Manuscript Details

Manuscript number	MGMREPORTS_2018_35_R1
Title	Diversity in the incidence and spectrum of organic acidemias, fatty acid oxidation disorders, and amino acid disorders in Asian countries: selective screening vs. expanded newborn screening
Article type	Research Paper

Abstract

Background: Expanded newborn screening (ENBS) utilizing tandem mass spectrometry (MS/MS) for inborn metabolic diseases (IMDs), such as organic acidemias (OAs), fatty acid oxidation disorders, (FAODs), and amino acid disorders (AAs), is increasingly popular but has not yet been introduced in many Asian countries. This study aimed to determine the incidence rates of OAs, FAODs, and AAs in Asian countries and Germany using selective screening and ENBS records. Materials and Methods: Selective screening for IMDs using gas chromatography-mass spectrometry and MS/MS was performed among patients suspected to be afflicted in Asian countries (including Japan, Vietnam, China, and India) between 2000 and 2015, and the results from different countries were compared. Similarly, ENBS results from Japan, South Korea, Taiwan, and Germany were compared. Additionally, the results of selective screening and ENBS in Japan were compared. Results: Among 39,270 patients who underwent selective screening, IMDs were detected in 1,170. Methylmalonic acidemia was frequently identified in several countries, including Japan (81/377 diagnosed IMDs), China (94/216 IMDs), and India (72/293 IMDs). In Vietnam, however, β-ketothiolase deficiency was particularly frequent (33/250 IMDs). ENBS yielded differences in overall IMD rates by country: 1:8,557 in Japan, 1:7,030 in Taiwan, 1:13,205 in South Korea, and 1:2,200 in Germany. Frequently discovered diseases included propionic acidemia (PPA) and phenylketonuria (PKU) in Japan, 3-methylcrotonyl-CoA carboxylase deficiency (MCCD) and PKU in Taiwan, MCCD and citrullinemia type I in South Korea, and PKU and medium-chain acyl-CoA dehydrogenase deficiency in Germany. Furthermore, in Japan, selective screening and ENBS yielded respective PPA frequencies of 14.7% and 49.4% among all organic acidemias. Conclusion: The incidence rates of IMDs vary by country. Moreover, the disease spectra of IMDs detected via selective screening differ from those detected via ENBS.

Keywords	organic acidemia; fatty acid oxidation disorder; amino acid disorder; inherited metabolic disease; expanded newborn screening; incidence rate
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May 8, 2018

Dr. Edward RB McCabe and Shawn McCandless
Co-Editor-in -Chief
Molecular Genetics and Metabolism Reports

We thank the editor and the reviewers for their constructive criticism and suggestions regarding our manuscript. We have carefully revised our original manuscript entitled " **Diversity in the incidence and spectrum of organic acidemias, fatty acid oxidation disorders, and amino acid disorders in Asian countries: selective screening vs. expanded newborn screening**" as per reviewers comment. All changes made in the manuscript are highlighted in red. Our responses to the reviewers are detailed in the attached response file.

We hope that we have adequately responded to the reviewers' comments and that our revised manuscript is now suitable for publication in Molecular Genetics and Metabolism Reports.

Sincerely yours,

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Response to the comments from reviewers

-Reviewer 1

Shibata et al. provide a large and interesting data collection. However, it presents with several difficulties that are often seen in retrospective multicenter studies.

'Symptomatic screening' is a strange term, perhaps selective screening would be more appropriate, at least more common).

Answer:

Thank you for your suggestion. We have changed the term from "symptomatic screening" to "selective screening".

The rational for selective screening should be provided in a more structured and more comprehensive way than just listing the features 'metabolic acidosis, ketosis, hyperammonemia, hypoglycemia, general fatigue, hypotonia, myopathy-like symptoms,

acute encephalopathy, and sudden infant death of unknown causes were analyzed'. How was the age distribution of the patients at the time of screening? How often was 'general fatigue' actually a reason for screening in children?

Answer:

As you indicated, the term "general fatigue" might be inappropriate. Accordingly, we changed this term to "lethargy". Among children, no patients complained of only general fatigue; although a few adults were included, most of our study subjects were newborns and pediatric patients. We have added this information to the manuscript, along with an explanation that request forms listing symptoms, clinical data, and administered medications were sent with blood/urine samples to Shimane University.

Was the MS/MS method exactly the same at Shimane University and Fukui University? How about the ENBS labs in Taiwan and South Korea? I am convinced that not all German laboratories which (indirectly) contributed newborn screening data to the paper performed butylation. The method section should either specify all the approaches or remain rather general, but not focus on one of several labs involved.

Answer:

Briefly, the butyl-derivatized method was used for selective screening at both Fukui and

Shimane Universities. In Japan, a non-derivatized method has been used for ENBS since at least 2014, although the pilot study used the derivatized method before 2013. Although the ENBS methods varied among countries or laboratories, the butyl-derivatization method was at least used consistently during the study period, as previously described by Niu DM, et al. (2010) in Taiwan; Yoon HR, et al. (2005) in South Korea; and Lindner M, et al. (2007) in Germany. DBS was collected 4–5 days after birth in Japan, 48–72 hours after birth in South Korea and Taiwan, and 36–72 hours after birth in Germany.

In Germany many of the listed diseases are not part of the national screening program. Therefore, I expect that several numbers are of limited value, as in agreement with the regulations valid for most of the time, many detectable cases probably have not been reported.

Answer:

All disorders included in the regular newborn screening panel used in Germany are listed in Table 3. Although several disorders such as MMA and PA were excluded from the German ENBS panel, we have included data of these disorders in Germany in Table 3. This nationwide German profile was obtained by collecting data from different screening laboratories. Therefore, this data was slightly larger than the incidence for only the

A main issue for this paper is that several diagnoses represent groups of inborn errors of metabolism rather than specific diseases. I am afraid, that not only in FAODs, where this has been done, but also in other diseases where biochemical data failed to provide a definitive diagnosis, 'enzyme activity measurements and/or gene mutations' need to be studied.

Answer:

Thank you for raising this important issue. We consider your point to be a limitation of our study. Although the diagnoses of most Japanese patients were confirmed based on genetic testing and/or enzyme activity analyses, these analyses were not performed for all foreign patients. However, the diagnoses of foreign patients could also be made according to the results of biochemical analysis indicating obvious and specific abnormal metabolites, along with clinical data (e.g., age, sex, clinical course, laboratory data, and medication) by several expert physicians familiar with IMDs. We have added these limitations to our revised manuscript.

The following points should be addressed in the manuscript:

The only tentative explanation given for MMA is the *MMACHC* mutation (p.W203X) in the Chinese populations. It is a problem not to distinguish between the different causes of MMA. Most importantly, many of the cases may reflect nutritional deficiency of vitamin B12 rather than a genetic disease.

Answer:

We agree with your comments. An MMA-like disease caused by vitamin B12 deficiency is well-known in South Asian countries such as Nepal and India. However, similar cases have never been reported in China (J Neurol Neurosurg Psychiatry. 2015 Apr;86(4):472-5), and we observed no episodes or clinical histories indicative of vitamin B12 deficiency, such as megaloblastic anemia and gastric resection. Therefore, we believed that none of the Chinese cases of MMA in our study could be attributed to a dietary vitamin B12 deficiency. However, because we did not conduct genetic testing in this study, we could not truly determine whether the incidence of Chinese MMA was influenced by genetic background factors or a vitamin B12 deficiency. We have described this as a limitation in our manuscript.

There are more such examples which should prompt the confirmation of the metabolite-based diagnoses. For instance, oxoprolinuria may not be due to oxoprolinase or

glutathione synthetase deficiencies, but may, e.g., also be due to diet or certain drug intake. It may be important to consider that, if oxoprolinuria is particularly frequent in one population.

Answer:

Some previous reports noted that metabolic acidosis with high anion gap in the presence of acetaminophen could be attributed to OXPA. We cannot eliminate the possibility that some medications have such effects in some OXPA cases. However, the diagnosed cases in our study exhibited huge OXPA peaks and presented with typical symptoms of glutathione synthetase deficiency, such as chronic metabolic acidosis and hemolytic anemia. We have added the above information to the manuscript.

2-hydroxy-glutaric acid may (depending on the enantiomer distribution) not only be the cause of several genetic metabolic disorders, but may also simply reflect bacterial degradation of the urine. 4-hydroxybutyric aciduria may also be caused by a sedative drug.

Answer:

As you noted, metabolites derived from the intakes of some drugs or from bacterial contamination can lead to the incorrect analysis of urinary organic acids. Accordingly,

diagnoses were based not only on biochemical profiles, but also on the subjects' clinical courses and the opinions of expert physicians. We have revised the description of this process in the Materials & Methods and Discussion sections.

It seems that all discussions in the paper on mutations underlying certain populations refer to the literature in general rather than in a specific way to the study population. It would be preferable to address the latter more.

Answer:

Few reports have addressed the prevalence of common mutations related to inherited metabolic diseases in East Asian countries, including Japan, Taiwan, and Korea. Therefore, we cannot discuss differences among the populations of individual countries. However, our results suggest considerable differences in the genetic backgrounds of Asian and Caucasian populations.

The disease character of MCC deficiency and its inclusion into newborn screening has been questioned.

Answer:

No consensus has been reached regarding the inclusion or exclusion of MCC deficiency

from the diseases targeted by ENBS. However, several reports have described symptomatic MCC deficiency with hyperammonemia or mental retardation in Japan, although the majority of these cases were asymptomatic. Therefore, we included MCC deficiency in this study.

Is it appropriate to summarize dietary induced biotin deficiency under the umbrella of MCD deficiency?

Answer:

Dietary biotin deficiency is not an IMD. However, cases of dietary biotin deficiency in Japan are sometimes detected by MS/MS as an elevated level of C5-OH. Accordingly, our final diagnoses of MCD deficiency were based on the relationship between the C5-OH level and follow-up data indicative of the clinical course.

In view of the Hmong ethnic group and a frequent ACADSB mutation among them, I am curious whether some of the cases of isovaleric acidemia may actually represent patients with methylbutyrylglycinuria. Has the diagnosis always been confirmed at least by organic acid analysis?

Answer:

For all cases of IVA, our final diagnoses were based on the urinary organic acid profile. We have never detected methylbutyrylglycinuria via GC/MS in IVA patients.

In tables 3 and 4, does 'n.d., not detected' mean that those diseases were no targets of the investigations or that they were not identified? Notably, β-ketothiolase deficiency deficiency was not identified in any newborn screening program. Does this reflect technical difficulties/ inappropriate methodology?

Answer:

According to your suggestions, we clarified with each author whether "n.d., not detected" or "n.a., not available" should be used in Tables 3 and 4.

Even if the birth rate in Japan is declining, 3.36 million seems to be a very low number for nationwide NBS from 1997 to 2015. Did the methodology not change over 18 years? Notably, nationwide ENBS is said to have started in 2014 only, while NBS was introduced in 1977 already. Thus, it is not clear, from where the data originate (certain regions?) and whether for some diseases the number of screened newborn should not be much higher than for others. Please clarify.

Answer:

Thank you for your criticism. The ENBS data of Japan included 1.95 million babies of the pilot study from 1997 to 2012, and the remaining data available babies of 1.41 million from 2013 to 2015. The annual birth number of Japan was approximately 1 million, but ENBS data of whole country was not available at least in the period between 2013 and 2015, because we could not approve the use of the data due to contractual problem. We revised the incorrect description of the duration and the subject in ENBS date.

It would be difficult to compare detection rates of selective screening and ENBS in Japan unless the methods are strictly defined/ standardized. Information on the applied quality control measures (including external quality controls) would be helpful.

Answer:

ENBS is controlled by several quality control programs, including external quality controls. Additionally, our ENBS and selective screening methods are consistently standardized. Nevertheless, a full comparison of the results of selective screening with those of ENBS might be difficult. However, we aimed to address differences in the detection incidence rates and spectra between selective screening and ENBS. We have included this issue in the manuscript as a limitation of our study.

Page 7:

'To investigate the variation in the incidence of IMDs by national or ethnic group…': The manuscript does not really address particular ethnic groups. I doubt that authors can really provide valid frequency data, considering that, e.g., several of the organic acidurias may be transient and have a number of possible explanations (see above).

Answer:

Thank you for your comments. We have deleted "ethnic group", which was not used for comparison in our study. This term was replaced with "country". However, we could not completely eliminate the secondary metabolic status, given the above-described limitation. We have added this point to the paragraph about study limitations.

Other issues:

Page 6: 'We have provided biochemical IMD diagnosis…' 'We' is probably the Shimane University lab?

Answer:

Yes, "We" indicates Shimane University. We have replaced this pronoun with "Shimane University".

Is SCHAD deficiency the appropriate name of the disease (see Mol Genet Metab. 2007 Jun;91(2):205-6)?

Answer:

According to your suggestion, SCHAD deficiency was changed to "HAD deficiency".

During which time interval has the 'symptomatic screening' been performed?

Answer:

Unfortunately, we do not understand what the reviewer mean by "interval". If "interval" indicates the time frame between sample collection and mass spectrometer analysis, this period varied from a maximum of a few weeks for foreign samples to a few days for Japanese samples.

How has HMGS deficiency been detected in 'symptomatic screening' (cite J Inherit Metab Dis. 2015 May;38(3):459-66, if appropriate). 'HMGA' is not a common abbreviation.

Answer:

Elevated levels of 4-hydroxy-6-methyl-2-pyrone and related metabolites and hypoketotic dicarboxylic aciduria in a urinary organic acid analysis are characteristic of HMGS

deficiency. According to your suggestions, we have changed "HMGA" to "HMGL deficiency" in our manuscript.

Reference [13] should be updated.

Answer:

Thank you. We have updated this reference.

-Reviewer 2

This manuscript reports the results of symptomatic screening of urine using GC/MS and MS/MS and ENBS using blood filter paper in several Asian nations. The results are compared to results from Germany. The data are interesting and important for considering the development of metabolic screening in Asian countries as well as nations in other areas of the world not currently covered by such screening. This is especially important for ENBS.

Thank you very much for your summary and positive feedback on our manuscript.

One addition could be a consideration of potential efficacy of screening in Asia. A proper

way to do this would be to separate those disorders which seem to be asymptomatic from those considered symptomatic. For instance, mild PA in Japan was not detected clinically but was the most frequent disorder detected by ENBS in Japan. Thus, if universal ENBS is established in Japan, should PA be reported or even identified?

Answer:

Thank you for your comments. Nationwide ENBS was implemented in Japan in 2014. Although mild PA can be distinguished from other types of PA by genetic testing, the natural history of mild PA remains to be determined; accordingly, this condition is currently reported in Japan. Although no report describes the presentation of symptoms in patients with mild PA, no evidence suggests that these patients remain asymptomatic throughout their lives. Therefore, we cannot conclude whether or not mild PA should be excluded.

The same could be asked of other disorders in Japan and other Asiatic nations. One way to at least begin this discussion would be to compare frequencies derived from the number of affected in Table 2 (symptomatic) with the listed frequencies in Table 3 (ENBS). An additional table could show these comparisons for at least some of the disorders.

Answer:

Per your comments, the observed differences of incidence rates from before to after ENBS implementation will help other Asian countries to determine which diseases should be included in ENBS panels. However, such data remain available. Therefore, we could only compare the results of symptomatic (selective) screening with those of ENBS in Japan, as shown in Table 4. Other Asian studies will need to conduct similar studies after implementing ENBS. These points have been added to the Discussions section.

A paragraph in Discussion would discuss these comparisons in relation to decisions about ENBS, i.e., should some be excluded from identification or reporting in ENBS?

Answer:

As noted above, our data could not clarify which diseases should be targeted by ENBS. Nevertheless, our results suggest that diseases not requiring treatment, such as mild PA, could potentially be excluded in the future after the natural disease histories have been determined. This point has been added to the Discussion.

Molecular Genetics and Metabolism Reports/Original Article

Diversity in the incidence and spectrum of organic acidemias, fatty acid oxidation disorders, and amino acid disorders in Asian countries: selective screening vs. expanded newborn screening

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Abbreviations: GC/MS, gas chromatography-mass spectrometry; MS/MS, tandem mass spectrometry; OA, organic acidemia; FAOD, fatty acid oxidation disorder; AA, amino acid disorder; UCD, urea cycle disorder; IMD, inherited metabolic disease; NBS, newborn screening; ENBS, expanded newborn screening; MMA, methylmalonic acidemia; PPA, propionic acidemia; MCD, multiple carboxylase deficiency; GA1, glutaric acidemia type I; MCCD, 3-methylcrotonyl-CoA carboxylase deficiency; MGA, 3-methylglutaconic aciduria; HMGL, 3-hydroxy-3-methylglutaryl-CoA lyase; 4-OH-BA, 4-hydroxybutyric acidemia; 2-OH-GA, 2-hydroxyglutaric acidemia; BKTD, β-ketothiolase deficiency; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthetase; OXPA,

5-oxoprolinemia; GA2, glutaric acidemia type II; VLCAD, very long-chain acyl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; SCAD, short-chain acyl-CoA dehydrogenase; PCD, primary carnitine deficiency; CPT1, carnitine palmitoyltransferase I; CPT2, carnitine palmitoyltransferase II; TFP, trifunctional protein; LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase; CACT, carnitine-acylcarnitine translocase; HAD, 3-hydoxyacyl-CoA dehydrogenase; PKU, phenylketonuria; MSUD, maple syrup urine disease; HCU, homocystinuria; CTLN1, citrullinemia type I; ASA, argininosuccinic aciduria

Abstract

Background: Expanded newborn screening (ENBS) utilizing tandem mass spectrometry (MS/MS) for inborn metabolic diseases (IMDs), such as organic acidemias (OAs), fatty acid oxidation disorders, (FAODs), and amino acid disorders (AAs), is increasingly popular but has not yet been introduced in many Asian countries. This study aimed to determine the incidence rates of OAs, FAODs, and AAs in Asian countries and Germany using selective screening and ENBS records.

Materials and Methods: Selective screening for IMDs using gas chromatography-mass spectrometry and MS/MS was performed among patients suspected to be afflicted in Asian countries (including Japan, Vietnam, China, and India) between 2000 and 2015, and the results from different countries were compared. Similarly, ENBS results from Japan, South Korea, Taiwan, and Germany were compared. Additionally, the results of selective screening and ENBS in Japan were compared.

Results: Among 39,270 patients who underwent selective screening, IMDs were detected in 1,170. Methylmalonic acidemia was frequently identified in several countries, including Japan (81/377 diagnosed IMDs), China (94/216 IMDs), and India (72/293 IMDs). In Vietnam, however, β-ketothiolase deficiency was particularly frequent (33/250 IMDs). ENBS yielded differences in overall IMD rates by country:

1:8,557 in Japan, 1:7,030 in Taiwan, 1:13,205 in South Korea, and 1:2,200 in Germany.

Frequently discovered diseases included propionic acidemia (PPA) and phenylketonuria

(PKU) in Japan, 3-methylcrotonyl-CoA carboxylase deficiency (MCCD) and PKU in

Taiwan, MCCD and citrullinemia type I in South Korea, and PKU and medium-chain

acyl-CoA dehydrogenase deficiency in Germany. Furthermore, in Japan, selective

screening and ENBS yielded respective PPA frequencies of 14.7% and 49.4% among

all organic acidemias.

Conclusion: The incidence rates of IMDs vary by country. Moreover, the

disease spectra of IMDs detected via selective screening differ from those detected via

ENBS.

Keywords:

Organic acidemia; fatty acid oxidation disorder; amino acid disorder; inherited

metabolic disease; expanded newborn screening; incidence rate

1. Introduction

In recent years, mass spectrometric techniques, including gas chromatography-mass spectrometry (GC/MS) and tandem mass spectrometry (MS/MS), have been used for the biochemical diagnosis of inherited metabolic diseases (IMDs) such as organic acidemias (OAs), fatty acid oxidation disorders (FAODs), and amino acid disorders (AAs). Newborn screening (NBS) for OAs, FAODs, and AAs utilizing MS/MS is becoming popular worldwide and is referred to as expanded NBS (ENBS) [1]. The prognoses of patients with diseases targeted by ENBS have markedly improved in countries that have implemented [2-4].

Japan introduced nationwide NBS in 1977 and implemented nationwide ENBS in 2014 following a pilot study performed between 1997 and 2012 [5]. The latter is also being implemented in other Asian countries, including Taiwan [6] and South Korea [7]. However, ENBS has yet to be introduced in several other Asian countries in which epidemiological data pertaining to IMDs remain limited [8, 9]. Accordingly, Shimane University has provided biochemical IMD diagnostic services using GC/MS and MS/MS for symptomatic patients (defined as selective screening) from several Asian countries, including Vietnam, China, and India, for more than 15 years.

To investigate variations in the incidence rates of IMDs by nation, we investigated the frequencies of OAs, FAODs, and AAs among Asian countries using our selective screening and ENBS records. Furthermore, we compared the detection rates using selective screening and ENBS in Japan with the aim of reevaluating the target diseases of ENBS.

2. Materials & Methods

2.1 Selective screening

2.1.1 Subjects

Screening for IMDs was performed for symptomatic patients upon request by medical institutes in Japan and other Asian countries, including Vietnam, China, India, Indonesia, Thailand, Mongolia, South Korea, Malaysia, Taiwan, and Turkey. The screening was performed using GC/MS and MS/MS at the Department of Pediatrics, Shimane University Faculty of Medicine, Japan between 2000 and 2015. Samples from patients with clinical findings suspected to indicate IMDs, such as metabolic acidosis, ketosis, hyperammonemia, hypoglycemia, lethargy, hypotonia, myopathy-like symptoms, acute encephalopathy, and sudden infant death of unknown cause, were analyzed. If the request included the above symptoms, blood and/or urine samples with

the patient's data (e.g., clinical course and administered medication) were sent to

Shimane University. The frequencies of detected IMDs were retrospectively compared

between countries. This study was approved by the Shimane University Institutional

Committee on Ethics (registration No. 20170920-2).

2.1.2 GC/MS analysis

Urine samples were delivered at room temperature from Asian countries using

dried urine filter paper [10], whereas frozen urine samples were sent from Japanese

medical institutes to Shimane University. The urinary organic acid analysis was

conducted as reported previously [10, 11]. A 'GCMS QP-2010 Plus' instrument

(Shimadzu, Kyoto, Japan) was used for the analysis.

2.1.3 MS/MS analysis

Dried blood filter papers were shipped from overseas at room temperature to

Shimane University, as used for ENBS. Blood serum samples from some Japanese

patients were analyzed. Only samples from India were analyzed at Fukui University,

Japan. Blood acylcarnitines and amino acids were analyzed with MS/MS using in butyl-

derivatized specimens [12] at both Shimane and Fukui Universities. An API-3000 or

API-4000 instrument (Applied Biosystems, Foster City, CA, USA) or an LC/MS/MS-

8040 instrument (Shimadzu, Kyoto, Japan) was used for MS/MS.

2.1.4 Diagnosis

Biochemical diagnoses were based on the results of GC/MS and/or MS/MS. Data of organic acid analysis were processed using a personal computer-based automated GC/MS data processing and diagnostic system [11]. Whereas the diagnoses of nearly all Japanese patients were finally confirmed based on enzyme activity measurements and/or gene mutations, the diagnoses of foreign patients were based on the results of biochemical analyses indicating obvious specific abnormal metabolites in accordance with clinical data (e.g., age, sex, clinical course, laboratory data, and medication use) by several expert physicians who were familiar with IMDs.

2.2 ENBS

To investigate the detection rate of IMDs using ENBS, we obtained nationwide ENBS data from the principal ENBS investigators in Japan, Taiwan, South Korea, and Germany (the latter is a representative European country).

The nationwide ENBS data for each country were acquired as follows. The screening data of approximately 3.36 million infants were screened between 1997 and 2015 in Japan; this population included 1.95 million infants screened during the pilot study period between 1997 and 2012 as described in a domestic journal [5] and available data of 1.41 million infants screened from 2013 to 2015. Nationwide ENBS

data were not available at least in the period, because ENBS was conducted on a province-by-province basis. Although the annual birth number in Japan was about 1.0 million and the coverage rate was >99.9%. In Taiwan, approximately 1.39 million babies (coverage rate was >99.9%; government-funded) participated in ENBS during the period between 2001 and 2014, which included a pilot study conducted from 2001 to 2009 [6]. In South Korea, approximately 3.34 million babies (coverage rate was approximately 40 to 80%; paid screening) were screened between 2000 and 2015 as described in a Korean-language domestic report. In Germany, approximately 7.51 million babies (coverage rate was >99.9%; government-funded) were screened between 2002 and 2015, as described in a domestic report [13].

In each country, ENBS was performed according to nationally standardized methods as follows. In Japan, the butyl-derivatization method was used during pilot study; subsequently, a non-derivatized method has been used since at least 2014. In other countries, the butyl-derivatization method was used during the study period, as previously described [4] [6] [7]. Dried blood spots were collected 4–5 days after birth in Japan, 48–72 hours after birth in South Korea and Taiwan, and 36–72 hours after birth in Germany.

3. Results

3.1 Selective screening

3.1.1 Total number of analyzed cases

Among the 39,270 patients analyzed during the study period, 30,625 and 8,645 were from Japan and other Asian countries, respectively (Table 1). A total of 58,463 patient samples were analyzed, including 28,469 and 29,994 samples examined using GC/MS and MS/MS, respectively. Of these samples, 43,983 were obtained from Japanese patients and 14,480 were from patients in other Asian countries. Although similar numbers of GC/MS and MS/MS examinations were performed, only the latter type of analysis was requested for patients in whom FAOD was strongly suspected.

Selective screening identified 1,170 patients with IMDs, of which 377 and 793 were from Japan and other Asian countries, respectively (Table 1). The overall detection frequency was 3.0% (1.2% in Japan and 9.2% in other Asian countries). The detection frequencies were 8.2%, 8.6%, 13.9%, 4.4%, 4.8%, 1.2%, and 1.6% in Vietnam, China, India, Indonesia, Thailand, Mongolia, and other countries, respectively.

Table 1. Profiles of patients subjected to selective screening

Country	Total	Japan	Asia*[1]	Vietnam	China	India	Indonesia	Thailand	Mongolia	Others*[2]
Patients analyzed	**39,270**	**30,625**	**8,645**	**3,054**	**2,519**	**2,105**	**413**	**251**	**241**	**62**
Samples analyzed	**58,463**	**43,983**	**14,480**	**6,004**	**2,912**	**4,137**	**795**	**257**	**302**	**73**
GC/MS	28,496	21,288	7,181	2,960	1,529	2,045	385	9	196	57
MS/MS	29,994	22,695	7,299	3,044	1,383	2,092	410	248	106	16
Cases detected	**1,170**	**377**	**793**	**250**	**216**	**293**	**18**	**12**	**3**	**1**
Detection frequency (%)	**3.0**	**1.2**	**9.2**	**8.2**	**8.6**	**13.9**	**4.4**	**4.8**	**1.2**	**1.6**

[1] "Asia" indicates Asian countries other than Japan. [2] "Others" includes 38 patients from South Korea, 16 from Malaysia, 6 from Taiwan, and 2 from Turkey.

GC/MS, gas chromatography-mass spectrometry; MS/MS, tandem mass spectrometry

3.1.2 Diseases detected using selective screening

The selective screening results are shown in Table 2. Among 377 identified

patients with IMDs in Japan, methylmalonic acidemia (MMA) was detected in 81, urea

cycle disorders (UCDs) in 60, and glutaric acidemia type II (GA2) in 30. Propionic

acidemia (PPA) and multiple carboxylase deficiency (MCD) were each identified in 24

patients. In Vietnam, IMDs were discovered in 250 of the 3,054 screened patients; here,

maple syrup urine disease (MSUD) was identified in 36, UCDs in 34, β-ketothiolase

deficiency (BKTD) in 33, PPA in 22, and 5-oxoprolinemia (OXPA) in 19 patients. In

China, IMDs were detected in 216 of the 2,519 examined patients; MMA was identified

in 94, UCDs in 20, PKU in 18, PPA in 12, and glutaric acidemia type I (GA1) in 10

patients. In India, 293 of the 2,105 examined patients were diagnosed with an IMD; the

most prevalent diseases were MMA in 72, UCDs in 48, PPA in 26, MSUD in 24, and

PKU in 23 patients. In other Asian countries, 34 of the 967 examined patients were

diagnosed with IMDs, although no particular diseases were prevalent.

Table 2. Results of selective screening in Japan and other Asian countries

Country	Japan	Vietnam	China	India	Others*[1]
Number of patients	**377**	**250**	**216**	**293**	**34**
OA	**184**	**98**	**140**	**166**	**8**
Methylmalonic acidemia	81	12	94	72	2

Propionic acidemia	24	22	12	26	1
MCD	24	2	8	6	0
Glutaric acidemia type I	17	1	10	16	0
MCCD	8	2	0	4	0
3-methylglutaconic aciduria	5	2	1	3	1
HMGL deficiency	5	0	3	2	0
Alkaptonuria	5	0	1	9	0
4-hydroxybutyric acidemia	4	1	2	0	0
2-hydroxyglutaric acidemia	4	0	1	4	1
isovaleric acidemia	2	6	2	4	2
β-ketothiolase deficiency	2	33	4	14	1
HMGS deficiency	2	0	0	0	0
5-oxoprolinemia	1	19	2	6	0
FAOD	**88**	**29**	**16**	**10**	**5**
Glutaric acidemia type II	30	10	8	2	2
VLCAD deficiency	23	5	3	3	0
MCAD deficiency	14	1	3	2	0
Primary carnitine deficiency	13	8	1	1	1
CPT2 deficiency	6	4	1	1	1
TFP/LCHAD deficiency	2	1	0	1	1
AA and UCD	**74**	**101**	**46**	**108**	**11**
Phenylketonuria	4	10	18	23	3
Maple syrup urine disease	2	36	5	24	3
Homocystinuria	2	12	3	4	3
Urea cycle disorder	60	34	20	48	1
Citrin deficiency	6	9	0	9	1
Other diseases	**31**[*2]	**20**[*3]	**14**[*4]	**9**[*5]	**10**

[1] "Other countries" includes 18 patients from Indonesia, 12 from Thailand, 3 from Mongolia, and 1 from Malaysia. [2] Thirty-one patients in Japan with other diseases included 16 strongly suspected of FAOD with non-ketotic dicarboxylic aciduria, 10 with peroxisomal diseases, and 4 with other diseases. [3] Twenty patients with other diseases in Vietnam included 16 strongly suspected of having FAOD with non-ketotic dicarboxylic aciduria, 2 with peroxisomal diseases, and 1 each with carnitine palmitoyltransferase I (CPT1) deficiency and short-chain acyl-CoA dehydrogenase deficiency (SCAD) deficiency. [4] Fourteen patients with other diseases in China included 9 suspected of having FAOD with non-ketotic dicarboxylic aciduria, 2 with SCAD deficiency, and 1 each with CPT1 deficiency, mevalonic acidemia, and tyrosinemia type I. [5] Nine patients from India with other diseases included 3 strongly suspected of having

FAOD with non-ketotic dicarboxylic aciduria, 4 with tyrosinemia type I, and 1 each with ethylmalonic encephalopathy and 3-hydoxyacyl-CoA dehydrogenase deficiency.

OA, organic acidemia; MCD, multiple carboxylase deficiency; MCCD, 3-methylcrotonyl-CoA carboxylase deficiency; HMGL, 3-hydroxy-3-methylglutaryl-CoA lyase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthetase; FAOD, fatty acid oxidation disorder; VLCAD, very long-chain acyl-CoA dehydrogenase, MCAD, medium-chain acyl-CoA dehydrogenase; CPT2, carnitine palmitoyltransferase II; TFP, trifunctional protein; LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase; AA, amino acid disorder; UCD, urea cycle disorder

3.2 ENBS

3.2.1 The incidence of IMDs detected through ENBS

As shown in Table 3, the overall IMD detection incidences with ENBS were 1:8,557 in Japan, 1:7,030 in Taiwan, 1:13,205 in South Korea, and 1:2,200 in Germany. In Japan, diseases with higher incidences included PPA (1:41,000), PKU (1:46,000), very-long chain acyl-CoA dehydrogenase (VLCAD) deficiency (1:93,000), citrin deficiency (1:96,000), MMA (1:120,000), and medium-chain acyl-CoA dehydrogenase (MCAD) deficiency (1:129,000). In Taiwan, 3-methylcrotonyl-CoA carboxylase deficiency (MCCD) was most frequently detected (1:41,000), followed by PKU (1:58,000), citrin deficiency (1:61,000), primary carnitine deficiency (PCD) (1:70,000), and MMA, GA1, and MSUD (1:107,000 each). In South Korea, MCCD was the most commonly identified disorder (1:111,000), followed by citrulinemia type I (CTLN1) (1:115,000), tyrosinemia type I (1:123,000), isovaleric acidemia and PKU (1:138,000

each), and MMA (1:246,000). In Germany, PKU (1:5,000) was most frequently

detected, followed by MCAD deficiency (1:10,000), CTLN1 (1:60,000), MCCD

(1:73,000), and VLCAD deficiency (1:76,000).

Table 3. Comparison of expanded newborn screening detection incidences of IMDs per

country

Country	Japan[*1]	Taiwan[*2]	Korea[*3]	Germany[*4]
Total No. of newborns screened	**3.36 million**	**1.39 million**	**3.44 million**	**7.51 million**
Total detection incidence	**1:8,557**	**1:7,030**	**1:13,205**	**1:2,200**
OA	**1:22,000**	**1:18,000**	**1:31,000**	**1:10,000**
Methylmalonic acidemia	1:120,000	1:107,000	1:246,000	1:125,000
Propionic acidemia	1:41,000	1:464,000	1:313,000	1:250,000
Isovaleric acidemia	1:672,000	1:696,000	1:138,000	1:96,000
MCD	1:1,121,000	1:464,000	n.a.	0
MCCD	1:153,000	1:41,000	1:111,000	1:73,000
HMGL deficiency	0	n.a.	1:861,000	1:550,000
Glutaric acidemia type I	1:280,000	1:107,000	1:492,000	1:127,000
β-ketothiolase deficiency	0	n.a.	n.a.	0
FAOD	**1:30,000**	**1:34,000**	**1:111,000**	**1:9,000**
CPT1 deficiency	1:420,000	1:696,000	n.a.	1:1,020,000
VLCAD deficiency	1:93,000	1:1,392,000	1:383,000	1:76,000
MCAD deficiency	1:129,000	1:350,000	1:492,000	1:10,000
TFP/LCHAD deficiency	1:840,000	n.a.	1:1,148,000	1:176,000
CPT2 deficiency	1:257,000	1:696,000	n.a.	1:3,060,000
CACT deficiency	0	n.a.	n.a.	1:7,510,000
GA2	1:480,000	1:696,000	n.a.	1:195,000
Primary carnitine deficiency	1:199,000	1:70,000	1:345,000	1:250,000
HAD deficiency	1:3,363,000	n.a.	1:1,723,000	0
AA and UCD	**1:26,000**	**1:17,000**	**1:29,000**	**1:5,000**
Phenylketonuria	1:46,000	1:58,000	1:138,000	1:5,000

Maple syrup urine disease	1:841,000	1:107,000	1:1,148,000	1:164,000
Homocystinuria	1:1120,000	n.a.	1:492,000	1:132,000
Tyrosinemia type I	0	n.a.	1:123,000	1:150,000
Citrullinemia type I	1:306,000	1:199,000	1:115,000	1:60,000
Argininosuccinic aciduria	1:1121,000	1:593,000	1:1,148,000	1:292,000
Citrin deficiency	1:96,000	1:61,000	1:3,445,000	0

[1] Data from Japan are from 1997 to 2015. [2] Data from Taiwan are from 2001 to 2014. [3] Data from Korea are from 2000 to 2015. [4] Data from Germany are from 2002 to 2015.

OA, organic acidemia; MCD, multiple carboxylase deficiency; MCCD, 3-methylcrotonyl-CoA carboxylase deficiency; HMGL, 3-hydroxy-3-methylglutaryl-CoA lyase; FAOD, fatty acid oxidation disorder; CPT1, carnitine palmitoyltransferase I; VLCAD, very long-chain acyl-CoA dehydrogenase, MCAD, medium-chain acyl-CoA dehydrogenase; TFP, trifunctional protein; LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase; CPT2, carnitine palmitoyltransferase II; CACT, carnitine-acylcarnitine translocase; GA2, glutaric acidemia type II; HAD, 3-hydoxyacyl-CoA dehydrogenase; AA, amino acid disorder; UCD, urea cycle disorder; n.a., not available

3.2.2 Comparison of selective screening and ENBS in Japan

According to selective screening, MMA was the most frequently identified disorder in Japan, occurring in 81 (50.9%) of all 163 patients with OA, followed by PPA and MCD (14.7% each) (Table 4). In contrast, ENBS identified PPA as the most frequently diagnosed disorder, occurring in 82 (53.9%) of all 152 patients with OAs, followed by MMA (18.4%), and MCCD (14.5%). MCD was identified in only 2.0% of patients.

Among patients with FAODs, GA2 was the most frequently identified by selective screening (30 of all 88 patients with FAODs, or 34.1%), followed by VLCAD deficiency (26.1%), MCAD deficiency (15.9%), and PCD (14.8%). In contrast, ENBS

most frequently detected VLCAD deficiency (36 of 112 patients with FAODs, 32.1%),

followed by MCAD deficiency (23.2%), PCD (15.2%), and carnitine

palmitoyltransferase II deficiency (11.6%).

Among patients with AA, CTLN1 and citrin deficiency were most frequently

discovered via selective screening (27.3% of all AA patients each). Although PKU was

identified in only 4 patients upon symptomatic screening, it was the most commonly

detected disorder by ENBS (73 of 129 patients, 56.5%).

Table 4. Comparison of the results of selective screening and expanded newborn

screening in Japan

	Selective screening	ENBS
Cases diagnosed	**377**	**393**
OA	**163 (100%)**	**152 (100%)**
Methylmalonic acidemia	81 (50.9)	28 (18.4)
Propionic acidemia	24 (14.7)	82 (53.9)
Isovaleric acidemia	2 (1.2)	5 (3.3)
MCD	24 (14.7)	3 (2.0)
MCCD	8 (4.9)	22 (14.5)
HMGL deficiency	5 (3.1)	0
Glutaric acidemia type I	17 (10.4)	12 (3.1)
β-ketothiolase deficiency	2 (1.2)	0
FAOD	**88 (100%)**	**112 (100%)**
CPT1 deficiency	0	8 (7.1)
VLCAD deficiency	23 (26.1)	36 (32.1)
MCAD deficiency	14 (15.9)	26 (23.2)

TFP/LCHAD deficiency	2 (2.3)	4 (3.6)
CPT2 deficiency	6 (6.8)	13 (11.6)
GA2	<u>30 (34.1)</u>	7 (6.3)
Primary carnitine deficiency	13 (14.8)	17 (15.2)
HAD deficiency	0	1 (0,9)
AA	**22 (100%)**	**129 (100%)**
Phenylketonuria	4 (18.8)	<u>73 (56.5)</u>
Maple syrup urine disease	2 (9.1)	4 (3.1)
Homocystinuria	2 (9.1)	3 (2.3)
Citrullinemia type I	6 (27.3)	11 (8.5)
Argininosuccinic aciduria	2 (9.1)	3 (2.3)
Citrin deficiency	6 (27.3)	35 (27.1)

(%) percentage of each group disease.

Selective screening was performed at Shimane University. Newborn screening was performed across Japan during the period from 1997 to 2015, as described in the text. Underlined values indicate the diseases in which large differences in incidence were observed between symptomatic screening and ENBS.

OA, organic acidemia; MCD, multiple carboxylase deficiency; MCCD, 3-methylcrotonyl-CoA carboxylase deficiency; HMGL, 3-hydroxy-3-methylglutaryl-CoA lyase; FAOD, fatty acid oxidation disorder; CPT1, carnitine palmitoyltransferase I; VLCAD, very long-chain acyl-CoA dehydrogenase, MCAD, medium-chain acyl-CoA dehydrogenase; TFP, trifunctional protein; LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase; CPT2, carnitine palmitoyltransferase II; GA2, glutaric acidemia type II; HAD, 3-hydoxyacyl-CoA dehydrogenase; AA, amino acid disorder

4. Discussion

Our study revealed differences in the incidence of IMDs among Asian countries; these differences were observed when using selective screening and ENBS (including when compared with a European country– Germany – using the latter).

Furthermore, the IMD frequencies and spectra revealed by selective screening differed from those identified by ENBS.

Notably, selective screening and ENBS demonstrated unique characteristics regarding the incidence of OAs in each country. Although MMA was frequently detected in several countries, a high number of patients with PPA was detected in Japan; this can be attributed to a common Japanese-specific mutation (p.Y435C) of *PCCB* [14, 15], which is generally associated with mild phenotypes and unlikely to be detected during selective screening. Compared to other Asian countries, BKTD and OXPA were more frequent in Vietnam, while MMA was more frequent in China. The high incidence of BKTD in the Vietnamese population can be attributed to a common Vietnamese-specific *ACAT1* mutation (p.R208X) [16]. OXPA was also frequently detected in this study, and many patients presented with typical symptoms of glutathione synthetase deficiency, such as chronic metabolic acidosis and hemolytic anemia. However, data on the genetic etiology of OXPA are presently unavailable, and further consideration will be needed.

The high incidence of MMA in China as detected via ENBS is consistent with previous reports [17-19] and might be attributable to a common Chinese population-specific *MMACHC* mutation (p.W203X) [20, 21]. Patients with this type of MMA

develop homocystinuria (HCU) consequent to a *Cbl* C defect. Although MMA-like disease caused by vitamin B12 deficiency is well-known in South Asian countries such as Nepal and India, no Chinese patients with MMA caused by dietary vitamin B12 deficiency were identified in our study, possibly because of the lack of cases involving episodes and histories indicating a dietary vitamin B12 deficiency (e.g., megaloblastic anemia and gastro resection). Although MMA, PPA, and BKTD were also frequently detected in India, the reasons for these relatively higher frequencies are currently unknown. Several Indian research institutes are now conducting pilot studies on ENBS [22, 23], which may clarify the genetic backgrounds of these OAs.

Regarding FAODs, GA2, VLCAD deficiency, MCAD deficiency, and PCD were frequently detected during the selective screening of Japanese children, whereas GA2 was relatively common in other Asian countries. Using ENBS, the incidence rate of MCAD deficiency detected in Germany was 1:10,000, which is over 10-fold higher than that of Japanese patients. Approximately 90% of the mutant alleles of *ACADM* in Caucasian patients with MCAD deficiency are known to harbor a common variant (p.K329E) [24], and the p.Y67H mutation of *ACADM* has been identified in asymptomatic European patients [25]. In Asian countries, MCAD deficiency was more frequent in Japan than in Taiwan and South Korea and was associated with the common

ACADM mutation p.T150Rfs (c.449-452delCTGA), which is found in 30–40% of

mutant alleles in Japanese patients with MCAD deficiency [26, 27]. This mutation was

also reported in some patients from South Korea [28]. Hence, further studies in other

Asian countries and beyond should clarify the genetic background of MCAD

deficiency.

Regarding AAs, the frequencies of PKU, MSUD, and UCDs identified by

selective screening were greater in other Asian countries than in Japan. MSUD was

particularly common in Vietnam and India, suggesting that this condition may be

prevalent in southeastern Asian countries. In contrast, the numbers of Japanese patients

with PKU, MSUD, and HCU detected via selective screening were very small. These

diseases are already included in Japanese NBS panels, and therefore are not normally

requested during selective screening. PKU and citrin deficiency were detected relatively

frequently during ENBS in Japan and Taiwan. Citrin deficiency is considered more

prevalent in East Asian countries [29]. Notably, the incidence of PKU in Germany was

10-fold higher than that in Japan and Taiwan. These findings suggest that the incidence

rates of AAs differ between European and Asian populations.

A comparison of IMD detection rates in Japan via selective screening versus

ENBS revealed different disease frequencies per screening method. PPA, MCCD,

VLCAD deficiency, MCAD deficiency, PKU, and citrin deficiency were discovered more frequently with ENBS than with selective screening. In particular, PPA was less frequently identified by selective screening than by ENBS. On the other hand, a larger number of patients with MCD were identified via selective screening, whereas very few were discovered with ENBS. Of the 24 Japanese patients with MCD, at least 7 were diagnosed with MCD secondary to dietary biotin insufficiency, which might have contributed to the large number of MCDs identified via selective screening. Moreover, a Japanese group first reported the cloning of the *HCS* gene, which encodes holocarboxylase synthetase, and the discovery of a mutation contributing to the underlying etiology of MCD [30, 31]. This discovery may have raised awareness of MCD along with its characteristic clinical features among practicing physicians in Japan. However, the actual MCD detection rate with ENBS was likely very low. Moreover, biotinidase deficiency has not been observed in the Japanese population.

This study had several limitations of note. During selective screening, the diagnoses of most patients were based mainly on the results of biochemical analysis. Because we did not examine genetic factors or enzyme activities, we could not conclude whether the incidence of each IMD was influenced by the genetic background or by other causes (e.g., dietary vitamin B12 and biotin deficiencies or the intakes of some

drugs). Indeed, it may be difficult to prove that the high prevalence rates of some diseases could be attributed solely to population-specific common mutations. Furthermore, it might be difficult to completely compare the results of selective screening with those of ENBS. Nevertheless, the observed differences in incidence rates from before to after the implementation of ENBS will help other Asian countries to determine which diseases should be included in ENBS panels.

During the last 40 years, NBS has played an important role in preventing neurological impairment by detecting diseases such as PKU in the pre-symptomatic phase. Although this is also true for ENBS [1, 2, 4], ENBS can also detect diseases that may not require treatment, such as mild PPA or asymptomatic VLCAD deficiency, which are relatively common in Japan and Europe [3]. Although no previous report has described symptoms among patients with mild PPA, no evidence that these patients remain asymptomatic throughout their lives has not yet reported. Therefore, we cannot conclude whether mild PA should or should not be excluded from screening at the present point. Our results, however, suggest that such diseases could potentially be excluded in the future after determining the natural disease history. Additionally, the diseases targeted by ENBS may be amended to reflect the individual country. Finally, the target diseases identified by ENBS are generally extremely rare. Therefore,

international collaborative activities such as the current study are important to the

clarification of the natural histories of these diseases, development of diagnostic

methods and therapies, and elucidation of genetic backgrounds.

5. Conclusion

Our study identified diverse IMD incidence rates and disease spectra among

Asian countries. The IMD disease spectra determined by selective screening differed

from those detected by ENBS. These data may facilitate improvements in ENBS

implementation, the development of diagnostic and therapeutic groundwork, and the

enhancement of welfare policies (including reductions in screening costs).

analysis and interpretation of data; writing of the report; or the decision to submit the article for publication.

Conflicts of interest

The authors have no conflicts of interest to declare regarding the publication of this manuscript.

Acknowledgments

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