formula: 30 + (age in years × 30).[10] Nocturnal polyuria is defined as nocturnal urine output >130% of expected bladder capacity.[1] To minimize evening fluid and solute intake, we instructed children to ensure enough fluid intake during the morning and early afternoon. All children were advised to empty the bladder and restrict fluid and fruit intake before going to bed at night. Initially all patients were given 120 μg ODM and the response was evaluated after 4 weeks. ODM were administered 30–60 min before bedtime daily, and all patients were informed and warned about fluid restriction.

Every 4 weeks following initiation of ODM, the response in each child was assessed according to the ICCS definitions: non-response, 0–49% decrease; partial response, 50–89% decrease; response, ≥90% decrease; full response, 100% decrease or <1 symptom occurrence monthly.

In patients who were still polyuric at 4 weeks, the dose was increased to 240 μg. After re-evaluation of the effects at 8 weeks, responders continued ODM until they achieved full response, while non-responders were treated with additional alternative therapy.

For the patients who achieved full response only with ODM, we proposed two protocols of ODM reduction as follows: group A, 240 μg ODM per day → 120 μg ODM per day → 120 μg ODM per alternate day → cessation; and group B, 240 μg ODM per day → 120 μg ODM per day → 60 μg ODM per day → 60 μg ODM per alternate day → cessation.

This clinical trial was approved by both institutional review boards. Forty-nine patients accepted the proposal and were randomized to undergo treatment with either of two protocols. In both protocols, tapering steps proceeded every 4 weeks, basically maintaining the status of full response, but some patients remained more than 4 weeks at some stages because they experienced more than one wet night. All the patients enrolled were scheduled for control after at least 12 weeks after the termination of ODM therapy and were questioned regarding bedwetting episodes. Relapse was defined as bedwetting occurring more than two nights monthly. The primary outcome measure was determination of relapse rate after cessation of ODM in the two protocols.

Statistical analysis

Paired-samples *t*-test and independent-samples *t*-test were used for continuous variables. Relapse rates were compared using Pearson's chi-squared test. Significance level was set at $P < 0.05$.

Results

Of 157 patients treated with ODM 120 μg, 79 patients (50.3%) started 240 μg for persistent polyuria at 4 weeks. At 8 weeks of ODM treatment, 92 patients (58.6%; 22 patients with ODM 120 μg and 70 patients with ODM 240 μg) failed to achieve full response and began additional treatment (anti-cholinergics for 51 patients, imipramine for 13 patients, enuresis alarm for 26 patients, and atomoxetine for two patients for managing comorbid attention-deficit/hyperactive disorder). For the remaining 65 patients (41.4%), we proposed continuation of treatment in either of the two protocols, and 49 of them were enrolled.

Table 1 Maximum ODM dose and time to FR

		Maximum ODM dose	Time to FR (days)
A	240→ 120 μg/day →off	120 μg: n = 15 240 μg: n = 1	114.0 ± 107.6
B	240→ 120 μg/day →120 μg/2 days →off	120 μg: n = 23 240 μg: n = 2	144.5 ± 87.8
C	240→ 120 μg/day →60 μg/day →60 μg/2 days →off	120 μg: n = 18 240 μg: n = 6	180.4 ± 97.3

No statistical significance was seen. FR, full response; ODM, oral desmopressin melt.

Twenty-five patients were allocated to group B and 24 patients to group C. The time to achieve full response on ODM therapy is given in Table 1. A total of 56% of the 25 group B patients and 17% of group C had subsequent relapse after the cessation of ODM (Table 2). When the groups were compared, the group C patients who were treated with the more gradual tapering of ODM had better outcome ($P = 0.026$).

Discussion

In this clinical study, we analyzed the results of two different withdrawal protocols of ODM therapy in polyuric MNE patients. We observed a better outcome in the more gradual withdrawal group.

Although desmopressin treatment has been regarded as the first-line therapy for polyuric MNE for many years, the high relapse rate after the discontinuation of treatment is problematic.[5]

In 2009, a report from Germany proved that desmopressin treatment followed by a structured withdrawal program is superior to regular treatment with abrupt termination in MNE children, based on a multicenter, retrospective survey of 487 patients.[8] In that study, patients were not treated with ODM, but desmopressin tablets (0.2 mg and 0.4 mg). In their tapering protocol, the dose of desmopressin was kept constant and daily treatment intervals were increased to every second evening for 2 weeks, twice weekly for 2 weeks and once weekly for 2 weeks, followed by termination. That structured protocol has long been recognized as a gold standard.

Table 2 Treatment outcome vs ODM tapering schedule

	Treatment outcome	Minirin tapering (days)	
B	Total (25)	101.1 ± 65.6	
240 → 120 μg/day → 120 μg/2 days → off	Relapse (14)	72.9 ± 42.8 → restart therapy	p=0.021
	No relapse (11)	137.1 ± 73.5 → off therapy	
C	Total (24)	102.1 ± 62.1	
240 → 120 μg/day → 60 μg/day → 60 μg/2 days → off	Relapse (4)	49.5 ± 20.6 → restart therapy	p=0.062
	No relapse (20)	112.6 ± 62.5 → off therapy	

ODM, oral desmopressin melt.

Since 2005, more patients have been treated with ODM, and very recently, two reports describing the structured withdrawal of ODM issues were published.[6,7] Ferrara *et al.* started treatment with ODM 120 μg per day in 81 patients for 3 months.[6] After 3 months of therapy, patients who were partial or full responders to ODM[1] were enrolled in the study. After the randomized allocation, their patients underwent either abrupt withdrawal or structured withdrawal (ODM 60 μg per day for 15 days and then 60 μg per alternate day for another 15 days). A total of 47.8% patients in the structured withdrawal process relapsed, and the authors failed to show superiority of structured withdrawal of ODM treatment.[6]

In contrast, Gokce *et al.* showed that structured withdrawal with ODM resulted in lower relapse rate.[7] They treated 421 MNE patients with ODM 120 μg or 240 μg per day for 12 weeks. A total of 284 patients who achieved full response were subsequently randomized into four different treatment groups. Patients in group 1 received half of the effective dose; those in group 2 received the effective dose on alternate days; those in group 3 underwent immediate cessation; and those in group 4 received placebo. The cessation programs were applied for 2 weeks, and 4 weeks after the cessation, 35.9% of group 1, 40.5% of group 2, and 55.3% of group 3 had relapsed. Although the patients who underwent structured withdrawal of ODM had significantly better outcome, no differences were seen between the two different withdrawal protocols.

Given that the treatment protocols in the two recent reports[6,7] are slightly different from the present ones, comparison of outcome is difficult. We propose, however, that more gradual withdrawal of desmopressin leads to better outcome in the treatment of MNE. Although the pathophysiological mechanism(s) behind this observation remain to be elucidated, one possible explanation is that the structured withdrawal of desmopressin would stimulate maturation of the innate production of antidiuretic hormones, as proposed previously by Marschall-Kehrel and Harms.[8]

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Original article

Efficacy and safety of fosphenytoin for benign convulsions with mild gastroenteritis

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Abstract

Objective: To clarify the efficacy and safety of fosphenytoin for seizures in children with benign convulsions and mild gastroenteritis.

Methods: Using the mailing list of the Annual Zao Conference on Pediatric Neurology, we recruited patients who met the following criteria: (1) clinical diagnosis of benign convulsions with mild gastroenteritis and (2) treatment with intravenous fosphenytoin. Benign convulsions with mild gastroenteritis were defined as a condition of (a) seizures associated with gastroenteritis without electrolyte imbalance, hypoglycemia, or dehydration in patients (b) between 6 months and 3 years of age with (c) no preexisting neurological disorders, (d) no impaired consciousness, and (e) a body temperature less than 38.0 °C before and after the seizures. The efficacy of fosphenytoin was categorized as effective when cessation of seizures was achieved.

Results: Data from 16 child patients were obtained (median age, 20 months). Seizures were completely controlled after the initial dose of fosphenytoin in 14 of 16 patients. The median loading dose of fosphenytoin was 22.5 mg/kg. In 10 patients, fosphenytoin was administered after other antiepileptic drugs such as diazepam and midazolam were used. Adverse effects of fosphenytoin, excessive sedation, or intravenous fluid incompatibility were not observed in any patients.

Conclusion: Fosphenytoin is effective and well tolerated among children with benign convulsions with mild gastroenteritis.

Keywords: Fosphenytoin; Benign convulsions with mild gastroenteritis; Efficacy; Safety

1. Introduction

Benign convulsions with mild gastroenteritis (CwG) were first reported by Morooka [1]. CwG is characterized by seizures, often occurring in clusters, associated with gastroenteritis in healthy children between 6 months and 3 years of age who have normal laboratory data, cerebrospinal fluid, interictal electroencephalogram results, and developmental outcomes [2,3]. CwG has been established as a benign condition with no neurological sequelae [3,4]. However, a diagnosis of CwG may not be easy during the first few hours after onset.

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A large majority of patients have a cluster of seizures, and clinicians may consider intensive antiepileptic treatment, even when CwG is suspected on the basis of clinical observation. In such situations, an effective and safe regimen of antiepileptic treatment is desirable. At present, the efficacies of carbamazepine (CBZ) and lidocaine (LD) have been confirmed [2,5,6].

Phenytoin (PHT) is an efficacious antiepileptic drug for status epilepticus or clustering seizures. One of the important advantages of PHT is its lesser effect on the level of consciousness [7]. There have been a few reports on the efficacy of PHT against CwG [8]. However, pediatricians and pediatric neurologists in Japan tend to avoid using PHT because of its adverse effects, including local irritation, phlebitis, intravenous fluid incompatibility, and purple glove syndrome. Fosphenytoin (fPHT) is a water-soluble prodrug of PHT with a neutral pH value that is expected to be as effective for CwG as phenytoin. Adverse effects of fPHT are presumed to occur less frequently than those of PHT.

We had planned a retrospective cohort study on the efficacy and safety of fPHT in children with acute encephalopathy using the mailing list of the Annual Zao Conference on Pediatric Neurology. Originally, we recruited patients who met the following criteria: (1) clinical diagnosis of acute encephalopathy, and (2) use of intravenous fPHT for treatment of seizures. However, we found that some children with CwG were included in the cohort. Because the efficacy and safety of fPHT for CwG have not been elucidated, we decided to summarize their data. Here we describe clinical data from children with CwG treated with fPHT.

2. Methods

We identified patients from the mailing list of the Annual Zao Conference on Pediatric Neurology who met the following criteria: (1) clinical diagnosis of CwG, and (2) treatment with intravenous fPHT. According to previous studies, CwG was defined as a condition of (a) seizures associated with gastroenteritis without electrolyte imbalance, hypoglycemia, or dehydration in patients (b) between 6 months and 3 years of age with (c) no preexisting neurological disorders, (d) no impaired consciousness, and (e) a body temperature less than 38.0 °C before and after the seizures [2,3]. The mailing list of the Annual Zao Conference includes more than 700 pediatric neurologists throughout Japan. From January 2012 to November 2013, we requested enrollment of the patients using the mailing list and provided a structured research form. The members of the mailing list were asked to fill out the research form if they had patients meeting the specified criteria. The completed research forms were returned to the first author by email. This study was approved by the institutional review board of Juntendo University Faculty of Medicine. The patients' data were collected anonymously.

The following items were included in the research form: age, gender, preexisting medical conditions, prodromal illness and its pathogen, onset of gastroenteritis, type of seizure (status epilepticus or clustering seizures), and fPHT outcome, efficacy, and adverse events. We also asked the participants to describe their seizure time course and use of antiepileptic drugs. According to our previous study [9], the efficacy of fPHT was categorized as follows based on clinical observations: effective: cessation of seizures; partially effective, 50% or more reduction in frequency and/or duration of seizures; ineffective, incompatible with the previous two conditions.

3. Results

Data from 16 child patients were obtained from three hospitals. The characteristics of these 16 patients are summarized in Table 1. All of them received a single fPHT administration course. The median loading dose of fPHT was 22.5 mg/kg (range, 20–25 mg/kg). The median rate of injection was 0.79 mg/kg/min (range, 0.67–3.0 mg/kg/min).

Among the patients (10 males and 6 females), the age at onset of CwG ranged from 12 to 49 months (median, 20 months). Five patients had a past history of febrile convulsions. No patient had a family history of epilepsy. Head CT and MRI were performed in one patient each and did not reveal any abnormalities. The median interval between the onset of gastroenteritis and that of seizure was 1 days (range, 0–4 days). The pathogens of gastroenteritis were rotavirus in three patients, norovirus in one, and unknown in 12. Before intravenous fPHT administration, no drugs were used in six patients, diazepam in five, midazolam in four, and diazepam plus thiopental in one. In four patients, fPHT was used after the occurrence of one seizure. The others experienced two or more seizures before administration of fPHT.

No seizures occurred in 14 of 16 patients after administration of fPHT. Thus, fPHT was deemed to be effective in these 14 patients. PHT levels in the serum were measured in five patients. The median concentration was 10.42 μg/mL (range, 1.0–10.7 μg/mL) 15–24 h after initial administration. Only the loading dose was given to 14 patients; the other two also received one maintenance dose of 7.5 mg/kg.

The clinical course of the two patients in whom fPHT was ineffective was as follows. Patient 5 was a 49-month-old boy with a past history of two febrile seizures and delayed psychomotor development. Diarrhea and vomiting due to rotavirus infection appeared on the day before seizure onset. He had a generalized seizure lasting for several minutes and was admitted to our hospital. He was treated with 22.5 mg/kg intravenous fPHT. Immediately afterward, he had a seizure with loss of

Table 1
Characteristics of patients.

Patient	Age (Mo)	Sex	Dose of fPHT (mg/kg)	Rate of injection (mg/kg/min)	Serum levels of fPHT (μg/ml) [sampling time*]	Number of diarrhea before fPHT	Number of vomiting before fPHT	Interval from onset of AGE to Sz (days)	Number of Szs before fPHT	Recurrence of Sz after fPHT	Causative virus of AGE	Drugs before fPHT
1	16	M	22.5	0.75	10.7 [19 h]	2	1	0	4	No	Unknown	DZP
2	17	F	20	0.67	N.M.	2	3	2	1	No	Noro	None
3	20	F	25	0.83	N.M.	2	1	2	4	No	Unknown	None
4	41	M	22.5	1.5	7.08 [24 h]	5	2	2	2	No	Rota	None
5	49	M	22.5	2.8	11.6 [15 h]	10	2	1	2	Yes	Rota	None
6	35	F	22.5	0.75	N.M.	2	3	1	1	No	Unknown	None
7	30	F	22.5	3.0	N.M.	2	2	1	2	No	Unknown	None
8	16	F	22.5	0.75	N.M.	3	3	2	2	No	Unknown	DZP
9	17	M	22.5	2.3	N.M.	3	3	1	3	No	Unknown	DZP
10	20	M	22.5	0.75	N.M.	3	2	1	1	No	Rota	DZP
11	42	M	22.5	2.25	N.M.	4	1	4	1	No	Unknown	MDZ
12	21	M	22.5	0.75	1.07 [21 h]	1	0	1	3	Yes	Unknown	MDZ
13	20	F	22.5	2.25	N.M.	2	7	3	2	No	Unknown	MDZ
14	21	M	22.5	0.75	N.M.	3	0	1	2	No	Unknown	MDZ
15	13	M	22.5	0.75	N.M.	1	1	0	1	No	Unknown	DZP
16	15	M	22.5	2.25	10.42 [21 h]	8	0	1	4	No	Unknown	MDZ, ThP

fPHT: fosphenytoin, AGE: acute gastroenteritis, Sz: seizure, N.M.: not measured, DZP: diazepam, MDZ: midazolam, ThP: thiopental.
* Sampling time was stated as the time at blood sampling after the loading dose of fPHT.

consciousness and right hemifacial twitch lasting for 3 min. In response, 0.1 mg/kg intravenous MDZ was added. Thereafter, no seizures were observed. Levels of fPHT 15 h after administration were 11.6 μg/ml. In this patient, the recurrence of seizure may have occurred before the concentration of fPHT reached the therapeutic level in the brain.

Patient 12 was a previously healthy 21-month-old boy. He had a seizure with loss of consciousness and increased muscle tone of the upper extremities lasting for 3 min in association with diarrhea. Although he was treated with 0.1 mg/kg intravenous MDZ, he had two more seizures within a few hours. Therefore, 22.5 mg/kg intravenous fPHT was used. He had additional two seizures 12 h after this administration, and 0.2 mg/kg intravenous MDZ was added. No further seizures were recognized. Levels of fPHT 21 h after administration were 1.07 μg/mL, far below the therapeutic level. The amount of fPHT may have been insufficient in this patient, although the dosage was not different from that given to the other patients.fPHT was administered via the peripheral venous line with extracellular fluid in all patients. Adverse effects of fPHT, excessive sedation, or intravenous fluid incompatibility were not observed in any patients.

4. Discussion

We found that fPHT was effective and well tolerated in children with CwG. Complete cessation of seizures was achieved in 14 of 16 patients in our study. fPHT was effective even after other antiepileptic drugs failed to control seizures. Adverse effects were not observed in any patients. These characteristics are very important for selecting the appropriate treatment for CwG. Since the prognosis of CwG is excellent, the treatment choice for CwG should be safe. fPHT meets these required conditions.

It is remarkable that no seizures occurred after the initial dose of fPHT in most patients with CwG. This implies that the efficacy of fPHT may be superior to that of benzodiazepines, although our study did not compare the efficacies of different antiepileptic drugs. Our previous study showed that the efficacy of the first dose for CwG was 38% with diazepam/bromazepam and 40% with phenobarbital, although some doses were administered rectally [2]. Li et al. reported that the efficacy rates of diazepam and phenobarbital as first-line treatments were 25% and 83.33%, respectively [10]. However, there have been no studies comparing the efficacy of antiepileptic drugs for CwG in a randomized controlled manner. Further studies are necessary to determine the efficacy of fPHT for CwG objectively.

No adverse effects of fPHT were recognized in our study. The lack of excessive sedation is a remarkable advantage of fPHT. The differentiation between CwG

and acute encephalitis/encephalopathy is critical when classifying repeated seizures with gastrointestinal symptoms. The presence or absence of altered mental state is a factor used for differentiation. Phenobarbital may be effective for CwG, but excessive sedation is not uncommon [11]. This makes it difficult to distinguish between CwG and acute encephalitis/encephalopathy. Water solubility and neutral pH value are important features of fPHT [12,13]. A neutral pH level is related to an infrequent occurrence of serious local reactions, such as purple glove syndrome and skin necrosis. The water solubility of fPHT reduces intravenous fluid incompatibilities. These adverse effects were not observed in this study. However, there is a potential risk for cardiovascular adverse effects such as hypotension and arrhythmia with fPHT as well as PHT. Continuous monitoring via electrocardiograms and blood pressure measurements should be performed, and the injection of fPHT should be administered slowly. Cardiovascular adverse effects were not observed in our patients, probably because the attending physicians were aware of the importance of monitoring and the appropriate infusion rate of fPHT.

Sodium channels may be involved in the pathogenesis of CwG, although the etiology has not yet been elucidated. Several previous studies have established the efficacy of CBZ and LD, and both drugs have been confirmed to block voltage-gated sodium channels. PHT also blocks these channels [2,5,6,14]. These facts suggest that functional alteration of sodium channels may be involved in the pathogenesis of CwG. However, there have been no reports on genetic mutations or single nucleotide polymorphisms associated with CwG. The pathogenesis of CwG remains a subject for future study.

There are some limitations to this study. Data on the efficacy and adverse effects may be inaccurate due to the retrospective design. The sample size in our study was too small to determine the efficacy and tolerance of fPHT. To determine the efficacy and safety of fPHT more accurately, prospective studies that include a larger number of patients are necessary. We did not compare the efficacy and safety of fPHT with those of other antiepileptic drugs. This is due to the lack of previous data on the efficacy and safety of PHT. The results of this study suggest that fPHT is useful in children with CwG. Comparative studies between fPHT and other epileptic drugs are essential to determine the appropriate use of antiepileptic drugs in children with CwG.

In summary, we showed that fPHT is effective and well tolerated and will be useful for the treatment of clustering seizures among children with CwG. Further evaluation of more patients is necessary to confirm the efficacy and safety of fPHT for CwG.

Disclosure

We have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. None of the authors have any conflict of interest to disclose.

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CASE REPORT

Use of targeted next-generation sequencing for molecular diagnosis of craniosynostosis: Identification of a novel *de novo* mutation of *EFNB1*

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ABSTRACT **Craniofrontonasal syndrome (CFNS; MIM#304110) is characterized by asymmetric facial features with hypertelorism and a broad bifid nose due to synostosis of the coronal suture. CFNS shows a unique X-linked inheritance pattern (most affected patients are female and obligate male carriers exhibit a mild manifestation or no typical features at all) associated with the ephrin-B1 gene (*EFNB1*) located in the Xq13.1 region. In this study, we performed targeted, massively parallel sequencing using a next-generation sequencer, and identified a novel *EFNB1* mutation, c.270_271delCA, in a Japanese female patient with craniosynostosis. Because subsequent Sanger sequencing identified no mutation in either parent, this mutation was determined to be *de novo* in origin. After obtaining molecular diagnosis, a retrospective clinical evaluation confirmed the clinical diagnosis of CFNS in this patient. Comprehensive molecular diagnosis using a next-generation sequencer would be beneficial for early diagnosis of the patients with undiagnosed craniosynostosis.**

Key Words: craniofrontonasal syndrome, craniosynostosis, ephrin-B1, massively parallel sequencing, next-generation sequencer, mutation

INTRODUCTION

Craniofrontonasal syndrome (CFNS; MIM#304110) is multiple congenital anomalies syndrome associated with craniosynostosis (Cohen 1979). Patients with this disorder present characteristic asymmetric facial features with hypertelorism and a broad bifid nose, due to synostosis of the coronal suture (Young 1987). CFNS shows a unique X-linked inheritance pattern; most patients are female, and obligate carrier fathers show no or only mild manifestations (Wieacker and Wieland 2005). This paradoxical inheritance pattern has been attributed to a pathomechanism referred to as "cellular interference", meaning the functional interference of mutant and wild-type cell populations in females (Twigg et al. 2013). In 2004, the ephrin-B1 gene (*EFNB1*), located in the Xq13.1 region, was identified as being responsible for CFNS (Twigg et al. 2004; Wieland et al. 2004). Subsequently, many *EFNB1* mutations have been identified in patients with CFNS (Wieland et al. 2005; Shotelersuk et al. 2006; Torii et al. 2007; Wallis et al. 2008; Hogue et al. 2010; Apostolopoulou et al. 2012; Ramirez-Garcia et al. 2013; Seven et al. 2013; Chacon-Camacho et al. 2014; van den Elzen et al. 2014; Goyal et al. 2015; Ozylmaz et al. 2015).

Molecular diagnosis is important for a final diagnosis, and without a final diagnosis, adequate genetic counseling cannot be provided. Thus, mutation detection is essential. However, molecular diagnosis is often challenging. Massively parallel sequencing using a next-generation sequencer enables easy identification of mutations at the genome-wide level. Here, we present a CFNS patient whose causative *EFNB1* mutation was confirmed by use of next-generation sequencing.

CASE REPORT

A 20-month-old girl was born with a birthweight of 3284 g (75th–90th centile), a length of 50.0 cm (75th–90th centile), and an occipitofrontal circumference of 32.5 cm (25th–50th centile). Her father and mother were 38 and 31 years old, respectively. Her 5-year-old elder sister was healthy. Thus, there was no remarkable family history. Due to her facial asymmetry and left preaxial polydactyly of the foot, she was referred to our institution. At 6 days after delivery, head computed tomography revealed craniosynostosis (Fig. 1). At 5 months, surgical operation was performed to repair the craniosynostosis and the polydactyly. She began to walk independently at an age of 17 months. Based on the craniosynostosis observed in this patient, we considered some differential diagnoses including Crouzon syndrome (MIM#123500), Greig cephalopolysyndactyly (MIM#175700), Saethre-Chotzen syndrome (MIM#101400), and Craniofrontnasal syndrome. Because we did not have enough confidence to determine the clinical diagnosis, we performed whole genomic copy number analysis and comprehensive analysis of all genes responsible for above potential disorders by use of targeted next-generation sequencing.

After obtaining molecular diagnosis, we re-evaluated the patient. At present, she can speak more than 10 words. Enjoji developmental scale evaluated her developmental quotient (DQ) as approximately 100, indicating normal development (Enjoji and Yanai 1961). Her manifestations including facial asymmetry, hypertelorism, the webbed neck, bilateral axillary pterygium, Sprengel deformity, left broad hallux, and nail splitting of the toes were compatible of the final diagnosis.

MOLECULAR ANALYSIS

This study was performed in accordance with the declaration of Helsinki, and was approved by the ethics committee of Tokyo Women's Medical University. After obtaining written informed

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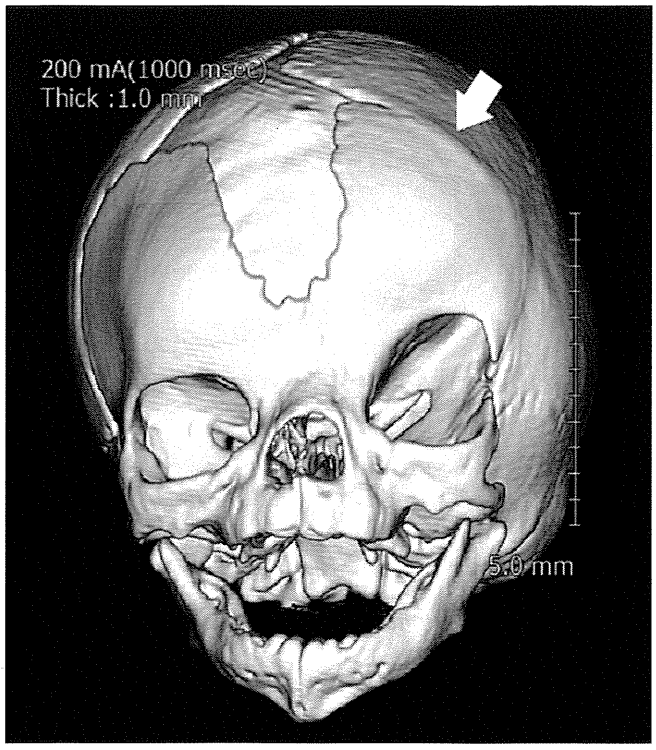

Fig. 1 Three dimensional computed tomography image examined at 6 days after delivery. Craniosynostosis of the left coronal suture (arrow) is noted.

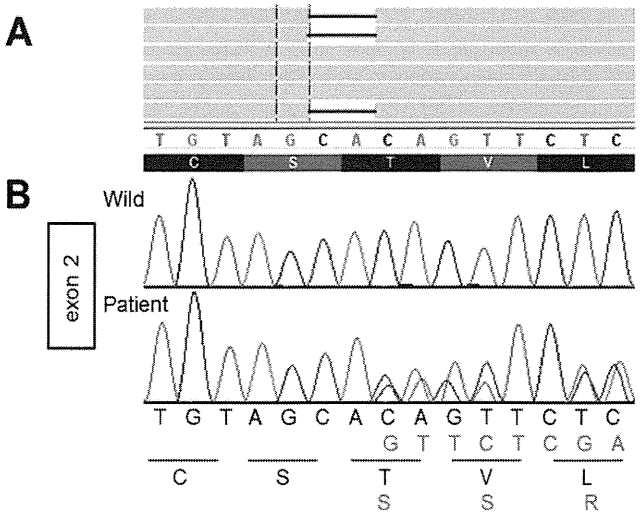

Fig. 2 Sequencing results for *EFNB1*. (A) Image view using Integrative Genomics Viewer (IGV). The 2-bp deletions are noted in about half of the read tags. (B) Electropherograms of Sanger sequencing. Compared to the wild type, the patient shows 2-bp deletion and subsequent frameshift.

consent from the patient's family, blood samples were obtained from the patient and her parents. DNA was extracted from the blood samples using a QIAamp DNA extraction kit (Qiagen, Hilden, Germany). Whole genome copy number was analyzed using Agilent SurePrint G3 Human CGH Microarray Kit 8×60K (Agilent Technologies, Santa Clara, CA, USA) as described previously (Shimojima et al. 2015), and there was no aberration. Targeted next-generation sequencing was performed using a TruSight One v1.0 sequencing panel (Illumina, San Diego, CA, USA), which targeted 4813 genes that are potentially associated with known clinical phenotypes ("Mendeliome") with capturing 11 946 514-bp targeted exon regions using 125 396 probes. The genes that can be responsible for craniosynostosis are also included in this panel, i.e., fibroblast growth factor receptor genes (*FGFR1*, *FGFR2*, and *FGFR3*), the twist basic helix-loop-helix transcription factor 1 gene (*TWIST1*), the GLI family zinc finger 3 gene (*GLI3*), and the ephrin-B1 gene (*EFNB1*) (Passos-Bueno et al. 2008). Prepared libraries were loaded onto a flow cell for sequencing, which was performed on a MiSeq device (Illumina) as described previously (Yamamoto and Shimojima 2015). Raw sequence reads were aligned to the hg19 human reference sequence.

The achieved coverage was >30× for more than 99% of the targeted exons and exon-intron boundaries. Mapping and variant calls were performed using cloud computing system of BaseSpace (Illumina), and annotation and filtering were performed using VariantStudio (Illumina). A total of 8354 variants remained after quality control. After exclusion of variants in introns more than 3-bp from exon-intron boundaries, common polymorphisms listed in the dbSNP129, variants with minor allele frequencies > 0.01, and variants listed in the Human Genetic Variation Database (HGVD) (http://www.genome.med.kyoto-u.ac.jp/SnpDB), we focused on identification of nonsynonymous variants, variants in splice acceptor/donor sites, and insertions and deletions (indels) causing frameshifts in coding regions. Following screening, 18 variants remained. Assessment of the functional relevance of these genes to the patient's phenotype suggested a 2-bp deletion in exon 2 of *EFNB1*, which was predicted to cause a frameshift creating a premature termination codon, i.e. NM_004429.4(EFNB1_v001):c.272_273del [NM_004429.4(EFNB1_i001):p.(Thr91Serfs*12)]. This variant was verified visually using the Integrative Genomics Viewer (IGV; http://www.broadinstitute.org/igv/) (Fig. 2A). The read depth of this region was 245 and altered reads were 119. Thus, altered variant frequency was 48.6, suggesting heterozygosity of this variant. This variant was confirmed by Sanger sequencing using primers, i.e., sense 5′-CCACCCAGTAGGCCCAG-3′ and antisense 5′-TGCACCACTTAGAAGCTCCC-3′ (Fig. 2B). Samples from both parents were also analyzed by Sanger sequencing and there was no cryptic change in both parents. This denied somatic mosaicism of this variant in both parents, indicating that this is a *de novo* mutation in a proband.

DISCUSSION

Although CFNS is distinctive and clinical diagnosis is straightforward (Zafeiriou et al. 2011), we could get this diagnosis after identification of *EFNB1* mutation using a comprehensive molecular analysis. Targeted next-generation sequencing disclosed a 2-bp deletion leading to a frameshift and a premature termination codon that would elicit nonsense-mediated decay. The same as this mutation, over 50% of previously reported *EFNB1* mutations were loss-of-function mutations; including nonsense, frameshift, and splicing error (Wieland et al. 2005). Therefore, this 2-bp deletion is definitely disease-causing. Finally, retrospective clinical evaluation of the patients confirmed clinical diagnosis of CFNS in this patient. Comprehensive molecular diagnosis using a next-generation sequencer would be beneficial for early diagnosis of the patients with undiagnosed craniosynostosis.

EFNB1 is located on the X-chromosome. Generally, disorders arising from mutations on the X-chromosome are typically characterized by affected males and unaffected carrier females (Dibbens

et al. 2008). In contrast, *EFNB1* mutations show a peculiar and rare pattern of inheritance, because most affected patients are female, and obligate male carriers exhibit either a mild manifestation or no typical features at all (Zafeiriou et al. 2011). This paradoxical finding is recognized as the attribution of "cellular interference" of wild type and mutant *EFNB1*. This mechanism is also observed in patients with epilepsy and mental retardation limited to females (EFMR) caused by the protocadherin 19 (*PCDH19*) gene mutations (Dibbens et al. 2008). Generally, EFMR can be observed only on carrier females of heterozygous *PCDH19* mutations, and hemizygous males are asymptomatic; however, mosaic males can be affected (Depienne et al. 2009; Depienne and LeGuern 2012). This finding supports the mechanism of "cellular interference" as the pathogenic mechanism. The "cellular interference" in severely affected female patients with CFNS will be caused by the altered coupling of ephrin receptors and their ligands derived from a functional mosaicism due to X-inactivation (Wieacker and Wieland 2005; Wieland et al. 2008; Zafeiriou et al. 2011; Twigg et al. 2013).

In this study, there was no cryptic change in Sanger sequencing for both parents. Thus, this 2-bp deletion is *de novo* origin and somatic mosaicisms in the parents were denied. According to the "cellular interference" theory, the father will show characteristic manifestation if he has mosaicism. In this study, the father showed no manifestation and no cryptic changes in Sanger sequence. However, we cannot exclude the possibility of germinal mosaicism in the parents (Twigg et al. 2006). Even if the mosaicism was absent in several tissues including hematologic cells and buccal epithelial cells of both parents, we cannot exclude germinal mosaicism, in which mosaicism is limited to germinal tissues (Dibbens et al. 2011; Tajir et al. 2013). This concept is very important because it complicates genetic counseling of this family who has a risk of recurrence of the mutation in subsequent pregnancies. Careful genetic counseling will be required in this case.

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DISCLOSURES

None.

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Current status of preimplantation genetic diagnosis in Japan

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Abstract:
This is a retrospective study aimingto clarify the current status of preimplantation genetic diagnosis (PGD) in Japan. Our data were collected from 12 facilities between September 2004 and September 2012, and entered into a database. A majority of PGD in Japan was performed for balanced structural chromosomal abnormalities in couples with recurrent miscarriage. PGD for monogenic diseases was performed only in two facilities. The average maternal age was 38 years for monogenic diseases and 40 years for chromosomal abnormalities. Overall there have been671 cycles to oocyte retrieval reported. Of these cycles, 85% (572 cycles)were for chromosomal abnormalities, and 15% (99 cycles) for monogenic diseases. Diagnosis rates in the current study were 70.8% for monogenic diseases and 94.0% for chromosomal abnormalities. Rates of embryo transfer of PGD were 62.7% for monogenic diseases and 25.5% for chromosomal abnormalities. Clinical pregnancy rates per embryo transfer were 12.0% for monogenic diseases and 35.6% for chromosomal abnormalities. Our study is the first PGD report from all facilities which had the approval of the ethics committee of the Japanese Society of Obstetrics and Gynecology. We have built a basis for gathering continuous PGD data in Japan.

Keywords: Chromosomalabnormalities, Genetic counseling, Japan, Monogenic diseases, Preimplantation genetic diagnosis (PGD)

Background:
Preimplantation genetic diagnosis (PGD) is a rapidly developing procedure for embryo genetic analysis **[1].** It allows couples carrying genetic diseases to have an unaffected child, without undergoing an invasive prenatal diagnosis and possible termination of pregnancy **[2].** The number of cases employing PGDincreases annually **[3, 4],** and indications for PGD have expanded to include identification of a broad variety of genetic diseases **[5].** Since 1997, the European Society of Human Reproduction and Embryology (ESHRE) PGD Consortium has been collecting data internationallyfrom the corresponding facilities where PGD was performed **[3].** Since 1999, 11 data sets of PGD-related information have been analyzed and published. The data of only a few Japanese facilities has been included **[3].**

PGD and prenatal diagnosis (PND) in Japan are not regulated by law but are governed by professional guidelines. In 1998, the Japanese Society of Obstetrics and Gynecology (JSOG) approved the clinical research of PGD and issued "The Guidelines on Preimplantation Genetic Diagnosis" **[6].** In 2003, 10 genetic-related medical societies published comprehensive guidelines to incorporate and expand on previously established guidelines. The "Guidelines for Genetic Testing" (2003) cover particular conditions for PND but do not mention PGD in particular **[7].** According to these guidelines, PGD is conducted as a "clinical research," and approval is required on a case-by-case basis, not only by the ethics committee of the facility but also by the ethics committee of JSOG. The report "Guidelines on Preimplantation Genetic Diagnosis" (1998) was amended in 2006 and revised in 2010. These guidelines indicate that this diagnostic procedure should be applied only to serious hereditary disorders and balanced structural chromosomal abnormalities in couples with recurrent miscarriages. Because of long-term discussions involved and these severe preconditions, no case of PGD had been

performed in Japan up to the end of 2004 despite the rapidly increasing number of PGD cycles worldwide **[8].** It has been 11 years since the initiation of clinical practices for PGD in Japan, and the number of live births has been increasing every year.

Although PGD data collection has been conducted by the ESHRE consortium, detailed data such as the technical background of facilities has not been evaluated. In addition, there is a need for continuous accumulation of information regarding the precision of genetic diagnosis, pregnancy outcome, and follow-up of deliveries. Furthermore, since PGD application in Japan is limited by JSOG, it is necessary to survey our present situation to expand PGD application. The aim of the present study was to monitor PGD treatment cycles and their outcome including the follow up of children born in order to clarify the current status of PGD in Japan.

Methodology:

Our data were collected retrospectively from 12 IVF laboratories. These facilities also have own PGD laboratories, and had the approval of the ethics committee of JSOG between September 2004 and September 2012, and entered into a FileMaker Pro 12 database (FilemakerInc, Santa Clara, California, USA). This included files for PGD referrals (patient history, PGD indications, and decisions taken by facilities and patients), ethical review and genetic counseling, PGD cycles (ART data and PGD analyses and results), and pregnancy and infant records. Indications for PGD are divided into two categories: (a) PGD for chromosomal abnormalities and (b) PGD for monogenic diseases. Data submitted were thoroughly assessed to identify omissions and any inconsistent data. Records with insufficient data (e.g., with no cycle, no patient identification, no clear indication, or from an incorrect time period) were excluded from subsequent data calculations. Following editing and correction of all data, the entire collection was separated into the two above-mentioned categories. We defined genetically transferable embryos as carrier and non-carrier embryos for monogenic diseases, and balanced and normal embryos for chromosomal abnormalities. Clinical pregnancies were defined as the presence of one or more fetal heartbeats by ultrasound at 6–7 weeks of gestation. Implantation rate was defined as the number of active fetal heartbeats per 100 transferred embryos. Delivery rate was defined as the percentage of pregnancies resulting in delivery per OR and per embryo transfer procedure. This clinical research was approved by the ethics committee of the Keio University School of Medicine (approval No. 20120089).

Results:

Data from 12 PGD facilities were included in this report. Any PGD data collected from facilities that did not have the approval of the ethics committee of JSOG were not included. In cases of PGD, ICSI was the most frequent method of fertilization, and cleavage-stage aspiration was the most common method of biopsy. Overall, laser drilling of the zona pellucida was most commonly performed.

Genetic counseling and the system of administrating genetic information

At three of 12 facilities, licensed medical geneticists were not in charge of genetic counseling. Four facilities had no separate room dedicated for genetic counseling. At seven of 12 facilities, genetic information was recorded on regular medical charts. In addition, at five facilities, genetic records were stored in usual medical records, and at three facilities, all staff were allowed access to genetic information. Further, at nine facilities, genetic testing results were concurrently disclosed to couples.

PGD for monogenic diseases

Table 1 (see supplementary material) summarizes data from 99 OR cycles, for which PGD was performed to identify monogenic diseases. Most common indications for this treatment were Duchenne muscular dystrophy (DMD, 59 cycles) and myotonic dystrophy type 1 (DM1, 31 cycles). Other indications were Ornithine transcarbamylase deficiency (OTCD, 6 cycles) and Fukuyama congenital muscular dystrophy (FCMD), adrenoleukodystrophy (ALD), and Leigh syndrome of mitochondrial disease, all with one case each. ICSI was used in all cycles, and the use of a noncontact diode infrared laser was the preferred method for biopsy (94.3% PGD cycles). Day 3 cleavage-stage embryo biopsy was used in all PGD cycles. A total number of 1050 cumulus–oocyte complexes (COCs) were collected, and 63.0% (661/1050) mature oocytes were fertilized. A total of 83.8% (554/661) embryos were biopsied, of which, 97.7% (541/554) were successful. Further, of successfully biopsied embryos, 70.8% (383/541) presented a diagnostic result, of which, 62.7% (240/383) were genetically transferable On an average, 10.6 COCs were collected per cycle that yielded 6.7 embryos, of which, 5.6 were suitable for biopsy. In addition, diagnosis was achieved for 3.9 embryos per cycle, of which, 2.4 embryos were deemed genetically transferable. On an average, 1.7 embryos were transferrable per cycle. A positive hCG was obtained in 19 cycles, with a positive heartbeat in 14 cycles [14.1% per OR (14/99), 12.0% per embryo transfer (14/117), with 15 active fetal heartbeats, giving an overall implantation rate of 9.0% (15/167)]. The delivery rate was 10.1% per OR (10/99) and 8.5% per embryo transfer (10/117). Eight miscarriages (42.1% per positive hCG, 21.4% per clinical pregnancy) and one ectopic pregnancy were reported.

PGD for chromosomal abnormalities

Table 2 (see supplementary material) summarizes 572 OR, for which PGD was performed for chromosomal abnormalities. PGD for reciprocal translocations was performed more often compared with that for Robertsonian translocations. The percentage of female carriers was higher for both reciprocal translocations and Robertsonian translocations (65% and 79%, respectively). In 72% cycles, ICSI was used for fertilization. Zona breaching was achieved by either laser drilling or mechanical techniques, in equal numbers. The aspiration of blastomeres from cleavage-stage embryos was the preferred biopsy method (86%). A total of 4140 oocytes were collected (mean, 7.2 per cycle). Of these, 66.3% (2744/4140) were fertilized. A total of 66.4% (1822/2744) embryos were biopsied, and of these, 99.8% (1819/1822) were successful. Of successfully biopsied embryos, 94.0% (1710/1819) presented a diagnostic result, of which, 25.5% (436/1710) were genetically transferable. On an average, 7.2 COCs were collected per cycle. This yielded 4.8 embryos, of which, 3.2 embryos were suitable for biopsy. Diagnosis was achieved for 3.0 embryos, of which, 0.8 embryos were deemed genetically transferable. On an average, 0.5 embryos were transferred per cycle. A positive hCG was obtained in 101ET cycles, with a positive heartbeat in

77 cycles [13.5% per OR (77/572), 35.6% per embryo transfer (77/216), with 80 active fetal heartbeats, giving an overall implantation rate of 27.4% (80/292)]. The delivery rate was 10.5% per OR (60/572) and 27.8% per embryo transfer (60/216). Moreover, 36 miscarriages (35.6% per positive hCG, 15.6% per clinical pregnancy) were reported, with approximately more than half of them (61%) occurring in the female reciprocal translocation group. In addition, two ectopic pregnancies were reported.

Prenatal and postnatal diagnosis

Table 3 (see supplementary material) summarizes prenatal and postnatal diagnoses of pregnancies. Excluding unknown cases, confirmation of the diagnosis was performed prenatally (47%, 27/57) and/or postnatally (7%, 4/57). Amniocentesis was performed for 45% cases (26/57), and 100% them (26/26) were normal. No misdiagnoses were reported.

Method of delivery, gestational age, and infants born

Table 4 (see supplementary material) summarizes the pregnancy outcome data. There were 70 deliveries of 75 babies. Caesarean section was performed for about 50% deliveries (31/63). In seven cases, the delivery method was not recorded. The mean birth weight of all singletons was 2964 g. The percentage of females (56%) born was higher than that of males.

Discussion:

We have built a basis for gathering continuous PGD data from which the status of PGD implementation can be tracked in Japan. Our study is the first PGD report from all facilitieswhich had the approval of the ethics committee of JSOG. Our results help to clarify the current status of PGD in Japan. A majority of PGD in Japan (98%) was performed for balanced structural chromosomal abnormalities in couples with recurrent miscarriage. PGD for monogenic diseases was performed only in two facilities because PGD for monogenic diseases warranted genetic counseling and tailor-made diagnoses for each case, which may not be feasible for outpatientclinics.

At three of 12 facilities, licensed medical geneticists were not in charge of genetic counseling. Although the genetic counseling system in Japan has been on the way to establish, it is still insufficient. Nishiyama *et al.* examined the current state of providing genetic counseling in Japan to pregnant women before they elected amniocentesis for prenatal diagnosis. The data revealed that pre-amniocentesis genetic counseling was usually provided by the obstetrician alone (73.8 %), by geneticists (18.4 %), including obstetricians certified as geneticists (12.6 %) and geneticists with other specialties (5.8 %), and by an obstetrician and nurse/midwife (7.8 %). These data reveal that most of the certified genetic counselors in Japan are not involved with prenatal genetic testing. Thus, we should consider the appropriate involvement of certified genetic counselors to assess and improve mental stress of pregnant women **[9].** In addition, the handling and management of genetic information should be regulated more strictly and sufficiently in the clinical field in Japan **[10].** Although limited access should be established in the management of genetic records, at three facilities, all staff were allowed to access genetic information.

Advanced maternal age was identified in this study (38 years for monogenic diseases and 40 years for chromosomal abnormalities). These maternal ages are higher compared with those documented in a recent ESHRE report (34 years for monogenic diseases and 35 years for chromosomal abnormalities) **[3].** Prolongation of the period before PGD initiation is regarded as one of the causes of advanced maternal age in Japan, which may be attributed to the two-step ethics examination; it is required to shorten this period by timeous ethics examinations.

For monogenic diseases, our diagnosis rates data were lower than those documented in the ESHRE report **[3].** On the other hand, our data on chromosomal abnormalities were similar to those reported in the ESHRE report. The primary reason for low diagnosis rates for DM1 was difficulties in diagnosis. The expanded myotonic dystrophy protein kinase (DMPK) allele is refractory to PCR amplification; therefore, the diagnosis is based on detection of the affected individual's unexpanded allele in the embryo. However, for couples who are either uninformative (sharing all alleles) or semi-informative (sharing one of their alleles) for the DMPK region, two parental alleles transmitted to an embryo may be of equal size; therefore, it may not be possible to confirm that the affected partner's unexpanded allele has been inherited. In these cases, diagnoses have been facilitated by the incorporation of linked markers to allow detection of contamination and/or the presence of phase alleles **[11].** Rates of embryo transferof PGD were 62.7% for monogenic diseasesand 25.5% for chromosomal abnormalities in Japan. These data are similar to those previously reported in ESHRE reports.Furthermore, clinical pregnancy rates per embryo transfer in the current study were 12.0% for monogenic diseases and 35.6% for chromosomal abnormalities. For monogenic diseases, our data were lower than previous reports ranging between 13% and 29% **[3, 12, 13].** In contrast, for chromosomal abnormalities, our data were similar to other previous reportsranging between 23% and 39% **[3, 14, 15].** Pregnancy rate is highly influencedby women's age. It is thought that advanced maternal age and two cell biopsies from day3 embryosare the primary reason for lower pregnancy rates following PGD for monogenic diseases in Japan.The miscarriage rate after PGD for monogenic diseases (44%, 8/18) and chromosomal abnormalities (36%,36/101) was higher than other reports **[3, 16, 17]**. We speculated that this high miscarriage rate was due to advanced maternal age.

Several problems about PGD in Japan were clarified in this study. First, it is imperative to reduce the prolonged period before implementation of PGD because of the two-step ethics examination. In addition, the genetic counseling system in Japan is still insufficientdue to the lack of medical geneticists and certified genetic counselors. Thus, we need to establish the system as quickly as we can.Finally, the introduction of the microarray-based PGD for chromosomal abnormalitiesshould be considered in the technical aspect **[17, 18].** To date, studies investigating neonatal outcomes following PGD have reported reassuring findings **[19, 20].** However, long-term effects of embryo biopsy have not been clarified. It is necessary to investigate the health of infants born following PGD to assess long-term effects of embryo biopsy. We wish to clarify the long-term negative effects of PGD in future by continuing investigation. Though the JSOG has so far approved only PGD,

clinical studies on preimplantation genetic screening (PGS) are likely to be launched by the JSOG. Behind its decision is the fact that the technical level of PGS has been improved remarkably, and that an increasing number of people have been calling for application of the latest genetic profiling.

Conclusion:

We have established a basis for gathering continuous PGD data from which the future status of PGD implementation can be tracked in Japan. Moreover, this system can be used to track the health of infants born following PGD. These data represent a valuable information resource with regard to the clinical application of PGDnot only from the social and ethical aspects but also from the technological and medical aspects.

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Supplementary material:

Table 1: Summary of the PGD data for monogenic diseases.

Indications	DMD	DM1	OTCD	FCMD	ALD	Leigh syndrome	Total
Cycles to OR	59	31	6	1	1	1	99
Number infertile	24	9	1	1	1	1	37
Female age (average)	38	36	42	38	41	39	38
Cancelled before IVF/ICSI	1	1	0	0	0	0	2
ART method							
IVF	0	0	0	0	0	0	0
ICSI	58	28	6	1	1	1	95
IVF+ICSI	0	0	0	0	0	0	0
Unknown	0	2	0	0	0	0	2
Cancelled after IVF/ICSI	1	0	1	0	0	0	2
Cycles to PGD	57	30	5	1	1	1	95
Zona breaching							
AT drilling	0	0	0	0	0	0	0
Laser drilling	57	23	0	1	1	1	83
Mechanical	0	0	0	0	0	0	0
Laser drilling + Mechanical	0	0	5	0	0	0	5
Unknown	0	0	0	0	0	0	0
Biopsy method							
Polar body biopsy	0	0	0	0	0	0	0
Cleavage aspiration	57	23	5	1	1	1	88
Cleavage extrusion	0	0	0	0	0	0	0
Blastocyst	0	0	0	0	0	0	0
Indications	**DMD**	**DM1**	**OTCD**	**FCMD**	**ALD**	**Leigh syndrome**	**Total**
Embryology							
COCs (mean / OR)	614 (10.4)	360 (11.6)	52 (8.7)	8 (8.0)	13 (13.0)	3 (3.0)	1050 (10.6)
Fertilized	395	224	29	5	7	1	661
Biopsied	331	186	25	4	7	1	554
Frozen before biopsy	0	0	0	0	0	0	0
Successfully biopsied	330	174	25	4	7	1	541
Diagnosed (mean / OR)	254	101	22	-	5	1	383
Transferable (mean / OR)	175	48	12	-	5	0	240
Transferable rate (% / diagnosed)	68.9	47.5	54.5	-	100	0	62.7
Transferred	125	30	7	-	5	0	167
Clinical outcome							
Cycles to ET(% / OR)	90 (153.0)	19 (61.3)	5 (83.3)	0 (0.0)	3 (300.0)	0 (0.0)	117.0 (118.2)
hCG positive	14	3	0	0	2	0	19
Positive FHB	12	1	0	0	1	0	14
Number of FHB	13	1	0	0	1	0	15
Clinical pregnancy rate(% / OR)	20.3	3.2	0	0	100	0	14.1
Clinical pregnancy rate(% / ET)	13.3	5.3	0	0	33.3	0	12.0
Implantation rate (FHB / embryos transferred)	10.4	3.3	0	0	20	0	9.0
Deliveries	9	0	0	0	1	0	10
Miscarriages	5	2	0	0	1	0	8
Ectopic pregnancies	0	1	0	0	0	0	1
Twins	1	0	0	0	0	0	1

DMD, Duchenne muscular dystrophy;DM1, myotonic dystrophy type 1;OTCD, Ornithine transcarbamylase deficiency; FCMD, Fukuyama congenital muscular dystrophy;ALD, Adrenoleukodystrophy; OR, oocyte retrieval; AT, acid Tyrode's solution; COC, cumulus–oocyte complex; ET, embryo transfer; ART, assisted reproduction technology; FHB, fetal heart beat.

Table 2: Summary of the PGD data for chromosomal abnormalities.

Indications	Rob, male carrier	Rob, female carrier	Rcp, male carrier	Rcp, female carrier	Total
Cycles to OR	13	48	179	332	572
Number infertile	4	16	67	87	174
Female age (average)	43	39	39	39	40
Cancelled before IVF/ICSI	0	2	8	14	24
ART method					
IVF	0	11	61	76	148
ICSI	13	37	105	239	394
IVF+ICSI	0	0	5	3	8
Unknown	0	0	0	0	0
Cancelled after IVF/ICSI	1	5	11	20	37
Cycles to PGD	12	41	160	298	511
Zona breaching					
AT drilling	0	1	5	13	19
Laser drilling	10	20	61	156	247
Mechanical	2	20	94	129	245
Unknown	0	0	0	0	0
Biopsy method					
Polar body biopsy	0	0	0	3	3
Cleavage aspiration	10	38	130	262	440
Cleavage extrusion	2	3	30	33	68
Blastocyst	0	0	0	0	0
Indications	**Rob, male carrier**	**Rob, female carrier**	**Rcp, male carrier**	**Rcp, female carrier**	**Total**
Embryology					
COCs (mean / OR)	63 (4.8)	350 (7.3)	1322 (7.4)	2405 (7.2)	4140 (7.2)
Fertilized	34	225	922	1563	2744
Biopsied	11	166	636	1009	1822
Frozen before biopsy	13	0	80	228	321
Successfully biopsied	11	166	635	1007	1819
Diagnosed (mean / OR)	11	148	626	925	1710
Transferable (mean / OR)	3	42	177	214	436
Transferable rate (% / diagnosed)	27.3	28.4	28.3	23.1	25.5
Transferred	2	23	114	153	292
Clinical outcome					
Cycles to ET (% / OR)	2 (15.4)	16 (33.3)	83 (46.4)	115 (34.6)	216 (37.8)
hCG positive	0	5	45	51	101
Positive FHB	0	4	40	33	77
Number of FHB	0	5	41	34	80
Clinical pregnancy rate (% / OR)	0	8.3	22.3	9.9	13.5
Clinical pregnancy rate (% / ET)	0	25	48.2	28.7	35.6
Implantation rate (FHB / embryos transferred)	0	21.7	36.0	22.2	27.4
Deliveries	0	3	28	29	60
Miscarriages	0	2	12	22	36
Ectopic pregnancies	0	0	0	2	2
Twins	0	1	1	1	3

Rob, Robertsonian translocation; Rcp, Reciprocal translocation; OR, oocyte retrieval; AT, acid Tyrode's solution; COC, cumulus-oocyte complex; ET, embryo transfer; ART, assisted reproduction technology; FHB, fetal heart beat.

Table 3: Prenatal and postnatal diagnosis.

Methods	Total	Results		
		Normal	Abnormal	Failed
Prenatal diagnosis				
Amniocentesis	26	26	0	0
CVS	0	0	0	0
Cordocentesis	1	1	0	0
Unexamined	30			
Unknown	13			
Postnatal diagnosis				
Karyotype	3	3	0	0
DNA test	1	1	0	0

CVS, chorionic villus sampling.

Table 4: Method of delivery, gestational age, and infants born.

	Singletons	Twins	Total
No. deliveries	65	5	70
Method of delivery			
Vaginal	32	0	32
Caesarian	26	5	31
Unknown	7	0	7
Term at delivery			
Preterm	6	4	10
Term	57	1	58
Post term	0	0	0
Unknown	2	0	2
Sex			
Male	30	2	32
Female	32	8	40
Unknown	3	0	3
Mean birthweight (g)	2964	2356	2919

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N- and O-glycan cell surface protein modifications associated with cellular senescence and human aging

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Abstract

Background: Glycans play essential roles in biological functions such as differentiation and cancer. Recently, glycans have been considered as biomarkers for physiological aging. However, details regarding the specific glycans involved are limited. Here, we investigated cellular senescence- and human aging-dependent glycan changes in human diploid fibroblasts derived from differently aged skin donors using a lectin microarray.

Results: We found that α2-6sialylated glycans in particular differed between elderly- and fetus-derived cells at early passage. However, both cell types exhibited sequentially decreasing α2-3sialylated *O*-glycan structures during the cellular senescence process and showed similar overall glycan profiles.

Conclusions: We observed a senescence-associated decrease in sialylation and increase in galactose exposure. Therefore, glycan profiling using lectin microarrays might be useful for the characterization of biomarkers of aging.

Keywords: Cellular senescence, Human aging, Glycan, Lectin microarray

Background

The cell surface is covered with various glycoproteins, which play crucial roles in biological functions such as cell–cell adhesion, maintenance of protein structure, and molecular recognition. Dynamic changes in cell surface glycosylation regulate cellular function during development, differentiation, and survival. Recently, glycans have been considered as biomarkers for physiological aging. Certain *N*-glycans of IgG and α1-antitrypsin have been associated with chronological age and the physiological parameters of inflammation or cardiovascular disease [1, 2]. In addition, *N*-glycan alteration associated with age and gender has been reported [3]. However, details of the changes of specific glycans including both *N*- and *O*-glycan forms on glycoproteins upon cellular senescence and their biological functions are unclear. Therefore, investigation of the cell surface glycan changes during the senescence process will be helpful to better understand their biological function in human aging.

Various human diploid fibroblasts have been used as model systems of cellular senescence. A series of human diploid fibroblasts (TIGs) have been well characterized with respect to morphological alteration, chromosome constitution, cellular life span, telomere attrition and length, cellular protein content, and glycosylation [4–8]. In addition, changes in the cell surface glycans of several of these lines during the senescence process have been analyzed using lectin, demonstrating a decrease of α2-6sialylation of *N*-glycan in senescent TIG-3 lung fibroblasts in vitro [9]. Furthermore, it has been suggested that the cell surface sialic acid level in senescent WI-38 human fetal lung diploid fibroblasts is low, and a great amount of sialic acid is transferred to asialo acceptors in the absence of exogenous acceptors as measured by a sialyltransferase assay [10]. The change of cell surface glycan composition during cellular senescence has also been demonstrated on the basis of lectin affinity in the human fetal lung fibroblast lines HSC172 and IMR-90 [11, 12]. Further, it has been suggested that the surface glycans of IMR-90 control both cell growth and function because they

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were observed to change prior to morphological alteration [13]. Based on these reports, it appears that various glycan changes on lung fibroblasts are associated with aging. However, these data reflected only partial analysis of glycosylation, examining alternately *N*- and *O*-glycan, and selected stepwise-aged cells. In order to analyze the glycome of cells, lectin microarrays have been developed [14, 15]. These arrays represent an emerging technology that can be applied to the ultrasensitive detection of multiplex lectin-glycan interactions [16–18]; such glycan profiles, for example, have been used to distinguish the developmental stage and differentiation of various cells [19–23].

In this study, we compared consecutive cell surface glycan profiles of three human skin diploid fibroblast lines (the fetus-derived fibroblasts TIG-3S and the elderly-derived fibroblast lines TIG-101 and TIG-102), during extended cell culture. In addition, we identified specific glycan profiles associated with cellular senescence and human aging. Clarification of the senescence-dependent glycan profile specific to each derived cell type will contribute to a better understanding of the aging process of the skin at the cellular level.

Results

Cell growth rate and morphological change of fetus-derived TIG-3S cells

To observe the cell lifespan and growth rate of the TIG-3S line, we investigated cellular proliferation under stable conditions. Figure 1a shows the growth curve for TIG-3S

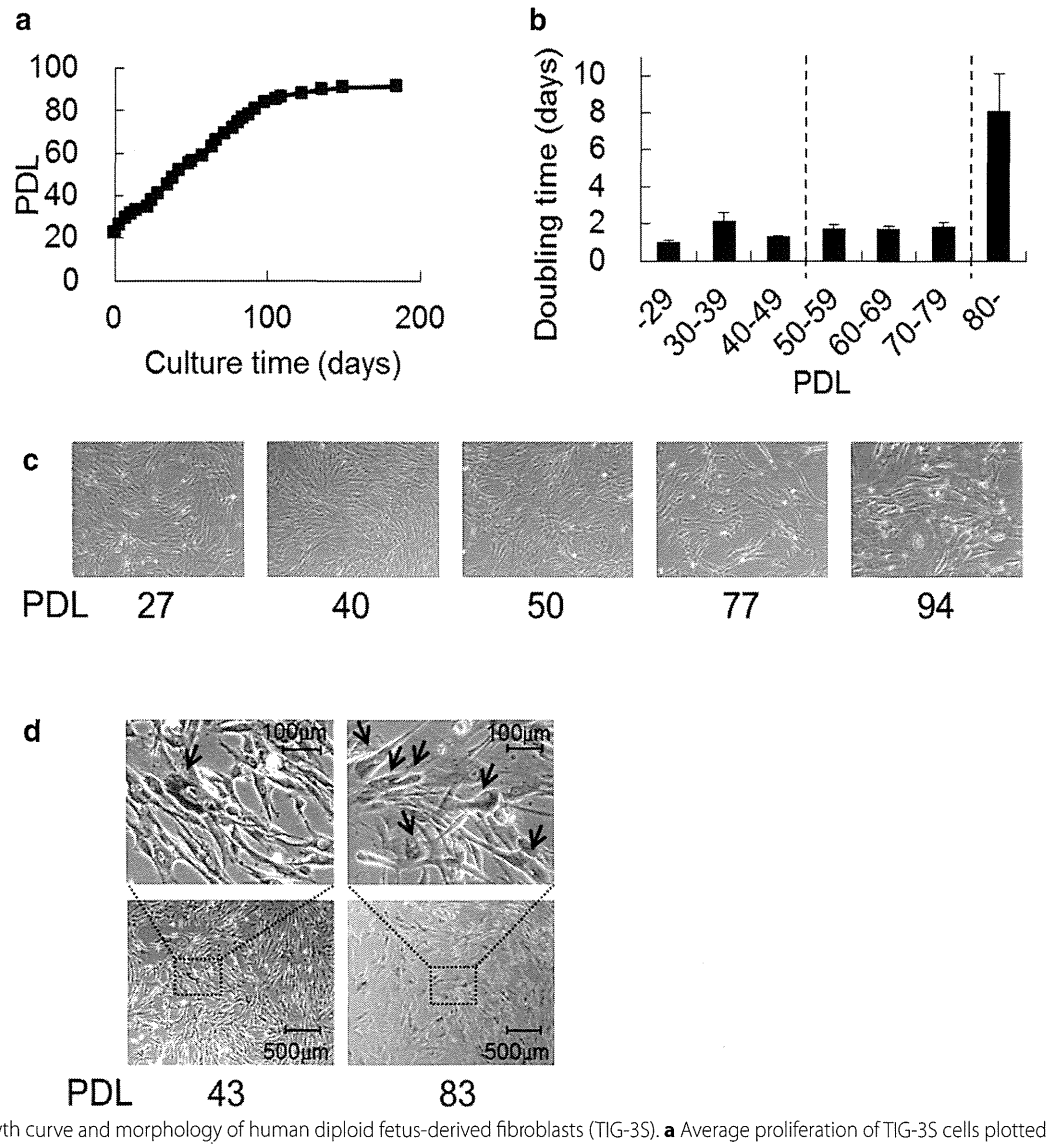

Fig. 1 Growth curve and morphology of human diploid fetus-derived fibroblasts (TIG-3S). **a** Average proliferation of TIG-3S cells plotted as PDL for approximately 180-day culture (n = 3). **b** *Bar graph* representation of the average doubling time of the cells at each PDL (mean + SE, n = 4–22). **c** Cell shapes are shown at PDLs 27, 40, 50, 77, and 94. **d** TIG-3S cells at PDLs 43 and 83 were stained with SA-β-galactosidase. The *upper panels* are a magnification of the squared area in the *lower panels*. The *arrows* indicate stained cells

(n = 3). Growth arrest was observed in the cells over population doubling level (PDL) 80 for 100-day culture. The doubling time of the TIG-3S line at early passages (PDL 27–50) was 1–2 days (Fig. 1b). At 80 % confluence, the cells exhibited an elongated shape (Fig. 1c, PDL 27, 40, and 50). Conversely, at late passages (>PDL 80), confluence was reached after a few weeks. The average doubling time after PDL 80 (n = 22) became approximately eight times as long as that observed during initial culture (Fig. 1b). At late passage, the cells appeared flat and expanded (Fig. 1c, PDL 94). In addition, they exhibited more senescence-associated (SA-)β-galactosidase activity than did early-passage cells, indicating that cellular senescence was induced (Fig. 1d).

Cellular senescence-dependent changes of cell surface glycans in TIG-3S cells

To investigate the glycan profiles associated with each PDL in TIG-3S fibroblasts, lectin microarray analysis was performed (Table 1). Figure 2a shows the heat map of TIG-3S lectin microarray signals, indicating that the signal intensities of some lectins significantly increased with passage. The signal intensity of WFA (Galβ1-3GalNAc- and GalNAcβ1-4GlcNAc-binder) gradually increased during cellular senescence process (Fig. 2b; Table 1). WFA is well known as a binder recognizing *O*-glycan. Signal intensities of three lectins, MPA (Galβ1-3GalNAc-binder), MAL-I (Siaα2-3Galβ1-4GlcNAc-binder), and Calsepa (High-Man- and Glc-binder) rapidly increased in late-passage cells (Fig. 2b; Additional file 1: Figure S1). Signal intensities of four other lectins, BPL (Galβ1-3GlcNAc-binder), TJA-II (Fucα1-2Galβ-binder), ECA (Galβ1-4GlcNAc-binder), and PHA-L (tri- and tetra-antennary complex type *N*-glycan-binder) slightly but significantly increased from middle passage (>PDL 50); these recognized *N*-glycan (Fig. 2b; Additional file 1: Figure S1). As the affinities of ECA and PHA-L increase with the branching number, the enhanced signals of ECA and PHA-L at middle and of MAL-I at late passages suggested that the large-antennary *N*-glycan increased, followed by a slight increase of the α2-3sialilated *N*-glycan form during cellular senescence process. Furthermore, the elevated WFA and MPA signals suggest that *O*-glycans such as the Galβ1-3GalNAc structure on the cell surface increased during cellular senescence as well.

Cell growth rate and morphological change of elderly-derived TIG-101 and TIG-102 cells

To compare the characteristics of fetal and elderly-derived adult cells, the growth rates of TIG-101 and TIG-102 cultures were observed. TIG-101 grew slowly, reaching approximately PDL 50 after 130-day culture, and TIG-102 reached approximately PDL 50 after 95-day culture (Fig. 3a). For both lines, the cells at approximately PDL 60 were in a state of growth arrest, suggesting cellular senescence. In fact, the average doubling time over PDL 50 (TIG-101: n = 5; TIG-102: n = 9) for both lines was about three times as long as that at PDLs 32–39 (Fig. 3b). In addition, although TIG-101 and TIG-102 exhibited spindle shapes at PDL 40, both cell types were flat after PDL 50 (Fig. 3c). TIG-102 was slightly stained with SA-β-galactosidase at PDL 45, but at late passage (>PDL 50) SA-β-galactosidase activity increased (Fig. 3d). However, although the growth of elderly-derived cell was slow, the early-passage cells (<PDL 40) were not senescence. These results suggested that senescence was substantively initiated for each elderly-derived cell line over PDL 50.

Cellular senescence-dependent changes of cell surface glycans in TIG-101 and TIG-102 fibroblasts

To investigate the glycan profiles associated with each PDL in TIG-101 and TIG-102 cells, lectin microarray analyses were performed (Table 1). Figure 4a shows the heat map of lectin microarray signals for both lines. The signal intensities of *O*-glycan-binders such as SBA (GalNAc-binder) and VVA (GalNAc-binder), and those of large-antennary *N*-glycan-binders such as PHA-L and ECA, initially decreased and then slightly increased in both lines (Fig. 4b; Additional file 2: Figure S2; Table 1). The signal intensities of *N*-glycan-binders such as TxLC-I [Manα1-3(Manα1-6)Man- and GalNAc-binder], and those of *O*-glycan-binders such as ACA (Galβ1-3GalNAc-binder) and MAH (Siaα2-3Galβ1-3GalNAc-binder) decreased with passage. The signal intensity of WFA initially decreased and then increased slightly compared to early-passage levels. These results suggested that the large-antennary *N*-glycan form and *O*-glycans such as the Tn-antigen (GalNAc-Ser/Thr) were decreased; however their glycan profile changes were not greatly.

Comparison of cell surface glycans between fetus- and elderly-derived cells

To examine whether the changes of total glycan profiles during the cell passage process correlated with cell source age, the microarray data for TIG-3S, TIG-101, and TIG-102 were compared (Additional file 3: Figure S3). Hierarchical clustering analysis of the total glycan profiles revealed that TIG-3S and TIG-101/TIG-102 had individual glycan characters, whereas the glycan character of TIG-3S increasingly resembled those of TIG-101 and TIG-102 with increasing passage number (Additional file 4: Figure S4). Figure 5 presents the principal component analysis (PCA) results for 24 lectins in a biplot. PC3 appeared to correlate to cellular passage in all three lines. The positions of each PDL in the PC3 axis show the degree

Table 1 Lectin microarray data of TIG-3S, TIG-101, and TIG-102

Lectin/PDL	TIG-3S (%)									TIG-101 (%)					TIG-102 (%)				
	27	40	43	50	57	65	77	89	94	40	41	43	46	51	40	43	47	49	52
LTL	0.2	0.1	0.1	0.2	0.2	0.3	0.2	0.3	0.2	0	0.1	0	0.1	0.1	0.2	0.2	0.1	1.4	0.1
PSA	13.7	14.4	11.3	13.5	11.5	13.1	14.8	17.5	19.6	29.4	24.5	18.8	20.4	24.9	32.1	26.6	14.7	22.0	21.6
LCA	17.6	19.9	16.8	16.9	14.7	16.3	19.4	20.4	21.3	26.0	26.0	20.9	22.3	22.9	31.2	29.7	15.8	23.5	20.2
UEA-I	0.1	0.1	0.1	0.1	0	0.3	0.1	0.2	0.1	0	0.1	0	0.1	0	0.1	0.2	0.1	0.5	0
AOL	8.0	8.8	8.0	8.6	8.3	8.8	9.2	11.7	13.6	11.2	17.8	11.5	15.9	11.7	12.5	18.7	9.0	14.2	8.3
AAL	10.9	11.2	12.4	12.8	12.6	12.9	11.7	14.8	16.9	23.3	19.8	14.6	18.1	20.2	20.8	17.7	10.9	14.9	15.0
MAL-I	9.7	10.1	8.8	8.6	11.0	11.9	12.6	15.3	22.5	25.7	19.6	12.5	17.9	19.0	26.9	21.1	12.5	18.6	18.0
SNA	19.7	17.7	17.7	14.5	16.0	19.3	15.2	11.4	22.0	10.0	8.9	14.4	10.4	13.2	10.5	11.3	8.3	11.0	11.8
SSA	15.6	15.4	14.5	13.2	13.7	16.1	12.8	12.1	21.5	9.9	8.1	12.0	9.2	12.0	11.8	11.6	8.0	11.1	12.5
TJA-I	35.6	30.5	34.2	32.6	33.4	36.3	29.6	26.4	39.3	24.7	20.3	30.4	23.3	28.7	24.6	25.8	21.4	25.7	28.2
PHA-L	3.4	4.2	3.6	3.4	4.5	5.8	5.5	6.2	7.2	11.2	5.7	4.2	5.3	8.8	12.4	7.4	4.2	6.8	8.4
ECA	1.8	2.4	2.4	2.3	3.1	3.9	3.6	4.3	4.5	9.7	6.4	5.3	5.8	7.0	11.2	8.5	4.8	8.0	8.0
RCA120	22.9	26.3	28.0	28.6	29.3	27.8	29.6	29.9	25.5	25.9	27.9	28.5	26.0	27.0	28.6	29.1	26.0	26.6	27.8
PHA-E	40.2	38.3	38.3	36.9	41.1	35.4	36.5	37.0	37.5	36.5	38.3	36.9	38.1	38.2	38.9	37.4	31.2	34.1	29.8
DSA	100	99.7	94.7	95.9	100	100	96.7	100	100	100	100	97.5	100	100	100	100	100	100	100
GSL-II	0.3	0.2	0.2	0.1	0.2	0.4	0.4	0.4	0.3	0.5	0.5	0.1	0.4	0.2	0.4	0.5	0.2	0.4	0.3
NPA	46.7	50.2	44.7	45.6	40.6	41.5	52.1	44.7	45.6	49.7	57.8	59.7	52.5	50.7	58.1	58.0	45.1	49.4	46.0
ConA	16.4	18.3	16.4	17.7	14.4	14.4	20.3	20.8	20.7	27.3	34.4	30.7	32.9	25.0	32.0	39.3	26.6	32.1	24.1
GNA	44.4	44.8	39.7	47.7	40.8	39.7	51.5	56.4	60.1	40.2	45.8	41.5	40.8	44.0	49.5	45.6	30.5	40.4	38.1
HHL	26.6	29.6	25.3	30.8	26.6	27.8	33.3	35.8	40.9	43.9	49.3	46.1	39.9	45.8	50.0	47.5	33.1	42.9	41.8
ACG	81.3	81.8	88.5	83.3	77.2	63.4	71.0	51.6	49.9	39.1	51.9	59.4	48.8	47.5	45.1	50.9	56.3	47.5	47.9
TxLC-I	11.8	11.2	11.0	10.1	12.6	11.4	9.1	10.2	9.9	18.4	17.0	15.4	15.9	15.0	17.0	15.2	9.5	11.0	8.8
BPL	1.8	2.0	2.1	2.4	3.0	3.8	3.5	5.2	4.9	6.8	4.1	3.7	3.8	5.6	6.5	4.2	2.7	5.2	5.6
TJA-II	3.5	3.0	4.4	4.7	5.3	7.6	5.7	7.0	8.8	8.4	6.2	6.3	5.5	7.5	9.2	7.2	5.6	7.6	9.1
EEL	0.4	0.2	0.3	0.2	0.2	0.6	0.7	0.5	0.5	0.7	0.4	0.8	0.5	0.3	0.6	0.4	0.3	0.4	0.3
ABA	15.0	15.9	16.7	15.3	14.8	18.1	20.2	19.7	22.3	22.5	20.4	17.0	19.3	18.2	26.5	23.3	15.7	19.7	17.9
LEL	91.5	96.3	94.6	97.5	85.9	82.2	98.2	90.1	87.6	86.8	92.3	95.2	86.3	77.5	82.4	86.9	83.7	74.3	73.5
STL	50.8	45.6	46.8	48.1	47.1	46.0	43.0	48.2	54.9	66.2	60.6	58.2	56.3	57.1	56.5	55.2	54.3	52.9	53.0
UDA	63.5	70.1	57.4	64.5	56.4	52.3	60.5	54.9	57.0	56.9	69.3	76.0	65.6	64.9	64.4	67.6	70.1	66.5	64.0
PWM	2.9	3.2	2.7	3.1	4.0	4.4	5.0	6.9	9.2	15.8	13.7	14.1	11.7	13.6	14.5	12.3	8.7	11.2	11.5
Jacalin	15.3	15.9	18.1	18.0	16.5	19.5	22.7	24.3	23.3	28.1	26.8	22.7	26.1	24.5	29.4	28.3	19.5	23.1	21.8
PNA	0	0	0	0	0	0	0	0	0.1	0	0	0	0	0	0.1	0.1	0	0.1	0
WFA	3.3	5.0	5.9	6.7	10.9	11.7	10.0	14.7	12.9	13.9	8.6	9.0	8.5	14.2	14.9	10.4	8.1	12.1	16.5
ACA	3.9	4.3	3.6	3.5	2.9	4.4	4.8	5.1	4.5	5.9	4.1	2.7	4.2	3.6	6.5	5.0	2.7	4.2	3.1
MPA	4.1	4.7	4.1	4.0	4.3	5.7	6.7	8.9	12.0	16.0	11.7	7.7	9.8	11.2	14.6	10.7	6.1	9.1	9.4
HPA	0.1	0	0	0	0	0	0	0.1	0	0.3	0.1	0	0	0	0.2	0.2	0.1	0.1	0
VVA	0.2	0.1	0.1	0.1	0.2	0.4	0.4	0.4	0.5	0.7	0.2	0	0.1	0.2	0.5	0.1	0	0.1	0.2
DBA	0	0	0	0	0	0	0.1	0	0	0	0	0	0	0	0.1	0.1	0	0.4	0
SBA	0.1	0.1	0.1	0	0.1	0.3	0.2	0.2	0.3	0.6	0.2	0	0	0.2	0.6	0.3	0.1	0.2	0.7
Calsepa	18.1	19.4	16.3	19.2	15.0	17.5	22.5	27.2	31.4	36.2	35.9	31.7	32.4	34.4	40.4	38.5	23.9	29.8	28.6
PTL-I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MAH	2.9	2.9	2.6	2.3	2.1	2.6	2.9	2.9	2.4	9.7	5.9	4.2	6.5	4.5	9.8	7.7	5.3	6.5	3.8
WGA	53.6	52.9	50.2	50.3	50.9	45.9	49.7	37.5	36.9	30.9	36.8	36.4	31.7	33.6	33.4	31.6	34.1	30.6	32.6
GSL-IA4	0.1	0.1	0	0	0	0.2	0.1	0.1	0	0	0.1	0	0	0	0.1	0.1	0.1	0.1	0.1
GSL-I B4	0.1	0	0	0	0	0.1	0.1	0.1	0	0	0	0	0	0	0	0.1	0	0	0

Each cell line at the indicated population doubling levels (PDLs) was applied for lectin microarray analysis