in life[4)].

The skeletal muscle is the primary site of insulin-stimulated glucose uptake, accounting for up to 70% of whole-body glucose disposal[5)], and is a key

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regulator of whole-body energy metabolism[6]. Furthermore, the skeletal muscle is among the tissues that are most sensitive to maternal nutritional restriction[7]. Upon binding to its receptor, insulin facilitates glucose uptake in skeletal muscle mainly through glucose transporters such as glucose transporter isoform 4 (GLUT4). In this process, distinct signaling cascades that include multiple enzymes, such as the phosphoinositide 3-kinase (PI3K)/protein kinase B (also known as Akt) pathway, are involved[8,9]. PI3K binds to insulin receptor substrate (IRS) proteins, resulting in the phosphorylation and activation of Akt, which translocate GLUT4 to the plasma membrane, enabling glucose uptake into the skeletal muscle.

Several FGR rat models have been established to investigate the mechanisms underlying intrauterine events and the eventual adult phenotype[10–13]. The maternal protein-restricted FGR model is one of the most extensively studied models, and the outcomes of offspring bear striking similarities to human diabetes, at both whole body and molecular levels[14]. Recently, it has been proposed that the epigenetic regulation of genes, particularly the methylation of clusters of CpG dinucleotides (islands) in the promoter regions of certain genes, may contribute to metabolic reprogramming[15]. Lillycrop et al.[16] demonstrated that feeding a protein-restricted diet to pregnant rats increased glucocorticoid receptor and peroxisome proliferator-activated receptor α (PPARα) expression in the livers of offspring by inducing the hypomethylation of constitutive promoters. These findings suggest that an epigenetic mechanism induced by prenatal nutrition may generate an altered phenotype in the offspring[17].

We hypothesized that the degeneration of skeletal muscle by FGR results in epigenetic changes and abnormal insulin signaling, which leads to the development of diabetes mellitus without obesity. The study was performed using a rat model of maternal protein-restricted FGR.

Materials and Methods

Animals and experimental designs

Female Sprague-Dawley rats (gestational day 11) were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan) and housed in individual cages in the same room at 24–25°C and 60% relative humidity under a 12-:12-h light-dark cycle with free access to food and water at Juntendo University Animal Care Facility (Tokyo, Japan). Pregnant rats were fed either a diet containing 20% protein (control group) or an isocaloric diet containing 7% protein (FGR group) until delivery. After delivery, each maternal rat was fed a normal diet during the 21-d lactation period. At 21 d of age, all offspring were fed a normal diet. In this study, only the male offspring were used to avoid the effects of sex and hormone differences. The study protocol was approved by the Animal Care Committee of Juntendo University (1455).

The control and FGR groups comprised six offspring each. We measured the body weight of the offspring at birth and at 4, 8, and 12 weeks of age. The oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were performed at 8 and 12 weeks of age, and dissection was performed at 12 weeks of age. Anesthesia was induced with 2%–2.5% isoflurane to reduce pain before dissection. The rat aorta was punctured and the organs were thoroughly perfused with saline to remove red blood cells. Thereafter, the soleus, gastrocnemius, and plantar muscles of the lower limbs were harvested. These three skeletal muscles were immersed in RNAlater liquid (Gene Keeper RNA & DNA stabilization solution; Nippon gene Co., Ltd, Tokyo, Japan) or snapped in liquid nitrogen and stored at −80 °C until further analysis.

Oral glucose tolerance test

Body weight and blood glucose and insulin levels were measured in overnight-fasted rats. After the initial blood collection, glucose solution (2 g/kg) was administered via oral gavage. Blood glucose level was measured at 15, 30, 60, 90, and 120 min after glucose administration using Precision Xceed (cat. no. 71085-80; ABBOTT Japan, Chiba, Japan). Blood insulin level was measured at 30, 60, and 120 min using an Ultra-sensitive Rat Insulin ELISA kit (cat. no. 49170-51; Morinaga Institute of Biological Science, Inc., Kanagawa, Japan). Blood samples for the analyses were collected from the tail veins, and the procedures were performed without sedation. Incremental areas under the curve (iAUCs) for both plasma insulin and glucose levels were calculated.

Insulin tolerance test

Body weight and blood glucose and insulin levels

were measured in overnight-fasted rats. After the initial blood collection, insulin (0.5 IU/kg) was administered via intraperitoneal injection, and blood samples were collected at 0, 30, 60, and 120 min to measure plasma glucose level. The iAUC for the plasma glucose level was then calculated.

Real-time quantitative reverse transcription-polymerase chain reaction

Real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) was performed to assess the expression of insulin signaling pathway molecules (*Akt2*, *PI3K*, *IRS1*, and *GLUT4*) in the skeletal muscles (plantar, soleus, and gastrocnemius) using the TaqMan® system (Applied Biosystems, Woburn, MA, USA) according to the manufacturer's instructions. The skeletal muscles were crushed, and RNA was extracted using the RNeasy® Mini Kit (cat. no. 74104; QIAGEN N.V., Hilden, Germany). The mRNA expression levels of *PI3K*, *Akt2*, *GLUT4*, and *IRS1* were normalized to those of the housekeeping gene *β-actin*. The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta Cq}$ method. Primers and probes for *SiC2a4* (*GLUT4*) (Rn01752377_m1; Applied Biosystems, Foster City, CA, USA), *Akt2* (Rn00690901_m1), *PiK3cg* (Rn00667869_m1), *IRS1* (Rn02132493_s1), and *β-actin* (Rn00667869_m1) were prepared using TaqMan gene expression assays.

Western blotting

The frozen skeletal muscle tissues were crushed using a homogenizer (TissueLyser II; Qiagen, Hilden, NRW Germany). Proteins were extracted from the precipitate using radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl buffer (pH 7.6), 150 mmol/L NaCl, 1% Nonidet® P40, 0.5% sodium deoxycholate, protease inhibitor cocktail, and 0.1% SDS) (cat. no. 08714-04; Nacalai Tesque, Kyoto, Japan), supplemented with phosphatase inhibitor cocktail (cat. no. 07574-61; Nacalai Tesque). Protein concentrations were quantified using the Pierce™ BCA Protein Assay Kit (cat. no. 23225; Thermo Fisher Scientific, Waltham, MA, USA). Polyvinylidene fluoride (PVDF) membrane was blocked with Bullet Blocking One for western blotting (cat. no. 13779-56; Nacalai Tesque) for 5 min, and then incubated overnight at 5°C with the following primary antibodies: rabbit anti-Akt2 monoclonal antibody (1:1000; cat. no. 9272s; Cell Signaling Technology, Danvers, MA, USA), mouse anti GLUT4 monoclonal antibody (1:1000; cat. no. 2213; Cell Signaling Technology), and rabbit anti-GAPDH monoclonal antibody (1:10000; cat. no. 5174S; Cell Signaling Technology). GAPDH was used as the internal reference. The PVDF membrane was washed three times with Tris-buffered saline containing 0.05% Tween-20 (TBST) and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10000; cat. no. 7074; Cell Signaling Technology) or HRP-conjugated goat anti-mouse IgG (1:10000; cat. no. 7076; Cell Signaling Technology) for 1 h at approximately 25°C. Subsequently, the PVDF membrane was washed three times with Tris Buffered Saline with Tween 20 (TBST), and the blots were developed using ImmunoStar LD (cat. no. 296-69901; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and the intensity of the bands was quantified using FUSION software (Vilber Lourmat, Collegien, France).

DNA methylation detection

DNA methylation in the promoters was detected using bisulfate sequencing PCR. Genomic DNA extracted from rat skeletal muscle was used as a template for methylation-specific PCR analysis of the target gene (*GLUT4*). All primers were designed according to previous studies[17, 18].

Statistical analysis

Results are presented as mean ± standard deviation. Differences between the groups were compared using Mann-Whitney *U* test. Pearson's correlation analysis was used to analyze the association between insulin and protein levels and FGR. Statistical significance was set at $p < 0.05$. Kendall rank correlation coefficient was used to analyze the association between insulin signaling and iAUC. All statistical analyses were performed using GraphPad Prism V.7.02 (GraphPad Software, San Diego, CA, USA).

Results

Weight trajectories of the FGR and control rats

The mean birthweight of rats in the FGR group (4.4 ± 0.4 g, n = 6) was lower than that of rats in control group (6.3 ± 0.7 g, n = 6) ($p < 0.05$). The mean body weight of rats in the FGR group was

also significantly lower than that of rats in the control group until 8 weeks of age. However, no significant difference was observed between the groups at 12 weeks of age (Figure 1).

Oral glucose tolerance test results

At 8 weeks of age, the iAUC 0–120 min of the blood glucose level of the FGR group was 9996 ± 3451 mg min^{-1} dL^{-1}, which was higher than that of the control group (6448 ± 1768 mg min^{-1} dL^{-1}) ($p < 0.05$). However, the iAUC of insulin level was not significantly different between the groups (68.51 ± 49.85 mg min^{-1} dL^{-1} for the FGR group and 42.14 ± 43.42 mg min^{-1} dL^{-1} for the control group; $p = 0.166$) (Figure 2). The iAUC 0–120 min of the blood glucose level at 12 weeks of the FGR group was 7786 ± 3511 mg min^{-1} dL^{-1}, which was higher than that of the control group (5034 ± 1689 mg min^{-1} dL^{-1}) ($p < 0.05$). Similarly, the iAUC of insulin level was significantly different between the groups (110.9 ± 47.1 mg min^{-1} dL^{-1} in the FGR group and 47.93 ± 69.89 mg min^{-1} dL^{-1} in the control group; $p < 0.05$) (Figure 2).

Insulin tolerance test results

At 8 weeks of age, the iAUC 0–120 min of the blood glucose level was −2146 ± 599 (mg min^{-1} dL^{-1}) for the FGR group and −2070 ± 2108 (mg min^{-1} dL^{-1}) for the control group; there was no significant difference between the groups. The iAUC 0–120 min of the blood glucose level of the FGR group at 12 weeks of age (−2530 ± 807 mg min^{-1} dL^{-1}) was significantly lower than that of the control group (−3421 ± 1216 mg min^{-1} dL^{-1}; $p < 0.05$) (Figure 3).

Insulin signaling in the skeletal muscles

The mRNA expression of *PI3K* in the soleus muscle and that of *Akt2* and *GLUT4* in the plantar muscles were lower in the FGR group ($p < 0.05$) than in the control. There were no significant differences in the expression levels of genes encoding other insulin signaling pathway molecules (Figure 4).

Western blot analysis of insulin signaling molecules in the skeletal muscles

The protein levels of Akt2 and GLUT4 in the plantar muscles were lower in the FGR group ($p < 0.05$) than in the control. There were no significant differences in the protein levels of other insulin signaling pathway molecules (Figure 5).

Association between *Akt2* and *GLUT4* expression levels and iAUC at 12 weeks of age

There was a negative correlation between the iAUC of ITT results and *Akt2* mRNA expression ($r = -0.61$, $p < 0.01$) in the plantar muscle at 12 weeks of age. Although not significant, there was a tendency toward a negative correlation between the iAUC of ITT results and *Glut4* mRNA ($r = -0.39$, $p = 0.07$), Akt2 protein ($r = -0.39$, $p = 0.07$), and GLUT4 protein ($r = -0.41$, $p = 0.06$) expression levels in the plantar muscle at 12 weeks of age (Table 1).

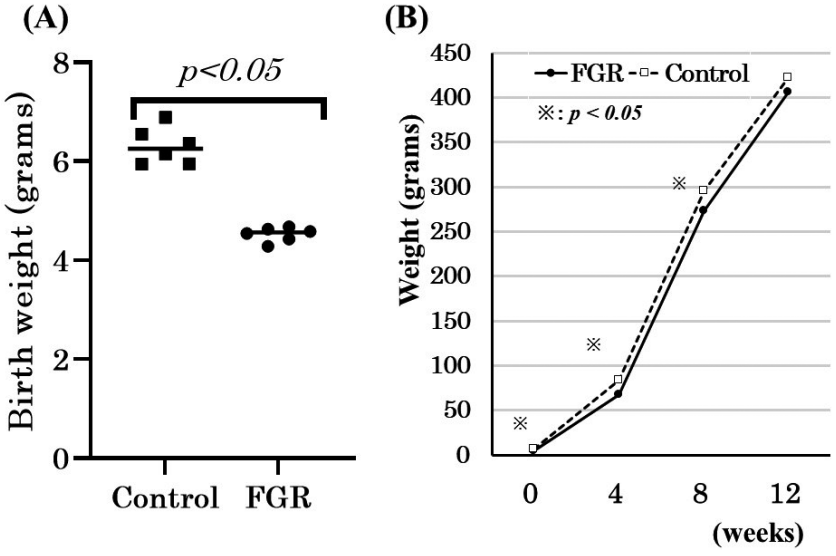

Figure 1 Weights of fetal growth restriction (FGR) and control rats from birth (A) to 12 weeks of age (B). Data are shown as mean ± SD. ※ $p < 0.05$ vs. control rats.

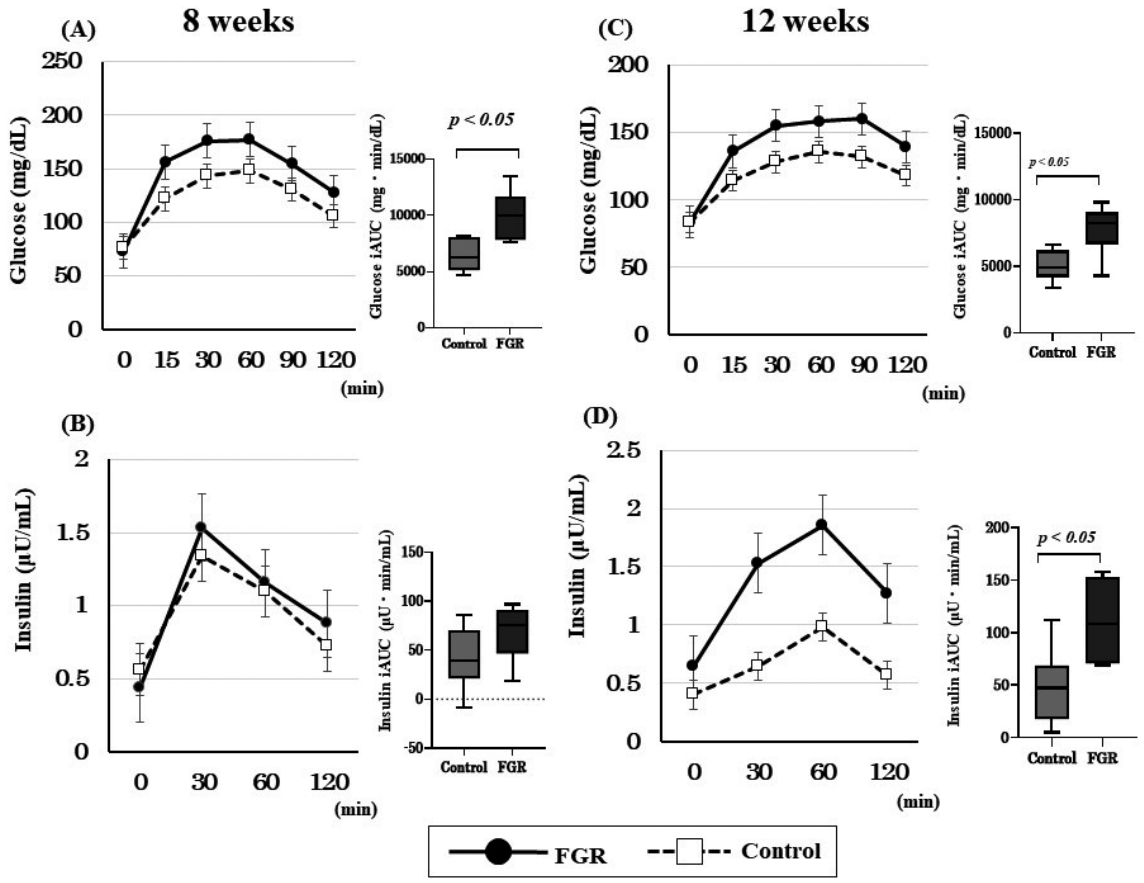

Figure 2 Results of oral glucose tolerance test (OGTT) at 8 and 12 weeks of age
(A) Glucose level and glucose incremental area under the curve (iAUC) at 8 weeks. (B) Insulin level and insulin iAUC at 8 weeks. (C) Glucose level and glucose iAUC at 12 weeks. (D) Insulin level and insulin iAUC at 12 weeks.

DNA methylation detection

We examined the DNA methylation rate of four CpG sites (56560017, 56560030, 56560221, and 56560284) in *GLUT4* between the FGR and control groups (Figure 6A). There were no significant differences in the methylation rate between the groups (Figure 6B).

Discussion

In this study, we investigated skeletal muscle insulin resistance in a rat model of maternal protein restriction during pregnancy. T2DM has been attributed to lifestyle and genetics; however, recent studies have indicated that a poor fetal environment is often associated with the development of glucose intolerance and insulin resistance later in life[19]. Furthermore, excessive catch-up and obesity in FGR are associated with insulin resistance[20, 21]. In this study, rats in the FGR group weighed less at birth than the controls; however, at 12 weeks of age, there was no significant difference between the groups. Moreover, the iAUCs of both blood glucose and insulin levels were significantly higher in the FGR group than in the control group at 12 weeks of age. Moreover, in the ITT, the iAUC of blood glucose level at 12 weeks of age was significantly higher in the FGR group than in the control group. Thus, protein-restricted FGR rats showed impaired blood glucose level-lowering ability in young adults without obesity.

In this study, we aimed to elucidate the mechanism of insulin resistance by analyzing the soleus and plantar muscles separately. Mammalian skeletal muscles are heterogeneous tissues composed of different fiber types identified by the expression of specific myosin heavy chain (MHC) isoforms. Muscle fibers can be classified into three distinct categories, namely, types I (slow twitch, oxidative), IIa (fast twitch, oxidative, glycolytic), and IIb (fast twitch, glycolytic). The action of insulin on glucose uptake and metabolism occurs in a muscle fiber-specific manner, with a greater response of insulin-

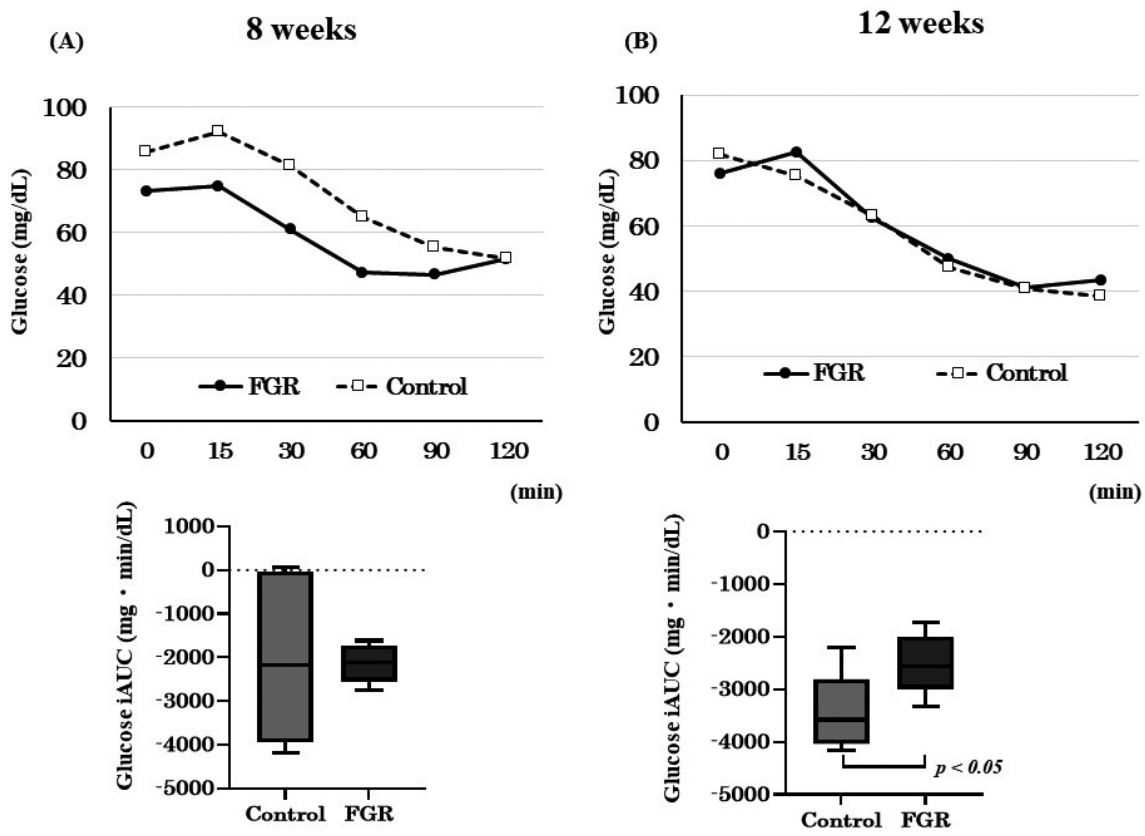

Figure 3 Results of insulin tolerance test (ITT) at 8 and 12 weeks of age
(A) Glucose level and glucose incremental area under the curve (iAUC) at 8 weeks. (B) Glucose level and glucose iAUC at 12 weeks.

stimulated glucose uptake observed in type I fibers than in type IIa or IIb fibers[22]. The soleus muscle is mainly composed of type I fibers, plantar muscle is composed of type IIb fibers, and gastrocnemius muscle has different fibers in different areas[23,24]. In skeletal muscles, the decrease in protein synthesis due to fasting is greater in type IIb fibers than in type I and IIA fibers[25]. Thus, in our study on a maternal protein-restricted diet model, the differences in the mRNA and protein levels of insulin signaling molecules can be seen more in the plantar muscle than in the soleus muscle and gastrocnemius muscle. Insulin-stimulated glucose transport is greater in skeletal muscle enriched in type I fibers[26], and this could be related to the higher GLUT4 level[27,28]. To the best of our knowledge, this study represents the first report to analyze the insulin-signaling molecules in the soleus and plantar muscles separately. Many other studies have analyzed the molecules in lower limb skeletal muscles of rat models without separating the muscles[17,18,29].

Reduced GLUT4 expression in skeletal muscles has been repeatedly observed in different experimental models of diabetes[30-33], similar to that in humans with insulin resistance and T2DM[34-36]. Insulin resistance in the muscle tissue is associated with reduced levels of GLUT4[37]. Some studies using FGR rats with maternal malnutrition have reported a decrease in GLUT4 levels in the skeletal muscles[11,37]. One study using a pig model also reported that offspring born to nutrient-restricted mothers showed reduced GLUT4 expression[38]. Our results suggest that the reduced expression of GLUT4 in the plantar muscle may play an important role in skeletal muscle insulin resistance in young adults.

In rats, insulin signaling via Akt is reduced in offspring of dams exposed to a hypoxic or malnourished environment during pregnancy[39]. Akt2 has been identified as the Akt isoform that is crucial for insulin-stimulated glucose uptake[40,41]. Xing et al.[29] reported that the reduction in GLUT4 expression is possibly mediated by decreased PI3K and phosphorylated Akt levels in maternal protein-restricted FGR models.

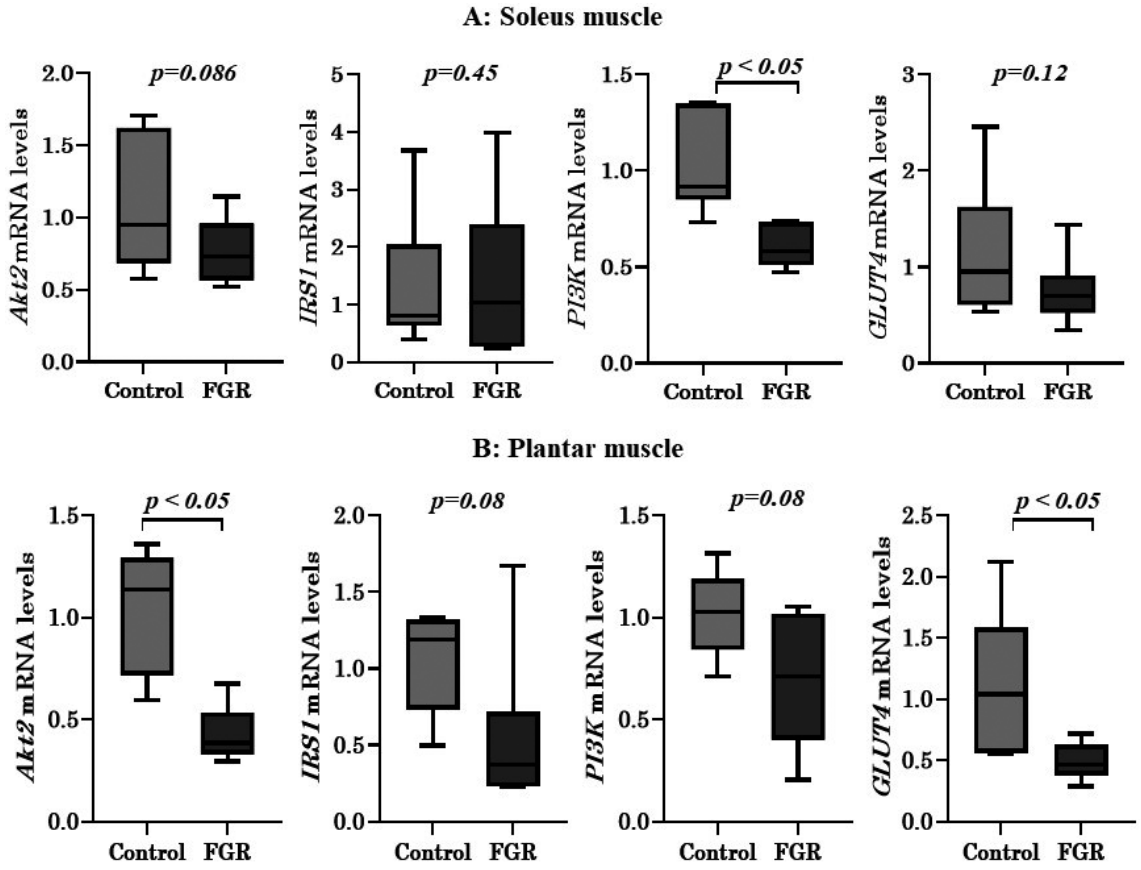

Figure 4 Results of real-time polymerase chain reaction analysis of protein kinase B (*Akt2*), phosphoinositide 3-kinases (*PIK3*), insulin receptor substrate 1 (*IRS1*), and glucose transporter type 4 (*GLUT4*) mRNA in the (A) soleus muscle and (B) plantar muscle of fetal growth restriction (FGR) and control rats. ※ $p < 0.05$ vs. control rats.

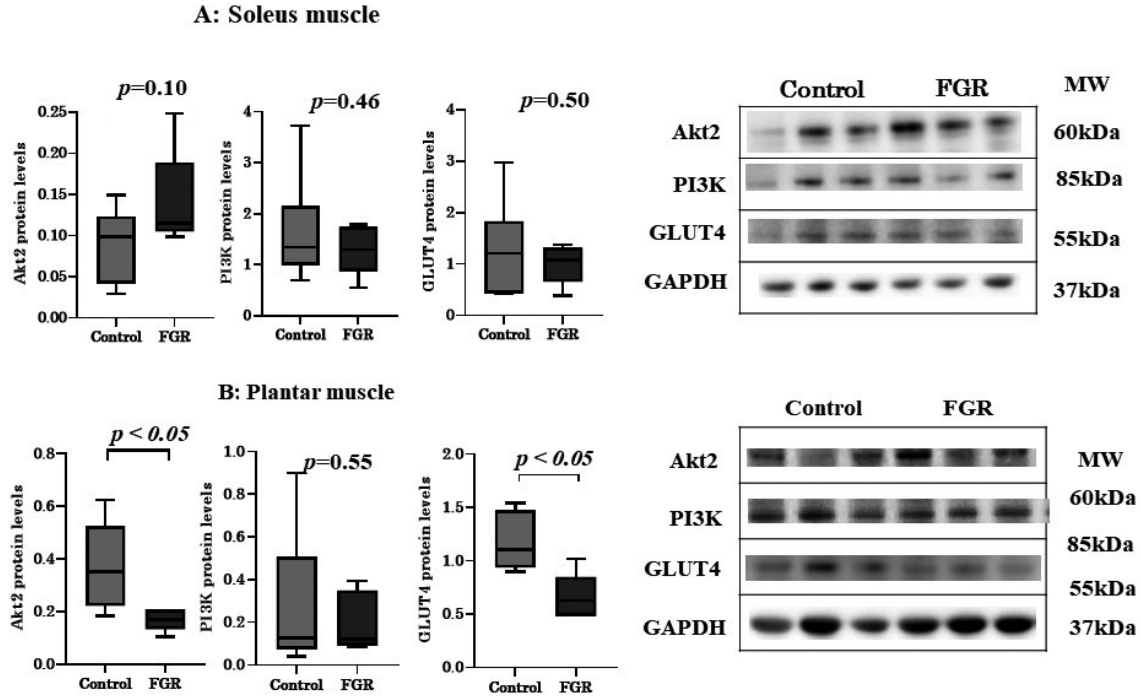

Figure 5 Results of western blot analysis of protein kinase B (Akt2), phosphoinositide 3-kinases (PIK3), and glucose transporter type 4 (GLUT4) in the (A) soleus muscle and (B) plantar muscle of fetal growth restriction (FGR) rats and control rats. ※ $p < 0.05$ vs. control rats.

Table 1 Association between Akt2 and GLUT4 expression and the incremental area under the curve (iAUC) in the plantar muscle at 12 weeks of age.

			mRNA expression		Protein expression	
			Akt2	*Glut4*	Akt2	GLUT4
iAUC	OGTT	r	−0.33	−0.12	−0.36	−0.17
		p	0.13	0.58	0.10	0.45
	ITT	r	−0.61	−0.39	−0.39	−0.41
		p	< 0.01	0.07	0.07	0.06

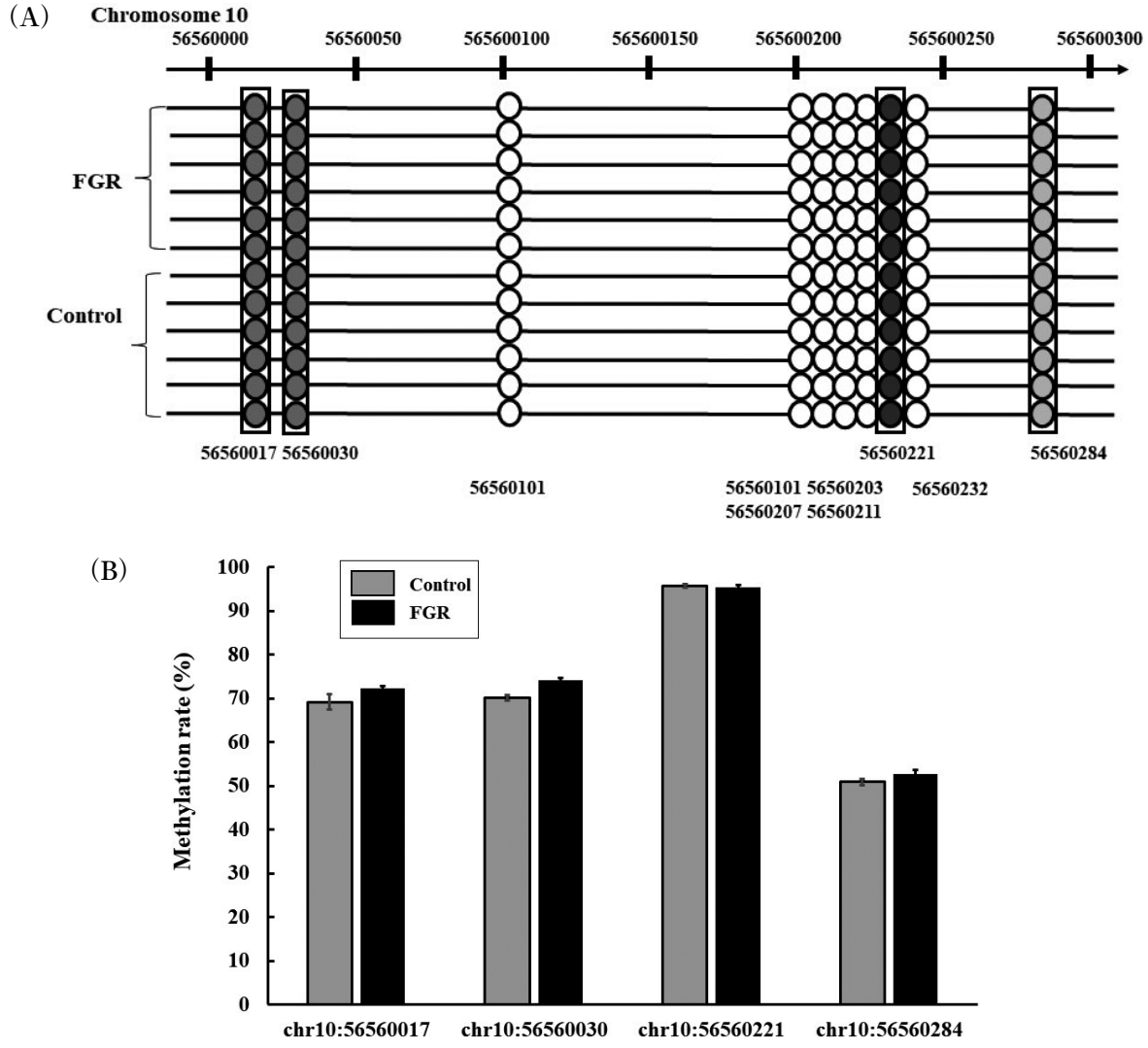

Figure 6 DNA methylation profiles in the promoter region of CpG sites of *GLUT4* in fetal growth restriction (FGR) and control rats (A). The methylation rate of *GLUT4* was not significantly different between the groups (B).

Several studies have reported DNA methylation due to nutritional abnormalities[25]. In this study, the mRNA levels of insulin signaling pathway molecules in the skeletal muscles were significantly lower in the FGR group than in the control group. However, *GLUT4* methylation was not significantly different between the groups. In another study, the insulin-like growth factor 2 gene was differentially methylated in regions upstream of the entire gene and was found to modify downstream gene transcription[42]. Another study reported that histone code modifications repress skeletal muscle GLUT4 transcription in the postnatal period, and that these changes persist in adult female FGR offspring[43].

Thus, we speculate that the reduced mRNA expression of *GLUT4* in the skeletal muscle of FGR rats may be related to causes other than methylation[17].

Our study has some limitations. We could not show the causal relationship between insulin resistance and altered insulin signaling in skeletal muscles in our model. Although an evaluation of the activation/phosphorylation levels of Akt and PI3K might shed further light on the potential mechanism underlying insulin resistance, we could not evaluate the activation/phosphorylation levels of these proteins in this study; we attempted these experiments, but the results were not informative. Furthermore, we could not examine the alteration of skeletal muscle fiber types in this model. We could not analyze DNA methylation of *Akt2* and *PI3K* in the skeletal muscles. It was not possible to observe the influence of insulin resistance after 12 weeks of age.

In conclusion, protein-restricted FGR model rats showed insulin resistance in the skeletal muscles at 12 weeks of age without obesity. This indicates that abnormal insulin signaling in the skeletal muscles may cause insulin resistance. However, we were unable to demonstrate the involvement of DNA methylation in this model.

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Author contributions

Research conception and design: KA and HS; experiments: KA, YA, SI, and KT; statistical analysis of the data: YM; interpretation of the data: SI and TS; writing of the manuscript: KA and HS

All authors read and approved the final manuscript.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Publication List

Juntendo Medical Journal
2024. 70(2), 152–173

Publications from Juntendo University Graduate School of Medicine, 2021 [6/6]

General Medicine

〈Original Articles〉

1) Naito T, Suzuki M, Fukushima S, Yuda M, Fukui N, Tsukamoto S, Fujibayashi K, Goto-hirano K, Kuwatsuru R: Comorbidities and co-medications among 28 089 people living with HIV: A nationwide cohort study from 2009 to 2019 in Japan. HIV Medicine, 2021.
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A dagger (†) denotes doctoral works by non-Japanese students.

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〈Reviews〉

1) Harada T, Miyagami T, Watari T, Kawahigashi T, Harada Y, Shikino K, Shimizu T: Barriers to diagnostic error reduction in Japan. Diagnosis (Berl), 2021.

Cell therapy & Transfusion Medicine

〈Original Articles〉

* 1) Ishii M, Ando J, Yamazaki S, Toyota T, Ohara K, Furukawa Y, Suehara Y, Nakanishi M, Nakashima K, Ohshima K, Nakauchi H, Ando M: iPSC-derived neoantigen-specific cytotoxic T-lymphocyte therapy for Ewing sarcoma. Cancer Immunology Research, 2021; 9: 1175-1186.
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(Updated: April 11, 2024)

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Abbreviations

Each abbreviation should be defined in parentheses together with its non-abbreviated term when it first appears in the text (except in the Title and Abstract).

Units

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Abstract

The second (and, if necessary, the third) page of the manuscript should contain only the abstract (maximum 250 words). The abstract must be fully comprehensible without reference to the text. Abstracts should be divided into sections as follows:

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2. Materials (or "Design")
3. Methods (or "Interventions")
4. Results
5. Conclusions

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Authors who used AI technology to conduct the study should describe its use in the methods section in sufficient detail to enable replication of the approach, including the tool used, version, and prompts where applicable.

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This section includes the results of the experiments. The Results and Discussion sections may be combined if this helps readers to understand and evaluate the study. Tables and figures, including photographs, can be used to present the experimental results (see below). Excessive explanations of the data presented in tables and figures should be avoided.

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The Conclusion or Discussion should be concise and should deal with the interpretation of the results. Novel models or hypotheses may be proposed in this section only if they are suggested by the results obtained in the experiments. Do not repeat the description of the experimental results in this section.

Acknowledgments

Contributors who do not meet the criteria for authorship should be listed in the Acknowledgements section. Examples of those who might be acknowledged include a person who provided purely technical help, or a department chair who provided only general support. If you do not have anyone to acknowledge, please write "Not applicable" in this section.

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Example citation list entries:

Journal article

1) You WC, Blot WJ, Li JY, et al: Precancerous gastric lesions in a population at high risk of stomach cancer. Cancer Res, 1993; 53: 1317- 1321.

Book

2) Matsumoto A, Arai Y: Hypothalamus. In: Matsumoto A, Ishii S, eds. Atlas of Endocrine Organs. Berlin: Springer-Verlag, 1992: 25-38.

Tables

Tables with suitable titles and numbered with Arabic numerals should be placed at the end of the text on separate sheets (one table per page). They should be understandable without referring to the text. Column headings should be kept as brief as possible, with units for numerical information included in parentheses. Footnotes should be labeled a), b), c), etc. and typed on the same page as the table they refer to.

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Figures should also be submitted online as separate files. They should be numbered in order of appearance with Arabic numerals (e. g. Fig. 1, Fig. 2). Author(s) must pay printing costs for color photographs. Electron micrographs should contain a scale. Individual figures may not exceed the size of a Journal page. Graphs or drawings containing typewritten characters are unacceptable. Numbers, letters and symbols must be large enough to be legible after reduction. In principle, figures should be suitable for publication, and jpg digital files preferred. Each figure must have an accompanying legend, which should be understandable without reference to the text. All figure legends are to be double spaced, and should be collected together as text page(s), rather than being attached to their respective figures.

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編 集 後 記

近年、包摂性、という言葉を多く使うようになりました。インクルージョンと言った方がその意味について理解しやすいかもしれません。包摂性はもともと、ヨーロッパにおける階級社会で生じていた社会的排除に対するカウンターメジャーとして概念化されたものですが、その後、貧困、障がい者、孤立・孤独、ひとり親、LGBTQ、など様々な社会的排除を是正する、多様性を理解し認め合う、といった考え方として広まりました。

私は、包摂性の低下が引き起こす問題の１つとして、日本人女性の痩せの問題があると考えています。日本では、痩せた若い女性が多いことが知られており、20 代の女性の 20% 程度で BMI が 18.5 未満の痩せとなっています。これは先進国で最も高い率で、月経異常、不妊や骨粗鬆症など様々な健康障害の原因となります。この背景にあるのが痩せ願望です。私はこの背景として、女性の美とはこういうもの、というメディアからの無自覚（あるいは自覚的な）な発信が、美意識への多様性を失わせ、次第に同調圧力的な力として作用し、痩せたい気持ちを過剰に作り出す社会を作り上げたのではないかと考えています。この社会的現象に対して、Juntendo Medical Journal のような学術雑誌が果たす役割は、それらの健康障害を客観的に伝えるだけではなく、その背景にある人文学的な背景について考察することにあり、それにより物事の問題の本質に迫り社会課題の解決に繋がることになると考えています。

これらの諸問題に対して、内閣府は戦略的イノベーション創造プログラムの１つとして「包摂的コミュニティプラットフォームの構築」をスタートさせ、私たちはそのプログラムの中の１つである「女性のボディイメージと健康改善のための研究開発」を産官学連携で進めております。包摂的なコミュニティの構築は、単に医学的な側面だけでなく、文化的および社会的な変化をもたらすために不可欠で、より良い QOL 実現に向けた活動となるように進めたいと考えています。

田村　好史
国際教養学部国際教養学科・医学研究科　代謝内分泌内科学／スポーツ医学・スポートロジー

イラスト作者より

毎年恒例の成田山初詣の時に歩く参道にずらっと並んでいる土産物店で、今年も面白い物を見つけました。ファンタジックな月のブランコに乗っている二匹のカエルです。早速、教室のウェルカムボードにしました。（宮道明子）

順天堂醫事雑誌の記事については既に明治８年の創刊号から電子化されており，J-STAGE（科学技術情報発信・流通総合システム）の電子ジャーナル公開システムにおいて閲覧することができます．順天堂医学会の Web サイトからもご覧いただけますので，ご活用頂ければ幸いです（https://www.juntendo.ac.jp/journal/）．

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抄　録

順天堂醫事雑誌
2024. 70(2), 186

小児眼疾患の長期予後

横　山　利　幸

順天堂大学医学部付属練馬病院眼科特任教授

小児眼疾患，特に先天眼疾患の治療には，全身疾患の合併や訴えがないなど成人とは違ういくつかの問題点がある．もっとも問題となるのは小児では解剖学的にも機能的にも発達途中で未熟であることである．このために疾患発症の時期，治療の時期が予後に大きく影響し，その予後も長期にみないとわからない．

片眼先天白内障の視力予後は不良だが，屈折矯正と厳格な弱視治療で良好な視力を得ることもある．当院での症例を提示するが，これには長期にわたる両親の熱意とアドヒアランスが必要と思われた．

小児の全層角膜移植の例はまれである．順天堂眼科での 28 年間のデータでは 15 歳以下の全層角膜移植は 25 眼あり，そのうちの 10 眼が先天角膜混濁であり，その予後は極めて不良であった．輪部デルモイドでは通常視力予後はよく，弱視治療が優先され，手術は視力が出てから行う．しかし瞳孔中心に腫瘍が近づくと視力は不良となり，瞳孔中心まで 8 割程度までくると視力は不良である．このような瞳孔中心にかかるような場合には術前の弱視治療に反応しないため，早期に切除と表層角膜移植をして，ハードコンタクトレンズ装用下で弱視治療をするのが必要と思われた．

小児眼疾患の予後は発症時期や治療の時期を疾患により大きく異なる．治療においては発達途上であることを考慮し，長期に経過を追うことが重要と思われる．

キーワード：小児眼疾患，先天白内障，先天角膜混濁，Peter 奇形，輪部デルモイド

この抄録は，順天堂醫事雑誌 70 巻 2 号，p104–111，2024 掲載の『Long-term Prognosis of Pediatric Ocular Disease』の和文抄録です．

抄　録

順天堂醫事雑誌
2024. 70(2), 187

自動血液算定器による敗血症時の好中球活性化の同定

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好中球は感染防御の最前線で活躍する細胞である．しかしながら好中球の活性化を評価する方法は未だ限られたものしかない．幼弱な好中球はサイズが大きく，細胞構造が複雑であり核酸の保有量が多いいことから自動血球算定器による cell population data で測定が可能である．すなわち forward scatter light, side fluorescent light, side fluorescence distribution width などの指標で同定することが可能である．さらに side fluorescence light の変化は neutrophil extracellular traps の放出を示唆することから，敗血症性 DIC の診断に役立つ可能性がある．

キーワード：敗血症，血液算定，好中球，細胞死，好中球細胞外トラップ

この抄録は，順天堂醫事雑誌 70 巻 2 号，p114–117，2024 掲載の『The Detection of Neutrophil Activation by Automated Blood Cell Counter in Sepsis』の和文抄録です．

抄　録

順天堂醫事雑誌
2024. 70(2), 188

COVID-19に対する抗血小板療法の有用性

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[3)] 順天堂大学救急・災害医学

COVID-19 において血小板は SARS-CoV-2 の主要な標的である．活性化された血小板は血栓形成を刺激する物質を放出し，接着分子を発現し，凝固を活性化する．しかし，抗血小板療法は現在の国際ガイドラインでは推奨されていない．われわれは，臨床試験において抗血小板療法の効果が検証されなかった原因は，開始のタイミングと対象症例の重症度にあると考える．抗凝固薬の効果を検討した臨床試験で示されたように，抗血栓療法を成功させるためには中等度重症度においての早期開始が必要な条件と考えられる．このような集団において抗血小板薬の効果を検討する臨床試験が必要であろう．

キーワード： COVID-19，血小板，血栓症，アスピリン，P2Y12 阻害薬

この抄録は，順天堂醫事雑誌 70 巻 2 号，p118-120，2024 掲載の『The Effect of Antiplatelet Therapy on COVID-19』の和文抄録です．

抄　録

順天堂醫事雑誌
2024. 70(2), 189

21世紀現在，DIC診断は希少診断となりつつある？

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播種性血管内凝固（DIC）は，20 世紀初頭においてはしばしば用いられる診断名であった．しかし現在では，DIC を起こしやすい臨床病態においても overt-DIC 診断がつけられることは稀となっている．これまで DIC の基礎疾患として一般的と考えられてきた 4 つの臨床病態，すなわち敗血症，外傷，産科疾患，癌の発生率は増加傾向にあるにもかかわらず，これらの病態において DIC と診断されることはむしろ稀となりつつあるのである．われわれは DIC と診断されることが少なくなってきた理由は，個々の病態生理の解明が進み，その多様性が理解されるようになったためと考えている．このパースペクティブスにおいては，このような概念変化を提示し，医療者がそれぞれの病態における新しい基準に基づいて早期に DIC 診断を行うことで，患者が治療の恩恵を受けることができるように啓蒙していきたい．

キーワード： 播種性血管内凝固，敗血症，診断基準，出血

この抄録は，順天堂醫事雑誌 70 巻 2 号，p121–124，2024 掲載の『Why is DIC a Rare Diagnosis in the 21st Century?』の和文抄録です．

抄　録

順天堂醫事雑誌
2024. 70(2), 190

敗血症性播種性血管内凝固異常における臨床試験デザインの将来を考える

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これまでほとんどの臨床試験が失敗に終わってきた敗血症治療の領域で，治療薬の効果を検証することは容易ではない．試験がうまくいかない理由はさまざまであるが，敗血症に起因する播種生血管内凝固（DIC）における試験では以下のような問題を挙げることができる．まず初期の試験ではDICではなく重症敗血症が対象とされたこと，用量設定が妥当ではなかった可能性があること，また治療開始が適切に行われなかったことなどである．さらに，敗血症試験では28日死亡がエンドポイントとされてきたが，死亡にかかわる要因は複雑であり，治療効果だけで決まるものではなく，妥当とは言えない．以上のような問題を解決しない限り，臨床試験が成功することは難しいと考える．

キーワード：敗血症，播種性血管内凝固異常，臨床試験，抗凝固薬，複合エンドポイント

この抄録は，順天堂醫事雑誌70巻2号，p125–128，2024掲載の『Designing Future Clinical Trials for Sepsis-associated Disseminated Intravascular Coagulation』の和文抄録です．

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順天堂医学会短期海外留学時助成金給付制度

順天堂医学会では短期海外留学時助成金給付制度を開始いたしました。

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(1) 順天堂大学（大学院を含む）の学生で 1 か月以上 12 か月未満の海外留学をする者

(2) 留学先の研究機関または財団などからの援助がない者

(3) 医学会の正会員として 1 年以上の経歴を有し、医学会費を完納している者

2. 申請書類

(1) 順天堂医学会短期海外留学時助成金申込書

(2) 所属長の推薦書

(3) 申請者の主な研究テーマ・研究業績

(4) 留学受け入れ機関の指導者からの推薦状

3. 助成金の給付金額

留学期間	助成金額
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4 か月以上 7 か月未満	20 万円
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4. 申請スケジュール（年 2 回）

申請期限	助成決定時期
6 月末	8 月
12 月末	2 月

5. 選考機関：順天堂医学会短期海外留学時助成金選考委員会

6. 助成後の義務

(1) 帰国後直近の順天堂医学会学術集会において研究成果の発表および、その内容を「順天堂醫事雑誌」に報告する。

(2) 帰国後は、順天堂大学またはその関連機関に原則として 3 年以上勤務する。

7. 本件の照会先

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