TANDEM MASS SPECTROMETRIC ANALYSIS FOR DISORDERS IN AMINO, ORGANIC AND FATTY ACID METABOLISM: TWO YEAR EXPERIENCE IN SOUTH KOREA

Hye-Ran Yoon[1], Kyung Ryul Lee[1], Hohyun Kim[1], Seungwoo Kang[1], Yunmi Ha[1], Dong Hwan Lee[2]

[1]Department of Biochemical Genetics, Seoul Medical Science Institute, Seoul Clinical Laboratories (SCL), Seoul 140-809, South Korea; [2]Department of Pediatrics, Soonchunhyang University School of Medicine, Seoul, South Korea

Abstract. Seoul Clinical Laboratories began screening newborns and high risk group blood spots with tandem mass spectrometry (MS/MS) in April 2001. The goal was to determine approximate prevalence of metabolic disorders and optimization of decision criteria for estimation of preventive effect with early diagnosis. Approximately 44,300 neonates and children were screened and the estimated prevalence (newborn/high risk group), sensitivity, specificity and recall rate amounted to 1:2000 / 1:1250, 94.1 %, 99.7 %, and 0.04 %, respectively. Confirmed 35 multiple metabolic disorders (newborn/high risk) were as follows; 16 amino acid disorders [classical PKU(3/4), BH4 deficient-hyperphenylalaninemia(0/1), Citrullinemia(2/0), Homocystinuria(0/2), Hypermethioninemia(0/1), Tyrosinemia(1/0)], OTC deficiency (0/1), MSUD (2/0), 10 organic acidurias [Propionic aciduria(2/1), Methylmalonic aciduria(0/1), Isovaleric aciduria(2/1), 3-methylcrotonylglycineuria(1/0), Glutaric aciduria type I(2/0)], 9 fatty acid oxidation disorders [LCHAD def. (2/2), Mitochondrial TFP def.(0/1), VLCAD def.(1/0), LC3KT def.(0/1), SCAD def (1/0), MADD def (0/1). The relatively normal development of 15 patients with metabolic disorders among newborns (except for the expired) demonstrates the usefulness of newborn screening by MS/MS for early diagnosis and medical intervention. However, close coordination between the MS/MS screening laboratory and the metabolic clinic/biochemical geneticists is needed to determine proper decision of screening parameters, confirmation diagnosis, follow-up scheme and additional tests.

INTRODUCTION

Newborn screening (NBS) for selected inherited metabolic disorders is well established (Millington *et al*, 1984, 1992; Rashed *et al*, 1995; Sniderman *et al*, 1999; Vreken *et al*, 1999). Worldwide, more than 10 million newborns are screened annually (Chace *et al*, 1992). There are assay methods such as enzyme immunoassays and radioimmunoassays for screening phenylketonuria (PKU) and congenital hypothyroidism in Korea. So far the most powerful tool for newborn screening for multiple metabolic disorders is tandem mass spectrometry (MS/MS) (Jones *et al*, 2002; Schulze *et al*, 2003; Shigematsu *et al*, 2003). Quantitative analysis of amino acids (AA) and acylcarnitines (AC) using MS/MS is an emerging technology and its utility for detecting amino, organic, fatty acids (including very long chain fatty acids and combinations of organic, amino and fatty acid metabolic disorders, etc), has been well described (Chace *et al*, 1992; Mueller *et al*, 2003). Early diagnosis and treatment can reduce the morbidity, mortality and social costs associated with these diseases (Carreiro-Lewandowski *et al*, 2002).

A pioneering newborn screening program originated at the Department of Pediatrics, Soonchunhyang University School of Medicine for PKU and CH. In 1985, the program was extended to cover more diseases: PKU, galactosemia, maple syrup urine disease, homocystinuria (HCY) and CH (but only for babies of low income mothers at provincial public health care centers) (Han *et al*, 2000). Central and local government funded nationwide newborn screening has been in operation at 65 small laboratories including two large non-profit medical foundation laboratories since 1997.

To evaluate the collective total incidence of disorders in the metabolism of amino acids, organic acids and fatty acids in South Korea, our lab has begun integrating testing for more than twenty metabolic disorders (using MS/MS) into the existing nationwide newborn screening program (see Table 1). Twenty-eight months' (April 2001- August 2003) experience of metabolic screening by MS/MS including newborn screening, and high risk screening are presented below.

MATERIALS AND METHODS

One spot of blood (3.2mm dia) was punched-out and put into a 96 well microplate (Costar, Cambridge, MA, USA). Extraction solvent (150μl of methanol containing isotopically labeled amino acid and acylcarnitine internal standards) was added and the solution was shaken for 30 minutes at 30°C. The supernatant (75μl) was transferred to a second plate and the wells were dried under nitrogen at 50°C. For butylation, 3 N butanolic hydrogen chloride (Regis Inc, IL, USA; 100μl/ each well) was added to the residue and heated at 65°C for 15 min. The reactant was dried under nitrogen (40°C) and redissolved in acetonitrile/water (4:1). The solution was directly injected into a turbo electrospray ion source of ESI-MS/MS (Perkin Elmer Life Science Inc, Boston, MA, USA).

Dried blood spots for newborn screening were generally collected at 2-7 days after an infant's birth. Specimens were randomly taken from children between 1 month and 18 years for high-risk screening. The laboratory analysis of metabolic markers of the disease began two days after the specimens were received from around the country. Initial test results were available within 24 hours following testing. Retesting of specimens above the cut-off level was completed 48 hours later and final results were generally available 72 hours after receipt of specimens.

RESULTS AND DISCUSSION

The annual birth cohort of approximately 550,000 newborns was the expected number that could potentially be tested. Approximately 44,300 newborn and high risk infants were tested for organic, amino and fatty acid metabolism disorders. Each micro-titer plate had 5 blanks and 3 blood spot controls with positive, negative controls and one control containing markers with concentrations

Table 1. Metabolic disorders screened using tandem mass spectrometry.

Grouping	Disorders	
Organic aciduria	Propionic acidemia	Isovaleric acidemia
	Methylmalonic acidemia	3-Methylcrotonylglycineuria
	Ethylmalonic encephalopathy	Multiple carboxylase def.
	Glutaric aciduria I	Methylmalonic aciduria with homocystinuria
Aminoacidopathy	Phenylketonuria	Argininosuccinic aciduria
	Atypical phenylketonuria	HHH syndrome
	Homocysteinemia	Argininemia
	Hypermethioninemia	Tyrosinemia Neonatal tyrosinemia
	Maple syrup urine disease	Non-ketotic hyperglycinemia
	Hypervalinemia	Hyperornithinemia
	Citrullinemia	
Fatty acid oxidation disorder	Carnitine uptake deficiency	Riboflavin responsive MADD
	Carnitine palmitoyl transferase deficiency I	Long chain acyl CoA dehydrogenase deficiency
	Carnitine palmitoyl transferase deficiency II	Very long chain acyl CoA dehydrogenase deficiency
	Acylcarnitine translocase deficiency	Long chain hydroxy acyl CoA dehydrogenase deficiency
	Short chain acyl CoA dehydrogenase def.	Mitochondrial trifunctional protein deficiency
	Short chain hydroxy acyl CoA dehydrogenase deficiency	B-Ketothiolase deficiency
	Medium chain acyl CoA dehydrogenase deficiency	2,4-Dienoyl CoA reductase deficiency
	Multiple acyl CoA dehydrogenase deficiency (MADD)	Hydroxy methyl glutaryl CoA lyase deficiency

Table 2. Reference range for amino acids in dried blood spots from full-term newborns.

Amino acids	Mass for Quantification	Newborn Population (n=2,500), Unit : μmol/l 2SD	Reference range (99.9 %tile)
Phenylalanine	222 / 227	12.5	19.4 - 164.0
Phenylalanine/Tyrosine	222 / 238	0.1	0.1 - 2.0
Methionine	206/ 209	6.3	9.7 - 84.0
Leucine/Isoleucine	189 / 191	19.7	50.0 - 328.0
Valine	174 / 182	41.8	47.5 - 368.0
Citrulline	232 / 234	5.3	4.4 - 97.0
Citrulline/Phenylalanine	232 / 222	0.1	0.1 - 1.0
Citrulline/Tyrosine	232 / 238	0.1	0.03 - 0.6
Arginine	231 / 236	8.8	2.1 - 37.1
Tyrosine	238 / 244	15.7	25.1 - 375.0
Glycine	132 / 134	29.5	137.0 - 752.4
Ornithine	189 / 191	8.6	18.8 - 182.3
Alanine	146 / 150	46.7	85.2 - 464.8
Glutamate	260 / 263	30.0	103.0 - 730.5
Aspartate	246 / 249	22.1	12.0 - 164.0

near the cut-off value. The precision of the mean of marker was: acylcarnitines (C3, C8, C16), 11%; amino acids Leu, 10%, Orn, 18%, Met, 9% , Phe, 2% , Cit, 3% and Tyr, 16%. Approximately 31,000 neonates and children were screened and the estimated prevalence (newborn/high risk group), sensitivity, specificity and recall rate amounted to 1: 2000 / 1:1250, 94.1%, 99.7%, and 0.04%, respectively. Newborns, infants and children diagnosed as having metabolic disorders over the two year will be described with abnormal (flagged) amino acid or acylcarnitine concentration.

The organic acid, amino acid, and fatty acid metabolic disorders included in screening are listed in Table 1.

The reference ranges for newborns (n=2,500) are presented in Table 2 and Table 3 for amino acids and acylcarnitines respectively. The cut-off value was set as the upper level of 99.9% + 4SD based on the range of 2,500 normal newborns and detected cases of metabolic disorders. The total number of newborns (37,817) and high risk group (6,483) screened totaled 44,300 from April 2001 to August 2003. At the onset of screening, the number of specimens was small, however, it dramatically increased to more than 3,500 a month in 2003. The 35 confirmed metabolic disorders (newborn/high risk) detected among 44,300 screened are listed in Table 4.

Timing of follow-up tests can be critical for many disorders because the abnormal markers may not be informative unless the infant is metabolically decompensated (Carpenter *et al*, 2002). Additionally, a decrease of acylcarnitines is expected when regular feedings are established.

The relatively normal development of 15 patients with metabolic disorders among newborns (except for the expired) demonstrates the usefulness of newborn screening by MS/MS for early diagnosis and medical intervention. Close coordination between the MS/MS screening laboratory and the metabolic clinic/biochemical geneticists is needed to determine proper decision of screening parameters, confirmation diagnosis, follow-up scheme and additional tests (Vianey-Saban *et al*, 1997; Abdenur *et al*, 1999).

Newborn Screening by MS/MS is well suited for high-throughput mass screening where a shorter analytical time is highly desirable and early diagnosis is indispensable for the quality of life of the newborn if affected (McLafferty, 1981; Coex *et al*, 1998; Carpenter *et al*, 1999).

Despite our limited experience, MS/MS newborn

Table 3. Reference value for acylcarnitines with blood spot in full-term newborns.

Acylcarnitines	Mass for Quantification	Newborn (n=2,500), Unit : μmol/l			
		2SD	Reference range (1-99%)		
Free carnitine	218 / 221	20.55	2.5	-	64.8
Acetylcarnitine	260 / 263	4.34	2.6	-	26.7
Propionylcarnitine	274 / 277	1.22	0.03	-	6.9
C3/C2	274 / 260	0.45	0.003	-	0.5
Methylmalonylcarnitine	374 / 375	1.27	0.008	-	0.72
Butyrylcarnitine	288 / 291	0.19	0.011	-	1.47
C4/C2	288 / 260	0.05	0.005	-	0.144
C4/C3	288 / 274	0.11	0.019	-	0.641
Tiglylcarnitine	300 / 311	0.04	0.005	-	0.380
Isovalerylcarnitine	302 / 311	0.07	0.006	-	0.326
Glutarylcarnitine	388 / 375	0.04	0.005	-	0.083
C5/C2	302 / 260	0.02	0.002	-	0.114
C5/C3	302 / 274	0.04	0.004	-	5.074
3-OH-Isovalerylcarnitine	318 / 311	0.50	0.008	-	0.376
Adipylcarnitine	402 / 403	0.04	0.003	-	3.800
Hexanoylcarnitine	316 / 319	0.09	0.005	-	0.560
Octanoylcarnitine	344 / 347	0.08	0.005	-	0.560
Decadienoylcarnitine	368 / 375	0.03	0.007	-	0.266
Decenoylcarnitine	370 / 375	0.09	0.005	-	0.228
Decanoylcarnitine	372 / 375	0.13	0.004	-	0.570
Dodecanoylcarnitine	400 / 403	0.05	0.005	-	0.318
Tetradecadienoylcarnitine	424 / 431	0.03	0.004	-	0.128
Tetradecenoylcarnitine	426 / 431	0.04	0.003	-	0.207
C14:1/C16	426 / 456	0.05	0.002	-	0.148
3-OH-Tetradecanoylcarnitine	444 / 431	0.02	0.003	-	0.087
3-OH-Hexadecenoylcarnitine	470 / 459	0.02	0.004	-	0.118
3-OH-Hexadecanoylcarnitine	472 / 459	0.02	0.003	-	0.083
3-OH-Linoleylcarnitine	496 / 487	0.02	0.005	-	0.158
3-OH-Oleylcarnitine	498 / 487	0.01	0.003	-	0.070
3-OH-Stearoylcarnitine	500 / 487	0.01	0.003	-	0.055
Myristoylcarnitine	428 / 431	0.04	0.007	-	0.820
Hexadecenoylcarnitine	454 / 459	0.06	0.003	-	0.178
Palmitoylcarnitine	456 / 459	0.87	0.201	-	9.140
Linoleylcarnitine	480 / 487	0.23	0.003	-	0.801
Oleylcarnitine	482 / 487	0.50	0.009	-	2.097
Methyl-Glutarylcarnitine	402 / 403	0.065	0.004	-	5.570

Table 4. Confirmed positive cases through screening by tandem mass spectrometry.

Number of Abnormal (Amino acid disorders)	Metabolic Disorders	Newborn (8)	High Risk (8)
16	Classical PKU	3	4
	BH4 def. -Hyper phe.		1
	Citrullinemia	2	
	Homocystinuria		2
	Hypermethioninemia		
	Tyrosinemia	1	
	OTC deficiency		1
	MSUD	2	
Number of Abnormal (Organic acidurias)		Newborn (7)	High Risk (3)
10	Propionic aciduria	2	1
	Methylmalonic aciduria		1
	Isovaleric aciduria	2	1
	3methylcrontonylglycineuria	1	
	Glutaric aciduria type1	2	
Number of Abnormal (Fatty acid oxidation disorders)		Newborn (4)	High Risk (5)
9	LCHAD def	2	2
	Mitochondrial TFP def		1
	VLCAD def	1	
	Long chain 3-keto acyl coA def		1
	SCAD def	1	
	MADD def		1
Total	35	19	16
Prevalence		1:2000	1:1250
Total screened 44,300	0.079%		

screening has provided valuable insights that may prove useful in the implementation of MS/MS and in determining future strategies for newborn screening.

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