Table 1
Clinical and biochemical data of published cases with atypical NKH

Sibships		Type/Gender *Neonatal*: <1m *Infantile*: 2m-2y *Late onset*: >2y	Symptomatology	Ratio CSF / plasma glycine CSF glycine	GCS activity Lymphoblasts (pmol/mg) Liver (nmol/mg) or Other testing	Genotype	Ancillary testing	Treatment	Reference
Confirmed diagnosis of NKH based on genetic or enzymatic testing									
1	a	Infantile/♀	-Infantile spasms (6m) -Mild MR (14y)	0.020 7.4 μmol/L	Lymph: normal Liver: 0.5 ↓ (2.1-3.8)		EEG(6m): Hyps MRI: normal	SB: ↓glycine levels, ↓ sz	[20]
	b	Late onset/ ♂	-Mild dd mostly expressive language (9y)	0.020 7.4 μmol/L			EEG: normal		
2		Infantile/ ♂	-Mild dd (7m) -Encephalitis-like episodes (2y) -Mild MR (11y)	0.060 51 μmol/L	Lymph: 0.8↓ (4.4-29) Liver: 0.4↓ (2.1-4.6)		EEG: Epil, IPDA (7m) MRI: ↑ T2 signal in thalamus b/l		[38]
3		Infantile/♀	-Hypotonia, infantile spasms (6m) -Mild global dd -Behavioral (irritability aggression)	0.07-0.08 66 μmol/L	Liver: 9,7↓ nkat/kg protein (>112)		EEG(6m): Hyps EEG(22m): Epil CT (6m): normal MRI (21m): normal	SB: improvement DMF: no change	[39]
4		Infantile/ ♀	-Episodes of lethargy (12m) -Temper tantrums -Global dd (expressive language) (10y)	0.041 41 μmol/L	Lymph: 0.22↓(0.61-1.6) P-protein component: 0.19 ↓ (0.87-1.5)		EEG(1m,13y): normal CT: normal (13y)	No treatment	[40]
5		Infantile/♂	-Global dd -Intractable ADHD, aggressiveness (9y) -Profound MR, behavioral problems (22y)	0.070 68 μmol/L	Liver: 0.14 ↓ P-protein: 3.5% of normal		CT (10y) : atrophy	Folic acid, SB, pyridoxine: no effect	[24]
6	a	Infantile/♀	-Global dd (6m) -Severe MR (15y)	0.097 42 μmol/L	Liver: 1.0↓ (3.9-5.2) T protein: 0.3↓ (52.1-77.9)				[41]
	b	Infantile/♀	-Moderate dd (27m)	0.097 72 μmol/L	Liver: 1.4 ↓(3.9-5.2) T protein (sibling)				
7		Infantile/♂	-Epilepsy (6m) -Global dd (language), microcephaly -Moderate MR, outbursts of rage (36y)	-	Lymph:36↓(700nkat/kg) Glycine load: seizure, B-S pattern on EEG			Low-glycine diet: no effect	[28]
8		Infantile/♂	-Myoclonic seizures (6wks) -Apneic spells	0.060 31 μmol/L	Liver: 0.7 ↓ (3.8-9.5)				[42]
9	a	Infantile/♀	-Myoclonic jerks hypotonia (6m) -Severe global dd (5y)	0.090 42 μmol/L	Liver: 1.0↓ (3.8-9.5) T-protein:0.3↓(30.2-77.9)			SB: no effect	[2,14]
	b	Infantile/♀	-Treatment with strychnine+ arginine started at 9d and SB was added at 15d -Normal development (1y) -Mild dd (27m), Mild MR (9y)	0.100 72 μmol/L	Liver: 1.4↓ (3.8-9.5)	*AMT* gene: G47R(exon 2) / R320H(exon 8)		SB, strychnine, arginine: ↓CSF glycine, better cognitive outcome	
10		Infantile/♂	-Hypotonia, global dd (language) -Ataxia, choreoathetoid movements -Mild MR (he can keep a job) (21y)	0.050 55 μmol/L		*GLDC* gene (exon 9): c.1166C>T A389V homozygous **Expression analysis** A389V: 8%			[32]
11		Infantile/♂	-Hypotonia, seizures (2m), global dd -Epilepsy, choreoathetoid movements -Severe MR, behavioral problems (25y)	0.090 150 μmol/L		*GLDC* gene (exon 9): c.1166C>T A389V homozygous **Expression analysis*** A389V: 8% *relative activity	EEG: slow MRI(21y): atrophy ^{1}HMRS(21y): no glycine peak		
12		Infantile/♂	-Hypotonia global dd (language) -ADHD, aggressive behavior (sexual impulsivity) (15y) -Mild MR, (20y)	0.056 36 μmol/L		*GLDC* gene (exon 9): c.2216A>C R739H homozygous **Expression analysis*** R739H: 6%	EEG(12y): IPDA MRI(12y): normal ^{1}HMRS: no glycine peak	SB: no effect DMF: no effect	
13		Neonatal/♀	-Hypotonia, apnea, global delay -DQ (age 32mo): 28 -Severe MR	0.310 270 μmol/L	Lymphoblast: 0 activity	*GLDC* gene: R790W(exon 20) / N150T(exon 3) **Expression analysis*** R790W: 14% N150T: 1%	EEG(neonate): b-s MRI: normal	SB: since age 16 d DMF: since age 16 d Imipr: since age 7y	[23]
14		Neonatal/♀	-Hypotonia, apnea, seizures -DQ (age53mo): 58 -Mild MR	0.090 160 μmol/L	Lymphoblast: 0 activity	*GLDC* gene: L82W(exon 1) / 1821-1831del(exon 15) **Expression analysis*** L82W: 11%	EEG: Epil MRI: normal	SB: since age 7 d Trp: since age 4 y	
15 16 17 18	a b c d e f g h i	Infantile (a,b,c,d,e,i) / ♀(3pt) and ♂(3pt) Neonatal (f,g,h) / ♀(2pt) ♂(1pt)	-Neonatal hypotonia, apnea (3pt) -Global dd -Choreoathetoid movements -Epilepsy: GTC seizures (5pt) infantile spasms (2pt) -Behavioral problems -Moderate MR	Ratio (Infantile) a 0.061 b 0.087 d 0.067 e 0.081 i 0.061 Ratio (Neonatal) f - g 0.058 h 0.058	CSF glycine (Infantile) a 38 μmol/L b 33 " d 43 " e 54 " i 56 " CSF glycine (Neonatal) f 72 μmol/L g 72 " h 78 "	*GLDC* gene (exon 22): c.2216A>C transversion that affects the 3rd base in codon 869 (proline) homozygous	EEG: Epil (5pt) Hyps (2pt)		[33]
19		Neonatal/♀	-Hypotonia, apnea, feeding difficulties -Expressive language delay -Mild MR	0.099 124 μmol/L		*GLDC* gene (exon 22): c.2216A>C transversion that affects the 3rd base in codon 869 (proline) homozygous	EEG: normal HCT: atrophy	SB (2mo) Ketamine (2mo)	

(*continued on next page*)

Table 1 (*continued*)

20	a	Neonatal / ♀	-Hypotonia, apnea (mechanical ventilation) -Normal cognition (10y)	0.103 124 μmol/L		*GLDC* gene (exon 20): c.2405C>T A802V homozygous **Expression analysis*** A802V: 32%	EEG: normal MRI: normal 1HMRS no glycine peak	SM (1d)	[11]
	b	Asymptomatic/♀	-Normal development -Normal cognition (6y)	0.056 127 μmol/L			EEG: normal	SB (8d) Ketamine (8d)	
	c	Asymptomatic/♀	-Normal development (10m)	0.077 78 μmol/L				SB (2d) Ketamine (2d)	
21		Neonatal/♀	-Hypotonia, apnea -Normal development (11m) -Neurological deterioration (14m)	0.074 93 μmol/L		*GLDC* (gene exon 20): c.2405C>T A802V homozygous **Expression analysis*** A802V: 32%			
Diagnosis of NKH based on increased CSF/Plasma glycine ratio									
22		Late onset/♀	- ↓visual acquitity (13y), seizure, - Normal cognition, optic atrophy (33y)	0.032 27 μmol/L			EEG(33y): Epil MRI: normal (33y)	B6, folic acid: no improvement	[43]
23		Late onset/♀	-dd (11y) -episodes of encephalopathy (31y and 42y) -Ataxia, choreoathetoid movements -Behavioral problems -Mild MR (41y)	0.071 68 μmol/L			EEG: IPDA HCT: cerebellar calcifications	DMF (42): improvement SB (42): improvement	[44]
24	a	Late onset/ ♀	-Mild global dd (language) (4y) -Intractable ADHD	0.060 36 μmol/L				SB: ↓glycine / no improvement Trp: no effect Imipr: improvement	[45]
	b	Infantile/ ♂	-Normal development -Behavioral problems	0.045 30 μmol/L				No treatment	
25	a	Infantile/ ♂	-Encephalitis-like episode (22m) with -Choreoathetoid movements -Mild global dd (expressive language) -9y WISC-R: IQ: 76	0.120 42 μmol/L	Oral glycine tolerance test: failure glycine to convert to serine		EEG(preload): normal EEG(afterload): Slow	Dietary modification (5 weeks): ↓glycine, improvement of behavior	[27]
	b	Infantile/ ♀	-Encephalitis like episode (lethargy irritability, dysmetria) (8m) -Choreoathetoid movements (3y) -Mild MR (expressive language) (7y)	0.160 52 μmol/L	Oral glycine tolerance test: failure glycine to convert to serine		EEG: normal (preload) Slow(after load)	Dietary modification (5 weeks): ↓glycine, improvement of behavior	
26		Late onset/♂	-Optic atrophy at (4y) -Spastic paraparesis (9y) -Spinocerebellar degeneration (15y)	0.060	Oral glycine tolerance test: failure glycine to convert to serine		CT: atrophy (pons, cerebellar)		[46]
27		Late onset/♀	-Mild MR -Progressive cerebellar ataxia at (40y) -Spastic paraparesis, Babinski (66y)	↑			MRI: hypoplasia of CC, mild diffuse atrophy		[16]
28	a	Infantile/ ♀	-Epilepsy 11m, -Global dd (expressive language) -Behavioral problems (13y) -Choreoathetosis, dementia (34y)	0.088 135 μmol/L			CT: normal	No treatment	[47]
	b	Infantile/♂	-Epilepsy 1y, -Uncontrolled behavior 14y -Moderate MR, slowly progressive (17y)	0.088 93 μmol/L			CT: normal	No treatment	
29		Infantile/♀	-Lethargy, hypotonia, hyperacusis (6m) -Epilepsy (9m) - Died at 15m: (cystic degeneration of WM, gliosis sparing the U fibers)	0.171 78 μmol/L	Oral glycine load: failure to convert to serine		EEG(9m): Epil	Strychnine: no effect	[15]
30	a	Late onset/♂	-Mild dd, expressive speech delay, (12y) -Episodes of ataxia, irritability	0.053 (baseline) 0.11 (episode) 32 μmol/L(baseline) 62μmol/L(episode)			EEG(during episode): slow		[13]
	b	Late onset/♂	-Normal intelligence (10y) -Expressive language, fine motor delay -Hyperactivity	0.042 25 μmol/L					
	c	Late onset/♂	-Normal intelligence (6y) -Marked expressive language delay -Hyperactivity	0.070 42 μmol/L					
31	a	Infantile/♀	-Mild dd (expressive language), mild hypotonia (29m)	0 .100 66 μmol/L			EEG(11m, 20m, 29m): normal	Low protein formula: no effect	[19]
	b	Asymptomatic/♂	-Normal at 15m	0.246 163 μmol/L			EEG(8m, 15m): normal		
32	a	Late onset/♂	-Spasticity and leg weakness (10y) -Normal cognition, spastic diplegia, peroneal muscle atrophy, pes cavus (19y)	0.035 160μg/100ml	Oral glycine load: failure glycine to convert to serine		EMG: Neuropathic changes		[17]
	b	Late onset/♂	-Spasticity and leg weakness -Normal cognition, spastic diplegia, peroneal muscle atrophy, pes cavus (23y)	0.025 180μg/100ml	Oral glycine load: failure glycine to convert to serine				
	c	Late onset/♂	-Abnormal gait (2y) -Spasticity and contractures (8y) -Normal cognition, spastic diplegia, peroneal muscle atrophy, pes cavus (10y)	-	Oral glycine load: failure glycine to convert to serine				

dd: developmental delay, **MR**: mental retardation, **Hyps**: Hypsarrhythmia, **IPDA**: Intermittent Polymorphic Delta Activity, **Epil**: Epileptiform discharges, [1]**HMRS**: proton MR spectroscopy, **CC**: corpus callosum, **SB**: Sodium Benzoate, **DMF**: Dextromethorphan, **Trp**: Tryptophan, **Imipr**: Imipramine, **m**: month, **y**: year, **sz**: seizures.

* Expression analysis of glycine decarboxylase in COS7 cells and measurement of the relatively activity of glycine decarboxylase.

Table 2
Comparison of clinical and biochemical features of neonatal, infantile, and late onset atypical NKH cases[a]

Biochemical marker	Neonatal ($n = 8$)	Infantile ($n = 27$)	Late onset ($n = 9$)
CSF/plasma glycine ratio[b]	Mean: 0.11	Mean: 0.08	Mean: 0.05
($\leqslant$0.02)[c]	Range: 0.06–0.25	Range: 0.02–0.17	Range: 0.03–0.07
CSF glycine[b]	Mean: 126	Mean: 56	Mean: 38
(3.8–8.0 μmol/L)[c]	Range:56–270	Range: 7–150	Range: 25–68
Symptomatology	Neonatal ($n = 8$)	Infantile ($n = 28$)	Late onset ($n = 11$)
Epilepsy	3(43%)	12 (39%)	1 (9%)
Hypotonia	8(100%)	18(69%)	—
Episodic presentation	—	4(14%)	2(18%)
Choreoathetosis	3(37%)	11(43%)	2(18%)
Optic atrophy	—	—	2(18%)
Spinocerebellar syndrome	—	—	6(54%)
Peripheral neuropathy	—	—	3(27%)
Behavioral problems	4(50%)	16(57%)	5(50%)
Cognitive impairment[d]			
None	1(12%)	2(7%)	5(45%)
Mild	3(37%)	9(32%)	6(55%)
Moderate-severe	4(50%)	17(61%)	—

[a] The reported asymptomatic cases are not included.
[b] Initial values as reported on the reference before any medical treatment.
[c] Normal control values may differ between laboratories.
[d] According to the clinical description or to a formal IQ testing.

per tantrums and irritability dominate the picture initially, followed by outbursts of aggressiveness and rage later on. Attention deficit hyperactive disorder (ADHD) resistant to treatment with psychostimulants and mild expressive language delay may be the only symptoms in some cases. An episodic presentation induced by a febrile illness has been described in some patients with lethargy, flaccidity, ataxia, choreoathetoid movements, gaze palsy, paroxysmal screaming, and delirium. A patient with an episodic presentation (case 30a) was studied during the episode and was found to have higher CSF glycine levels and CSF/plasma glycine ratio than during the baseline [13]. The reason for the intermittent glycine increase is unknown but may be related to the metabolic stress during an illness that can further compromise the deficient mitochondrial GCS activity. Overall the phenotype is consistent with a static encephalopathy and, although the outcome is better than the classical one, 61% of cases will have a moderate to severe cognitive impairment. In addition, neurological deterioration has been described in some cases (cases 9b, 21, 29) [11,14,15] despite the mild initial symptomatology.

The *late onset* form is less common and more heterogeneous. The clinical presentation is after the second birthday and even during adulthood, mainly with mild cognitive decline and behavioral problems. In a few cases, a slowly progressive spinocerebellar syndrome including ataxia, upper-motor neuron signs, optic atrophy, and peripheral neuropathy has been described. Persons with late onset NKH tend to have a normal life span and the oldest reported case is 66 years old [16]. A sibship of three males has been described with a peculiar phenotype of progressive lower extremity spasticity, leg weakness, peroneal muscle wasting, and neuropathic changes on EMG; the diagnosis of NKH was based on mild elevation of CSF/plasma glycine ratio and on abnormal glycine tolerance test [17]. Although a deficient GCS is speculated, the cause of the hyperglycinemia in these *late onset* cases is unknown, since they all lack enzymatic or genetic confirmation.

In classical NKH, on a retrospective natural history study [18], females were found to have a more severe disease. However, no such gender predilection was found in published cases of atypical NKH. Siblings are expected to be concordant in clinical and biochemical phenotypes. However, discordance for age at presentation, clinical severity, and glycine levels, which may [14] or may not [19,20] be related to treatment, has been reported.

Metabolic characteristics and pathogenesis

A CSF/plasma ratio higher than 0.08 is considered diagnostic of classical NKH [8] but atypical NKH cases can have lower ratios. The mean CSF/plasma glycine ratios, in *neonatal* form of atypical NKH of 0.11 (range 0.06–0.25) and in *infantile* form of 0.08 (range 0.02–0.17), differ substantially from the mean ratio of 0.23 (range 0.07–0.70) described by Applegarth and Toone[8], in 25 classical NKH cases with enzymatic confirmation. However, there is much overlap and, when the clinical presentation is during neonatal period, the CSF/plasma glycine ratio alone is not a reliable test to identify those rare patients with a potentially favorable

outcome. Moreover, the few reports on transient NKH with severe disease in the newborn period and subsequent clinical and biochemical resolution [21] complicate even more the decision-making about aggressive supportive management and early therapy. In *late onset* form of atypical NKH the mean CSF/plasma glycine ratio is even lower at 0.04 (range 0.03–0.07) and none had a ratio above 0.08, that is considered a diagnostic threshold for the classical form.

There is debate whether the CSF/plasma glycine ratio is correlated with the prognosis in classical NKH. For atypical NKH, all cases described with "normal cognition" had a ratio less than 0.10 except for one extreme outlier (case 31b) with a ratio of 0.25 and "normal development" at the age of 15 months [19]. The lowest ratio level described in an atypical NKH with moderate or severe mental retardation is 0.06. Hence, we consider a ratio lower than 0.06 a promising factor for a better outcome. The stratification of atypical cases by cognitive outcome shows that there is a trend for cognitive impairment to be associated with higher CSF/plasma glycine ratio (Fig. 1). *Late onset* atypical NKH have lower ratios and better cognitive outcome. The above picture, extrapolated by the literature review, is a gross estimate since a validated measurement of the cognitive outcome and the timing of therapeutic intervention are often lacking.

The metabolic defect in *neonatal* and *infantile* forms of mild NKH is usually an abnormality in the P-protein or less commonly in the T-protein of GCS, whereas the metabolic defect in *late onset* form is unknown. In the classical NKH, the overall GCS activity is usually very

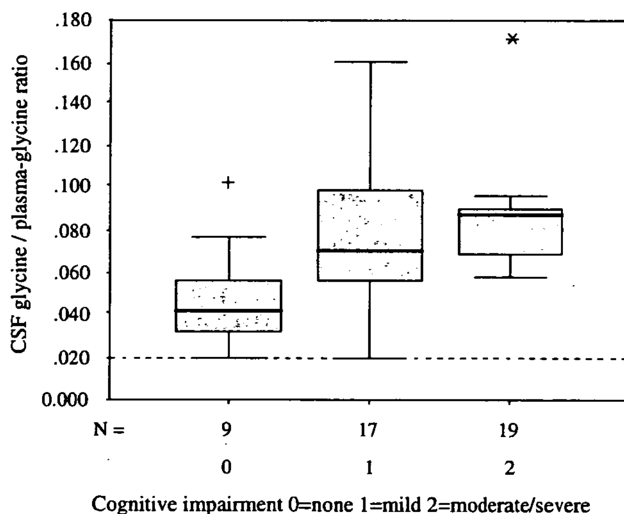

Fig. 1. CSF/plasma glycine ratio and cognitive outcome. Published atypical NKH cases were stratified grossly by measures of cognitive function (see text), and CSF/plasma glycine ratios were compared, excluding case 31b, an extreme outlier whose cognitive function could not be assessed reliably at 15 months (apparently was normal). A trend of cognitive impairment to associated with higher CSF/plasma glycine ratios was observed ($p = 0.07$). Notice the overlap of the CSF/plasma glycine ratios between groups. +(case 20a), *(case 29).

low or undetectable in liver tissue and brain [22]. Generally in atypical forms a higher hepatic and lymphoblast GCS activity is observed. However, there are reported cases with low hepatic (case 1a) [20] or lymphoblast (cases 13, 14) [23] GCS activity, in the classical NKH range, and mild clinical phenotype. This discrepancy exists presumably because liver and lymphoblast GCS activity does not absolutely reflect brain GCS activity [24].

Glycine accumulation in the CNS has been assumed to exert its toxic effect primary through *N*-methyl-D-aspartate (NMDA)-mediated excitotoxicity in the cerebral cortex, hippocampus, and cerebellum as well as through enhanced direct inhibition in the spinal cord and brain stem [25]. Much research supports the toxic effect of glycine on neuronal activity. Intraventricularly administered glycine potentiates seizures in animals [26] and an oral glycine load can cause lethargy, precipitate seizures, and induce slow activity and even burst-suppression EEG patterns in patients with *infantile* NKH [27,28]. Common manifestations of atypical NKH patients are the mental retardation and behavioral problems. Since the NMDA receptor complex is involved in many functional processes including neural development, synaptic plasticity and learning and memory [29], a cognitive dysfunction is not surprising with NMDA dysregulation caused by the abundant CNS glycine. Studies in animals showed a causative role of glycine binding site of the NMDA receptor in inducing defensive behavior and anxiogenic-like reaction, which may be reversed by non-selective glycine B receptor antagonists [30]. Two observations support the idea that the neuropathogenesis may arises in part from the deficient GCS activity per se and not merely from the toxic effect of glycine. First, there are a few cases with low hepatic CGS activity and neurological impairment despite normal CSF glycine [20] and second, successful lowering of the CSF glycine concentration with therapy, does not always improve neurological function. The GCS is abundantly expressed in neural stem cells in developing brain, and generates one-carbon unit (5,10-methylenetetrahydrofolate) that is required for DNA synthesis [31]. Deficiency of the GCS activity may cause irreversible damage to embryonic brain, resulting in poor long-term neurological outcome.

Molecular studies

The first genetic characterization in atypical NKH was in a patient with T-protein defect harboring two different missense mutations G47R and R320H in the *AMT* gene [2]. To date three missense mutations (A389V, R739H, and A802V) on the *GLDC* gene have been identified in homozygous state in *neonatal* and

infantile form of atypical NKH. The mutations are not detected in control subjects from the same geographic origin indicating that they are not prevalent polymorphisms [11,32]. When, these mutations were transfected into COS7 cells in vitro, a significant higher glycine decarboxylase activity was expressed compared with the classical NKH Finnish mutation. Mutant *GLDC* with the A389V and R739H mutations showed 6–8% of the wild-type glycine decarboxylase activity and that with the A802V mutation had 32% activity. Clinically, the A802V mutation is associated with a *neonatal* form of NKH and transient or absent symptoms with early therapeutic intervention. Two other missense mutations (R790W and L82W) on *GLDC* gene were found in two compound heterozygotes with *neonatal* form of atypical NKH [23]. Both mutations had a substantial higher glycine decarboxylase activity when expressed in COS7 cells (14 and 11% of the wild-type, respectively). Despite the limited data it seems that there is a genotype–phenotype correlation with certain mutations associated with a more favorable outcome. These mutations, even in a compound heterozygous state, permit some residual enzyme activity that may be responsible for the milder phenotype. Finally a pathogenic exonic *GLDC* splice mutation (silent transversion c.2607C>A), that does not change the amino acid sequence but does cause reduced *GLDC* mRNA levels on lymphoblast, was identified in homozygous state in a large kindred with atypical NKH [33].

Diagnosis and treatment

Identification of patients with atypical NKH will require increased clinical suspicion, especially in populations with non-specific mild mental retardation, in whom a laboratory work up is usually omitted. The presence of behavioral problems especially outbursts of rage and medically intractable ADHD, choreoathetosis, and ataxic episodes should raise suspicion for atypical NKH. Simultaneous plasma and CSF glycine measurements are required in patients with isolated elevations in urine or plasma glycine levels. The CSF/plasma glycine ratio (normal $\leqslant 0.02$) in atypical cases is usually lower than the diagnostic cut-point for classical NKH (0.08) but results require careful interpretation because other conditions and drugs can cause hyperglycinemia and hyperglycinorachia, and sampling errors may occur [34]. Difficulties also may arise in separating out the rare cases of transient neonatal NKH, so enzymatic or genetic confirmation is of paramount importance. Measurement of GCS activity can be done in liver tissue or transformed lymphoblasts [35]; the measurement of the liver GCS activity is more sensitive, but because it is more invasive, it should be reserved for the selected, indeterminate cases. Identification of the causative mutation helps for diagnostic confirmation, accurate genetic counseling, and prenatal diagnosis. Unfortunately there is not an immediately available test to predict with reliability the outcome. The expression analysis of the mutation can identify favorable mutations with residual enzyme activity that may help in future decision-making.

Ancillary testing such as EEG and brain imaging studies add little to the diagnosis of atypical NKH, since they show no specific abnormalities. Proton MR spectroscopy (^{1}HMRS) allows non-invasive measurement of glycine concentrations and has been described as a useful tool for diagnosis and monitoring the therapeutic interventions in classical NKH [36]. However, in three atypical cases (cases 11, 12, 20a) ^{1}HMRS failed to show increased glycine, which presumably reflects lower parenchymal brain glycine concentrations, below ^{1}HMRS detection level [11,32].

The traditional treatment of classical NKH involves agents that decrease glycine concentration, as well as agents that antagonize the overstimulation of NMDA receptor. In atypical NKH cases treatment such as low protein diet, sodium benzoate, dextromethorphan, strychnine, ketamine, tryptophan, and imipramine have been tried alone or in combination. Clinical improvement has been described but it is not consistent. Several investigators also used various single carbon donors (methionine, folate, and leucovorin) in an attempt to correct the presumed deficiency of single carbon units in NKH [37]. Overall, it is difficult to infer the value of treatment since all data are from small case series and the age of initiation of treatment varies. For specific "favorable" mutations the timing of therapeutic intervention is crucial, and NMDA receptors antagonists and glycine reducing agents may have a beneficial impact on the outcome.

Conclusions and future aspects

The identification of mutations on *GLDC* gene in patients with *neonatal* and *infantile* form of NKH demonstrates that this form represents a continuum with the classical NKH and that the milder atypical phenotype is presumably the result of the residual GCS function. The *late onset* NKH may represent a different entity and more studies are needed to identify the cause of the hyperglycinemia. The true incidence of atypical NKH is unknown; it is likely underdiagnosed. Since all patients have increased plasma and urine glycine, testing plasma, and urine amino acids in patients with mild mental retardation, particularly those with behavioral problems and abnormal movements or seizures, may be of diagnostic use. The real challenge is the early and reliable prediction of those patients who will have a mild phenotype and of those who an early therapy will

produce a favorable outcome before irreversible damage occurs. Identification of pathogenic mutations and information of their expression analysis, in a number of atypical NKH patients, may give us a more accurate genotype–phenotype correlation and help with the decision making in clinical setting.

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References

[1] M. Takayanagi, S. Kure, Y. Sakata, Y. Kurihara, Y. Ohya, M. Kajita, K. Tada, Y. Matsubara, K. Narisawa, Human glycine decarboxylase gene (GLDC) and its highly conserved processed pseudogene (psiGLDC): their structure and expression, and the identification of a large deletion in a family with nonketotic hyperglycinemia, Hum.Genet. 106 (2000) 298–305.

[2] K. Nanao, K. Okamura-Ikeda, Y. Motokawa, D.M. Danks, E.R. Baumgartner, G. Takada, K. Hayasaka, Identification of the mutations in the T-protein gene causing typical and atypical nonketotic hyperglycinemia, Hum. Genet. 93 (1994) 655–658.

[3] S. Kure, K. Kojima, T. Kudo, K. Kanno, Y. Aoki, Y. Suzuki, T. Shinka, Y. Sakata, K. Narisawa, Y. Matsubara, Chromosomal localization, structure, single-nucleotide polymorphisms, and expression of the human H-protein gene of the glycine cleavage system (GCSH), a candidate gene for nonketotic hyperglycinemia, J. Hum. Genet. 46 (2001) 378–384.

[4] S.W. Scherer, G. Otulakowski, B.H. Robinson, L.C. Tsui, Localization of the human dihydrolipoamide dehydrogenase gene (DLD) to 7q31-q32, Cytogenet. Cell Genet. 56 (1991) 176–177.

[5] K. Hiraga, H. Kochi, K. Hayasaka, G. Kikuchi, W.L. Nyhan, Defective glycine cleavage system in nonketotic hyperglycinemia. Occurrence of a less active glycine decarboxylase and an abnormal aminomethyl carrier protein, J. Clin. Invest. 68 (1981) 525–534.

[6] S. Kure, M. Takayanagi, K. Narisawa, K. Tada, J. Leisti, Identification of a common mutation in Finnish patients with nonketotic hyperglycinemia, J. Clin. Invest. 90 (1992) 160–164.

[7] J.R. Toone, D.A. Applegarth, M.B. Coulter-Mackie, E.R. James, Biochemical and molecular investigations of patients with nonketotic hyperglycinemia, Mol. Genet. Metab. 70 (2000) 116–121.

[8] D.A. Applegarth, J.R. Toone, Nonketotic hyperglycinemia (glycine encephalopathy): laboratory diagnosis, Mol. Genet. Metab. 74 (2001) 139–146.

[9] J.R. Toone, D.A. Applegarth, S. Kure, M. Coulter-Mackie, P. Sazegar, K. Kojima, A. Ichinohe, Novel mutations in the P-protein gene in patients with glycine encephalopathy (non-ketotic hyperglycinemia), Mol. Genet. Metab. 76 (2002) 243–249.

[10] T. Gerritsen, E. Kaveggia, H.A. Waisman, A new type of idiopathic hyperglycinemia with hypo-oxaluria, Pediatrics 36 (1965) 882.

[11] S.H. Korman, A. Boneh, A. Ichinohe, et al., Persistent NKH with transient or absent symptoms and a homozygous GLDC mutation, Ann. Neurol. 56 (2004) 139–143.

[12] A. Hamosh, J.W. McDonald, D. Valle, C.A. Francomano, E. Niedermeyer, M.V. Johnston, Dextromethorphan and high-dose benzoate therapy for nonketotic hyperglycinemia in an infant, J. Pediatr. 121 (1992) 131–135.

[13] D.E.C. Cole, D.C. Meek, Juvenile non-ketotic hyperglycinemia in three siblings, J. Inherit. Metab. Dis. 2 (1985) 123–124.

[14] E. Haan, D.M. Kirby, K. Tada, K. Hayasaka, D.M. Danks, Difficulties in assessing the effect of strychnine on the outcome of non-ketotic hyperglycinemia. Observations on sisters with a mild T-protein defect, Eur. J. Pediatr. 145 (1986) 267–270.

[15] D.A. Trauner, T. Page, C. Greco, L. Sweetman, S. Kulovich, W.L. Nyhan, Progressive neurodegenerative disorder in a patient with nonketotic hyperglycinemia, J. Pediatr. 98 (1981) 272–275.

[16] T. Hasegawa, Y. Shiga, A. Matsumoto, A. Takeda, Y. Itoyama, Late-onset nonketotic hyperglycinemia: a case report, No To Shinkei 54 (2002) 1068–1072.

[17] W.J. Bank, G. Morrow, Familial neuromuscular disease with nonketotic hyperglycinemia, Trans. Am. Neurol. Assoc. 96 (1971) 21–23.

[18] J.E. Hoover-Fong, S. Shah, J.L. Van Hove, D. Applegarth, J. Toone, A. Hamosh, Natural history of nonketotic hyperglycinemia in 65 patients, Neurology 63 (2004) 1847–1853.

[19] G. Holmgren, H. Kison Blomquist, Nonketotic hypergycinemia in two sibs with mild psycho-neurological development, Neuropadiatrie 8 (1977) 67–72.

[20] A.H. Jackson, D.A. Applegarth, J.R. Toone, S. Kure, H.L. Levy, Atypical nonketotic hyperglycinemia with normal cerebrospinal fluid to plasma glycine ratio, J. Child Neurol. 14 (1999) 464–467.

[21] D. Aliefendioglu, A. Tana Aslan, T. Coskun, A. Dursun, F.N. Cakmak, M. Kesimer, Transient nonketotic hyperglycinemia: two case reports and literature review, Pediatr. Neurol. 28 (2003) 151–155.

[22] K. Hayasaka, K. Tada, G. Kikuchi, S. Winter, W.L. Nyhan, Nonketotic hyperglycinemia: two patients with primary defects of P-protein and T-protein, respectively, in the glycine cleavage system, Pediatr. Res. 17 (1983) 967–970.

[23] S. Kure, A. Ichinohe, K. Kojima, K. Sato, Z. Kizaki, F. Inoue, C. Yamanaka, Y. Matsubara, Mild variant of nonketotic hyperglycinemia with typical neonatal presentations: mutational and in vitro expression analyses in two patients, J. Pediatr. 144 (2004) 827–829.

[24] H.S. Singer, D. Valle, K. Hayasaka, K. Tada, Nonketotic hyperglycinemia: studies in an atypical variant, Neurology 39 (1989) 286–288.

[25] M.A. Zorbin, J.K. Wamsley, M.J. Kiehor, Glycine receptor: light microscopic localization with ^{3}H-strychnine, J. Neurosci. 1 (1981) 532.

[26] A.A. Larson, A.J. Beitz, Glycine potentiates strychnine-induced convulsions: role of NMDA receptor, J. Neurosci. 8 (1988) 3822.

[27] D.M. Frazier, G.K. Summer, H.R. Chamberlin, Hyperglycinuria and hyperglycinemia in two siblings with mild developmental delays, Am. J. Dis. Child 132 (1978) 777–781.

[28] J.E. Wraith, Non-ketotic hyperglycinemia: prolonged survival in a patient with a mild variant, J. Inherit. Metab. Dis. 19 (1996) 695–696.

[29] R.G.M. Morris, Synaptic plasticity and learning in rats and blockade of long term potentiation in vivo by NMDA receptor antagonist AP5, J. Neurosci. 9 (1989) 3040–3057.

[30] A.P. Carobrez, K.V. Teixeira, F.G. Graeft, Modulation of defensive behavior by periaqueductal gray NMDA/glycine-B receptor, Neurosci. Behav. Rev. 25 (2001) 697–709.

[31] A. Ichinohe, S. Kure, S. Mikawa, T. Ueki, K. Kojima, K. Fujiwara, K. Iinuma, Y. Matsubara, K. Sato, Glycine cleavage system in neurogenic regions, Eur. J. Neurosci. 19 (2004) 2365–2370.

[32] A. Dinopoulos, S. Kure, G. Chuck, K. Sato, D.L. Gilbert, Y. Matsubara, T. Degrauw, Glycine decarboxylase mutations: a distinctive phenotype of nonketotic hyperglycinemia in adults, Neurology 64 (2005) 1255–1257.

[33] H. Flusser, S.H. Korman, K. Sato, Y. Matsubara, A. Galil, S. Kure, Mild glycine encephalopathy (NKH) in a large kindred due to a silent exonic GLDC splice mutation, Neurology 64 (2005) 1426–1430.

[34] S.H. Korman, A. Gutman, Pitfalls in the diagnosis of glycine encephalopathy (non-ketotic hyperglycinemia), Dev. Med. Child Neurol. 44 (2002) 712–720.
[35] S. Kure, K. Narisawa, K. Tada, Enzymatic diagnosis of nonketotic hyperglycinemia with lymphoblasts, J. Pediatr. 120 (1992) 95–98.
[36] A. Viola, B. Chabrol, F. Nicoli, S. Confort-Gouny, P. Viout, P.J. Cozzone, Magnetic resonance spectroscopy study of glycine pathways in nonketotic hyperglycinemia, Pediatr. Res. 52 (2002) 292–300.
[37] A. Hamosh, M.V. Johnston, C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, Nonketotic Hyperglycinemia, The Metabolic and Molecular Bases of Inherited Disease, McGraw-Hill, New York, 2001.
[38] R.D. Steiner, D.A. Sweetser, J.R. Rohrbaugh, S.B. Dowton, J.R. Toone, D.A. Applegarth, Nonketotic hyperglycinemia: atypical clinical and biochemical manifestations, J. Pediatr. 128 (1996) 243–246.
[39] J.M. Neuberger, S. Schweitzer, M.O. Rolland, R. Burghard, Effect of sodium benzoate in the treatment of atypical nonketotic hyperglycinaemia, J. Inherit. Metab. Dis. 23 (2000) 22–26.
[40] J. Christodoulou, S. Kure, K. Hayasaka, J.T. Clarke, Atypical nonketotic hyperglycinemia confirmed by assay of the glycine cleavage system in lymphoblasts, J. Pediatr. 123 (1993) 100–102.
[41] K. Hayasaka, K. Tada, N. Fueki, Y. Nakamura, W.L. Nyhan, K. Schmidt, S. Packman, M.R. Seashore, E. Haan, D.M. Danks, et al., Nonketotic hyperglycinemia: analyses of glycine cleavage system in typical and atypical cases, J. Pediatr. 110 (1987) 873–877.
[42] C. Borrone, C. Bachmann, M. DiRocco, K. Tada, K. Hayasaka, R. Gatti, Atypical non-ketotic hyperglycinemia, J. Inherit. Metab. Dis. 9 (1986) 402–403.
[43] Y. Tanaka, M. Miyazaki, M. Tsuda, K. Murai, S. Kuzuhara, Blindness due to non-ketotic hyperglycinemia: report of a 38-year-old, the oldest case to date, Intern. Med. 32 (1993) 641–642.
[44] D.A. Hall, S.P. Ringel, Adult nonketotic hyperglycinemia (NKH) crisis presenting as severe chorea and encephalopathy, Mov. Disord. 19 (2004) 485–486.
[45] E.J. Wiltshire, N.K. Poplawski, J.R. Harrison, J.M. Fletcher, Treatment of late-onset nonketotic hyperglycinaemia: effectiveness of imipramine and benzoate, J. Inherit. Metab. Dis. 23 (2000) 15–21.
[46] G.S. Steiman, M. Yudkoff, P.H. Berman, B. Blazer-Yost, S. Segal, Late-onset nonketotic hyperglycinemia and spinocerebellar degeneration, J. Pediatr. 94 (1979) 907–911.
[47] D.B. Flannery, J. Pellock, D. Bousounis, P. Hunt, C. Nance, B. Wolf, Nonketotic hyperglycinemia in two retarded adults: a mild form of infantile nonketotic hyperglycinemia, Neurology 33 (1983) 1064–1066.

based on a thorough search of the medical literature and a poison control center consultation. Tamoxifen infrequently causes reversible neurotoxicity, but only at much higher doses ($\geq$160mg/m^2 daily).[5]

Recent investigations into the use of artemisinin compounds in cancer treatment[6] have not been substantiated in clinical trials. Because of pervasive reports of animal brainstem toxicity, and the gradual emergence of similar patient cases, it becomes imperative to ascertain the safety of prolonged courses of artemisinin for cancer prophylaxis.

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References

1. Kissinger E, Hien TT, Hung NT, et al. Clinical and neurophysiological study of the effects of multiple doses of artemisinin on brain-stem function in Vietnamese patients. Am J Trop Med Hyg 2000;63:48–55.
2. Miller LG, Panosian CB. Ataxia and slurred speech after artesunate treatment for falciparum malaria. N Engl J Med 1997;336: 328–1329.
3. Brewer TG, Grate SJ, Peggins JO, et al. Fatal neurotoxicity of arteether and artemether. Am J Trop Med Hyg 1994;51: 251–259.
4. Schmuck G, Roehrdanz E, Haynes RK, Kahl R. Neurotoxic mode of action of Artemisinin. Antimicrob Agents Chemother 2002;46:821–827.
5. Bergan RC, Reed E, Myers CE, et al. A phase II study of high-dose tamoxifen in patients with hormone-refractory prostate cancer. Clin Cancer Res 1999;5:2366–2373.
6. Singh NP, Lai H. Selective toxicity of dihydroartemisinin and holotransferrin toward human breast cancer cells. Life Sci. 2001; 70:49–56.

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Adult Alexander's Disease without Leukoencephalopathy

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Diagnosis of Alexander's disease is based on demonstration of Rosenthal fibers in astrocytes but can be supported by detection of mutations in the gene encoding the glial fibrillary acidic protein (GFAP).[1,2] Three clinical subtypes of Alexander's disease are identified, namely, infantile, juvenile, and adult, which usually occur sporadically, arising through de novo mutations.[2]

A 71-year-old man was referred for a complex neurological syndrome that began at age 52 years with muscle pain and unsteady gait. At age 61 years, he developed progressive lower limb weakness, dysarthria, and dysphagia. Neurological examination showed palatal myoclonus, gaze-evoked nystagmus, cogwheel phenomenon on smooth pursuit, diffuse bradykinesia, and dysmethria and bilateral Babinski sign. Magnetic resonance imaging (MRI; Fig) showed thinning of the medulla and cervical spinal cord and a cross-like hyperintensity in the medulla in T2-weighted images. Few small foci of

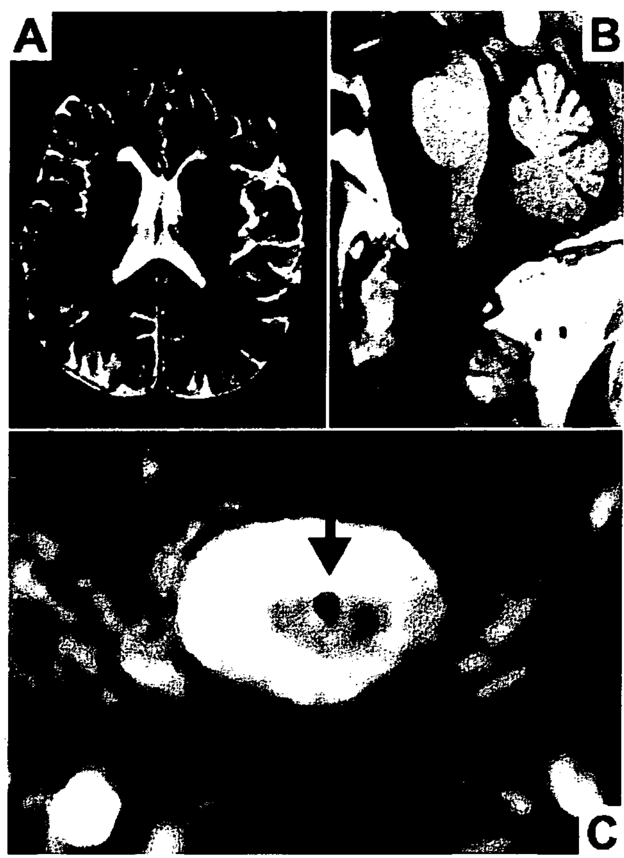

Fig. Axial T2-weighted (TR, 2,400; TE, 100) (A) spin-echo image shows symmetric hypointensity of the caudate and putamen and along the lateral ventricular walls without signal abnormality of the periventricular white matter. Sagittal T1-weighted (TR, 500; TE, 14) (B) spin-echo image shows marked thinning of the medulla and of the upper cervical spinal cord. Axial T2-weighted gradient-echo image (TR, 500; flip angle, 20 degrees; TE, 25) of the spine at C2 (C) demonstrates dot-like hypointensity* (arrow) *in the anterior sulcus of a thinned spinal cord.*

age-related signal changes were present in the cerebral white matter. A symmetric T2 hypointensity of the caudate and putamen was present, as well as a line of T2 hypointensity along the walls of the lateral ventricles and in the anterior sulcus of the cervical spinal cord. Molecular investigation[1] showed a C to T substitution at nucleotide 208 in exon 1 of the *GFAP* gene in the heterozygous form resulting in change of arginine to tryptophan at position 70 (R70W).

The prominent MRI finding in our patient was atrophy of the medulla and cervical spinal cord. The linear T2 hypointensity along the lateral ventricular walls and in the anterior sulcus of the spinal cord might be related to accumulation of Rosenthal fibers in these cerebrospinal fluid recesses.[3] Interestingly, small heat shock proteins and ubiquitin, which with GFAP are components of Rosenthal fibers, are increased in the cerebrospinal fluid of patients with Alexander's disease.[3] We did not observe any leukoencephalopathy consistent with the view that Alexander's disease is not an obligate leukodystrophy; this is in line with adult Alexander's disease cases

previously reported.[4] However, the MRI features of adult Alexander's disease are not homogeneous. In fact, adults with GFAP mutation exhibiting leukoencephalopathy were reported.[5] We submit that the MRI features of Alexander's disease can range from a pure leukoencephalopathy (predominant in the infantile variant[3]) to a medullary-spinal cord atrophy without leukoencephalopathy. The mechanisms underlying this spectrum deserve further studies. Search of the GFAP mutation is recommended in adults with progressive bulbar palsy and atrophy of the medulla and the spinal cord.

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References
1. Brenner M, Johnson AB, Boespflug-Tanguy O, et al. Mutations in GFAP encoding glial fibrillary acidic protein are associated with Alexander disease. Nat Genet 2001;27:117–120.
2. Moser HW. Alexander disease: combined gene analysis and MRI clarify pathogenesis and extend phenotype. Ann Neurol 2005;57:307–308.
3. Van der Knaap MS, Naidu S, Breiter SN, et al. Alexander disease: diagnosis with MR imaging. AJNR Am J Neuroradiol 2001;22:541–552.
4. Namekawa M, Takiyama Y, Aoki Y, et al. Identification of GFAP gene mutation in hereditary adult-onset Alexander's disease. Ann Neurol 2002;52:779–785.
5. Li R, Johnson AB, Salomons G, et al. Glial fibrillary acidic protein mutations in infantile, juvenile, and adult forms of Alexander disease. Ann Neurol 2005;57:310–326.

Is Intracranial Monitoring Dispensable for Neocortical Epilepsy with Normal Magnetic Resonance Imaging?

Richard Wennberg, MD, FRCPC

I was interested to read in the article of Lee and colleagues[1] that no prognostic value could be attributed to the localization or morphology of ictal onsets recorded with subdural electrodes in their patients with neocortical epilepsy. This finding comes as no surprise to me. Notwithstanding, the authors repeat the current assumption that "intracranial monitoring is indispensable for neocortical epilepsy with normal MRI" and they do not follow through on their results by drawing this assumption of indispensability into question.

There is a modern paradox in the logical processes underlying the planning of surgical management in patients with neocortical epilepsy. As is typically the case, surgical resection in the patients described by Lee and colleagues[1] was guided by the margins of the "intracranial ictal onset zone," the same zone that the authors subsequently found to have no relation to surgical outcome. In fact, from the techniques reported, it was only the findings of noninvasive presurgical investigations (mainly interictal electroencephalography and positron emission tomography) that were independently related to surgical outcome.[1] All this is puzzling when one considers that the same noninvasive evaluations are used to determine the placements of the subdural electrodes for intracranial monitoring.[1]

From the results of studies of acute and chronic intracranial electrocorticography,[2,3] I have suggested previously that surgery for neocortical epilepsy could be contemplated without chronic invasive recording.[4] In that intracranial monitoring is expensive, time-consuming, and not without risk, our inability to clearly demonstrate a prognostic value in neocortical epilepsy should, at the very least, raise the question of dispensability in this particular setting.

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References
1. Lee SK, Lee SY, Kim K-K, et al. Surgical outcome and prognostic factors of cryptogenic neocortical epilepsy. Ann Neurol 2005;58:525–532.
2. Wennberg R, Quesney F, Olivier A, Rasmussen T. Electrocorticography and outcome in frontal lobe epilepsy. Electroenceph Clin Neurophysiol 1998;106:357–368.
3. Bautista RED, Cobbs MA, Spencer DD, Spencer SS. Prediction of surgical outcome by interictal epileptiform abnormalities during intracranial EEG monitoring in patients with extrahippocampal seizures. Epilepsia 1999;40:880–890.
4. Wennberg R. Poor surgical outcome in patients with neocortical epilepsy is correlated with interictal epileptiform abnormalities outside the area of surgical resection. Epilepsia 2000;41:355–357.

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Reply

Necessity of Invasive Monitoring in Neocortical Epilepsy with Normal Magnetic Resonance Imaging

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Dr Wennberg suggested that surgery for neocortical epilepsy with normal magnetic resonance imaging (MRI) could be contemplated without chronic invasive recording. We think that this is an interesting and important point. Our results did not find any relationship between a specific intracranial ictal onset pattern, such as focal or regional onset, and surgical outcome. However, this does *not* mean that the resection of this zone did not relate to surgical outcome or that intracranial monitoring is dispensable for these patients. It only means that a specific onset pattern (the number of electrodes with intracranial ictal onset rhythm or the frequency of intracranial ictal onset rhythm) did not relate to surgical outcome. We resected the area with intracranial ictal onset irrespective of onset pattern, and many patients have benefited from this. Only by sparing some intracranial ictal onset area and showing a good surgical outcome could we prove that intracranial monitoring is indeed dispensable. However, we could not claim this because our resection margin always included the intracranial ictal onset zone.

The necessity of using intracranial electrodes depends on various situations and treatment policy. There should be pros and cons. However, we consider that intracranial monitoring

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MUTATION UPDATE

Mutations in the Holocarboxylase Synthetase Gene *HLCS*

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Holocarboxylase synthetase (HLCS) deficiency is an autosomal recessive disorder. HLCS is an enzyme that catalyzes biotin incorporation into carboxylases and histones. Since the first report of the cDNA sequence, 30 mutations in the *HLCS* gene have been reported. Mutations occur throughout the entire coding region except exons 6 and 10. The types of mutations are one single amino acid deletion, five single nucleotide insertions/deletions, 22 missense mutations, and two nonsense mutations. The only intronic mutation identified thus far is c.1519+5G>A (also designated IVS10+5G>A), which causes a splice error. Several lines of evidence suggest that c.1519+5G>A is a founder mutation in Scandinavian patients. Prevalence of this mutation is about 10 times higher in the Faroe Islands than in the rest of the world. The mutations p.L237P and c.780delG are predominant only in Japanese patients. These are probably founder mutations in this population. Mutations p.R508W and p.V550M are identified in several ethic groups and accompanied with various haplotypes, suggesting that these are recurrent mutations. There is a good relationship between clinical biotin responsiveness and the residual activity of HLCS. A combination of a null mutation and a point mutation that shows less than a few percent of the normal activity results in neonatal onset. Patients who have mutant HLCS with higher residual activity develop symptom after the neonatal period and show a good clinical response to biotin therapy. Hum Mutat 26(4), 285–290, 2005. © 2005 Wiley-Liss, Inc.

KEY WORDS: holocarboxylase synthetase; HLCS; multiple carboxylase deficiency; biotin

INTRODUCTION

Holocarboxylase synthetase (HLCS; MIM# 609018; EC 6.3.1.10) is an enzyme that catalyzes biotin incorporation into carboxylases [Achuta Murthy and Mistry, 1972; Wolf, 2001]. In humans, four carboxylases are known to be biotinylated by HLCS. They are pyruvate carboxylase, propionyl-CoA carboxylase, and methylcrotonyl-CoA carboxylases, which are mitochondrial matrix enzymes, and acetyl-CoA carboxylase, located in both the cytosol and the mitochondrial membrane. There are two acetyl-CoA carboxylases genes. The ACACA gene (MIM# 200350) gene encodes only a cytosolic enzyme whereas the ACACB gene (MIM# 601557) gene produces both the cytosolic and the mitochondrial enzymes [Pacheco-Alvarez et al., 2002]. Biotinylation of these carboxylases is essential for their enzymatic activities. Recently, HLCS has been thought to biotinylate histones, although the physiological significance has not been elucidated [Narang et al., 2004; Peters et al., 2002; Stanley et al., 2001].

Holocarboxylase synthetase deficiency (MIM# 253270) is an autosomal recessive disorder. It is also called (biotin-responsive) multiple carboxylase deficiency (MCD), because deficient HLCS activity results in reduced activity of multiple carboxylases. The exact incidence of this disease is not known. Our laboratory has diagnosed one or two new cases per year in Japan, where approximately 1.2 million babies are born every year. We suspect the incidence of HLCS deficiency is less than 1 in 100,000 live births per year in Japan. Most patients with HLCS deficiency manifest symptoms in the newborn to early infantile period [Narisawa et al., 1982; Wolf, 2001]. Symptoms of HLCS deficiency include metabolic acidosis, a characteristic organic aciduria, lethargy, hypotonia, convulsions, and dermatitis. Many symptoms of HLCS are also seen in biotinidase deficiency (MIM# 253260), another type of MCD. Some patients become symptomatic in the later infantile period, at the age of several months to years [Chikaoka et al., 1992; Gibson et al., 1996; Suormala et al., 1997, 1998]. All patients with HLCS deficiency reported thus far have responded to biotin administration. In some patients, however, the response was only partial as manifested by continued excretion of abnormal metabolites in the urine. Developmental abnormalities have also been reported in some cases in spite of high-dose biotin therapy [Baumgartner and Suormala, 1997; Santer et al., 2003; Suzuki et al., 1996; Wolf et al., 1981].

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BIOLOGICAL RELEVANCE

Isolation of human cDNA for HLCS from the liver enabled the investigation of HLCS at the molecular level [Leon-Del-Rio et al., 1995; Suzuki et al., 1994]. The cDNA encoded 726 amino acids with in-frame stop codons lying at 5′ upstream of the first methionine. The deduced protein sequence contains a homologous region (amino acids numbers 448–701) to BirA, the biotin apo-carboxyl carrier protein ligase of *Escherichia coli*. This homologous portion of human HLCS is thought to be the putative biotin-binding region [Suzuki et al., 1994]. The importance of the domain outside the biotin-binding region of HLCS for enzymatic activity was examined in an expression study with HLCS deletion mutants. The N-terminal amino acid region up to Ile117 had much less effect on the enzymatic activity than the amino acid sequence between Ile117 and Met234 [Sakamoto et al., 1999]. The data are consistent with another experiment [Hiratsuka et al., 1998] and the observation that the mutations identified in patients so far are located after the amino acid residue Arg183 (Table 1). Multiple cDNA clones were isolated from the human lymphocyte cDNA library [Leon-Del-Rio et al., 1995]. They differed from human liver cDNA mainly at their 5′ ends. Using an exon-trapping method, another type of cDNA was isolated from the human myeloid cell line KG-1 [Ohira et al., 1997]. This cDNA has the longest 5′ upstream and 3′ downstream untranslated regions. Because the first and second methionine codons found in other types of cDNAs were skipped, this cDNA should produce only the shortest form of HLCS protein [Hiratsuka et al., 1998].

Comparisons of sequences of multiple cDNAs and the 21q22.1 genomic region determined in the human genome project [Hattori et al., 2000] has elucidated the organization of the exons and introns of the human HLCS gene [Yang et al., 2001). The HLCS gene spans approximately 240 kb and comprises 14 exons (Fig. 1). The first methionine codon is located in exon 6, and a stop codon in exon 14. Of the 14 introns, intron 9 is the longest (about 130 kb). It contains two polymorphic tetra-nucleotide repeats that are useful for determining a haplotype of the HLCS gene [Yang et al., 2000]. On the basis of results reported for several cDNA species, the HLCS gene probably generates multiple transcripts, although the physiological significance of this is not yet clear. We demonstrated at least three mRNA types with different transcription starting sites in human cultured cells [Yang et al., 2001]. Type 1 mRNA starts at exon 1. Type 2 mRNA starts at exon 3 and type 3 mRNA starts at exon 2. The variations at the 5′ end of the mRNA so far identified do not change the amino acid sequence of HLCS. Substantial variations were also present in the 3′ untranslated region (UTR) sequence of HLCS, where no consensus sequence for the polyadenylation signal was found [Yang et al., 2001].

MUTATIONS AND POLYMORPHISMS

Mutations

During the last 10 years since the first report of the cDNA sequence [Suzuki et al., 1994], 30 mutations in the HLCS gene have been reported (Table 1). The table includes novel mutations identified in four Japanese, two Faroese patients, one Danish, and one Spanish patient whose cases were not reported previously. Mutations occur throughout the entire coding region except exons 6 and 10 (Fig. 1). The types of mutations are one single amino acid deletion, five single nucleotide insertions/deletions, 22 missense mutations, and two nonsense mutations. The only intronic mutation identified was a single nucleotide mutation in intron 10, c.1519+5G>A (also designated IVS10+5G>A), that causes a splice error [Sakamoto et al., 2000]. Two mutations, L237P and 780delG, account for 50% of the Japanese mutant alleles [Yang et al., 2001], but are never found in other ethnic groups. The IVS10+5G>A mutation was found in north European countries; details of this will be discussed in the next section. The mutations R508W, V550M, G581S, D571N, and R565X were found in several ethnic groups.

Polymorphisms

The current public SNP database (dbSNP) shows three SNPs in the coding region of HLCS. We identified two other polymorphisms during the investigation of patients with HLCS deficiency and their family members (Table 1).

CLINICAL RELEVANCE

Founder Mutations

The IVS10+5G>A mutation was first identified in a Swedish patient who showed her first symptoms at the age of 8 years. The amelioration of her biochemical and clinical abnormalities after biotin treatment started was relatively slow among patients with HLCS [Holme et al., 1988]. This mutation resulted in abnormal splicing with a decreased level of normal mRNA [Sakamoto et al., 2000]. Holocarboxylase synthetase activity in the patient's fibroblasts was 4% of the normal level [Sakamoto et al., 2000]. Subsequently, a Danish patient and two patients from the Faroe Islands who are homozygous for this mutation have been reported [Yang et al., 2001]. In addition, one French patient and one German patient were reported to have IVS10+5G>A in a heterozygous form [Santer et al., 2003; Yang et al., 2001]. We further analyzed the cases of two unrelated Faroese patients and another Danish patient and found that all of the patients were homozygous for the mutation; so far, 16 alleles have been identified (Table 1). To determine the origin of the mutations, we investigated polymorphic microsatellite markers in the *HLCS* gene and determined the haplotypes of patients [Yang et al., 2000]. All IVS10+5G>A alleles were associated with the 2-3 haplotype.

Geographically, the Faroe Islands are located in the North Atlantic, north of Scotland, and west of Norway [Ewald et al., 1999]. The Faroese immigrated from Norway about 1,000 years ago. They have expanded in number from a few thousand to about 47,000 during the last two centuries. Because we have already confirmed five patients from distinct families on this island, the incidence may be estimated to be at least 1 in 10,000. This value may be 10 times higher than those of countries in the rest of the world. These observations strongly suggest that IVS10+5G>A is a founder mutation in Scandinavian patients with HLCS deficiency [Yang et al., 2001]. In a Faroese family with an HLCS-deficient child, we found an asymptomatic father who was also homozygous for this mutation. He has never shown clinical symptoms throughout his life, suggesting that there may be other asymptomatic IVS10+5G>A homozygotes.

The mutations L237P and 780delG are predominant only in Japanese patients [Aoki et al., 1995; Sakamoto et al., 1998; Yang et al., 2000, 2001]. We investigated the haplotype of these two mutations and found that both mutant alleles were exclusively associated with haplotype 2-2. This finding is consistent with the notion that L237P and 780delG are founder mutations in the Japanese population [Yang et al., 2000]. Although patients were found who were homozygous for L237P or compound heterozygous for L237P and 780delG, none were found who were homozygous for 780delG, suggesting that homozygous 780delG mutations may be lethal.

TABLE 1. List of Mutations and Polymorphisms in the *HLCS* Gene*

Exon/intron	Nucleotide change in cDNA	Effect on coding region	Number of alleles	Patient origin	References
Mutations					
Exon 7	c.548G>C	p.R183R	2	Unknown	Sakamoto et al. [1999]
Exon 7	c.647T>G	p.L216R	3	Australian Maori	Dupuis et al. [1996] Morrone et al. [2002]
Exon 7	c.655_656insA	p.I219NfsX58	2	Japanese	Yang et al. [2001]
Exon 7	c.710T>C	p.L237P	9	Japanese	Aoki et al. [1995] Yang et al. [2001]
Exon 7	c.780delG	p.G261VfsX20	7	Japanese	Aoki et al. [1995] Yang et al. [2001]
Exon 8	c.998T>A	p.V333E	1	German	Suormala et al. [1997] Aoki et al. [1999]
Exon 8	c.1053_1054insC	p.L353AfsX7	1	Spanish	Briones et al. [1989] Yang et al. [2001]
Exon 8	c.1080A>C	p.R360S	2	Japanese	Yang et al. [2001]
Exon 8	c.1088T>A	p.V363D	2	Unknown	Dupuis et al. [1996]
Exon 9	c.1367A>G	p.Y456C	1	Malaysian	Yang et al. [2001]
Exon 9	c.1385C>T	p.T462I	1	Spanish	Aoki et al. [1999] Yang et al. [2001]
Exon 9	c.1409T>C	p.L470S	1	Japanese	Yang et al. [2001]
Intron 10	c.1519+5G>A	Splice defect (IVS10+5G>A)	16	Spanish Danish Faroese Swedish French German	Holme et al. [1988] Sakamoto et al. [2000] Yang et al. [2001] This study
Exon 11	c.1522C>T	p.R508W	15	Japanese Taiwanese Chinese Iranian Other	Dupuis et al. [1996] Sakamoto et al. [1998] Yang et al. [2001] Morrone et al. [2002] Tang et al. [2003]
Exon 11	c.1533T>A	p.N511K	1	Italian	Morrone et al. [2002]
Exon 11	c.1553G>A	p.G518E	1	Unknown	Dupuis et al. [1996]
Exon 11	c.1589delT	p.L529RfsX15	1	German	Aoki et al. [1999]
Exon 11	c.1640T>G	p.V547G	1	German	Yang et al. [2001]
Exon 11	c.1648G>A	p.V550M	8	Japanese African Other	Dupuis et al. [1996] Aoki et al. [1997] Morrone et al. [2002]; Tang et al. [2003]
Exon 12	c.1693C>T	p.R565X	2	Japanese African	Sakamoto et al. [1998] Yang et al. [2001] Tang et al. [2003]
Exon 12	c.1711G>A	p.D571N	3	German Spanish	Aoki et al. [1999] Dupuis et al. [1996] This study
Exon 12	c.1741G>A	p.G581S	4	Italian Turkish	Fuchshuber et al. [1993] Suormala et al. [1997] Morrone et al. [2002]
Exon 12	c.1744G>A	p.G582R	1	Italian	Morrone et al. [2002]
Exon 13	c.1828_1830del	p.T610del	2	Lebanese	Aoki et al. [1999] Touma et al. [1999]
Exon 13	c.1843G>T	p.D615Y	1	Spanish	This study
Exon 13	c.1990G>T	p.D634Y	1	German	Suormala et al. [1997]
Exon 13	c.1990G>A	p.D634N	2	Japanese Chinese	This study Tang et al. [2003]
Exon 13	c.1992delC	p.R665DfsX41	1	French	Suormala et al. [1997]
Exon 13	c.1993C>T	p.R665X	1	Japanese	This study
Exon 14	c.2144A>G	p.D715G	1	Japanese	This study
Polymorphisms of the coding region					
Exon 6	c.285C>T	p.P95P			dbSNP# rs2230182
Exon 7	c.126G>T	p.E42D		Malaysian	Yang et al. [2001]
Exon 7	c.843C>T	p.S278S			Aoki et al. [1999] dbSNP# rs1065758
Exon 7	c.971G>A	p.R324H		Spanish	This study
Exon 8	c.1053T>C	p.N351N			dbSNP# rs1065759

*Intronic sequence of c.1519+5G>A is based on AB063285; others are based on NT_000411.4 (A of ATG is +1).

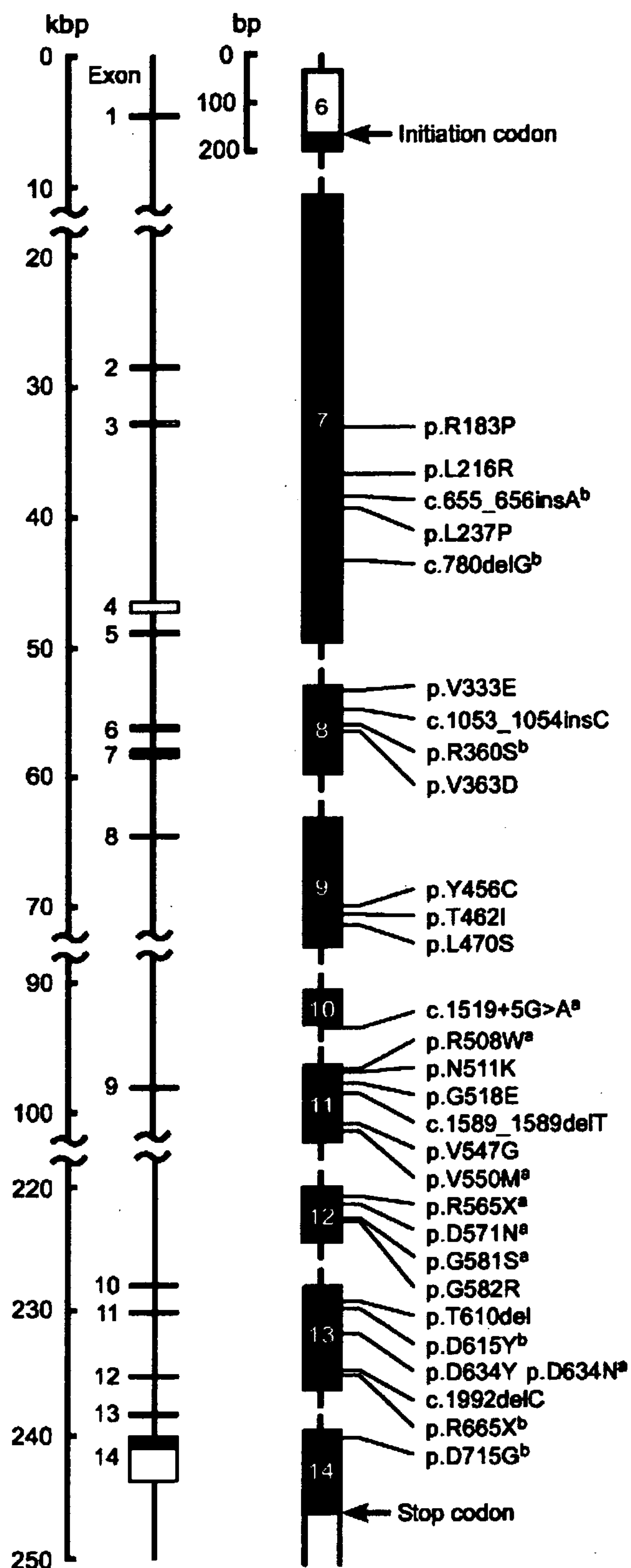

FIGURE 1. Structure of the human holocarboxylase synthetase gene and a summary of its mutations. The *HLCS* gene spans approximately 240 kb and comprises 14 exons. The initiation codon is located in exon 6 and the stop codon is located in exon 14. Filled squares are coding exons. [a]Mutations found in several ethnic groups. [b]Mutations found in Japanese patients.

Recurrent Mutations

The R508W mutation was found in a heterozygous form in three Japanese patients and one Chinese patient [Sakamoto et al., 1998; Tang et al., 2003; Yang et al., 2001] and in the homozygous form in three Chinese patients and one Iranian patient [Hwu et al., 2000; Morrone et al., 2002; Tang et al., 2003]. This mutation was also found in five mutant alleles in four U.S. patients whose ethnic backgrounds were not described [Dupuis et al., 1996]. The R508W mutation has been shown to have low activity in the cells [Dupuis et al., 1999]. Another mutation found in several ethnic groups is V550M. One Japanese patient was homozygous for this mutation [Aoki et al., 1997]. Dupuis et al. [1996] reported that three U.S. patients had this mutation (four alleles). Two Italian patients and one African patient were also reported to have the V550M mutation [Hwu et al., 2000; Morrone et al., 2002; Tang et al., 2003]. The mutation resulted in a decreased affinity of HLCS to biotin (i.e., a Km mutant); the mutant HLCS shows its enzymatic activity to some extent at high biotin concentrations [Aoki et al., 1997]. The R508W mutant alleles in Japanese patients were associated with either haplotype 2-3 or haplotype 1-4 [Yang et al., 2000]. The homozygous R508W mutation in the Taiwanese patient was associated with haplotype 2-3. The haplotype of the V550M mutation in the Japanese patient was 1-4, whereas that in the Jewish patient was 2-3. The nucleotide change in the R508W and the V550M mutations is a C-to-T transition at the CpG dinucleotides. These data suggest that R508W and V550M are not founder mutations but recurrent mutations occurring at CpG dinucleotide mutation hotspots [Yang et al., 2000].

Genotype–Phenotype Correlation

From an early kinetic study of mutant HLCS, patients were thought to be responsive to biotin treatment because they have mutant HLCS with a decreased affinity for biotin (i.e., a Km mutant) [Burri et al., 1985]. Using a transient expression system in HLCS-deficient fibroblasts, we further investigated the enzymatic characters of mutant enzymes [Aoki et al., 1997; Sakamoto et al., 1999]. The enzymes containing mutations that are located outside the biotin-binding region (R183P, L216R, L237P, V333E, and V363D) showed normal to higher affinity for biotin but decreased Vmax (i.e., they were non-Km mutants). The mutations located in the biotin-binding region (Gly581Ser and Thr610del) showed lower affinity for biotin (i.e., Km mutants). We usually expect that a "Km mutant" recovers its activity with high biotin concentration but a "non-Km mutant" does not. However, patients with non-Km mutants responded clinically to pharmacological doses of biotin in a manner similar to that of patients with Km mutants. The observation may be explained by our experimental data that the Km for biotin of normal HLCS is higher than the concentration of biotin in human cells under ordinary nutritional conditions [Aoki et al., 1997; Sakamoto et al., 1999].

In 16 Japanese patients from apparently unrelated families whose clinical pictures were well known, nine patients developed symptoms within a week after birth. Eight of these were compound heterozygotes of a point mutation and a null mutation (insertion/deletion and nonsense mutations). The point mutations were L237P and L470S. The null mutations were 780delG, 6556insA, and R665X.. Only one patient was homozygous for L237P. We studied mutations in two European patients who displayed symptoms before they were 1 week old. One was found to be a compound heterozygote of T462I and 1053_1054insC and the other was found to be a homozygote of G581S [Yang et al., 2001].

The expression study suggested that mutant HLCS with T462I or G581S showed less than 5% of the normal activity [Aoki et al., 1999]. We never found HLCS-deficient patients who harbor two null mutations. From these observations, we can speculate that homozygous null mutations are lethal in utero and combination of a null mutation and a point mutation that shows less than a few percent of the normal activity results in neonatal onset.

In eight clinically well-documented patients who have at least one allele of R508W, none displayed symptoms before the age of 2 months. Patients are biotin responsive and the prognosis was fine except in one case [Morrone et al., 2002]. This mutant enzyme's favorable response to biotin was also demonstrated by in vitro experiments [Dupuis et al., 1999]. Although the number of cases to study is still limited, patients with V550M are also expected to show good prognosis [Aoki et al., 1997; Morrone et al., 2002]. The IVS10+5G>A mutation shows a unique clinical character. Onset of the homozygotes of this mutation ranges from 2 months to 8 years (median 5 months) [Santer et al., 2003; Yang et al., 2001]. As previously noted, some patients homozygous for this mutation may be asymptomatic. In spite of a rather mild phenotype associated with IVS10+5G>A among HLCS-deficient patients at the onset, the clinical responses to biotin treatment are slow and partial in some cases [Holme et al., 1988; Santer et al., 2003].

In conclusion, there is a relationship between clinical biotin responsiveness and the residual activity of HLCS. We can predict the response to biotin therapy from genotypes in a patient who has a combination of well-characterized mutations stated above. However, we should be aware that some variation of outcome exists between patients with the same mutation.

DIAGNOSTIC RELEVANCE

Clinical pictures of HLCS deficiency and those of biotinidase deficiency are often indistinguishable. It is essential to perform enzymatic or DNA assays to identify the primary defect of patients who show MCD. Out of 16 HLCS-deficient Japanese patients from independent families, 15 have at least one allele of the four major mutations (del780C, L237P, R508W, and V550M). We routinely screen for these four mutations in our laboratory as the initial step for diagnosis of MCD in Japanese patients. If none of the four mutations are identified, we perform an enzyme assay [Suzuki et al., 1996]. In patients from ethnic groups other than the Faroese, mutations appear to be diverse. Thus, an enzyme assay is the first choice for the definitive diagnosis of HLCS deficiency in many ethnic groups. In the Faroese, DNA examination to detect IVS10+5G>T is useful.

FUTURE PROSPECTS

Information on HLCS mutations is still limited and further investigation into the mutation spectrum of each ethnic group is required to facilitate the diagnostic DNA examination of patients with MCD.

As exemplified by the recent works, roles of HLCS other than the biotinylation of carboxylases may exist [Narang et al., 2004; Solorzano-Vargas et al., 2002]. Newly identified functions of HLCS may be responsible for various symptoms observed in patients with HLCS deficiency.

Recently, a patient with MCD and a deficiency of the biotin transporter in the peripheral blood cells was reported [Mardach et al., 2002]. No abnormalities were found in biotinidase or HLCS, indicating that MCD can be caused by defects in three different primary substances: HLCS, biotinidase, and biotin transporter. We encountered patients who showed biotin-responsive MCD symptoms with normal HLCS and biotinidase activity (unpublished results). The *SMVT* gene coding for biotin transport had been investigated as a candidate disease-causing gene. However, the reported patient and our patients had no mutation in the gene (unpublished results) [Mardach et al., 2002]. Identification of the third gene that causes MCD remains to be elucidated in the field of human biotin metabolism.

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REFERENCES

Achuta Murthy PN, Mistry SP. 1972. Synthesis of biotin-dependent carboxylases from their apoprotein and biotin. J Sci Ind Res (India) 31:554–563.

Aoki Y, Suzuki Y, Sakamoto O, Li X, Takahashi K, Ohtake A, Sakuta R, Ohura T, Miyabayashi S, Narisawa K. 1995. Molecular analysis of holocarboxylase synthetase deficiency: a missense mutation and a single base deletion are predominant in Japanese patients. Biochim Biophys Acta 1272:168–174.

Aoki Y, Suzuki Y, Li X, Sakamoto O, Chikaoka H, Takita S, Narisawa K. 1997. Characterization of mutant holocarboxylase synthetase (HCS): a Km for biotin was not elevated in a patient with HCS deficiency. Pediatr Res 42:849–854.

Aoki Y, Li X, Sakamoto O, Hiratsuka M, Akaishi H, Xu L, Briones P, Suormala T, Baumgartner ER, Suzuki Y, Narisawa K. 1999. Identification and characterization of mutations in patients with holocarboxylase synthetase deficiency. Hum Genet 104:143–148.

Baumgartner ER, Suormala T. 1997. Multiple carboxylase deficiency: inherited and acquired disorders of biotin metabolism. Int J Vitam Nutr Res 67:377–384.

Briones P, Ribes A, Vilaseca MA, Rodriguez-Valcarcel G, Thuy LP, Sweetman L. 1989. A new case of holocarboxylase deficiency. J Inherit Metab Dis 12:329–330.

Burri B, Sweetman L, Nyhan WL. 1985. Heterogeneity of holocarboxylase synthetase in patients with biotin-responsive multiple carboxylase deficiency. Am J Hum Genet 37:326–337.

Chikaoka H, Nagano M, Takita S, Shinka T, Kuhara T, Inoue Y, Matsumoto I. 1992. Multiple carboxylase deficiency: a case report. In: Matsumoto I, editor. Advances in chemical diagnosis and treatment of metabolic diseases. New York: John Willy & Sons. p 31–36.

Dupuis L, Leon-Del-Rio A, Leclerc D, Campeau E, Sweetman L, Saudubray JM, Herman G, Gibson KM, Gravel RA. 1996. Clustering of mutations in the biotin-binding region of holocarboxylase synthetase in biotin-responsive multiple carboxylase deficiency. Hum Mol Genet 5:1011–1016.

Dupuis L, Campeau E, Leclerc D, Gravel RA. 1999. Mechanism of biotin responsiveness in biotin-responsive multiple carboxylase deficiency. Mol Genet Metab 66:80–90.

Ewald H, Wang AG, Vang M, Mors O, Nyegaard M, Kruse TA. 1999. A haplotype-based study of lithium responding patients with bipolar affective disorder on the Faroe Islands. Psychiatr Genet 9:23–34.

Fuchshuber A, Suormala T, Roth B, Duran M, Michalk D, Baumgartner ER. 1993. Holocarboxylase synthetase deficiency: early diagnosis and management of a new case. Eur J Pediatr 152:446–449.

Gibson KM, Bennett MJ, Nyhan WL, Mize CE. 1996. Late-onset holocarboxylase synthetase deficiency. J Inherit Metab Dis 19:739–742.

Hattori M, Fujiyama A, Taylor TD, Watanabe H, Yada T, Park HS, Toyoda A, Ishii K, Totoki Y, Choi DK, Soeda E, Ohki M, Takagi T, Sakaki Y, Taudien S, Blechschmidt K, Polley A, Menzel U, Delabar J, Kumpf K, Lehmann R, Patterson D, Reichwald K, Rump A, Schillhabel M, Schudy A. 2000. The DNA sequence of human chromosome 21. The chromosome 21 mapping and sequencing consortium. Nature 405:311–319.

Hiratsuka M, Sakamoto O, Li X, Suzuki Y, Aoki Y, Narisawa K. 1998. Identification of holocarboxylase synthetase (HCS) proteins in human placenta. Biochim Biophys Acta 1385: 165–171.

Holme E, Jacobson CE, Kristiansson B. 1988. Biotin-responsive multiple carboxylase deficiency in an 8-year-old boy with normal serum biotinidase and fibroblast holocarboxylase-synthetase activities. J Inherit Metab Dis 11:270–276.

Hwu W, Suzuki Y, Yang X, Li X, Chou S, Narisawa K, Tsai W. 2000. Late-onset holocarboxylase synthetase deficiency with homologous R508W mutation. J Formos Med Assoc 99:174–177.

Leon-Del-Rio A, Leclerc D, Akerman B, Wakamatsu N, Gravel RA. 1995. Isolation of a cDNA encoding human holocarboxylase synthetase by functional complementation of a biotin auxotroph of Escherichia coli. Proc Natl Acad Sci USA 92: 4626–4630.

Mardach R, Zempleni J, Wolf B, Cannon MJ, Jennings ML, Cress S, Boylan J, Roth S, Cederbaum S, Mock DM. 2002. Biotin dependency due to a defect in biotin transport. J Clin Invest 109:1617–1623.

Morrone A, Malvagia S, Donati MA, Funghini S, Ciani F, Pela I, Boneh A, Peters H, Pasquini E, Zammarchi E. 2002. Clinical findings and biochemical and molecular analysis of four patients with holocarboxylase synthetase deficiency. Am J Med Genet 111:10–18.

Narang MA, Dumas R, Ayer LM, Gravel RA. 2004. Reduced histone biotinylation in multiple carboxylase deficiency patients: a nuclear role for holocarboxylase synthetase. Hum Mol Genet 13:15–23.

Narisawa K, Arai N, Igarashi Y, Satoh T, Tada K. 1982. Clinical and biochemical findings on a child with multiple biotin-responsive carboxylase deficiencies. J Inher Metab Dis 5:67–68.

Ohira M, Seki N, Nagase T, Suzuki E, Nomura N, Ohara O, Hattori M, Sakaki Y, Eki T, Murakami Y, and others. 1997. Gene identification in 1.6-Mb region of the Down syndrome region on chromosome 21. Genome Res 7:47–58.

Pacheco-Alvarez D, Solorzano-Vargas RS, Del Rio AL. 2002. Biotin in metabolism and its relationship to human disease. Arch Med Res 33:439–447.

Peters DM, Griffin JB, Stanley JS, Beck MM, Zempleni J. 2002. Exposure to UV light causes increased biotinylation of histones in Jurkat cells. Am J Physiol Cell Physiol 283:C878–C884.

Sakamoto O, Suzuki Y, Aoki Y, Li X, Hiratsuka M, Yanagihara K, Inui K, Okabe T, Yamaguchi S, Kudoh J, Shimizu N, Narisawa K. 1998. Molecular analysis of new Japanese patients with holocarboxylase synthetase deficiency. J Inherit Metab Dis 21:873–874.

Sakamoto O, Suzuki Y, Li X, Aoki Y, Hiratsuka M, Suormala T, Baumgartner ER, Gibson KM, Narisawa K. 1999. Relationship between kinetic properties of mutant enzyme and biochemical and clinical responsiveness to biotin in holocarboxylase synthetase deficiency. Pediatr Res 46:671–676.

Sakamoto O, Suzuki Y, Li X, Aoki Y, Hiratsuka M, Holme E, Kudoh J, Shimizu N, Narisawa K. 2000. Diagnosis and molecular analysis of an atypical case of holocarboxylase synthetase deficiency. Eur J Pediatr 159:18–22.

Santer R, Muhle H, Suormala T, Baumgartner ER, Duran M, Yang X, Aoki Y, Suzuki Y, Stephani U. 2003. Partial response to biotin therapy in a patient with holocarboxylase synthetase deficiency: clinical, biochemical, and molecular genetic aspects. Mol Genet Metab 79:160–166.

Solorzano-Vargas RS, Pacheco-Alvarez D, Leon-Del-Rio A. 2002. Holocarboxylase synthetase is an obligate participant in biotin-mediated regulation of its own expression and of biotin-dependent carboxylases mRNA levels in human cells. Proc Natl Acad Sci USA 99:5325–5330.

Stanley JS, Griffin JB, Zempleni J. 2001. Biotinylation of histones in human cells. Effects of cell proliferation. Eur J Biochem 268:5424–5429.

Suormala T, Fowler B, Duran M, Burtscher A, Fuchshuber A, Tratzmuller R, Lenze MJ, Raab K, Baur B, Wick H, Baumgartner ER. 1997. Five patients with a biotin-responsive defect in holocarboxylase formation: evaluation of responsiveness to biotin therapy in vivo and comparative biochemical studies in vitro. Pediatr Res 41:666–673.

Suormala T, Fowler B, Jakobs C, Duran M, Lehnert W, Raab K, Wick H, Baumgartner ER. 1998. Late-onset holocarboxylase synthetase-deficiency: pre- and post-natal diagnosis and evaluation of effectiveness of antenatal biotin therapy. Eur J Pediatr 157:570–575.

Suzuki Y, Aoki Y, Ishida Y, Chiba Y, Iwamatsu A, Kishino T, Niikawa N, Matsubara Y, Narisawa K. 1994. Isolation and characterization of mutations in the human holocarboxylase synthetase cDNA. Nat Genet 8:122–128.

Suzuki Y, Aoki Y, Sakamoto O, Li X, Miyabayashi S, Kazuta Y, Kondo H, Narisawa K. 1996. Enzymatic diagnosis of holocarboxylase synthetase deficiency using apo-carboxyl carrier protein as a substrate. Clin Chim Acta 251:41–52.

Tang NL, Hui J, Yong CK, Wong LT, Applegarth DA, Vallance HD, Law LK, Fung SL, Mak TW, Sung YM, Cheung KL, Fok TF. 2003. A genomic approach to mutation analysis of holocarboxylase synthetase gene in three Chinese patients with late-onset holocarboxylase synthetase deficiency. Clin Biochem 36: 145–149.

Touma E, Suormala T, Baumgartner ER, Gerbaka B, Ogier de Baulny H, Loiselet J. 1999. Holocarboxylase synthetase deficiency: report of a case with onset in late infancy. J Inherit Metab Dis 22:115–122.

Wolf B, Hsia YE, Sweetman L, Nyhan WL. 1981. Multiple carboxylase deficiency: clinical and biochemical improvement following neonatal biotin treatment. Pediatrics 68:113–118.

Wolf B. 2001. Disorders of biotin metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The metabolic and molecular basis of inherited disease. New York: McGraw-Hill. p 3935–3962.

Yang X, Aoki Y, Li X, Sakamoto O, Hiratsuka M, Gibson KM, Kure S, Narisawa K, Matsubara Y, Suzuki Y. 2000. Haplotype analysis suggests that the two predominant mutations in Japanese patients with holocarboxylase synthetase deficiency are founder mutations. J Hum Genet 45:358–362.

Yang X, Aoki Y, Li X, Sakamoto O, Hiratsuka M, Kure S, Taheri S, Christensen E, Inui K, Kubota M, Ohira M, Ohki M, Kudoh J, Kawasaki K, Shibuya K, Shintani A, Asakawa S, Minoshima S, Shimizu N, Narisawa K, Matsubra Y, Suzuki Y. 2001. Structure of human holocarboxylase synthetase gene and mutation spectrum of holocarboxylase synthetase deficiency. Hum Genet 109:526–534.

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Review

Genetic testing for pharmacogenetics and its clinical application in drug therapy

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Abstract

There is wide individual variation in drug responses and adverse effects. As the main causes of the variation in drug responses, attention has focused on the genetic polymorphisms that encode metabolic enzymes regulating pharmacodynamics and receptors modulating the affinity with the responsive sites. Tailor-made drug therapy analyzes genetic polymorphisms involved in drug responses before drug administration and selects drugs and doses suitable for the individual genetic background. Establishment of tailor-made drug therapy is expected to contribute to medical economy by avoiding wasteful drug administration. To promote such medical practice, it is necessary to use simple genetic testing that is clinically convenient. Currently, genetic testing using real-time PCR has been frequently employed at laboratories with its clinical application anticipated. As to the many genes involved in drug responses, to date, the application of patient genetic information to tailor-made drug therapy has been achieved at the practical level. Information on pharmacogenetics will be a critical factor in medical practice in the near future.
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Keywords: Genetic testing; Genetic polymorphism; Pharmacogenetics; Real-time PCR; Single nucleotide polymorphism; Tailor-made drug therapy

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1. Introduction

There is wide individual variation in the pharmacodynamics of the administered drugs, effectiveness, and the appearance rates of adverse effects, and they are regulated by functions of the liver, kidneys, and heart, age, gender, circadian rhythm, diet, concomitantly administered drugs, and healthy foods. Recently, genetic polymorphism has drawn attention as one of such factors [1]. In particular, a single nucleotide polymorphism (SNP) of DNA is thought to produce a variation in drug responses and has become a representative research target in pharmacogenetics. In some cases, a difference in only one nucleotide results in a several- to ten-fold increase in maximum drug concentrations in the blood and frequent incidences of unexpected critical adverse effects. Today, it is anticipated that such genome information will be used to provide safer and more efficient tailor-made medicine for patients. If patient genome information is investigated before drug administration and appropriate drugs, doses, and administration timing can be predicted, a minimum drug dose will yield the maximum effect with the minimum side effects. If such medical practice is available, low efficiency in selecting drugs on a trial and error basis and drug administration as symptomatic therapy in cases of the emergence of side effects are expected to greatly change, which will contribute to medical economy. To date, polymorphisms of genes encoding drug metabolism enzymes, drug transporters, and drug receptors, which are involved in drug responses, have been reported and in some of them the association between pharmacodynamics and drug efficacy has been clarified [1,2].

In the future the expansion of tailor-made medicine using genome information, the development of simple and rapid genetic testing will be critical. Especially in the case of outpatients, unless results are returned within some tens of minutes to a few hours after the order of genetic testing, genome information cannot be used for prescription on the same day. Recently, new systems have been developed to detect genetic polymorphisms involved in drug responses in about 30 min to 2 hours by using real-time PCR.

In this review, we summarize the recent information on genetic polymorphisms involved in drug responses and demonstrate recent genetic testing including real-time PCR, the clinical application of tailor-made drug therapy, and the impact on medical economy.

2. Target genes of pharmacogenetics

2.1. P450 enzymes

A number of enzymes are involved in drug metabolism, and cytochrome P450 (CYP, P450) is regarded as the most important enzyme involved in drug metabolism, because many drugs are the substrate for the enzyme. P450 forms a superfamily consisting of a variety of molecules, and almost 20 kinds of enzymes involved in drug metabolism have been confirmed in humans (http://drnelson.utmem.edu/CytochromeP450.html). Of them, CYP3A4, CYP2D6, CYP2C9, CYP2C19, CYP2B6, and CYP1A2 play the most critical role in drug metabolism, and account for more than 90% of drugs metabolized by P450 [1,3]. P450 enzymes involved in the metabolism of the drugs currently available in medical practice are shown in Table 1. These molecules have proven genetic polymorphisms (Table 2), and a number of reports have shown the association between drug responses and genetic polymorphisms, in particular, as to CYP2D6, CYP2C9, and CYP2C19. Updated information can be found on the Human CYPallele Nomenclature Website (http://www.imm.ki.se/cypalleles). Phenotypes of P450 are divided into the extensive metabolizer (EM) that shows regular metabolic capacity and the poor metabolizer (PM) that shows low metabolic capacity. The PM phenotype carrying gene alterations on both alleles is inherited in an autosomal recessive manner. In addition, the categorization includes the intermediate metabolizer (IM) that shows the metabolic capacity between the PM and the EM, and the ultra-rapid metabolizer (UM) that shows higher metabolic capacity than the EM.

2.1.1. CYP2D6

In cases with CYP2D6, 5–10% of Caucasians are the PMs that have little enzymatic activity [4]. The frequency of the PM has racial diversity and the frequency of the CYP2D6 PM is less than 1% in Mongolian-origin races including Japanese [5,6]. Metabolism is delayed in a variety of drugs in the PM, and even the same drug dose is more likely to cause side effects, because plasma concentrations of the drug last longer at higher than normal levels. For example, in cases with the CYP2D6 PM, tricyclic antidepressants cause arrhythmia and thirstiness at higher rates [7]. To date, almost 50 genetic polymorphisms for CYP2D6

Table 1
Substrates of drug-metabolizing enzymes

Enzymes		Substrates
CYP1A2	Antidepressants	amitriptyline, clomipramine, fluvoxamine, imipramine
	Miscellaneous	clozapine, cyclobenzaprine, estradiol, haloperidol, mexiletine, naproxen, olanzapine, ondansetron, propranololriluzole, ropivacaine, tacrine, theophylline, verapamil, *R*-warfarin, zileuton, zolmitriptan
CYP2B6	Chemotherapeutic agents	cyclophosphamide, ifosfamide
	Miscellaneous	alfentanil, bupropion, efavirenz, ketamine, methadone, nevirapine, propofol, tamoxifen,
CYP2C9	Anticoagulants	*S*-warfarin
	Sulfonylureas	glibenclamide, glimepiride, glipizide, tolbutamide.
	NSAIDs	celecoxib, diclofenac, ibuprofen, mefenamic acid, meloxicam, naproxen, piroxicam, suprofen
	Antidepressants	amitriptyline, fluoxetine, imipramine
	Antihypertensives	irbesartan, losartan
	Miscellaneous	fluvastatin, nateglinide, phenytoin, rosiglitazone, tamoxifen, torsemide
CYP2C19	Benzodiazepines	diazepam
	Antidepressants	amitriptyline, clomipramine, imipramine
	Proton pump inhibitors	lansoprazole, omeprazole, pantoprazole, rabeprazole
	Miscellaneous	carisoprodol, citalopram, cyclophosphamide, hexobarbital, indomethacin, *S*-mephenytoin, mephobarbital, nelfinavir, nilutamide, phenobarbitone, phenytoin, primidone, progesterone, proguanil, propranolol, teniposide
CYP2D6	Antiarrhythmics	amiodarone, aprindine, encainide, fmexiletine, idocaine, lecainide, lsparteine, propafenone, *N*-propylajmaline
	Opiates	codeine, dextromethorphan, dihydrocodeine, ethlmorphine, hydrocodone, norcodeine, oxycodone, tramadol
	Antihypertensives	alprenolol, carvedilol, bufuralol, bunitrolol, bupranolol, debrisoquine, guanoxan, indoramin, *S*-metoprolol, propranolol, timolol
	Antidepressants	amiflamine, amitriptyline, brofaromine, citalopram, clomipramine, desipramine, desmethylcitalopram, fluoxetine, fluvoxamine, imipramine, maprotiline, minaprine, moclobemide, nortriptyline, paroxetine, tomoxetine, trimipramine, venlafaxine
	Antipsychotics	chlorpromazine, clozapine, haloperidol, perphenazine, risperidone, sertindole, thioridazine, zuclopentixol
	Miscellaneous	cinnarizine, dolansetron, methoxyamphetamine, methoxyphenamine, metoclopramide, nicergoline, ondansetron, phenformin, promethazine, tamoxifen, tolterodine, tropisetron
CYP3A4	Immune modulators	cyclosporine, tacrolimus
	Benzodiazepines	alprazolam, diazepam, midazolam, triazolam
	Ca channel blockers	amlodipine, diltiazem, felodipine, lercanidipine, nefedipine, nisoldipine, nitrendipine, verapamil
	Chemotherapeutic agents	busulfan, docetaxel, etoposide, irinotecan, paclitaxel, tamoxifen, vinblastine, vincristine
	HMG-CoA reductase inhibitors	atorvastatin, cerivastatin, lovastatin, simvastatin
	Estrogens, corticosteroids	estradiol, hydrocortisone, progesterone, testosterone
	Macrolide antibiotics	clarithromycin, erythromycin
	Protease inhibitors	indinavir, nelfinavir, ritonavir, saquinavir
	Antihistamines	astemizole, chlorpheniramin, terfenadine
	Miscellaneous	buspirone, cisapride, cilostazol, cocaine, dapsone, dextromethorphan, domperidone, fentanyl, finasteride, imatinib, lidocaine, methadone, nateglinide, pimozide, ondansetron, quinine, salmeterol, sidenafil, sirolimus, trazodone, zaleplon, zolpidem
TPMT	Chemotherapeutic agents	mercaptopurine, thioguanine
	Immune modulators	azathiprine
DPD	Chemotherapeutic agents	carmofur, doxifluridine, 5-fluorourasil, tegafur
NAT2		isoniazid, hydralazine, procainamide, sulfamethoxazole
UGT1A1		bilirubin, irinotecan
COMT		levodopa

have been reported (http://www.imm.ki.se/cypalleles). Of them, CYP2D6*3, CYP2D6*4, and CYP2D6*5 are the major genetic polymorphisms involved in the PM of Caucasians [8]. More than 90% of the PMs in Caucasians are ascribable to these three genetic polymorphisms. Moreover, the existence of CYP2D6*10, a genetic polymorphism of the IM that shows lower CYP2D6 metabolic capacity but not as low as the PM, has been identified [9]. This genetic polymorphism is markedly prevalent in Japanese and Chinese.

2.1.2. CYP2C9

CYP2C9 is involved in the metabolism of an antiepileptic agent phenytoin and an anticoagulant warfarin. To date, 12 CYP2C9 variant alleles have been reported (http://www.imm.ki.se/cypalleles). Of these, a decrease in activity was confirmed in cases with CYP2C9*3 by the expression system using COS cells and yeast and in vivo tests on the normal volunteers and patients whose genetic polymorphisms were known [10,11]. For example, in cases with phenytoin, oral clearance decreased to one quarter in the

Table 2
Pharmacogenetics of drug-metabolizing enzymes (P450)

Gene	Genotypes	Major allelic variants	Phenotypes: Frequency	
			Caucasian	Asian (Japanese)
CYP1A1	*1-*11			
CYP1A2	*1-*14	*1F, *2, *3, *4, *5, *6		
CYP1B1	*1-*26			
CYP2A6	*1-*17	*2, *4, *7, *9, *10		
CYP2A13	*1-*9			
CYP2B6	*1-*15			
CYP2C8	*1-*5			
CYP2C9	*1-*13	*3	EM: 97% PM: 3%	
CYP2C19	*1-*16	*2, *3	EM: 94-98% PM: 2-6%	EM: 80% PM: 20%
CYP2D6	*1-*51	*2×N, *3, *4, *5, *6, *10, *17	EM: 90-97% PM: 3-10%	EM: 99% PM: 1%
CYP2E1	*1-*7	*2, *3, *4		
CYP2J2	*1-*7			
CYP2R1	*1-*2			
CYP2S1	*1-*3			
CYP3A4	*1-*19	*4, *5, *6		
CYP3A5	*1-*10	*3, *6		
CYP3A7	*1			
CYP3A43	*1-*3			
CYP4B1	*1-*7			

A description of the alleles can be found on the human cytochrome P450 allele nomenclature committee home page (http://www.imm.ki.se/CYPalleles/).
EM = extensive metabolizer; IM = intermediate metabolizer; PM = poor metabolizer.

subjects with homozygous polymorphism for CYP2C9*3 [12]. Oral clearance for (S)-warfarin decreased to less than half in the subjects with heterozygous polymorphism for CYP2C9*3 (CYP2C9*1/*3) and to less than 10% in the patients with homozygous polymorphism for CYP2C9*3 [13].

2.1.3. CYP2C19

CYP2C19 is an enzyme involved in the hydroxylation of the S form of an anti-epileptic agent mephenytoin, and also in the metabolism of a series of proton pump inhibitors such as omeprazole. The PM for CYP2C19 is found in about 20% of Japanese and about 3% of Caucasians [14]. To date, 15 variant alleles responsible for the PM for CYP2C19 have been identified (http://www.imm.ki.se/cypalleles), and almost all PMs in Japanese are ascribable to the two genetic polymorphisms CYP2C19*2 and CYP2C19*3. Omeprazole concentrations in the blood after oral intake of omeprazole were significantly different in each genetic polymorphism for CYP2C19, and the concentration in the blood 10 h after the intake in the PM was comparable to the peak concentration in the EM [15]. When the area under the plasma concentration-time curve was compared, the PM was reported to be different from the EM by about 13 times [16].

2.2. Non-P450 enzymes

2.2.1. Dihydropyrimidine dehydrogenase (DPYD)

Dihydropyrimidine dehydrogenase (DPD) is a rate-limiting enzyme for the metabolism of an anticancer drug 5-FU, and more than 85% of administered 5-FU is metabolized by DPD. The gene encoding DPD is called DPYD, and 13 genetic polymorphisms have been reported [17,18]. In particular, the genetic polymorphism that decreases DPD activity has been reported to be DPYD*2 with a polymorphism at the splicing recognition site [19]. Administration of 5-FU to the patients with decreased DPD activity increases the adverse events such as leukocytopenia, stomatitis, diarrhea, nausea and vomiting, and cerebellum disorder [20].

2.2.2. Thiopurine S-methyltransferase (TPMT)

Thiopurine *S*-methyltransferase (TPMT) is involved in the detoxification and metabolism of an anti-leukemia drug 6-mercaptoprine (6-MP) and an immunosuppressant azathioprine. In Caucasian infant patients with acute myeloid leukemia, those who carried TPMT*2, TPMT*3A, TPMT*3B, or TPMT*3C showed significantly higher concentrations of the 6-MP metabolite in the red blood cells. In addition, a dose reduction or termination of the administration was reported to be necessary in all patients due to the adverse effects such as myelosuppression [21].

2.2.3. N-acetyltransferase 2 (NAT2)

An individual variation in *N*-acetylation activity of an anti-tuberculosis drug isoniazid has been reported to be ascribable to the genetic polymorphism for *N*-acetyltransferase 2 (NAT2). NAT2 is categorized into the rapid acetylator (RA), the intermediate acetylator (IA), and the slow acetylator (SA) according to the acetylation activity. The frequency of the SA is about 50% in Caucasians and about 10% in Japanese [22,23]. To date, more than 10 variant alleles have been identified (http://www.louisville.edu/medschool/pharmacology/NAT.html), and three genetic polymorphisms NAT2*5, NAT2*6, and NAT2*7, but not NAT2*4 (wild type alleles), are responsible for almost all SAs in Japanese [23]. Drug-induced hepatitis by isoniazid occurs more than twice in the SA for NAT2 than in the RA [24,25].

2.2.4. Other non-P450 enzymes

Other than the above-mentioned enzymes for drug metabolism, genetic polymorphisms for UDP-glucuronic acid transferase (UGT) 1A1 [26], glutathione-*S*-transferase (GST) [27], and thymidylic acid synthase (TS) [28] are thought to be responsible for the individual variation in drug responses. The relation between these genes and drug responses is shown in Table 3.

Table 3
Pharmacogenetics of drug-metabolizing enzymes (non-P450)

Drug-metabolizing enzymes	Gene	Genotypes	Major allelic variants	Phenotypes: Frequency	
				Caucasian	Asian (Japanese)
Thiopurine S-methyltransferase	TPMT	*1–*15	*2, *3	EM: 73–89% IM: 11–27% PM: 0–4%	EM: 97% IM: 3%
Dihydropyrimidine dehydrogenase	DPYD	*1–*12	*2	EM: 97% PM: 3%	
N-acetyltransferase 2	NAT2	*4–*19	*5, *6, *7, *14	EM: 25% IM: 25% PM: 50%	EM: 45% IM: 45% PM: 10%
UDP-glucuronosyltransferase 1A1	UGT1A1	*1–*64	*6, *7, *27, *28, *29	EM: 90% PM: 10%	EM: 99% PM: 1%
Catechol O-methyltransferase	COMT		Val158Met	EM: 75% PM: 25%	
Glutathione S-transferase M1	GSTM1		null		
Glutathione S-transferase M3	GSTM3		*A, *B		
Glutathione S-transferase-P1	GSTP1		Ile105Val		
Glutathione S-transferase-T1	GSTT1		null		

EM = extensive metabolizer; IM = intermediate metabolizer; PM = poor metabolizer.

2.3. Non-enzymes

Recently, genetic polymorphisms, other than those for enzymes for drug metabolism, have drawn attention as important factors for drug responses. They include those for drug transporters, receptors, transport protein, and mitochondria DNA (mtDNA) (Table 4). Especially, the focus has been on genetic polymorphisms for receptors that can be the molecular target for drugs. An anti-lung cancer drug gefitinib binds the site for epidermal growth factor receptor-tyrosine kinase (EGFR-TK) and blocks the signal transduction for cancer proliferation. An individual variation in the effect of gefitinib was reported [29–31], and good response was observed in the patients with a polymorphism in the EGFR-TK domain. On the other hand, gefitinib was not effective in the patients without polymorphisms in the EGFR-TK domain.

3. Genotyping methods

Recent progress in genetic testing technologies is remarkable, and they are applied for the analysis of genetic mutations and polymorphisms that regulate disease vulnerability and drug responses. Classically, the PCR-RFLP analysis and the allele-specific amplification method had been used for the detection of SNP [32]. However, these methods require rather troublesome maneuvers such as electrophoresis after PCR and staining with ethidium bromide. A few years ago a new method was developed to cover the shortcomings of these genetic tests. The method is called real-time PCR because it uses a fluorescent probe or dye in the PCR reaction to detect DNA amounts and characteristic base compositions over time [33,34]. These methods allow the detection of SNP within about 40 min to 2 h of DNA extraction. For real-time PCR, the TaqMan probe and Hybridization probe methods are used in a number of institutes to detect various SNPs (Table 5).

3.1. Real-time PCR

3.1.1. TaqMan probe

The TaqMan probe method employs 5′-exonuclease activity of Taq DNA polymerase for PCR. The TaqMan probe consists of an oligo-nucleotide of about 20–30-mer and labeled with a reporter fluorescent dye at the 5′-terminus and a quencher fluorescent dye at the 3′-terminus. The two fluorescent dyes on the TaqMan probe are in a state of decreased fluorescent intensity due to fluorescence resonance energy transfer (FRET) phenomenon when they are close in physical distance. However, the 5′-exonuclease of Taq DNA polymerase cleaves the binding of the fluorescent dye along the extension reaction. The fluorescent dye is released from the influence of the quencher and fluoresces. In general, the TaqMan probe is designed to be at the center of SNP.

Recently, we developed a new real-time PCR method using allele-specific amplification [38]. We designed PCR primers at the site of SNP so that not only the TaqMan probe, but also less expensive SYBR Green I is available [38,54]. ABI PRISM7000, 7300, 7500, PRISM7700, 7900Fast (Applied Biosystems), LightCycler (Roche), Mx4000, Mx3000P (STRATAGENE), and Smart Cycler II (TaKaRa) can be used for detection.

3.1.2. Hybridization probe

The Hybridization Probe method allows real-time detection of PCR products and SNP using two fluorescent probes. Firstly, PCR primers are designed to amplify the target sequence including SNP. Secondly, a probe labeled with fluorescein at the 3′-terminus is designed so that it