Table 4
Blood and urine acylcarnitines in cases with a blood carnitine deficit

Case	Age	Cr (mol/l)	Urine (mmol/mol creatinine)					Blood (nM)		Carnitine clearance (%)
			AC	C0	C2	C5	C5-OH	C0	Acyl carnitine (increase)	
Carnitine transporter defect										
45	2y0m	1.21	6.34	22.15	29.27	0.27	0.22	8.00		4.30
Secondary carnitine deficiency antibiotics (cephem pivoxil)										
46-1	2y1m	3.32	22.41	0.79	0.53	21.17	0.08	0.73	C5(1.12)	1.40
46-2	2y2m	2.58	2.45	1.58	0.97	0.12	0.11	9.78	C5(0.13)	0.30
47	4y2m	3.72	82.59	0.53	0.81	80.83	0.18	0.50	C5(2.13)	0.12
malnutrition (severe milk allergy)										
48	5m	5.36	10.01	1.72	0.75	0.08	6.81	8.59	C5-OH(3.72)	0.10
Reference value										
1–5 years			<22.84	5.67 −56.09	<60.48	<0.89	<0.54	20 −80	See*	2.00

* Reference values of blood acylcarnitines: C5-OH, <1; C5, <1.0 42-1 and 42-2 are the same case. Values over cut-off are underlined.

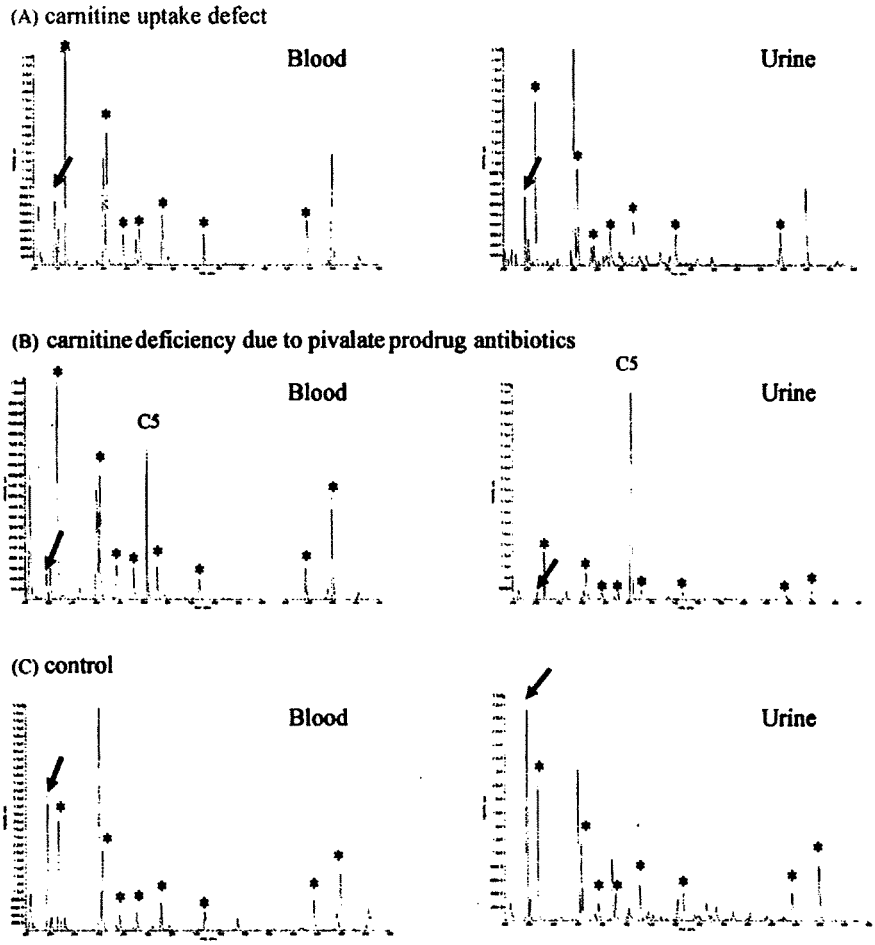

Fig. 2. Blood and urine acylcarnitine profiles in carnitine deficiency (cases 45 and 46). Patient with CUD (A) excreted a large amount of free carnitine in urine (arrows) compared to free carnitine in blood. Following treatment with pivalate prodrug antibiotics (B), patients with secondary carnitine deficiency showed a large peak for pivaloylcarnitine in both blood and urine, whereas only a very small amount of free carnitine was present in urine. (C) Control data from a 2-year-old child. (*) Peaks from the mixture of internal standards.

chemical diagnosis of GA1. Increased excretion of C5DC in urine was observed in at least three cases in the current study.

Diagnostic acylcarnitine markers for each OA showed an increase with carnitine loading. Even in the mild form of MMAemia, the levels of C3 and C3/C16 increased in urine after carnitine loading, despite the absence of abnormalities in blood. Therefore, in cases of OAs with ambiguous metabolic profiles or borderline marker levels, urinary acylcarnitine analysis may be helpful for confirmation of diagnosis, particularly after carnitine loading. In FAODs, urinary acylcarnitine profiles in SCAD deficiency and MCAD deficiency were similar to blood acylcarnitine profiles. Additionally, it appears unlikely that urinary acylcarnitine analysis will be useful for cases of long-chain fatty acid disorders including VLCAD deficiency, TFP deficiency or CPT2 deficiency.

In MS/MS screening, a free carnitine deficit was found in four patients who presented clinically with an acute encephalopathy-like illness. In such cases, urinary acylcarnitine analysis was helpful for differential diagnosis of disorders. Carnitine treatment is important in cases with systemic carnitine deficiency, whereas administration of drugs [15,16] or nutritional approaches should be reconsidered in other cases. MS/MS screening of samples from newborns has the potential to detect OAs, FAODs and amino acid disorders in pre-symptomatic stage, and early diagnosis and intervention is essential for a favorable outcome in such children. Precise diagnosis of disease or clinical types is also required, and other diagnostic tools, including urinary organic acid analysis, enzyme determination, or molecular analysis, should also be part of the screening system. Our results suggest that urinary acylcarnitine analysis is also useful for evaluation of some OAs and FAODs, and further investigation of urinary acylcarnitines in more cases and other disorders will determine the significance of this approach. Latest manuscripts [17,18] promote measurement of acylcarnitines without derivatization. Current method remains potential to develop simpler method by non-derivatized method. Further investigation will also be needed.

Acknowledgements

We are grateful to Mrs. Kimoto for her technical assistance. This study was partly supported by grants from The Ministry of Health, Labor and Welfare of Japan, from the Ministry of Education, Culture, Sports, Science and Technology, and from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

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Comprehension of abstract words among hearing impaired children

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Abstract word;
SCTAW;
Academic language

Summary

Introduction: This study examines the ability and development in the comprehension of abstract words with hearing impaired children. The ability to understand abstract words is quite important for their academic learning and adaptation in their school life. Here, we qualitatively and quantitatively analyzed the development of abstract vocabulary in hearing impaired children using The Standardized Comprehension Test for Abstract Words (SCTAW).
Subjects and methods: We examined 75 hearing impaired children (hearing aid users, 61; cochlear implant users, 14; 1st to 10th grade) and 188 children with normal hearing (1st to 6th grade) using the Picture Vocabulary Test (PVT) and SCTAW.
Results: The PVT and SCTAW results closely correlated ($r = 0.87$). The SCTAW scores of the hearing impaired group were lower than those of their peers with normal hearing, but the scores improved as their school grade advanced. In particular, their abstract ability began to catch up from the fifth grade. The error trends of abstract vocabulary in the two groups did not significantly differ.
Conclusions: The SCTAW was useful as an abstract lexical evaluation of hearing impaired children. The development of an abstract vocabulary did not qualitatively differ between children with or without a hearing impairment.

1. Introduction

Prelingual hearing impairment can secondarily cause several different disabilities affecting hearing

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ability, speech intelligibility, as well as language development. Impaired language development may result in academic or occupational problems and easily hamper their quality of life especially after their adolescence period. Therefore, several different vocabulary testing can be available for this age group including Clinical Evaluation of Language Fundamentals, Fourth Edition (CELF-4), Peabody Picture Vocabulary Test (PPVT-III), Expressive One Word Picture Vocabulary Test, Third Edition (EOWPVT), Receptive One Word Picture Vocabulary Test (ROWPVT), Comprehensive Assessment of Spoken Language (CASL), and Reynell Developmental Language Scales III (RDLS III). One of the very important aspect of vocabulary is that include abstract concept. The ability to comprehend abstract words and ideas are essential in the development of academic language skills for hearing impaired children. Bebko [1,2] classified the characteristics of the levels of language proficiency into three levels and language of children starts from more experience-depending language proficiency (Levels 1 and 2), and later develops to abstract usage of language (Level 3 skills) by means of more intended learning process.

Because the acquisition of this abstract vocabulary plays an important role in development of their learning skills, comprehension of abstract words is essential for education in school-aged children. However, no suitable methods have been established to evaluate abstract vocabulary [3]. Recently, a new approach has been developed to examine the ability to comprehend abstract words. The Standardized Comprehension Test for Abstract Words (SCTAW), reported by Haruhara et al. [4], tests abstract words alone. This test enables the assessment of developmental delay or learning difficulties across a wide spectrum of the population, including children with hearing impairment. The present study is the first to evaluate the usefulness of this newly developed test.

In this study, we used the SCTAW to qualitatively and quantitatively evaluate the development of abstract vocabulary among hearing impaired children.

2. Patients and methods

2.1. Patients

Seventy-nine hearing impaired children were asked to enroll in this evaluation. After preliminary evaluation with the Raven Colored Progressive Matrix Test (RCPM), one child with pervasive developmental disorder (PDD) and three with significant mental

Table 1 Profiles of hearing aid users

Grade	CA	N	Hearing levels of better hearing ear			Hearing aids	
			Moderately severe	Severe	Profound	Analog	Digital
2	7	8	0	2	6	4	4
3	8	8	0	1	7	5	3
4	9	5	1	1	3	3	2
5	10	5	1	0	4	2	3
6	11	6	0	0	6	4	2
7	12	5	0	2	3	4	1
8	13	5	0	0	5	4	1
9	14	10	0	1	9	9	1
10	15	9	1	1	7	9	0
Total		61	3	8	50	44	17

Grade	Communication methods		Educational environment		
	Sign auditory–aural	Auditory–verbal	School for the deaf	Hard-of hear school	Mainstream
2	7	1	8	0	0
3	7	1	7	0	1
4	4	1	4	0	1
5	5	0	5	0	0
6	6	0	6	0	0
7	5	0	3	2	0
8	5	0	5	0	0
9	10	0	10	0	0
10	5	4	9	0	0
Total	54	7	57	2	2

retardation were excluded. The remaining 75 children (41, male; 20, female; 7–15 years old served as subjects. Characteristics of the cases are summarized in Table 1. Sixty-one children wore hearing aids (44 analogue and 17 digital). The communication and educational status of these children are also summarized in Table 1. Fourteen cochlear implant users were also enrolled in this study (7–15 years old); they included 12 children with prelingual deafness, which was due to meningitis in 2 children. Implants were Nucleus 22 or Nucleus 24 models in which all 22 electrodes could be stimulated. They had been implanted from the age of 3 years and 4 months to 14 years and 1 month. Communication and educational status of these children are summarized in Table 2. Controls comprised 188 school-aged children (aged 6–11 years) with neither hearing impairment nor developmental delay. Some findings from the hearing impaired cases have reported previously [5,6]. This project was approved by the Ethical committee for each of deaf school.

2.2. Vocabulary tests

All these evaluations were conducted by teachers of Okayama Deafness School.

Table 2 Profiles of cochlear implant users

	School grades at the first test	Age at deafness	Age at implantation	Pre/postoperative periods at the first test	Implant type and coding strategy	Additional handicap
A	2	0 year	3 years and 6 months	3 years and 8 months	CI24M ACE	
B	2	0 year	3 years and 4 months	3 years and 4 months	CI22M SPEAK	
C	2	0 year	5 years and 3 months	2 years and 10 months	CI24M ACE	Semantic disorder
D	2	0 year	7 years and 10 months	Preoperative: before 3 months	CI24M ACE	
E	2	0 year	8 years and 2 months	Preoperative: before 4 months	CI24M ACE	Mild mental retardation and ADHD
F	4	0 year	9 years and 6 months	0 year and 2 months	CI24M ACE	
G	4	0 year	8 years and 2 months	1 year and 7 months	CI24M ACE	
H	4	0 year	4 years and 9 months	5 years and 9 months	CI22M SPEAK	Mental retardation
I	4	0 year	9 years and 4 months	0 years and 3 months	CI24M ACE	
J	5	0 year	7 years and 2 months	4 years and 6 months	CI22M SPEAK	
K	7	0 year	7 years and 7 months	4 years and 10 months	CI22M SPEAK	Mental retardation dyslexia
L	8	0 year	12 years and 5 months	0 years and 4 months	CI24M ACE	
M	9	1 year and 5 months	4 years and 10 months	11 years and 10 months	CI22M SPEAK	
N	10	2 years and 0 month	14 years and 1 month	0 year and 11 months	CI24M ACE	

	Communication methods	Education environment
A	Auditory–verbal	Hard-of hear school
B	Auditory–verbal	Mainstream
C	Auditory–verbal	Hard-of hear school
D	Auditory–verbal	Deafness school
E	Sign auditory–aural	Deafness school
F	Auditory–verbal	Hard-of hear school
G	Auditory–verbal	Mainstream
H	Auditory–verbal	Hard-of hear school
I	Auditory–verbal	Mainstream
J	Auditory–verbal	Mainstream
K	Sign auditory–aural	Deafness school
L	Auditory–verbal	Hard-of hear school
M	Auditory–verbal	Hard-of hear school
N	Auditory–verbal	Deafness school

2.3. PVT

For comparison with other vocabulary tests, Picture Vocabulary Test (PVT) [7] was conducted in a one-on-one setting. The test words were presented both visually on cards and phonetically (read by the examiner) and the subject selected the most suitable of four pictures presented on cards (Fig. 1). Additional information was presented by cued speech or finger alphabet according to the subject's need. The test results of PVT in normal hearing children were drawn from the manual of PVT [7].

2.4. SCTAW

In The Standardized Comprehension Test for Abstract Words (SCTAW), six pictures are presented to examinees and they were asked to choose the most suitable picture for the presented word and marked the corresponding number in the response sheet individually (Fig. 2). The test words were presented both phonetically and visually in the present study, whereas the original report did not use both methods. Normal hearing controls were therefore subjected to the following analysis. One hundred eighty-nine collaborative elementary school children from first grade to sixth grade underwent SCTAW. For these hearing peer children, test words were simultaneously presented by projector (visual presentation) in the classroom and by the teacher's real-time voice (phonetic presentation).

In the hearing impaired group, the test was conducted both using a message board (visual presentation) and by real-time voice (phonetic presentation) in a one-on-one setting. For younger children, the test words presented visually were given in both Kana and Kanji letters. Otherwise, the methods strictly adhered to those of the original study.

3. Results

3.1. PVT

The results of PVT for each school grade are summarized in Fig. 3. Hearing aid users exhibited a strong correlation between school grade and vocabulary ($y = 0.9593x + 2.0726$, $r = 0.8846$, $p < 0.0001$). However, vocabulary age was generally lower than chronological age, particularly in the lower grades (the first and second grades) when the discrepancy was two to three years. Wide inter-personal variation was apparent from around the fourth grade, and a vocabulary surge was observed in the ninth grade. Cochlear implant users also demonstrated wide inter-personal differences in vocabulary age.

3.2. SCTAW in hearing peers

As school grade advanced, SCTAW test scores increased and mean scores correlated significantly

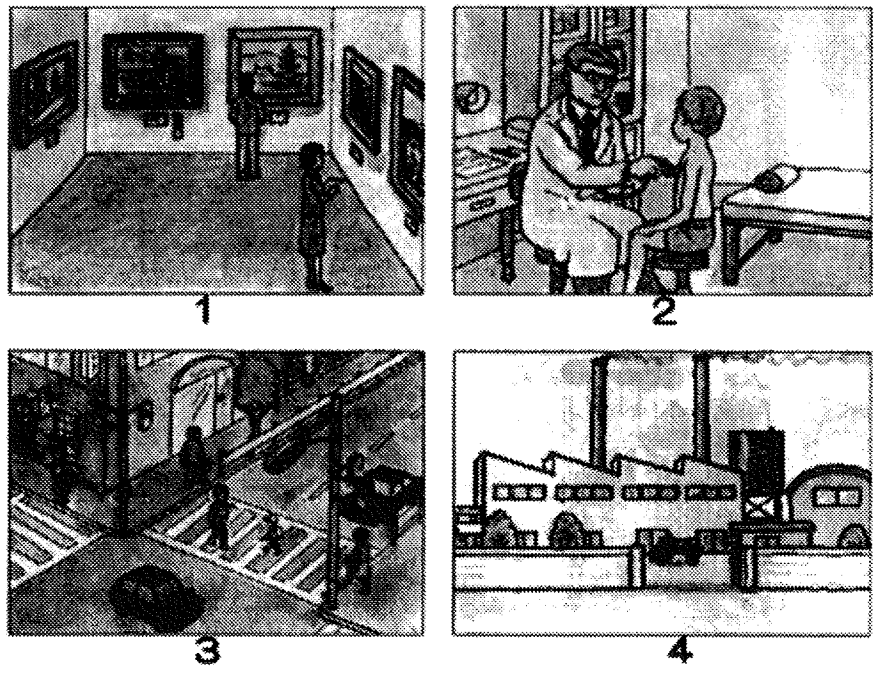

Fig. 1 An examples of Picture Vocabulary Test (PVT): children are asked to choose one most suitable picture for the stimulating word in one to four choices. The words used for this example are including "Art", "Industry", "Doctor", "Paintings", and "Production".

Example:

Presentation word [Japanese]	Phonological related stimulus		Semantic related stimulus		Irrelevant stimulus
Rescue	Stadium	School provided lunch	Submergence	Courtesy	Kettle
[Kyuu-jyo]	[Kyuu-jyou]	[Kyuu-syo-ku]	[Chin-bo-tsu]	[Sin-se-tsu]	[Ya-kan]

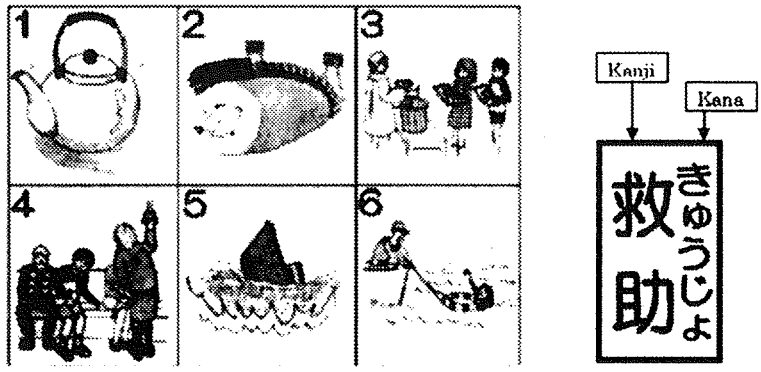

Fig. 2 An example of SCTAW: children are asked to choose one most suitable picture for the stimulating word in one to six choices. Right: examples of stimulating word, "*Kyuu-jyo*" (rescue in English) and left: one to six choice board.

with grade (Table 3, Fig. 4) ($y = 3.198x + 8.987$, $r = 0.84$, $p < 0.0001$). These findings were similar to those obtained in a trial of phonetically presented test words, as described in the original results. The error tendencies among collect answer, semantic mistake, phonological mistake, irrelevant answer or no choice were summarized in Fig. 5. "No choice" was observed 46% in first grade, 23% in second grade, 10% in third grade and less than 10% in older grades.

Error analysis revealed the following tendencies (Fig. 6). Generally, lower grade children were more prone to phonologic errors. As grade advanced, semantic mistakes became more frequent and this tendency was also similar to the original data. More precisely, phonologic mistakes

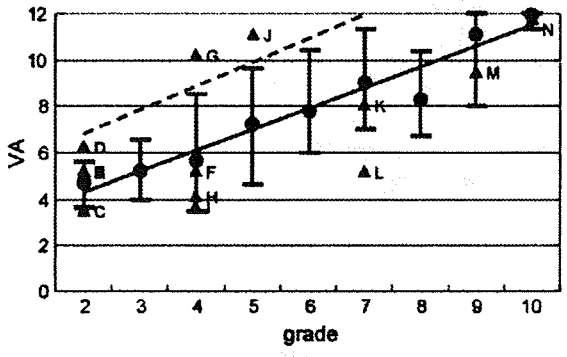

Fig. 3 PVT scores (vocabulary age: VA) and the grade among hearing aid users and cochlear implant users. Best (upper wing), worst (lower wing) and mean (closed circle) scores of hearing aid users were indicated in the figure. Cochlear implant users were indicated as triangle (▲). Two children (D and E) were classified as cochlear implant user, but both of them were actually hearing aid users at this point. They receive operations for cochlear implant during this study periods. Broken line indicated the regression line of PVT scores of hearing peers.

Table 3 SCTAW among hearing peers

Grade	N	Mean	S.D.	df	t-value	
1	33	10.636	3.855			
				66	5.950	$p < 0.0001$
2	35	16.029	3.618			
				66	4.140	$p = 0.0001$
3	33	19.515	3.308			
				60	4.490	$p < 0.0001$
4	29	23.172	3.071			
				59	1.910	$p = 0.0611$
5	32	24.719	3.235			
				56	2.595	$p = 0.0121$
6	26	26.846	2.935			

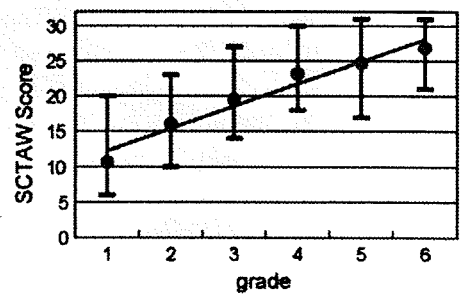

Fig. 4 The result of SCTAW in hearing peers. The best (upper wing), the worst (lower wing) and mean (closed circle) were demonstrated in the figure.

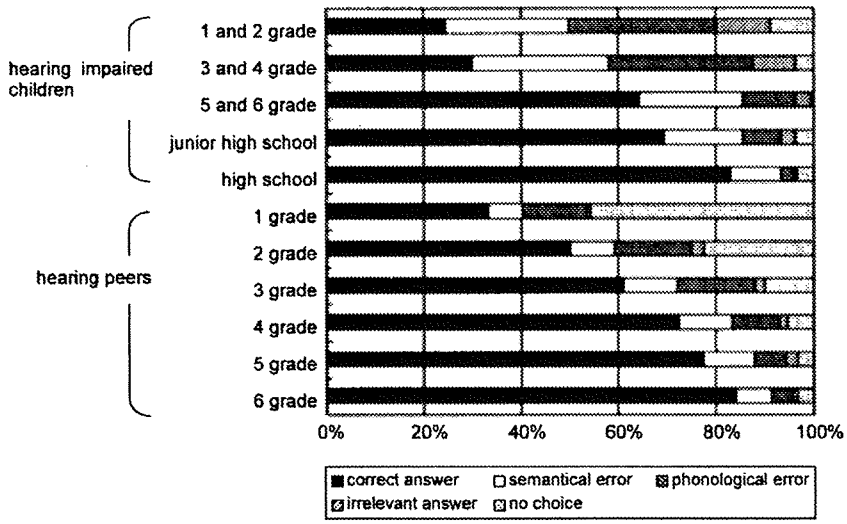

Fig. 5 Total results of SCTAW in hearing and hearing impaired children.

were more frequent than semantic mistakes among first grade to third grade students. On the other hand, nearly equal frequencies of phonologic and semantic mistakes were observed for fourth grade students, while semantic mistakes outnumbered phonological mistakes in fifth and sixth grade students.

These conditions were again consistent regardless of how test words were presented. As each kana letter corresponds to one monosyllabic resonant sound, we initially assumed that phonological mistakes might be detected less frequently, because sounds and kana letters were presented simultaneously. However, closer analysis revealed that the method of presenting the test words did not seriously affect the tendency for errors. For error analysis described later in this manuscript, the data originally obtained by Haruhara et al. [4] were used as standard data of SCTAW.

3.3. SCTAW in hearing impaired children

In hearing impaired children, a strong correlation between the results of PVT and SCTAW was observed obtained ($y = 2.313x + 0.843$, $r = 0.87$, $p < 0.0001$) (Fig. 7). Although lower than for their hearing peers, scores improved steadily as school grade advanced, with a surge observed in the fifth grade (Fig. 8). Indeed, SCTAW scores of some cochlear implant users noticeably caught up with those of their hearing peers. However, a ceiling effect was observed in the seventh to ninth grades. Interpersonal differences widened with advancing school grade; with the exception of the seventh grade.

Among cochlear implant users, three children (A, F, G) exhibited higher scores than the standard deviation of hearing aid users, while two children (H, K) demonstrated lower scores. The others had

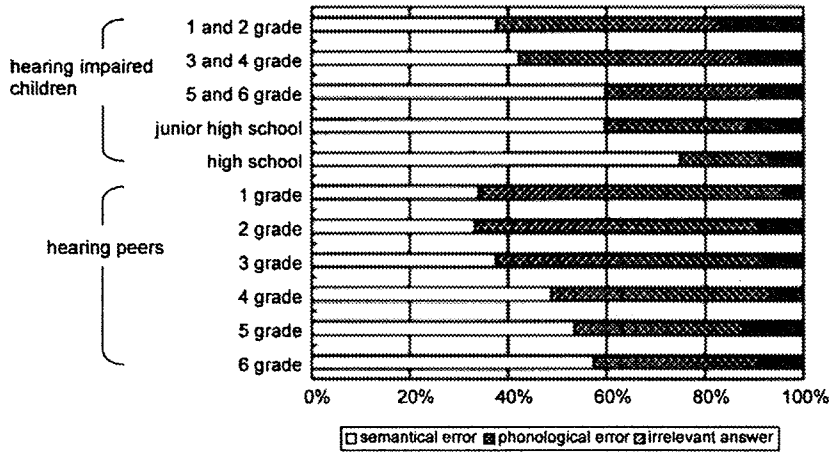

Fig. 6 Error analysis of SCTAW scores in hearing and hearing impaired children.

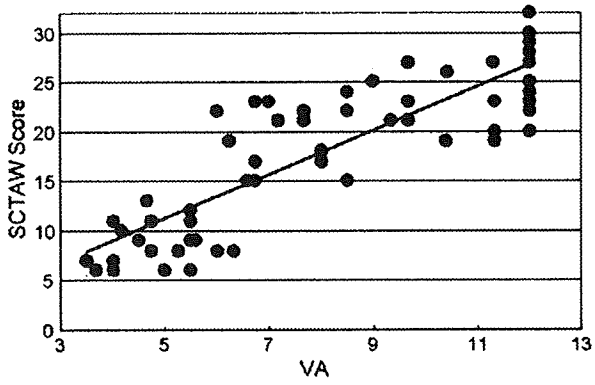

Fig. 7 Relationship between PVT (VA) and SCTAW in hearing impaired children.

similar results to hearing aid users. A second test obtained after a one-year interval revealed fair progression of SCTAW scores in seven cochlear implant users (A, B, C, G, J, L, M), four of whom (A, G, J, M) demonstrated results similar to those of their hearing peers. The total increase in SCTAW scores was similar among children with cochlear implants and those with hearing aids.

SCTAW errors are summarized in Fig. 6. Lower grade children (first and second graders) were more likely to display phonologic errors (45%) than semantic errors (38%). As grade advanced, the semantic errors were more frequently observed (42% of errors were semantic in the middle grades, vs. 60% in the senior grades). Similar to their hearing peers, the frequency of phonological errors are gradually reduced as the grade advanced. Instead, the semantic errors are coming to be more frequent.

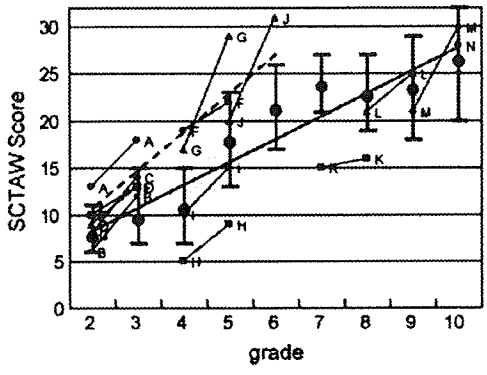

Fig. 8 SCTAW scores and grades in school among hearing aid and cochlear implant users. SCTAW scores in each grade was indicated as upper wing: best score, lower wing: worst score closed circle mean value. Triangle (▲) indicated cochlear implant users. Only one data was obtained from Case *N*. Dot line indicated the regression line obtained from hearing peers.

4. Discussion

PVT is widely applied in the vocabulary evaluation of hearing impaired children, because it can be used for younger children and can be completed relatively quickly. Moreover, presentation method can be modified according to the communication mode. In the present study, a strong correlation was confirmed between PVT and SCTAW, suggesting that the ability to understand abstract words can be a good indicator of language development for hearing impaired children. Although PVT has the benefit of simultaneously screening several types of vocabulary including concrete and abstract words, SCTAW has many advantages over PVT. First, unlike PVT, it can be applied in adults. The present PVT results for 10th grade children demonstrated narrow variance, possibly due to the ceiling effect observed in this test, i.e. this test is too easy for this age group and older. Hence, in terms of being able to reflect inter-personal differences or the effect of particular intervention or their learning ability, PVT is not suitable for older age groups. On the other hand, SCTAW exhibited wide variance of test results in the same age group. This was particularly apparent for cochlear implant users, two of whom (H and K) demonstrated similar but relatively low PVT scores but much lower SCTAW scores when compared to hearing aid users. These two children actually exhibited mild mental retardation on RCPM, and the presence of additional handicaps thus appears to be adequately demonstrated on SCTAW. For older children, SCTAW is a highly sensitive evaluation procedure that does not exhibit the ceiling effect, and it is accordingly useful for evaluating senior grade students' vocabulary and acquired language.

In addition, the sophisticated design of SCTAW enables error analysis. Phonetic errors can be distinguished from semantic errors and their relative frequency can yield additional information or indicate background neurological deficits in the children tested. This is potentially important for the evaluation of hearing impaired children because hearing impairment can affect phonetic processing abilities, at least initially. In this study, we presented words simultaneously both visually and vocally in an attempt to avoid additional effects stemming from severity of hearing impairment or procedures for hearing intervention. Interestingly, the error analysis of hearing impaired children demonstrated a similar tendency of that observed among hearing peers. Although a quantitative difference in vocabulary was apparent, the qualitative aspect of language development did not differ between hearing impaired children and their hearing peers. In other words, it might be possible to use

SCTAW to evaluate educational outcomes of several hearing interventions by sound-only presentation.

Hearing impaired children demonstrated lower SCTAW scores than their hearing peers. A similar finding was also reported by Blamey et al., however, they also found that hearing impaired children began to catch up with their hearing peers from the fifth grade of school and improved further as school grade advanced [8]. Bollard et al. reported that the Vocabulary Age (VA) from the Peabody Picture Vocabulary Test (PPVT) can be improved by cochlear implantation [9] and a similar tendency was observed in some of the cochlear implant users in the present study; hence, advances in hearing interventions could be one factor responsible for this catching-up. However, not all implant user demonstrated steep "surge" of language development and, conversely, many long-term hearing aid users also demonstrated such a surge in the fifth grade. The pace of vocabulary development in hearing impaired children may be affected by many factors and those responsible for the surge should be further examined.

Only a limited number of standardized vocabulary tests: Picture Vocabulary Tests (PVT) and Japanese MacArthur Communicative Development Inventories (JCDLs), are presently available for Japanese language users. JCDLs are primarily used for the evaluation of language development and communication in infants and toddlers. In contrast, for English users, PPVT and the Clinical Evaluation of Language Fundamentals (CELF) are widely used to test vocabulary for hearing impaired children [10]. For these tests, some authors have concluded that hearing impaired children generally score lower than their hearing peers [11,12]. On the other hand, some have found no apparent correlation between severity of hearing loss and the amount of acquired vocabulary. In reports describing comparisons with hearing peers, no consistent tendencies were observed for deaf children; while some have demonstrated reduced scores [13], others have reported a variable gap between hearing peers and hearing impaired children. Similarly, PPVT has been widely applied to evaluate the vocabulary of cochlear implant users [14–16]. These inconsistencies might result from age at evaluation, severity of hearing loss and educational intervention methods. As is frequently experienced in educational settings, reading ability can positively affect vocabulary regardless of the presence of hearing impairment. Because visually presented information such as reading materials could not disadvantage hearing impaired children, such children tend to acquire vocabulary more rapidly after they start to read. In this regard, vocabulary of hearing impaired children should eventually catch up with their hearing peers as they progress through elementary school. Accordingly, final evaluation of vocabulary should be performed as late in school as possible. For such long-term evaluations of vocabulary, more complex tests such as SCTAW could be useful.

5. Conclusions

The results of the SCTAW and PVT that are also widely used for hearing impaired children closely correlated. Hence, SCTAW is useful as a lexical evaluation of such children. The SCTAW scores were lower in the children with, than without a hearing impairment, but the scores improved as their school grade advanced. Individual differences among children with cochlear implants were large. Ultimately, the acquisition of an abstract vocabulary did not qualitatively differ between children with normal or impaired hearing.

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先天代謝異常症13例における新生児期ろ紙血を用いたタンデムマス分析による後方視的検討

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要　旨

タンデムマスによる新生児マススクリーニングの有用性を評価するため，ガスクロマトグラフィー質量分析計（GC/MS）などで診断した先天代謝異常症13症例について新生児期の血液ろ紙を用い，後方視的にタンデムマスによるアシルカルニチン分析を行った．患者は有機酸代謝異常症6例，脂肪酸代謝異常症5例，アミノ酸代謝異常症2例であった．発症時期は日齢0から1歳8か月であった．検討した全例で新生児期ろ紙血の分析によって発見できることを確かめた．13例のうち4例は新生児期早期の発症であったが，そのうち2例は早期に死亡した．1か月以降に発症した症例9例のうち6例が感染を契機に代謝不全として発症したものであった．新生児期早期発症の先天代謝異常症はタンデムマススクリーニングによって予後改善に限界があるものの，今回の研究によって，新生児期以降に発症する代謝異常症は早期発見により予後改善が期待できることが示唆された．

キーワード：タンデムマス，先天代謝異常症，マススクリーニング，新生児

はじめに

1990年代中頃からタンデム型質量分析計（タンデムマス）を導入した新生児マススクリーニングが世界的に普及している[1)~3)]．この方法は従来から新生児マススクリーニングに用いられてきた血液ろ紙を用いて，これまで行われてきたアミノ酸代謝異常症3疾患を含む20種類以上の先天代謝異常症をスクリーニングすることが可能である．ランニングコストは現行スクリーニングのそれに劣らず，かつ短時間で多数の検体を分析できる[1)~4)]ため，現在では欧米諸国を中心としてアジア諸国でもタンデムマスによる新生児マススクリーニングが導入されつつある．本邦においては1997年から重松らが試験研究を行っており，タンデムマスによって発見される日本人患者の頻度は約7,000～9,000人に1人といわれている[5)~7)]．島根大学小児科ではタンデムマス分析とともに有機酸血症の診断を目的にGC/MSを用いた尿中有機酸分析を行ってきた[8)]．今回我々は新生児期におけるタンデムマスによるスクリーニングの有用性を検討するために，最近2年間に先天代謝異常症と診断した症例のうち，新生児期のろ紙血が入手できた13症例について，後方視的にタンデムマスによるアシルカルニチン分析を行った．

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対　象

対象は2004年4月から2006年3月までに島根大学小児科で診断した先天代謝異常患者のうち新生児期の血液ろ紙を入手できた13症例を対象とした．発症年齢は日齢0から1歳8か月までであった．症例の内訳は，マルチプルカルボキシラーゼ欠損症（MCD），プロピオン酸血症（PPA），シトルリン血症1型（CIT-1）がそれぞれ2例，メチルマロン酸血症（MMA），グルタル酸血症1型（GA1），グルタル酸血症2型（GA2），ミトコンドリア三頭酵素（TFP）欠損症，中鎖アシルCoA脱水素酵素（MCAD）欠損症，極長鎖アシルCoA脱水素酵素（VLCAD）欠損症，カルニチンパルミトイルトランスフェラーゼ-2（CPT-2）欠損症がそれぞれ1例であった．MCD，PPA，MMA，GA1については尿中有機酸分析の結果をもって確定診断とした．それ以外の疾患については特徴的な化学診断のプロフィールに加え，酵素診断もしくはイムノブロッティング，遺伝子解析により診断を確定した．

新生児期の血液ろ紙は新生児期早期の発症例については発症時の血液ろ紙を分析し，それ以降の発症例については，発症時の血液ろ紙とともに現行の新生児マススクリーニングで使用済みの血液ろ紙を分析した．

方　法

血液ろ紙の分析はタンデムマスを用いたアシルカル

表1　13例の発症形態と転帰

症例　　診断	発症時期	臨床症状および検査所見	転帰
有機酸代謝異常症			
1. マルチプルカルボキシラーゼ欠損症＊	0d	代謝性アシドーシス	
2. メチルマロン酸血症＊	5d	哺乳不良，体重減少，代謝性アシドーシス 高アンモニア血症	死亡
3. プロピオン酸血症	1m	嘔吐，傾眠，高アンモニア血症 代謝性アシドーシス	
4. マルチプルカルボキシラーゼ欠損症	4m	感染後の多呼吸，代謝性アシドーシス 高乳酸血症，高アンモニア血症	
5. プロピオン酸血症	1y4m	発達遅滞，感染後の嘔吐，代謝性アシドーシス 高グリシン血症	
6. グルタル酸尿症1型	8m	頭囲拡大，発達遅滞	発達遅滞
脂肪酸代謝異常症			
7. TFP or LCHAD 欠損症	1m	呼吸障害，高 CK 血症，低血糖 高アンモニア血症	死亡
8. VLCAD 欠損症	3m	哺乳不良，不機嫌，意識障害，肝腫大 高アンモニア血症	死亡
9. グルタル酸尿症2型	4m	低血糖，肝腫大，低体温，高乳酸血症 代謝性アシドーシス，高 TG 血症	
10. CPT2 欠損症	5m	嘔吐，意識障害，肝障害，高アンモニア血症	
11. MCAD 欠損症	1y8m	感染を契機に突然死，痙攣，意識障害，低血糖 高アンモニア血症，代謝性アシドーシス	死亡
アミノ酸代謝異常症			
12. シトルリン血症1型＊	0d	高アンモニア血症	
13. シトルリン血症1型＊	3d	高アンモニア血症	死亡

＊：新生児期早期の発症例

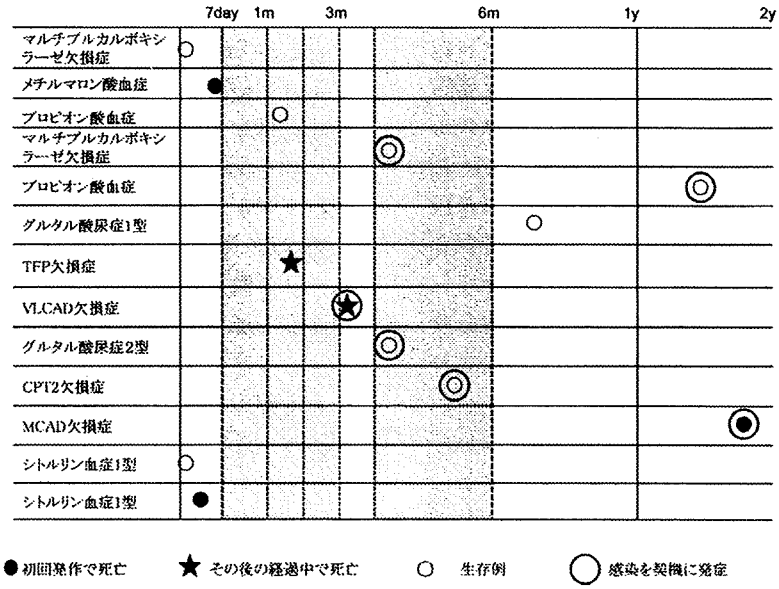

図1　診断時期と発症形態

ニチン・アミノ酸分析を行った．分析方法は新生児マススクリーニングとして標準化された方法[1~3]によって試料を調整した後，Applied Biosystems 社の API 3000 を用いて分析を行った．検査値の判定は既報[1~3,6]を参考に島根大学小児科で新生児用スクリーニング用に設定した基準値を用いて評価した．

結　果

1）発症時期と発症形態

発症時の臨床症状を表1に，診断時期と発症形態における関係を図1に示した．新生児期早期の発症例では，哺乳不良，呼吸障害，嘔吐などの非特異的症状とともに発症する傾向がみられた．それ以降に急性発症した症例については，高アンモニア血症やケトアシ

ドーシス発作などの急性脳症様の臨床症状で発症する例が9例中8例にみられた．残りの1例は頭囲拡大，発達遅滞に気が付かれたGA-1の8か月乳児例であった．脂肪酸代謝異常症は全例において重度の低血糖かそれを示唆する所見が見られた．

発症時期は，新生児期早期の発症例は13例中4例であり，その内訳はCIT-1が2例，MCD，MMAが各1例であった．CIT-1の1例とMMAの症例は治療に反応せず短期間のうちに死亡した．日齢7から生後6か月までに発症した症例は6例あった．このうち有機酸代謝異常症が2例(PPAおよびMCD)であったのに対し，脂肪酸代謝異常症は4例（TFP欠損症，VLCAD欠損症，GA-2，CPT-2が各1例）であった．この群で死亡したのはTFP欠損症とVLCAD欠損症の2例であり，いずれも2回目以降の代謝不全のエピソードの際に突然死した．6か月齢以降の発症は3例あった(PAおよびGA-1，MCAD欠損症)．1歳8か月で発症したMCAD欠損症例では生来健康であったにも関わらず，感染に引き続いて急性脳症様の代謝不全に陥り，初回の発作で死亡した．また，新生児期以降の発症例9例のうち6例が感染を契機に代謝不全を起こしていた．

2) タンデムマス分析結果

発症時および新生児期のアシルカルニチン分析結果を表2に示した．分析した13例全例において急性期の血液ろ紙の分析によって特徴的な異常を検出することができた．また，新生児期のろ紙血分析においても，新生児マススクリーニングで使用した血液ろ紙を検討した9例全例においてタンデムマス分析で特徴的な異常を検出することが出来た．13症例中4例において，発症時に遊離カルニチンの低下が認められた．

有機酸代謝異常症

MCD（症例1および症例4）の発症時はイソバレリルカルニチン(C5-OH)およびプロピオニルカルニチン(C3)，C3/C2，C3/C16の上昇がみられたが，症例4の新生児期検体ではC5-OHの上昇のみであった．MMA（症例2）は新生児期発症例であり，発症時すでに遊離カルニチン（C0）の低下がみられた．PPAの2例においては（症例3および症例5），発症時，新生児期ともにC3およびC3/C2，C3/C16の上昇として異常を検出可能で，症例3では発症時および新生児期ともに遊離カルニチンの低下を認めた．また，症例3では無症状であった新生児期においてより顕著なC3の上昇がみられ，臨床症状とC3値は必ずしも一致していなかった．GA1（症例6）ではグルタリルカルニチン（C5DC）の上昇として異常を検出できた．

脂肪酸代謝異常症

TFP欠損症（症例7）は典型的には3-ヒドロキシーパルミチルカルニチン(C16-OH)，3-ヒドロキシーステアロイルカルニチン(C18-OH)，3-ヒドロキシーオレイルカルニチン(C18：1-OH)の上昇が特徴的であるが，急性期はミリストレイルカルニチン(C14：1)，3-ヒドロキシミリストイルカルニチン（C14-OH）も上昇していた．VLCAD欠損症（症例8）の新生児期ではC14：1は4.63μmol/*l*と著明な上昇を認めたが，二次性遊離カルニチン欠乏をきたしていた発症時にはC14：1は0.70μmol/*l*と上昇の程度が小さかった．GA2（症例9）では発症時，新生児ともに短鎖から中鎖アシルカルニチンの上昇を認め，発症時により強く上昇していた．CPT2欠損症（症例10）では発症時，新生児期ともに特徴的なC16上昇と(C16+C18)/C0上昇を認めたが，発症時においては遊離カルニチンの著明な低下に加え，C14からC18までの広範なアシルカルニチンの上昇を認めた．MCAD欠損症（症例11）においては発症時，新生児期ともに同程度の中鎖アシルカルニチンの上昇を認めた．

アミノ酸代謝異常症

シトルリン血症1型（症例12および症例13）ではともに著明なシトルリンの高値と比較的低値なアルギニンおよびアルギニノコハク酸が特徴的であった．症例13では遊離カルニチン欠乏も同時に認めた．

考　察

今回の研究結果からタンデムマスによる新生児マススクリーングによって十分な成果が期待できることが示唆された．新生児期早期の発症を除く9症例の発症形態は発症時期に関わらず，いずれも生命を脅かす重篤な症状，もしくは不可逆的な精神運動発達遅滞であった．また，9例中6例は感染を契機に発症していた．先天代謝異常症の急性期の治療は一般に十分なカロリーの補給や輸液を必要とすることが多く，感染に伴う急性脳症等の初期臨床診断に基づく治療では代謝不全を加速する可能性もある．また，新生児期早期に発症した例は現行のマススクリーニングの採血前にタンデムマスもしくはGC/MSで診断されていた．このような場合にでもタンデムマスは一度に多種類の疾患をスクリーニング出来るという特徴を活かして，短時間で効率的な鑑別診断の手段として活用されるべきである．

タンデムマスによる新生児マススクリーニングの報告によると異常が発見されるのは5,000～9,000人に1人といわれている[1-3,5,7]．一方，タンデムマスによるスクリーニングで発見された患者の中にも無症状で経過する例も少なからずあると考えられている．これらについては全ての症例で治療が必要か否かは議論がある[4]．さらに新生児期早期に発症するような例ではスク

表2　13症例のタンデムマスによる分析および尿中有機酸分析所見

症例	診断	タンデムマス分析結果			尿中有機酸分析（GC/MS）
		分析指標	発症時	新生児期	
1.	マルチプル カルボキシラーゼ欠損症＊	C5-OH	1.92	—	乳酸・ピルビン酸，3-OH-butyrate,
		C3	7.04	—	3-OH-propionate, methylcitrate,
		C3/C2	0.32	—	3-methylcrotonylglycine の上昇
		C3/C16	5.48	—	
2.	メチルマロン酸血症＊	C3	15.47	—	methylmalonate, 3-OH-propionate 上昇
		C3/C2	0.61	—	methylcitrate の上昇
		C3/C16	8.25	—	
		C0 低値	13.93	—	
3.	プロピオン酸血症	C3	8.85	18.22	3-OH-propionate, propionylglycine 上昇
		C3/C2	1.48	4.2	methylcitrate の上昇
		C3/C16	5.17	29.5	
		C0 低値	7.68	7.21	
4.	マルチプル カルボキシラーゼ欠損症	C5-OH	8.82	1.91	乳酸・ピルビン酸，3-OH-butyrate,
		C3	5.18	NL	3-OH-propionate, methylcitrate,
		C3/C2	0.24	NL	3-methylcrotonylglycine の上昇
		C3/C16	9.11	NL	
5.	プロピオン酸血症	C3	21.38	11.9	3-OH-propionate, propionylglycine 上昇
		C3/C2	2.89	0.85	methylcitrate の上昇
		C3/C16	15.34	5.57	
6.	グルタル酸尿症1型	C5DC	1.79	0.68	glutarate, 3-OH-glutarate の増加 非ケトン性ジカルボン酸尿
7.	TFP 欠損症	C14：1	0.56	NL	非ケトン性ジカルボン酸尿
		C14-OH	0.14	NL	
		C16-OH	0.51	0.82	
		C18-OH	0.29	0.31	
		C18：1-OH	0.49	0.4	
8.	VLCAD 欠損症	C14：1	0.7	4.63	非ケトン性ジカルボン酸尿
		C0 低値	2.74	2.52	乳酸，ピルビン酸の排泄増加
9.	グルタル酸尿症2型	C6	0.63	NL	ジカルボン酸尿，ethylmalonate,
		C8	0.66	0.32	methylsuccinate, 2-OH-glutarate,
		C10	0.9	0.6	hexanoylglycine, isovalerylglycine の増加
		C12	0.55	1.19	
		C14	NL	0.8	
		C14：1	NL	0.62	
10.	CPT2 欠損症†	C0 低値	1.01	NL	非ケトン性ジカルボン酸尿
		C2	2.22	NL	
		(C16+C18) /C0	2.63	0.6	
		C14：1	0.25	NL	
		C16	2.08	8.13	
		C18	0.58	0.97	
		C18：1	2.05	NL	
		C18：2	0.36	NL	
11.	MCAD 欠損症	C6	1.57	0.74	低ケトン性ジカルボン酸尿,
		C8	4.75	4.43	hexanoylglycine, suberylglycine の上昇
		C10	0.58	0.42	
		C10：1	0.41	0.35	
12.	シトルリン血症1型＊	シトルリン	968	—	ウラシル，オロット酸の排泄増加
		アルギニン	1.48	—	
		アルギニノコハク酸	0.5	—	
13.	シトルリン血症1型＊	シトルリン	1,594	—	ウラシル，オロット酸の排泄増加
		アルギニン	3.2	—	非ケトン性ジカルボン酸尿
		アルギニノコハク酸	1.13	—	
		C0 低値	11.77	—	

（　）内は測定値，単位は μmol/l.
＊：新生児期早期の発症例，†：発症時は血清検体，NL：基準範囲内，—：マススクリーニング前発症
血液ろ紙分析における基準値：C0, ＜10（新生児），＜20（乳幼児）；C2, ＜5；C3, ＜5.25；C5, ＜1.2；C5-OH, ＜0.8；C5DC, ＜0.15；C6, ＜0.25；C8, ＜0.35；C10, ＜0.42；C10：1, ＜0.32；C14, ＜0.7；C14：1, ＜0.41；C14-OH, ＜0.12；C16, ＜7；C16-OH, ＜0.1；C18, ＜2；C18-OH, ＜0.1；C18：1-OH, ＜0.1；C3/C2, ＜0.2；C3/C16, ＜3；(C16+C18)/C0, ＞0.3；シトルリン，＜65；アルギニン，＜80；アルギニノコハク酸，＜1.5
血清分析における基準値：C0, ＜20；C2, ＜5；C14：1, ＜0.1；C16, ＜0.5；C16：1, ＜0.1；C18, ＜0.3；C18：1, ＜0.45；C18：2, ＜0.3

リーニング結果が発症に間に合わない場合や治療も困難である場合も多く，スクリーニングによる予後改善効果には限界がある．さらにタンデムマスによる血液ろ紙分析では疾患によって検出率に差があることも議論されている[9][10]．例えば軽症メチルマロン酸血症やグルタル酸尿症2型などである．これらは特に安定期には異常の検出が困難な事が多い．

今回の我々の研究によって，何らかの症状で発症するような中等症以上の先天代謝異常症の多くは新生児期に症状の有無に関わらず発見できることが明らかになった．今回の検討は後方視的研究であり，分析対象疾患にも偏りがあるものの，タンデムマスによるスクリーニングの有用性は確認された．発症前診断，早期治療，生活指導によって感染などのストレスに充分な対応を行い，生命予後の改善および障害予防に結びつけることが期待される．

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Retrospective Tandem MS Analysis of Newborn Blood Spots from Thirteen Patients with Organic and Fatty Acid Disorders Who Became Symptomatic and Diagnosed in Infancy or Childhood

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<OBJECTIVE> To evaluate the efficacy of the neonatal mass screening using tandem mass spectrometry (TMS), acylcarnitines on Guthrie papers obtained in the neonatal period from children with organic acidemias (OAs) or fatty acid disorders (FAODs) were analyzed retrospectively. <METHODS> Acylcarnitines on Guthrie papers from 13 children were analyzed using TMS. They had not received newborn screening with TMS, and became symptomatic in infancy, and diagnosed as having OAs or FAODs during the period between 2004 and 2006. Diseases diagnosed were as follows：2 cases each of multiple carboxylase deficiency (age at onset, 0 d and 4 mo), citrullinemia type 1 (0 d and 3 d), and propionic acidemia (1 mo and 16 mo), one each of methylmalonic academia (5 d), glutaric aciduria type 1 (8 mo), medium-chain acyl-CoA dehydrogenase deficiency (20 mo), very-long-chain acyl-CoA dehydrogenase deficiency (3 mo), CPT2 deficiency (5 mo), glutaric aciduria type 2 (4 mo), and trifunctional protein deficiency (1 mo). <RESULTS> In all 13 cases tested, acylcarnitine profiles were specific for each disease. Two of four cases, whose onset was within 5 days of age, died during neonatal period. Metabolic crisis was triggered by infection in 6 of 9 infantile-onset cases. <CONCLUSION> It is suggested that the newborn screening with TMS should detect all the patients who at least become symptomatic in childhood and thus be essential for detection of pre-symptomatic patients for their favorable prognoses.

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Clinical and molecular investigations of Japanese cases of glutaric acidemia type 2

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Abstract

Glutaric acidemia type 2 (GA2) is an autosomal recessive disorder resulting from a deficiency of electron transfer flavoprotein (ETF) or ETF dehydrogenase (ETFDH) that manifests from most severe neonatal to late-onset forms. However, the genetic defect responsible for the disease and clinical severity is not well-characterized. In order to understand the relationship between the phenotype and genetic defect, we investigated the clinical and molecular features of 15 Japanese patients, including 4 previously reported cases. Three patients had the neonatal form and 8 patients had the late-onset form, 1 of whom presented an extremely mild phenotype. Immunoblot analysis showed that either ETFα, ETFβ, or ETFDH was significantly reduced or absent in all patients. However, no specific enzyme deficiency predominated, and there were no associations with the clinical severity. Genetic analyses identified 15 mutations including non-sense, missense, splice site mutations, and small deletions, in *ETFA*, *ETFB* and *ETFDH* genes. Although almost all mutations were unique to Japanese patients and no common mutations were found, some of them appeared to be associated with a specific phenotype. Our results suggest that clinical and mutational spectrums of Japanese GA2 patients are heterogeneous and that genetic diagnoses may help to predict a prognosis and provide more accurate diagnostic information for patients and families with GA2.

Keywords: Glutaric acidemia type 2 (GA2); Electron transfer flavoprotein (ETF); Electron transfer flavoprotein dehydrogenase (ETFDH); Organic acidemia; Fatty acid metabolism disorder

Glutaric acidemia type 2 (GA2) is an inherited autosomal recessive disorder of fatty and organic acid metabolism caused by a defect of electron transfer flavoprotein (ETF) or ETF dehydrogenase (ETFDH) [1,2]. GA2 is roughly divided into 2 clinical forms: a neonatal onset form (severe form) and a late onset form (milder form). In the severe form, hypotonia, hypoglycemia, metabolic acidosis and/or hyperammonemia are present with/without anomalies such as dysplastic kidney and/or congenital heart disease. Children with the severe form show a fatal course in the neonatal period regardless of intensive treatments. In the mild form, the age at onset is often after infancy, and the symptoms are variable with intermittent episodes of hypotonia, tachypnea, hypoglycemia and/or hyperammonemia, which are often trigged by metabolic stress [3,4].

In Japanese cases of fatty acid disorders, GA2 is relatively common, followed by VLCAD deficiency and CPT2 deficiency in terms of frequency [5]. In the acute phase of the disease, increased excretions of suberylglycine, isovalerylglycine, hexanoylglycine, ethylmalonic acid, and hypoketotic dicarboxylic aciduria are noted on urine organic acid analysis using GC/MS. However, they may not be detected in the stable phase of many cases, making a precise diagnosis difficult. With ESI-MS/MS, an increase of some acylcarnitines with specific carboxylases ranging from medium to long chains (C4, C5, C8:1, C8, C12, C14, C16, and C5DC) is detected, but in the stable asymptomatic phase, no abnormalities may be seen.

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A defective protein in GA2, ETF, is a heterodimetric mitochondrial matrix enzyme consisting of alpha and beta subunits (ETFα and ETFβ, respectively). ETFDH is a monomer integrated in the inner mitochondrial membrane. At the molecular level, the defective enzyme in GA2 is either ETFα, ETFβ or ETFDH. These enzymes are required for electron transfer from at least 9 mitochondrial flavin-containing dehydrogenases to the main respiratory chain [6,7]. The genes for ETFα, ETFβ and ETFDH proteins (*ETFA*, *ETFB* and *ETFDH*, respectively) were cloned and mapped to 15p23–25, 19q13.1 and 4q33, respectively [8–10]. Up to now, 55 mutations have been reported in the literature, including 4 Japanese mutations [11–14], although GA2 is one of the most common defects in fatty acid oxidation and organic acids. Since one of the three genes is affected in GA2, it is essential to accumulate information on genetic mutations to determine any genotype/phenotype correlation and to identify defective enzymes for an accurate diagnosis/prenatal diagnosis of GA2.

In this study, we investigated the relationship between clinical and molecular aspects of Japanese patients with GA2, in which typical profile of urinary organic acids were observed at least in the acute stage, and found 15 mutations in 11 Japanese patients, referring 4 previously reported cases [11,12].

Materials and methods

Patients

GA2 was diagnosed in 11 Japanese children, based on the characteristic metabolic profiles of urinary organic acids analyzed using gas chromatography/mass spectrometry (GC/MS). Informed consent was obtained from all patients' families. Our study protocol was approved by the Ethics Committee of the Shimane University Faculty of Medicine. Previously reported Japanese patients (cases 12–15) were compared.

Cell culture

Skin fibroblasts obtained from the patients were cultured in Eagle's essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (10% FCS/MEM) and antibiotics (penicillin-streptomycin: 100 μg/mL).

Immunoblotting

The pellets of cultured fibroblasts were dissolved in 0.1 M potassium phosphate buffer, pH 7.2, 1% Triton X-100 and 0.2 M NaCl, and then sonicated. After centrifugation, the supernatant was subjected to 12.5% SDS/PAGE. Immunoblot analysis was carried out as previously described [15]. The protein concentration was determined by the method of Lowry et al. [16], according to the Bio-Rad protein assay protocol (Bio-Rad Laboratories, Hercules, CA, USA). Fifty micrograms of protein of fibroblasts were subjected to each lane.

DNA sequencing

The genomic DNAs were isolated from fibroblasts using a Qiamp DNA Microkit (QIAGEN GmbH, Hilden, Germany). Control genomic DNA from 50 unaffected Japanese individuals was obtained from peripheral blood lymphocytes using Blood and Cell Culture DNA Midi Kits (QIAGEN GmbH, Hilden, Germany). Each exon of *ETFA*, *ETFB*, and *ETFDH* including intron/exon boundaries was PCR-amplified for 30 cycles using the conditions shown in Table 1. Primers of *ETFDH* were prepared as previously reported [17]. The PCR products were purified by a QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany) and sequenced using ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) or CEQ 8000 Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA, USA). The structures of human *ETFA*, *ETFB*, and *ETFDH* genes were obtained from the GenBank database (*ETFA*: AF436646–AF436657, *ETFB*: AF436658–AF436663, and *ETFDH*: AF449432–AF449444).

Results

Clinical aspects

Clinical phenotypes of 11 Japanese patients (cases 1–11) and 4 reported cases (cases 12–15) with GA2 are summarized in Table 2. Three patients showed neonatal onset with a severe phenotype (cases 1–3) and 8 showed the late onset form (cases 4–11). Case 12 had neonatal onset, whereas cases 13, 14, and 15 had late onset [11,12].

None of the patients had a familial history of consanguineous marriage. All neonatal patients died upon the initial development of symptoms, demonstrating the lethality of neonatal GA2. Polycystic kidney and congenital heart anomaly were seen in 2 cases with the neonatal form (cases 1 and 3). In contrast, 7 out of 8 cases with the late onset form remain alive even after the onset of disease. At least 2 patients with the late onset form (cases 4 and 5) responded to vitamin B2 (riboflavin-responsive). Case 7 and his father were affected with osteogenesis imperfecta. While polycystic kidney and congenital heart anomaly have been previously documented, GA2 with osteogenesis imperfecta has never been reported. It is not clear whether these combinations are merely coincidental or are closely associated.

Among the 8 late onset patients, cases 4–8 showed the onset of symptoms before 2 years of age, whereas cases 9, 10, and 11 showed a later onset compared to the other patients. Of note, case 11 demonstrated an extremely late onset with the mildest phenotype. He stayed asymptomatic until his early 40's, when muscle pain, hypotonia, and CPK elevation were noticed. He had 8 siblings, but his younger brother died suddenly at the age of 30 due to an unidentified disease. All other patients with late onset form developed symptoms upon infection or metabolic stress.

Protein defect in ETF or ETFDH

In order to confirm the diagnosis of GA2 of these patients, immunoblot analyses were performed using antibodies against ETF and ETFDH in the present cases along with 4 previously reported patients (Fig. 1). ETF or ETFDH protein was absent or significantly reduced in all patients, validating the GA2 diagnosis. ETF protein was absent in cases 1–3 and 6 whereas ETFDH was observed in these patients. In contrast, ETFDH was significantly

Table 1
Primers and conditions to amplify genes of *ETFA*, *ETFB*, and *ETFDH*

Gene	Exon	Sense primer	Antisense primer	Annealing temperature (°C)	Product length (bp)
ETFA	1	ttatctcgttcgcgcgtct	cagtgatctttgcaagacc	53	227
	2	ctgtgacaacattctttacc	tcctgtctcttgatggagat	53	251
	3	tcttaactggccctttgct	caagcgtaatttagacactac	55	268
	4	aaggcctacttccaggtga	ccagattggctacataaacag	55	303
	5	agtcttccatcactatgtatgtg	gggccaccagcaattctagactaa	55	312
	6	gagttagaagacaactggtc	tgtaggcagaggtcatctct	53	325
	7	gtctttccattatcagagag	cttcccaacttcttacatttg	51	380
	8	ggtgacatgcttaatatggc	ccatttacttggaggcttaag	53	359
	9	cagtgaatatcctaactagc	cactgagatcactgttcaac	51	230
	10	acagcaaacactttggaaag	acaaggtaagccaaatccta	55	199
	11	agaactatcagtctgtgtga	cctaaccatttctgagagtt	51	215
	12	ctgtgaccatatcacagtga	ctctagaggctaggcatatt	53	389
ETFB	1	attgtatcaccggcggaagcggagacc	gttaaagcggcagaggtcg	51	300
	2	ttgcctgtttcctcctgacc	aatccaggctatcagcccat	57	347
	3	tgaggatagcagccaagtca	tcagcctggagtggtgaatg	55	318
	4	tgaatggtccatgagctcca	ttccaggaggagagaacag	55	222
	5	tcggacctgaattagcctca	gctggcaatgtgcttgcca	55	266
	6	gagttccacagccctgtgaa	tgggtctctaggaaataag	57	282
ETFDH	1	ctgcagcagagttcttgctt	gcctgagaaagctgatgaga	60	271
	2	tttcagtctactgaggaaaac	caaagtatccagaaaagtctc	50	253
	3	gggttatattaatcccag	gggaacaattactgaaat	50	330
	4	cacttgcaaatataaact	cctccagctgtggaattc	50	178
	5	gtgaccatcaatgtagcact	catgaggaattcaagtactc	57	401
	6	gaaacctaaggctgtttactgttttt	tttcactttgatgcccacac	55	235
	7	tctgaccagatgtgaatgtatttt	ccccttgaaaaatatcgcaaa	55	374
	8	ttatgcattgtggtgacataaa	aatataactctagcagcagaat	55	280
	9	cctacatgttttctgata	acatacttttcctattcc	48	351
	10	gggtattctgttgttctt	aaatacacataacccagc	53	288
	11	cagtttcgcacttaacat	atcatgtcactcatactc	53	251
	12	gggctagtcatatttctttggtg	cacattcctaaaatgtttaaagcaaa	55	390
	13	aagttaggcacttcaata	aaactggctagctgcagt	50	221

reduced or barely detectable in cases 4, 5, 7, 8, 9, 10, and 11, all of whom showed the late onset form. ETF was observed at comparable levels in these patients (cases 4, 5, 7, 8, 9, 10, and 11) with controls. These results suggest that cases 1, 2, 3, and 6 are deficient in ETF, while patients 4, 5, 7, 8, 9, 10, and 11 exhibit ETFDH deficiency.

Gene mutations in ETFA, ETFB or ETFDH

ETFA and ETFB

Genetic defects in these patients were determined by DNA sequencing (Table 3). Direct sequencing of all the intron/exon boundaries of *ETFA* and *ETFB* genes in patients 1, 2, 3, and 6 with ETF deficiency revealed 3 different mutations in the *ETFA* gene and 4 mutations in the *ETFB* gene. Patients 1 and 2 with the neonatal phenotype were compound heterozygotes of 77delG/R174stop and K19stop/80delC in the *ETFB* gene, respectively. A novel homozygous IVS6-1G>C mutation at the splice acceptor site of intron 6 in the *ETFA* gene was identified in case 3 with the neonatal phenotype. The mRNA of the patient lacked the N-terminus in the first 7 nucleotides of exon 7, resulting in a truncated peptide as a consequence of a frame shift. In addition, a shorter transcript that lacks exons 6 and 7 was identified by direct sequencing of the whole PCR product, although this shorter product was barely detectable in the RT-PCR gel. A normal transcript was not detected. Familial analysis revealed that her parents were heterozygous carries for IVS6-1G>C. A novel homozygous mutation of L95V was found in the *ETFA* gene in patient 6 with the late onset phenotype. A homology search of the ETFA protein in different species demonstrated that leucine 95 was highly conserved from zebrafish, Xenopus, rats, and mice, to humans.

ETFDH

All patients with ETFDH deficiency had the late onset form. Sequencing of their *ETFDH* gene demonstrated 9 mutations. A homozygous C to T transition in exon 11 that substitutes proline at 456 with leucine (P456L) was found in patient 11 with an extremely mild phenotype. Patient 10, who manifested liver dysfunction during early adolescence, harbored a G to A transition at nucleotide 524 and a T to C transition at 1774. They introduced a substitution of arginine 175 in the FAD binding domain with histidine (R175H) and cysteine 592 with arginine (C592R), respectively. Patient 7 with osteogenesis imperfecta who experienced a hyperammonemia attack at 1.4 years old was compound heterozygous for G362R and P534L. Patient 4 who showed hypotonia and hypoglycemia at 5

Table 2
Clinical manifestations of Japanese patients with GA2

Patient		Age at onset	Family history of sudden death	Congenital anomaly	Clinical findings at onset	Outcome
Neonatal onset form						
1	F	0 d	+	PCK, CHD	Dyspnea, hypoglycemia, and liver dysfunction	Dead (3d)
2	F	0 d	+	?	Sudden death	Dead (5d)
3	F	0 d	+	PCK	Liver dysfunction and cardiomyopathy	Dead (21d)
Late onset form						
4	M	5 m	−	—	Hypotonia and hypoglycemia, and riboflavin-responsive	Alive n.d. (1y)
5	M	6 m	−	—	Hypotonia and riboflavin-responsive	Alive n.d. (15y)
6	M	8 m	−	—	Poor feeding, CPK elevation, and cardiomyopathy	Dead (1y)
7	M	1y4m	+	OI	Hypotonia, hypoglycemia, and hyperammonemia	Alive n.d. (5y)
8	F	1y10m	−	—	Vomiting, hypoglycemia, and liver dysfunction	Alive n.d. (17y)
9	F	5 y	+	—	Convulsion, hypoglycemia, and liver dysfunction	Alive n.d. (7y)
10	F	13 y	−	—	Vomiting, hypotonia, and liver dysfunction	Alive (14y)
11	M	58 y	+	—	Hypotonia, muscle pain, and CPK elevation	Alive n.d. (60y)
Previously reported cases (Neonatal onset form)						
12*	M	0 d	+	PCK	Dyspnea, hypoglycemia, and hyperammonemia	Dead (3d)
(Late onset form)						
13**	M	4 m	+	—	No remarkable symptoms but died suddenly	Dead (3y)
14**	M	5 m	+	—	Poor feeding, hypoglycemia and liver dysfunction	Dead (2y)
15*	M	1y0m	−	—	Reye-like illness and riboflavin-responsive	Alive n.d. (2y)

PCK, polycystic kidney; CHD, congenital heart disease; OI, osteogenesis imperfecta; n.d., normal development.
* Purevjav E. et al. [12].
** Colombo I. et al. [11]; sibling case.

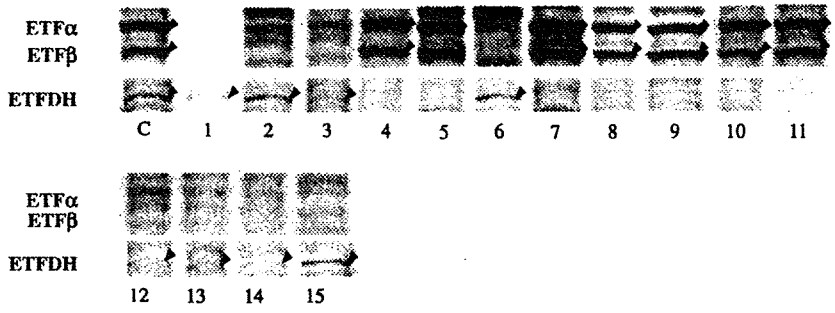

Fig. 1. Immunoblotting of electron transfer flavoprotein (ETF) and ETF dehydrogenase (ETFDH) in the fibroblasts. Fifty micrograms of protein were loaded per lane. ETFα and ETFβ represents α- and β-subunit of ETF, respectively; ETFDH represents ETF dehydrogenase; C, a normal control; Patient numbers are shown at the bottom of the gel. Arrowheads indicate the protein detected by immunoblot analyses.

months old was heterozygous for the F308V mutation in exon 9, but no other mutation was identified. Patient 5 was homozygous for the A403V mutation in exon 10 and developed hypotonia at 6 months old. A heterozygous codon termination mutation (R559stop) and L366F mutation were identified in exons 12 and 9, respectively, in patient 8. L366F was also identified in patient 9, but no other mutation was detected. All mutations identified in this study were novel except for P456L in *ETFDH*, that was reported in the late onset form [17]. A homology search of the EST data base for human ETFDH identified several potential *ETFDH* transcripts in the beetle, pea aphid, and zebrafish and demonstrated that the mutated amino acids are highly conserved among insects, zebrafish, mice, rats, and humans. Screening of normal Japanese individuals revealed the presence of none of these mutations in 100 alleles of Japanese controls.

Discussion

This study identified 3 patients with the neonatal onset form and 8 cases with the late onset form in the Japanese population. Biochemical and genetic analyses including 4 previously reported cases revealed that there were 4 cases with ETFα deficiency, 4 with ETFβ deficiency, and 7 with ETFDH deficiency. There were 15 genetic alterations: 14 novel and 1 reported mutations were identified. Eight missense mutations (R175H, F308V, G362R, L366F, A403V, P534L, P456L, and C592R) and a nonsense mutation (R559stop) were identified in the *ETFDH* gene, respec-