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Applying targeted next generation sequencing to dried blood spot specimens from suspicious cases identified by tandem mass spectrometry-based newborn screening

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Abstract

Background: Tandem mass spectrometry (TMS)-based newborn screening has been proven successful as one of the public healthcare programs, although the practicability has not yet been specifically addressed.
Methods: Sixty residual dried blood spot (DBS) specimens from confirmation/diagnosis-insufficient cases discovered by TMS screening were analyzed by targeted next generation sequencing (TNGS) assay.
Results: In total, 26, 11, 9, and 14 cases were diagnosed as positive, high risk, low risk, and negative, respectively.
Conclusions: Applying the DBS-based TNGS assay for the accurate and rapid diagnosis of inborn errors of metabolism (IEMs) is feasible, competent, and advantageous, enabling a simplified TMS screening-based, TNGS assay-integrated newborn screening scheme highlighting an efficient, executable, and one-step screening-to-diagnosis workflow.

Keywords: dried blood spot; inborn errors of metabolism; newborn screening; tandem mass spectrometry; targeted next generation sequencing.

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Introduction

As one of the most successful public health prevention programs, newborn screening (NBS) aims at reducing neonatal morbidity and mortality caused by birth defects [1]. Through various tests, NBS identifies dozens of life-threatening inborn errors of metabolism (IEMs) in newborns within a few days of life, enabling timely diagnosis and proper healthcare for improvement of neonatal survival and health [1–3].

The application of the tandem mass spectrometry (TMS) technique since 1990s greatly expanded the number of screenable conditions due to its sensitive, specific, and quantitative performance upon monitoring dozens of analytes simultaneously. Via the detection of amino acids and acylcarnitines, up to 40 IEMs – mainly categorized into amino acid, organic acid, and fatty acid disorders – are able to be identified in a single 2–3 min run [2]. Broader applications on other IEMs and genetic diseases such as lysosomal storage disorders and severe combined immune deficiency were also reported [2–5].

Nevertheless, the TMS screening faces bottlenecks similar to those of other conventional tests. The TMS results are prone to be affected by gestational age, birth weight, nutrition, medication, geographical/ethnical differences, etc. To reduce false-positives and false-negatives, repetitive, confirmatory and second-tier testings are always necessarily following positive results from primary screening, resulting in over-recall, lengthy workflow, and delayed diagnosis, not to mention the varying recall measurement and execution efficiency at confirmatory and diagnostic stages across different regions, countries, territories, etc. [2, 3, 6–9]. Although series of tests and examinations are available, these combined results may still not be pathogenically differential or diagnostically indicative; thus, more definitive tests such as enzymatic or genetic assays are usually introduced to reduce unambiguity in diagnosis [1, 4, 6].

Enzymatic assays have long been regarded as the gold-standard diagnostic method for IEMs because of their exploration of phenotypic pathogenicity. However, these assays are highly diverse, low-throughput, laborious, time-consuming, and rigorous on sample requirement [1, 6]. As an alternative,

genetic assays have been adopted to diagnose suspicious IEMs by exploring genotypic pathogenicity. Rapid technical development has enabled sophisticated clinical applications of next-generation sequencing (NGS) techniques, such as whole genome sequencing (WGS), whole exome sequencing, and targeted NGS (TNGS). Because of their precise, accurate, sensitive, scalable, and high-throughput properties, and due as well to the decreasing costs and diminishing technological barriers to their implementation, genetic assays are gradually moving from downstream diagnosis to upstream screening of IEMs [10–12].

In this paper, we investigated the feasibility of incorporating TNGS assay into the current TMS screening scheme by measuring residual dried blood spot (DBS) specimens from confirmation/diagnosis-insufficient (CDI) cases and attempted to propose a simplified screening-confirmation/diagnosis workflow after a brief evaluation of cost-effectiveness.

Materials and methods

Subjects and samples

Between May 2013 and February 2015, 81,664 infants were screened by TMS in BGI-Tianjin Clinical Testing Laboratory, China. However, there still have been a number of CDI cases, including (a) those with positive primary screening but recall and diagnosis failure (RF group) and (b) those with positive primary screening and recall but diagnosis unclear (DU group). To investigate the diagnosability of these CDI cases, we adopted a targeted multigene panel NGS assay (called AngelCare) to genotypically diagnose TMS-detectable disorders based on residual DBS samples left from the TMS screening.

Cases in the RF group with highly abnormal results (n = 82) and cases in the DU group (n = 19) were enrolled in this study. Meanwhile, to ensure sufficient yield and concentration of extracted DNA required for exon capturing, library preparation, and Sanger validation, only cases with residual DBS capable of punching at least three discs (id = 3.2 mm) were accepted in this study. Eighty-three cases were preliminarily sorted out, and 60 cases finally met the sample preparation criteria. The summary of the current TMS screening-diagnosis yield, including an overview schematic of sample sorting for AngelCare assay, was shown in Supplemental Figure 1 and Table 1.

The present study has been approved by the Ethics Committee of Beijing Genomic Institute (BGI), Shenzhen, China. The parents of subjects were all informed about the research and consented on the behalf of participants to this study.

Design of the gene panel

The capture panel, developed by BGI, contains 169 known causative genes for 87 common inherited diseases, in which 49 TMS-detectable IEMs were included (Supplemental Table 2). Capture regions included the translated regions whose genes have been chosen, and also include 50 bases within the upstream and downstream areas. The whole target region size is approximately 1 M. The probe is 90 bp in length with a 5- to 10-bp overlap in restricted regions. As a reference sample, the in-house YH cell line was used to evaluate the platform. After mapping to the reference (GRCh37/hg19), 67.17% of the yielded clean data were uniquely matched to target regions, with 99.45% of the targeted region covered in YH. The mean depth of the coverage was 158.94 X. More than 95% targeted regions were covered at a depth of 30× in YH. The variant calling accuracy of the reference sample was 98.104% for the YH. The false-positive and false-negative rates of YH were 0.952% and 0.803%, respectively.

DNA extraction, target region capture, and next-generation sequencing

Genomic DNA was extracted from DBS, using the MagPure DNA Kit (Magen, China). Next, 50 ng genomic DNA was fragmented by transposes and tag sequence was added. Then index labeling and sequencing components were added to the paired end of the target DNA fragments using polymerase chain reaction (PCR). The target region fragments were enriched by an in-house capture chip (BGI) through hybridization and capture procedures. The distribution of the fragment size of tagged DNA (200–500 bp) was analyzed utilizing the 2100 Bioanalyzer. Final captured DNA libraries were sequenced using the Illumina HiSeq 2500 platform.

Data filtering and functional annotation

Primary reads were generated using the Hiseq 2500. Low-quality reads were removed from the primary data using an in-house filtering algorithm, and the remaining reads were further aligned using the Burrows-Wheeler Aligner program version 0.5.9-r16 to the human genome assembly hg19 [13]. Sequence variants were called using the Genome Analysis Toolkit software package (version 2.4) carefully following the best practice guidelines recommended by GATK [14–16]. Finally, variant calls were annotated according to BGI internal reference panels and public databases including the dbSNP, 1000 genome variants database, CGD, ESP6500, and ENSEMBL prediction database, using in-house annotation pipeline scripts.

Mutation and genotype interpretation

Functional predictions for the mutations detected were made using the Mutation taster, SIFT, and PolyPhen software packages. Mutations were classified as either pathogenic (P), likely pathogenic (LP), or variants of uncertain significance (VUS), based on the ACMG Guidelines [17]. Novel mutations were assessed for the possibility of pathogenicity, and sequence conservation was evaluated using SIFT and PolyPhen.

Sanger sequencing

Identified pathogenic single-nucleotide variants and small insertions/deletions variants were confirmed by Sanger sequencing. Primers were designed using Primer 5.0 software. We amplified the target sites and the flanking regions of each DNA template individually using TAKARA

ExTaq. The final volume used was 25 μL, consisting of 20 ng DNA template in the liquid, and 10 μL (10 μmol/L) of forward and reverse primer. The PCR product was purified using ExoSAP-IT, followed by sequencing with the ABI 3500 genetic analyzer. Mutation Surveyor software was used to analyze the results.

Molecular diagnostic model

To improve the diagnostic confidence of AngelCare, the TMS screening results were integrated for diagnostic decision making. Based on the association of TMS results with TNGS results, both concordant and discordant (including irrelevant) outcomes were evaluated for each genotype. A molecular diagnostic model was briefly built, in which individual genotype was first categorized by mutations, zygosity, and heredity, then aligned with association outcome, and finally assigned a molecular diagnostic decision (positive, high risk, low risk, and negative).

For P/P or P/LP genotype, a diagnostic decision was made to declare the sample positive, regardless of zygosity, heredity, and TMS association. LP/LP, P/VUS, LP/VUS, or VUS/VUS genotype results the same decision as P/P and P/LP, except that discordant association degrades the decision from positive to high risk. For dominant heredity, P/-, LP/-, or VUS/- genotype likewise gets the same decision as above, whereas for recessive heredity, P/-, LP/-, or VUS/- genotype was designated as low risk except that concordant association upgrades the decision from low risk to high risk. If no mutation was found, diagnostic decision was made as negative except that the presence of confirmatory TMS results upgrades the decision from negative to low risk. The detailed molecular diagnostic decision made on each genotype is summarized in Supplemental Table 3.

Results

AngelCare assay

A total of 53 non-redundant mutations were identified and confirmed. Based on the ACMG classification, 38 (72%), 8 (15%), and 7 (13%) mutations were classified as P, LP, and VUS type, respectively, the last of which consisted entirely of novel mutations. Among the 11 mutations displaying redundancy, 9, 1, and 1 were found in 2, 3, and 4 samples, respectively, and 10 and 1 were found in P and VUS type, respectively (Table 1).

The identified mutations came from 17 non-redundant genes that are relevant to 11 discrete metabolite signatures, indicative of five amino acid, six organic acid and six fatty acid disorders, and four genes that correlate to four disorders that defied TMS screening. All of the genes follow autosomal recessive heredity, except for one that presents a partial autosomal dominance property (Table 1).

Out of all the 60 samples, 41 were found containing at least one mutation. To further classify, 36 were found to have genes with just a single mutation, and five were found containing several concurrently mutated genes. If the genotype was taken from the one that potentially gives the most severe outcome for the latter, five homozygotes, 20 compound heterozygotes, and 16 heterozygotes were identified (Table 1).

Molecular diagnostic outcome

A total of 43% (26/60), 18% (11/60), 15% (9/60), and 23% (14/60) of cases were diagnosed as positive, high risk, low risk, and negative, respectively (Table 1), based on the molecular diagnostic model; 42% (19/45)/47% (7/15) of positive, 18% (8/45)/20% (3/15) of high risk, 7% (3/45)/40% (6/15) of low risk, and 31% (14/45)/0% (0/15) of negative cases were found in RF/DU group, respectively.

The 26 positive cases cover 10 kinds of diseases, including 3, 3, 3, and 1 types of amino acid (n = 12), organic acid (n = 5), fatty acid (n = 7), and lysosomal (n = 2) disorders, respectively (Supplemental Figure 1). All the diseases are included in the U.S. federally recommended uniform screening panel, with five belonging to the core panel, and the other five to the secondary panel, consisting of common rare diseases that have an average prevalence of 1:10,000 ~ 1:50,000 except tyrosinemia type III (TYRIII) that was reported very rare [18–20].

Phenylalanine hydroxylase (PAH) deficiency is the most frequently diagnosed disease (n = 8), and all came from the RF group. Because the conventional fluorometric screening program of PAH was independently conducted by our clients, the diagnostic results were compared between each other, and one case (NBS45) was found missed by the conventional screening (data not shown). Because of the above reason, only one positive PAH case was counted for the cost-effectiveness evaluation. Additionally, one methylmalonic acidemia (MMA) case, as well as the propionic acidemia (PA) case, was independently confirmed by our clients, with the former consistent and the latter reported as MMA.

In summary, the molecular diagnostic outcome demonstrates the robust competence of TNGS assay integration into the TMS screening scheme. Not only were a considerable number of positive cases and diseases identified, the diagnostic accuracy of TNGS is also superior to that of TMS.

Cost-effectiveness evaluation

Based on the previously retrieved diagnostic information, the TMS screening conducted in our lab during May 2013–February 2015 has identified 20 cases of IEMs

Table 1: Summary of TNGS and MD results.

Sample no.	Genes	Mutations	ACMG category	Zygosity	Diseases	Heredity	TMS signature	Group	Association	Molecular diagnosis
NBS55	PAH	c.721C>T	P	ComHet	PAH	AR	Phe	RF	Con	P
		c.755G>A	P				Phe/Tyr			
NBS51	PAH	c.208_210delTCT	P	ComHet	PAH	AR	Phe	RF	Con	P
		c.505C>T	P				Phe/Tyr			
NBS52	PAH	c.331C>T	P	ComHet	PAH	AR	Phe	DU	Con	P
		EX6 del	LP				Phe/Tyr			
NBS45	PAH	c.1238G>C	P	ComHet	PAH	AR	Phe	RF	Con	P
		c.158G>A	P				Phe/Tyr			
NBS46	PAH	c.158G>A	P	ComHet	PAH	AR	Phe	RF	Con	P
		c.728G>A	P				Phe/Tyr			
NBS47	PAH	c.1222C>T	P	ComHet	PAH	AR	Phe	RF	Con	P
		c.532G>A	P				Phe/Tyr			
NBS56	PAH	c.694C>T	P	ComHet	PAH	AR	Phe	RF	Con	P
		c.441+3G>C	P				Phe/Tyr			
NBS57	PAH	c.728G>A	P	ComHet	PAH	AR	Phe	RF	Con	P
		c.1238G>C	P				Phe/Tyr			
NBS12	SLC22A5	c.1400C>G	P	Hom	CUD	AR	C0	DU	Con	P
NBS58	SLC22A5	c.51C>G	P	ComHet	CUD	AR	C0	RF	Con	P
		c.1400C>G	P							
NBS11	SLC22A5	c.1198C>T	VUS	ComHet	CUD	AR	C0	DU	Con	P
		c.1363C>A	VUS							
NBS26	SLC22A5	c.51C>G	P	ComHet	CUD	AR	C0	RF	Con	P
		c.1363C>A	VUS							
NBS10	MMACHC	c.609G>A	P	Hom	MMA-HCY	AR	C3	DU	Con	P
							C3/C2			
NBS60	MMACHC	c.609G>A	P	ComHet	MMA-HCY	AR	C3	RF	Con	P
		c.452A>G	P				C3/C2			
NBS15	MMACHC	c.658_660delAAG	P	ComHet	MMA-HCY	AR	C3	DU	Con	P
		c.689G>A	VUS				C3/C2			
NBS53	ACADVL	c.553G>A	P	ComHet	VLCAD	AR	C16	RF	Con	P
		c.1276G>A	P				C12			
							C14			
							C18			
NBS50	ACADVL	c.664G>C	P	ComHet	VLCAD	AR	C14:1	RF	Con	P
		c.1349G>A	P				C12			
							C14			
NBS44	MAT1A	c.791G>A	P	Het	MET	AD/AR	Met	RF	Con	P
NBS48	MAT1A	c.791G>A	P	Het	MET	AD/AR	Met	RF	Con	P
NBS13	MAT1A	c.769G>A	LP	Het	MET	AD/AR	Met	DU	Con	P
	PAH	c.442-1G>A	P	Het	PAH	AR				
NBS37	GAA	c.761C>T	P	ComHet	GSD II	AR	C0	RF	Dis	P
		c.752C>T	P							
NBS3	GNMT	c.451+1G>C	LP	Het	MET	AD/AR	Arg	RF	Dis	P
	GAA	c.761C>T	P	ComHet	GSD II	AR				
	GAA	c.752C>T	P							
NBS49	ACADS	c.1031A>G	P	Hom	SCADD	AR	C4	RF	Con	P
							C4/C2			
NBS9	ACAD8	c.749A>G	VUS	ComHet	IBD	AR	C4	DU	Con	P
		c.1156_1158delCAG	P				C4/C2			
NBS54	PCCA	c.305delA	LP	ComHet	PA	AR	C3	RF	Con	P
		c.1288C>T	P				C3/C2			
NBS59	HPD	c.97G>A	P	Hom	TYR III	AR	Tyr	RF	Con	P
NBS21	SLC22A5	c.1400C>G	P	ComHet	CUD	AR	C0	RF	Con	HR
	PAH	c.158G>A	P		PAH	AR				
NBS25	SLC22A5	c.695C>T	P	Het	CUD	AR	C0	RF	Con	HR

Table 1 (continued)

Sample no.	Genes	Mutations	ACMG category	Zygosity	Diseases	Heredity	TMS signature	Group	Association	Molecular diagnosis
NBS24	SLC22A5	c.1400C>G	P	Het	CUD	AR	C0	RF	Con	HR
NBS23	MCCC1	c.639+2T>A	P	Het	3-MCCD	AR	C5OH C5OH/C2	RF	Con	HR
NBS22	MCCC1	c.1829delA	LP	Het	3-MCCD	AR	C5OH C5OH/C2	RF	Con	HR
NBS14	CPT2	c.1711C>A	VUS	Hom	CPT II	AR	C0	DU	Dis	HR
NBS27	MCEE	c.286A>G	VUS	Het	MMA	AR	C3 C3/C2	RF	Con	HR
NBS32	ATP7B	c.525dupA c.722A>G	P VUS	ComHet	WD	XR	C5	RF	Dis	HR
NBS7	SLC25A13	c.1092delT	P	Het	NICCD	AR	Cit Met Tyr Arg	DU	Con	HR
NBS8	MCCC2	c.1025G>A	LP	Het	3-MCCD	AR	C5OH C5OH/C2	DU	Con	HR
NBS29	PAH	c.611A>G	P	Het	PAH	AR	Phe Phe/Tyr	RF	Con	HR
NBS28	SLC25A13	c.2T>C	P	Het	NICCD	AR	Phe	RF	Dis	LR
	SLC26A4	c.919-2A>G	P	Het	DFNB1	AR	Phe/Tyr			
NBS41	SLC25A13	c.2T>C	P	Het	NICCD	AR	Tyr	RF	Dis	LR
	IDUA	c.1225G>C	LP	Het	MPS I	AR				
	CPT1A	c.1683delA	LP	Het	CPT1A	AR				
NBS16	MUT	c.323G>A	P	Het	MMA	AR	C8 C10	RF	Dis	LR
NBS35	ATP7B	c.2333G>T	P	Het	WD	XR	Tyr	RF	Dis	LR
NBS4							C5OH	DU	Dis	LR
NBS5							C5OH	DU	Dis	LR
NBS2							Arg	DU	Dis	LR
NBS1							Arg	DU	Dis	LR
NBS6							C4 C4/C2	DU	Dis	LR
NBS30							C16OH C16:1OH	RF	Dis	N
NBS31							Cit	RF	Dis	N
NBS34							C3DC	RF	Dis	N
NBS36							C3DC	RF	Dis	N
NBS39							Phe Phe/Tyr	RF	Dis	N
NBS33							Val Phe Orn Tyr Arg	RF	Dis	N
NBS17							C5	RF	Dis	N
NBS18							Orn	RF	Dis	N
NBS19							Tyr	RF	Dis	N
NBS38							C5	RF	Dis	N
NBS40							C8C10	RF	Dis	N
NBS42							C8C10	RF	Dis	N
NBS43							C0	RF	Dis	N
NBS20							C3C3/C2	RF	Dis	N

Summary of TNGS and molecular diagnosis results. RF, recalled-and-diagnosis failure; DU, diagnosis unclear; Con, concordant; Dis, discordant. Molecular diagnosis: P, positive; HR, high risk; LR, low risk; N, negative.

(Supplemental Figure 1), accounting for an overall screening rate of 1:3712 that was similar to the reported. The current study additionally diagnosed 19 cases due to the complementation of TNGS assay, resulting in an overall screening rate of 1:2094.

A simplified calculation was made to evaluate the cost-effectiveness of incorporating TNGS into the current TMS screening program [7]. We suppose that TMS screening costs 500 RMB per capita, and that TNGS assay costs about 10,000 RMB per capita. Considering the total expense average of an IEM subject to be 3 ~ 9 million RMB if not found, diagnosed, and managed properly [7], the current TMS screening program would have an estimated cost-effectiveness rate of 1:1.47 ~ 4.41. If we integrate the TNGS assay into the current TMS screening program, which would mean that all of the cases in the RF and DU group would be subjected to the TNGS assay, and still the 19 cases were correctly diagnosed, the conservatively estimated cost-effectiveness rate would be 1:2.64 ~ 7.92, a nearly 80% improved health-economic benefit. Therefore, a simplified TMS screening-based, TNGS assay-integrated screening-diagnosis workflow under the current TMS-based NBS scheme is proposed (Figure 1).

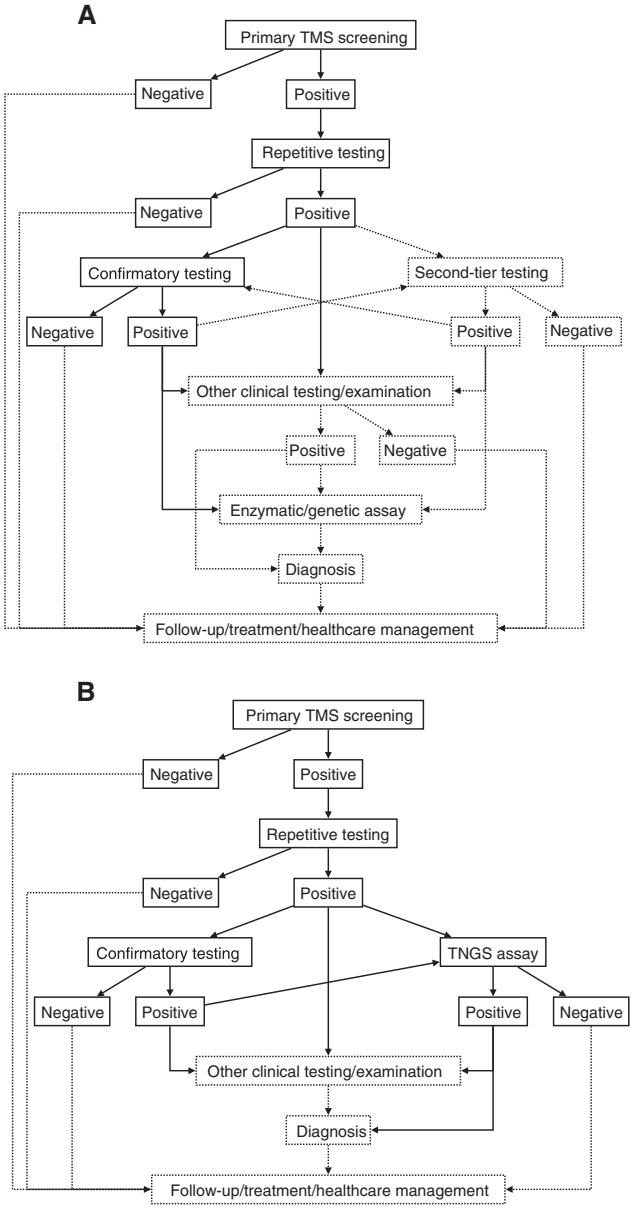

Figure 1: Current and proposed screening schemes.
(A) Current TMS-based method goes with redundant procedures and low-efficiency. (B) TNGS assay-integrated screening-diagnosis workflow is more time and cost-effective.

Discussion

The current situation of TMS screening in China

Compared to the sophisticated TMS applications in expanded NBS in developed countries [1, 3, 4], China has been making great efforts to explore, evaluate, and implement TMS screening since 2003 [18, 21, 22]. At present, China has an estimated national coverage of 20%, concentrated in well-developed provinces and cities [21, 22].

Unlike the conventional NBS of PAH and congenital hypothyroidism (CH), current TMS screening is neither mandatory nor has uniform technique guidelines provided by the Ministry of Health (MOH) [23]. It is usually a joint effort of provincial and municipal NBS centers (mostly affiliated with Maternal and Children Healthcare [MCH] centers or hospitals), renowned hospitals, third-party independent clinical testing laboratories, and others to promote local pilot studies, screening, diagnosis, management, and follow-up [3, 18, 24]. Because most independent clinical testing labs are non-governmental medical organizations and just commissioned with TMS screening, the suspicious subject recall and diagnostic information retrieval are neither straightforward nor efficient. Besides this complication, the participated institutions involved in the TMS screening also face challenges like limited governmental recognition and support, insufficient professional and public education, decentralized administration, early hospital discharge, etc. [3, 24, 25], making implementation of the current TMS screening scheme less efficient and beneficial to health economics. Because of incomplete recall and insufficient confirmation or diagnosis, we utilized a customized TNGS assay to investigate residual DBS samples of CDI cases from the

current TMS screening scheme in order to complement the screening-confirmation-diagnosis workflow.

TNGS assay

The AngelCare assay is designed for DBS-adoptable, fast, and reliable confirmation and diagnosis of well-characterized Mendelian genetic disorders such as IEMs, immunological disorders, and miscellaneous genetic conditions, which are commonly discovered between birth and late childhood. It has been successfully applied in the molecular diagnosis of high-risk infants suspected of having IEMs [26]; however, the utility of TNGS assay integration into the TMS screening scheme has not yet been systematically evaluated. Because all TMS-screenable disorders were covered and residual DBS samples from TMS screening available, the exploration of a TNGS-integrated, simplified, and more executable TMS screening scheme seems plausible and feasible.

Unlike P or LP genetic variants with relatively strong pathogenic confidence, VUS variants are not readily used for positive diagnosis unless concordant association with TMS screening results is obtained. Functional prediction (Supplemental Table 4) revealed that all but one of the seven VUS mutations present a certain degree of pathogenic tendency, among which four have coincident biochemical features that result in positive diagnosis, one (c.286A>G) has coincident biochemical features that result in high-risk diagnosis (recessive carrier), and one (c.1711C>A) has discrepant biochemical features that result in high-risk diagnosis (homozygote). Interestingly, the (c.1711C>A) mutation came from the CPT2 gene, corresponding to carnitine palmitoyltransferase II deficiency (CPTII) that is usually suspected with elevated C16 and/or C18:1 acylcarnitine [19, 20]. However, the homozygous (c.1711C>A) was found correlating to a measured decrease (in both primary and confirmatory tests) of free carnitine that is indicative of carnitine uptake defect (CUD), resulting in a CUD-negative diagnosis made by our client (data not shown). This scenario implies either an updated interpretation of CPTII/CUD through TMS screening or a proof-of-evidence of irrelevant pathogenicity, meriting further follow-up and confirmation.

Molecular diagnosis

The molecular diagnostic model was built to consolidate the TNGS results. Unlike the integrated screening model reported by others that mainly focused on the TNGS-TMS concordant cases [6], the algorithm assigns each case with either concordant or discordant association to a diagnostic decision through leveraging the TNGS and TMS results against one another. The positive outcome indicates a definitive molecular diagnosis of highly certain disease that deserves immediate response. The high-risk outcome indicates potential risk of underdiagnosis that necessitates further confirmation, interpretation, and clinical examination before making a definitive diagnosis. The low-risk outcome indicates caution against morbidity predisposition and long-term follow-up. The negative outcome indicates a relatively safe and healthy status.

According to the publications of several expanded NBS programs, this study signifies considerable underestimation and territorial variation of prevalence of identified diseases although a limited population size was investigated. For instance, the highest published prevalence of CUD in the Southern China was 1:32,354, whereas four cases have already been identified merely from the CDI cases out of a population of 81,664 in the Northern China [18]. Several other identified diseases follow similar prevalence pattern [18, 21, 22].

Proposition of screening-diagnosis workflow

The current TMS screening scheme follows a screening-confirmation-diagnosis workflow that involves multiple institutions and departments, with our lab mainly getting involved in primary, repetitive, and confirmatory testings, and with others in miscellaneous clinical testings, examinations, and diagnosis. Although certain diseases (such as MUSD, TYRI, IVA, etc.) could follow a relatively short turnaround time (TAT) from screening to diagnosis-in part due to clear primary and confirmatory indications and guidelines – a considerable amount of diseases that share similar biochemical traits may require more laborious and time-consuming rounds of confirmation and discrimination, and finally end up with TNGS assays to make definitive diagnosis to facilitate treatment and follow-up, resulting in excessive resource utility and extended TAT. Take the elevation of propinoylcarnitine (C3) and/or propinoylcarnitine/acetylcarnitine (C3/C2) as an example [27]; this biochemical trait usually indicates both MMA and PA. To discriminate the two diseases as well as different types of MMA (isolated and homocystinuria-combined), routine clinical testings and biochemical assays such as urine/plasma amino acids, organic acids, and vitamins had to be conducted at separately qualified facilities. The vitamin B12 response assay was then followed to further discriminate subjects into subtypes that correspond to distinct outcome, treatment, and follow-up

before definitive diagnose was made. The estimated TAT could be 12 ~ 25 days since the beginning of primary screening depending on the testing relay as well as the recall/transfer delay, which would not be affordable for cases that present neonatal-onset manifestations [27]. In our study, 3 MMACHC-mutated MMA and 1 PCCA-mutated PA, 1 MCEE-mutated MMA, and 1 MUT-mutated MMA were retrospectively diagnosed as positive, high risk and low risk respectively, implying the necessity of timely and unambiguous diagnosis.

The proposed workflow highlights direct TNGS assay following suspicious primary TMS screening based on the same DBS specimen, thus enabling streamlining and substitution of the confirmatory/second-tier/diagnosis procedure that both shortened TAT and improved service quality. Furthermore, as far as the TAT of TNGS assay was concerned, we can now achieve a throughput for 20 samples in 5 days, comprising 2-day sample preparation, 2-day sequencing and analysis, and 1 day of confirmation and validation [28]. Because the primary screening rate is about 1:200, a maximum daily TMS screening throughput of 4000 could be processed in accordance with the TNGS assay. Therefore, a total TAT of 8 ~ 10 days for the one-stop screening-diagnosis workflow is reasonable and acceptable for diseases that otherwise would necessitate comprehensive testings.

Limitation

Although this pilot study demonstrates a successful application of TNGS assay toward integrating into the TMS-based NBS scheme, there are three limitations that need to be addressed:

First, cases with only mildly abnormal results in the RF group were not included in this study though the underdiagnosis upon this subgroup is presumably true. From our data, we found 1 out of 7 diagnostic positive cases and 1 out of 3 diagnostic high-risk cases that have mildly abnormal results on primary TMS screening in the DU group, implying improved diagnostic yield if all of the cases in the RF group were TNGS assayed. On the other hand, insufficient sample quantity as well as unqualified sample preparation (which takes 16% of sort-out cases), probably owing to sample contamination or degradation (data not shown), also resulted in the rejection of several cases from enrollment. Nevertheless, underdiagnosis might still be inevitable, even when all of the confirmation/diagnosis-insufficient cases are included since the false-negativity of primary TMS screening was not taken into account [1, 4, 7].

Second, IEMs might be caused by variants located outside of exons, and thus are not detectable by TNGS [29, 30]. Although the majority of functionally critical and disease-causing mutations occur in protein-coding regions, most of the genome is noncoding and may contain overlooked pathogenic variants. Besides, the TNGS assay is insensitive to detection of copy number variation (CNV) and other structural variants [28, 31]. In our study, 9 out of 11 high-risk cases were identified as having a recessive carrier mutation with concordant association, among which 2 even have confirmatory TMS results. Because one case was independently diagnosed as PKU, we infer that more positive cases could be identified if genetic assays such as WGS, Q/M PCR, and Sanger sequencing were complementarily applied to explore potential pathogenic mutations and variations [32].

Third, molecular diagnosis could not completely represent clinical diagnosis. On the one hand, not all the genotype/phenotype correlation of each mutation/variation is supportively confident. That's why VUS mutations and others deserve further observation, annotation, interpretation, confirmation, and validation, and more retrospective studies have to be conducted to lead prospectively consolidated applications [33]. On the other hand, many IEMs are clinically heterogeneous that the molecular diagnosis is not always practically helpful [34] because of the imperfect penetrance of each individual genotype. It has been reported that the implementation of TMS screening program has led to more identified cases bearing certain disorders of which most subjects are asymptomatic throughout their life [9, 20–22]. The environmental/maternal/ethnic effect as well as miscellaneous manifestation also complicated the genotype/phenotype correlation [35–38]. Therefore, enhanced cooperation and communication among fundamental, experimental, and clinical medicine cannot be addressed more.

Conclusions

By measuring the residual DBS specimens of the CDI cases from the current TMS screening program, we demonstrated the feasibility, competency, and advantage of applying TNGS assay upon accurate and rapid diagnosis of IEMs, enabling a simplified TMS screening based. TNGS assay-integrated NBS scheme highlighting efficient and executable one-step screening-diagnosis workflow.

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References

1. Almannai M, Marom R, Sutton VR. Newborn screening: a review of history, recent advancements, and future perspectives in the era of next generation sequencing. Curr Opin Pediatr 2016;28:694–9.
2. Wilcken B, Wiley V, Hammond J, Carpenter K. Screening newborns for inborn errors of metabolism by tandem mass spectrometry. N Engl J Med 2003;348:2304–12.
3. Therrell BL, Padilla CD, Loeber JG, Kneisser I, Saadallah A, et al. Current status of newborn screening worldwide: 2015. Semin Perinatol 2015;39:171–87.
4. Ombrone D, Giocaliere E, Forni G, Malvagia S, la Marca G. Expanded newborn screening by mass spectrometry: new tests, future perspectives. Mass Spectrom Rev 2016;35:71–84.
5. Jannetto PJ, Fitzgerald RL. Effective use of mass spectrometry in the clinical laboratory. Clin Chem 2016;62:92–8.
6. Park KJ, Park S, Lee E, Park JH, Park JH, et al. A population-based genomic study of inherited metabolic diseases detected through newborn screening. Ann Lab Med 2016;36:561–72.
7. Schulze A, Lindner M, Kohlmuller D, Olgemoller K, Mayatepek E, et al. Expanded newborn screening for inborn errors of metabolism by electrospray ionization-tandem mass spectrometry: results, outcome, and implications. Pediatrics 2003;111(6 Pt 1):1399–406.
8. Pollitt RJ. Different viewpoints: international perspectives on newborn screening. J Med Biochem 2015;34:18–22.
9. Mak CM, Lee HC, Chan AY, Lam CW. Inborn errors of metabolism and expanded newborn screening: review and update. Crit Rev Clin Lab Sci 2013;50:142–62.
10. Hollegaard MV, Grauholm J, Nielsen R, Grove J, Mandrup S, et al. Archived neonatal dried blood spot samples can be used for accurate whole genome and exome-targeted next-generation sequencing. Mol Genet Metab 2013;110:65–72.
11. Bhattacharjee A, Sokolsky T, Wyman SK, Reese MG, Puffenberger E, et al. Development of DNA confirmatory and high-risk diagnostic testing for newborns using targeted next-generation DNA sequencing. Genet Med 2014;17: 337–47.
12. Francescatto L, Katsanis N. Newborn screening and the era of medical genomics. Semin Perinatol 2015;39:617–22.
13. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 2009;25:1754–60.
14. DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nature Genet 2011;43:491–8.
15. Van der Auwera GA, Carneiro MO, Hartl C, Poplin R, Del Angel G, et al. From FastQ data to high confidence variant calls: the Genome Analysis Toolkit best practices pipeline. Curr Protoc Bioinformatics 2013;43:11.10.1–33.
16. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010;20:1297–303.
17. Richards S, Aziz N, Bale S, Bick D, Das S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015;17:405–24.
18. Shi XT, Cai J, Wang YY, Tu WJ, Wang WP, et al. Newborn screening for inborn errors of metabolism in mainland china: 30 years of experience. JIMD Rep 2012;6:79–83.
19. Dietzen DJ, Rinaldo P, Whitley RJ, Rhead WJ, Hannon WH, et al. National academy of clinical biochemistry laboratory medicine practice guidelines: follow-up testing for metabolic disease identified by expanded newborn screening using tandem mass spectrometry; executive summary. Clin Chem 2009;55:1615–26.
20. Lim JS, Tan ES, John CM, Poh S, Yeo SJ, et al. Inborn error of metabolism (IEM) screening in Singapore by electrospray ionization-tandem mass spectrometry (ESI/MS/MS): an 8 year journey from pilot to current program. Mol Genet Metab 2014;113:53–61.
21. Huang X, Yang L, Tong F, Yang R, Zhao Z. Screening for inborn errors of metabolism in high-risk children: a 3-year pilot study in Zhejiang Province, China. BMC Pediatr 2012;12:18.
22. Han LS, Ye J, Qiu WJ, Gao XL, Wang Y, et al. Selective screening for inborn errors of metabolism on clinical patients using tandem mass spectrometry in China: a four-year report. J Inherit Metab Dis 2007;30:507–14.
23. Zhong K, Wang W, He F, Wang Z. Neonatal screening external quality assessment in China, 2014. J Med Screen 2015;22: 175–81.
24. Yang H, Chan K. Newborn screening and the relaxation of one-child policy in mainland China. Public Health 2015;129:1304–6.
25. Therrell BL, Padilla CD. Barriers to implementing sustainable national newborn screening in developing health systems. Int J Pediatr Adolesc Med 2014;1:49–60.
26. Bing-bing W, Wei L, Lin. Y, Ping-ping J, Gang L, et al. Application of targeted next generation sequencing in the molecular diagnosis of abnormal mass spectrometry analysis findings. Chin J Evid Based Pediatr 2015;10:40–5.
27. Baumgartner MR, Horster F, Dionisi-Vici C, Haliloglu G, Karall D, et al. Proposed guidelines for the diagnosis and management of methylmalonic and propionic acidemia. Orphanet J Rare Dis 2014;9:130.
28. Yohe S, Hauge A, Bunjer K, Kemmer T, Bower M, et al. Clinical validation of targeted next-generation sequencing for inherited disorders. Arch Pathol Lab Med 2015;139:204–10.
29. Mornet E. Hypophosphatasia: the mutations in the tissue-nonspecific alkaline phosphatase gene. Hum Mutat 2000;15:309–15.

30. Fauvert D, Brun-Heath I, Lia-Baldini A-S, Bellazi L, Taillandier A, et al. Mild forms of hypophosphatasia mostly result from dominant negative effect of severe alleles or from compound heterozygosity for severe and moderate alleles. BMC Med Genet 2009;10:51.
31. Kidd JM, Cooper GM, Donahue WF, Hayden HS, Sampas N, et al. Mapping and sequencing of structural variation from eight human genomes. Nature 2008;453:56–64.
32. Spentchian M, Brun-Heath I, Taillandier A, Fauvert D, Serre JL, et al. Characterization of missense mutations and large deletions in the ALPL gene by sequencing and quantitative multiplex PCR of short fragments. Genet Test 2006;10:252–7.
33. Greenblatt MS. Sequence variants of uncertain significance: what to do when genetic test results are not definitive. Surg Oncol Clin N Am 2015;24:833–46.
34. Wortmann SB, Duran M, Anikster Y, Barth PG, Sperl W, et al. Inborn errors of metabolism with 3-methylglutaconic aciduria as discriminative feature: proper classification and nomenclature. J Inherit Metab Dis 2013;36:923–8.
35. Niu DM, Chien YH, Chiang CC, Ho HC, Hwu WL, et al. Nationwide survey of extended newborn screening by tandem mass spectrometry in Taiwan. J Inherit Metab Dis 2010;33(Suppl 2): S295–305.
36. El-Hattab AW, Li FY, Shen J, Powell BR, Bawle EV, et al. Maternal systemic primary carnitine deficiency uncovered by newborn screening: clinical, biochemical, and molecular aspects. Genet Med 2010;12:19–24.
37. Fonseca H, Azevedo L, Serrano C, Sousa C, Marcao A, et al. 3-Methylcrotonyl-CoA carboxylase deficiency: Mutational spectrum derived from comprehensive newborn screening. Gene 2016;594:203–10.
38. Botkin JR, Rothwell E. Whole genome sequencing and newborn screening. Curr Genet Med Rep 2016;4:1–6.

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