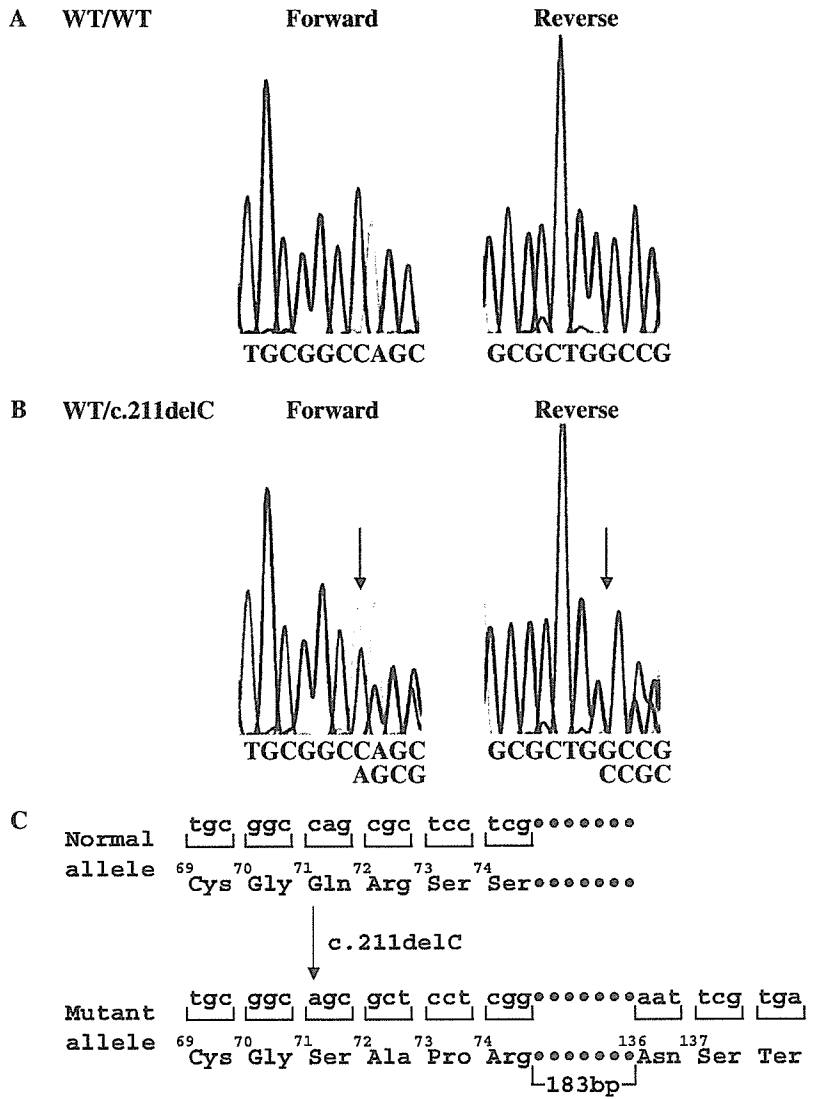


**Fig. 2** Sequence analysis of the *KCNQ4* in a an unaffected (WT/WT) and **b** an affected member (WT/c.211delC) of the pedigree. The *arrow* indicates the c.211delC mutation, shown in **c**. The *arrow* shows the deletion of C at nucleotide position 211 of the *KCNQ4* cDNA sequence. The deletion results in a frameshift after Gly70 (FS71), followed by 67 novel amino acids and a premature stop codon at amino acid position 138 (p.Q71fsX138)



**Table 2** Clinical features of family members with c.211delC

Family members	Age	Onset (years)	Sex	Hearing threshold (dB)										Age at the test	
				500 Hz		1,000 Hz		2,000 Hz		4,000 Hz		8,000 Hz			
				Right	Left	Right	Left	Right	Left	Right	Left	Right	Left		
II-5	78	22	Male	50	40	75	65	85	>95	>95	>95	>95	>95	>95	71
II-7	71	8	Male	85	85	>95	>95	>95	>95	>95	>95	>95	>95	>95	64
II-9	69	50	Female	30	40	40	35	>95	>95	>95	>95	>95	>95	>95	62
II-11	64	N/A	Female	25	20	30	35	85	75	>90	>90	>90	>90	>90	58
III-3	48	20	Male	10	20	30	25	75	80	>95	>95	>95	>95	>95	41
III-5	52	30	Male	30	20	30	40	80	80	>95	90	>95	>95	>95	45
III-8	51	N/A	Male	20	10	10	10	80	>90	>90	>90	>90	>90	>90	45
III-12	34	N/A	Female	10	15	15	10	15	20	60	60	80	70	34	34
III-13	46	N/A	Female	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
IV-2	23	17	Female	15	15	5	10	10	15	45	45	65	65	18	
IV-3	21	14	Male	10	5	10	10	40	45	70	60	85	70	20	
IV-4	18	15	Male	15	15	10	10	40	30	50	45	65	65	18	
IV-5	20	15	Male	10	10	10	10	10	10	10	10	30	20	15	

N/A No information was available

ment in this family was progressive, starting with high frequencies and including middle and low frequencies later in life. According to the anamnestic data, the age at onset varied from 8–50 years.

## Discussion

We have identified a novel *KCNQ4* mutation in a large Japanese pedigree with hearing loss by a genome-wide linkage analysis followed by the mutational analysis of candidate genes. The identified mutation was a one-base deletion in exon 1 of the *KCNQ4* gene, c.211delC, which caused a profoundly truncated peptide without the transmembrane portion of the potassium channel (p.Q71fsX138). Individuals with the apparently null mutation c.211delC had late-onset and pure high-frequency hearing loss, which did not resemble the phenotypes of the patients with the *KCNQ4* missense mutations. The individuals with c.211delC had phenotypes similar to those of patients with the c.211\_223del (Topsakal et al. 2005). Dominant-negative mechanism has been suggested to underlie the pathogenesis of hearing loss caused by the missense *KCNQ4* mutations. Concordance of the mild phenotypes in two different null mutations, c.211\_223del and c.211delC, suggests the genotype–phenotype correlation in which *KCNQ4* deletions are associated with later-onset and milder hearing loss than the missense mutations.

The phenotypic difference may be caused by the difference in the pathogenic mechanism. Two null mutations (c.211\_223del and c.211delC) are expected to yield a *KCNQ4* protein that is truncated before the first transmembrane region. Although deleterious mutations result in haploinsufficiency, the function of normal *KCNQ4* protein produced from the intact allele may be spared. Fifty-percent decrease in normal *KCNQ4* channels may lead to mild hearing impairment. Haploinsufficiency has been suggested to underlie the pathogenesis of both long QT syndrome and benign familial neonatal convulsions caused by *KCNQ1* and *KCNQ2* or *KCNQ3* mutations, respectively (Gouas et al. 2004; Rogawski 2000). In contrast, the dominant-negative effect due to missense mutations significantly interferes with the normal channel subunit. Six missense mutations have been identified in the *KCNQ4* gene to date. Five mutations are located in the *KCNQ4* P-loop domain, and one mutation is located in the sixth transmembrane domain. Because these missense mutations are located in the critical domain of the protein, they may cause the dominant-negative effect.

The LOD score estimated at the early stage of this study was 3.0, which was far lower than the final estimation. The NPL score at the early stage was estimated to be 8.0, suggesting the possibility of incomplete penetrance. The identified *KCNQ4* mutation, c.211delC, was also present in a family member III-12, who had been classified as an unaffected individual. She did not complain of any difficulty in hearing. After the identifi-

cation of the causative mutation we performed the audiological examination on her. It turned out that she had mild hearing impairment only in high-frequency, in concordance with her genotype. Re-analysis using this information showed an LOD score of 5.42. Thus, the deafness caused by the c.211delC appeared to be transmitted at a level of complete penetrance. The current study illustrates the potential difficulty of linkage analysis in slowly progressive hearing loss in a limited frequency range. Thorough otological examinations may be required even in individuals who are seemingly not affected upon interview.

Although more than 30 deafness genes have been so far identified, the genotype–phenotype relation in each allelic mutation remains largely unidentified. Elucidation of the genotype–phenotype correlations would facilitate informative genetic counseling.

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## RESEARCH ARTICLE

Comprehensive Mutation Analysis of *GLDC*, *AMT*, and *GCSH* in Nonketotic Hyperglycinemia

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Nonketotic hyperglycinemia (NKH) is an inborn error of metabolism characterized by accumulation of glycine in body fluids and various neurological symptoms. NKH is caused by deficiency of the glycine cleavage multi-enzyme system with three specific components encoded by *GLDC*, *AMT*, and *GCSH*. We undertook the first comprehensive screening for *GLDC*, *AMT*, and *GCSH* mutations in 69 families (56, six, and seven families with neonatal, infantile, and late-onset type NKH, respectively). *GLDC* or *AMT* mutations were identified in 75% of neonatal and 83% of infantile families, but not in late-onset type NKH. No *GCSH* mutation was identified in this study. *GLDC* mutations were identified in 36 families, and *AMT* mutations were detected in 11 families. In 16 of the 36 families with *GLDC* mutations, mutations were identified in only one allele despite sequencing of the entire coding regions. The *GLDC* gene consists of 25 exons. Seven of the 32 *GLDC* missense mutations were clustered in exon 19, which encodes the cofactor-binding site Lys754. A large deletion involving exon 1 of the *GLDC* gene was found in Caucasian, Oriental, and black families. Multiple origins of the exon 1 deletion were suggested by haplotype analysis with four *GLDC* polymorphisms. This study provides a comprehensive picture of the genetic background of NKH as it is known to date. *Hum Mutat* 27(4), 343–352, 2006. © 2006 Wiley-Liss, Inc.

KEY WORDS: *GLDC*; *AMT*; *GCSH*; glycine encephalopathy; nonketotic hyperglycinemia; NKH; glycine cleavage system; mutation spectrum; genotype–phenotype

## INTRODUCTION

Nonketotic hyperglycinemia (NKH, MIM# 605899), also termed glycine encephalopathy, is an inborn error of amino acid metabolism characterized by the accumulation of a large amount of glycine in body fluids [Hamosh and Johnston, 2001]. Glycine levels are elevated to a much greater extent in cerebrospinal fluid (CSF) than in plasma; hence, an abnormally high value for the CSF/plasma glycine ratio is observed. NKH is clinically classified (by onset of symptoms) as three types: neonatal, infantile, or late-onset. Later onset appears to be associated with a better prognosis. The vast majority of patients fall into the neonatal category, which involves a stereotypic presentation with severe hypotonia, apnea requiring assisted ventilation, and intractable seizures. Approximately 30% of such patients die in the neonatal period. Survivors often have severe psychomotor retardation, although 15–20% of survivors achieve developmental milestones such as head control, independent sitting, or walking [Hoover-Fong et al., 2004]. Patients with the infantile type of NKH are often asymptomatic in the neonatal period and the phenotype is characterized by mild to moderate psychomotor retardation, behavioral problems, seizures, and chorea. The clinical presentations of late-onset

NKH are heterogeneous. In previous studies, two families did not have seizures or mental retardation, but exhibited progressive paraplegia and optic atrophy [Bank and Morrow, 1972; Steiman et al., 1979]. Another family was reported to present with mental retardation and choreoathetosis [Singer et al., 1989].

The fundamental defect lies in the glycine cleavage system (GCS; EC2.1.2.10) [Tada et al., 1969]. The GCS is a mitochondrial complex enzyme system that consists of four

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individual proteins [Kikuchi, 1973]: glycine decarboxylase (also called P-protein), aminomethyltransferase (T-protein), hydrogen carrier protein (H-protein), and dihydrolipoamide dehydrogenase (L-protein). The enzymatic analysis of NKH patients revealed that approximately 80% of patients with NKH have a *GLDC* deficiency and the rest have an *AMT* deficiency [Tada and Hayasaka, 1987]. Mitochondrial precursors of human P, T, H, and L-proteins consist of 1,020, 403, 173, and 509 amino acids, respectively. Dihydrolipoamide dehydrogenase is a housekeeping enzyme that serves as an E3 component of other  $\alpha$ -keto acid dehydrogenase complexes, such as pyruvate dehydrogenase. Deficiency of dihydrolipoamide dehydrogenase causes progressive neurological deterioration with lactic acidosis but not hyperglycinemia [Hong et al., 1996]. The three GCS-specific components (P, T, and H-proteins) are encoded by distinct genes: *GLDC* (MIM# 238300) on chromosome 9p24 [Isobe et al., 1994], *AMT* on 3p21.1-21.2 [Nanao et al., 1994], and *GCSH* (MIM# 238330) on 16p24 [Kure et al., 2001], respectively.

To date, a limited number of NKH mutations have been reported (Human Gene Mutation Database, Cardiff; <http://www.hgmd.cf.ac.uk>). The *GLDC* mutations reported to date include the S564I mutation that is prevalent in Finnish patients [Kure et al., 1992], the R515S mutation found in 5% of Caucasian patients [Toone et al., 2000], microdeletions [Kure et al., 1991], large deletions [Takayanagi et al., 2000; Sellner et al., 2005], one abnormal splicing [Flusser et al., 2005], one nonsense mutation [Sellner et al., 2005], and 10 missense mutations [Toone et al., 2002; Korman et al., 2004; Kure et al., 2004; Boneh et al., 2005; Dinopoulos et al., 2005]. The *AMT* gene (MIM# 238310) mutations identified to date include nine missense mutations [Nanao et al., 1994, 1994a; Kure et al., 1998; Toone et al., 2000, 2001, 2003], one microdeletion [Kure et al., 1998, 1998b], and one splicing mutation [Toone et al., 2000]. In *GCSH* we have found one abnormal splicing in a patient with a transient form of NKH [Kure et al., 2002]. Since multiple genes are responsible for NKH, previous studies screened only a small number of *GLDC* and *AMT* exons and/or a few patients, which hampered elucidation of the genetic background of NKH.

The purpose of the present study was to establish the mutation spectrum of NKH by performing a comprehensive screening for mutations in *GLDC*, *AMT*, and *GCSH* in 69 families with three different types of NKH. The structure of *AMT* has been determined [Nanao et al., 1994, 1994b]. Also, we previously reported the exon-intron organizations of *GLDC* [Takayanagi et al., 2000] and *GCSH* [Kure et al., 2001], which provided us with basic information to amplify the entire coding regions for the three genes. To increase the sensitivity of mutational screening, we directly sequenced all amplicons without employing prescreening scanning methods such as single-strand conformation polymorphism.

## MATERIALS AND METHODS

### Patients

Patients were examined in the metabolic disease clinics of a number of referring hospitals. NKH was clinically suspected based on the presentation of symptoms characteristic of each disease type and electroencephalograms (EEG) recordings, and were subsequently confirmed by amino acid analysis. The CSF/serum glycine ratio at diagnosis was  $>0.04$  in all patients, whereas it was  $<0.03$  in normal neonates. Patients were classified into three clinical subtypes (neonatal, infantile, and late-onset) based on the onset of clinical symptoms.

**Neonatal type.** We studied 56 families with neonatal onset. Initial symptoms, including hypotonia, apnea, and coma, devel-

oped within 7 days after birth—in most cases within 3 days. Almost all of the patients showed a burst suppression pattern on EEG within 2 weeks after birth, and hypsarrhythmia thereafter.

**Infantile type.** Six families were enrolled. Symptoms started at 3–12 months of age. Five of the six patients had no symptoms in the neonatal period. Very mild hypotonia and apnea were observed in patient P107 [Dinopoulos et al., 2005]. Mild mental retardation and abnormal behaviors, such as aggressiveness, developed in adolescence.

**Late-onset type.** We studied seven patients with late-onset NKH. All seven patients had elevated glycine concentrations in repeated amino acid analysis of CSF and/or plasma. Spastic paraplegia without mental retardation developed in three of the seven patients. These patients resembled those in previously reported families [Bank and Morrow, 1972; Steiman et al., 1979]. The rest of the patients presented with mental retardation after they entered school or during adolescence. There is some confusion in terms of phenotypic classification of the mild form of the infantile type and the late-onset type, since there are some reports of patients in whom developmental delay or mild hypotonia started in the middle or late infantile periods [Flannery et al., 1983; Singer et al., 1989]. We classified such patients as having the infantile type—not the late-onset type. In the present study we classified patients as having the late-onset type when they were free of any symptoms during infancy.

### Mutational Screening

Exons and flanking intron sequences of *GLDC* (GenBank NM\_000170, NT\_008413.16), *AMT* (GenBank NM\_00481, NT\_086638.1), and *GCSH* (GenBank NM\_004483, NC\_00016.8) were amplified by PCR from genomic DNA, followed by direct sequencing analysis using the dye-primer sequencing method as previously described [Kure et al., 2001]. The 18-mer oligonucleotides of the M13 and reverse sequencing primers were added to the 5' end of the forward and reverse PCR primers, respectively. We initially screened for a large deletion involving *GLDC* exon 1 by semiquantitative PCR using the pseudogene of *GLDC* (*GLDCP*) as a gene dose control [Takayanagi et al., 2000]. PCR primer sequences for amplifying *GLDC* exons 1–6 were previously reported [Takayanagi et al., 2000], and those for other exons are described in Supplementary Table S1 (available online at <http://www.interscience.wiley.com/jpages/1059-7794/suppmat>). Each PCR cycle consisted of denaturing at 98°C for 10 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, with repetition for 35 cycles. For PCR amplification of *GLDC* exon 1, 10% dimethylformamide was added to the reaction mixture. Fifty control subjects were subsequently screened for any detected base changes to exclude noncausative polymorphisms. If no mutations were detected in the sequencing analysis of *GLDC*, we screened for mutations in *AMT* by amplifying all of the nine exons. When no mutations were identified in either *GLDC* or *AMT*, we sequenced all of the five exons in *GCSH*. For characterization of *GLDC* and *AMT* missense mutations, each mutated amino acid residue was compared with the corresponding amino acid in rat [Sakata et al., 2001], chicken [Kume et al., 1991], pea [Turner et al., 1992], and *E. coli* [Okamura-Ikeda et al., 1993], as shown in Tables 2 and 3.

### Characterization of the *GLDC* Deletion

We identified the minimum deleted region of both alleles of each homozygous patient of the *GLDC* exon 1 deletion. Fifteen sequence tagged sites (STSs) were used for this deletion mapping,

as illustrated in Fig. 1. PCR primer sequences and amplification conditions for D9S281 and RH92434 were obtained from the website of the UCSC Genome Browser (<http://genome.ucsc.edu>). The PCR primer sequences and amplicon sizes of STSs 1–8 were presented in Supplementary Table S1. STSs 1–7 were located 5' upstream of the *GLDC* gene, and STS 8 was located in intron 2. Amplification primers for *GLDC* exons 1–5 were also used in the deletion mapping. We amplified these 15 STSs by using genomic DNA of Patients P5 and P36 as a template in order to test whether each STS was involved in the homozygously deleted region. Structural information about the 5' upstream region of *GLDC* and the location of *Alu* motifs was obtained from the UCSC Genome Browser (Fig. 1).

**RESULTS**

**Mutation Screening**

We performed mutational screening in 69 NKH families (56 neonatal type, six infantile type, and seven late-onset type). First, the *GLDC* exon 1 deletion was screened by semiquantitative PCR amplification using the *GLDCP* as a control of the gene copy number. This deletion was found in six families (Table 1). Subsequent extensive sequencing of *GLDC*, *AMT*, and *GCSH* coding exons revealed that 42 of the 56 neonatal-type families (75%) had *GLDC* or *AMT* mutations in at least one of two alleles. *GLDC* mutations were found in 31 (74%) and *AMT* mutations were detected in 11 (26%) of the 42 families. No differences were

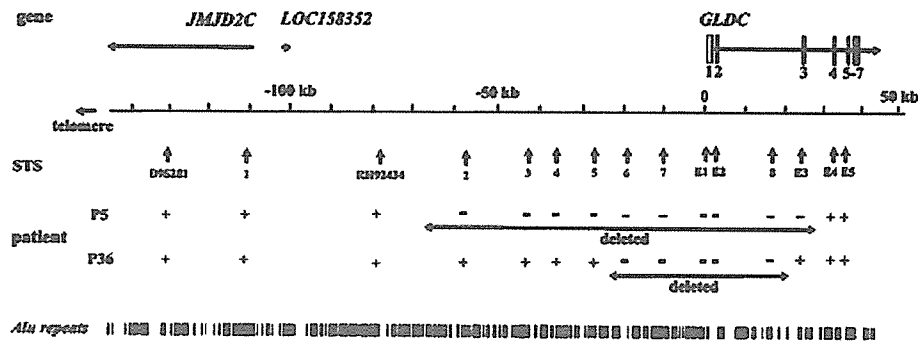


FIGURE 1. Mapping of the *GLDC* deletions. Genomic regions that were homozygously deleted in patients P5 and P36 were defined by amplification of 13 STS markers. The *JMJD2C* gene and the gene-like structure, *LOC158352*, are shown based on the information of the UCSC Genome Browser. E1–5 indicate amplicons including the *GLDC* exons 1–5, respectively. Amplicons (+) indicate that the STS was successfully amplified, while (-) means that the STS failed to be amplified.

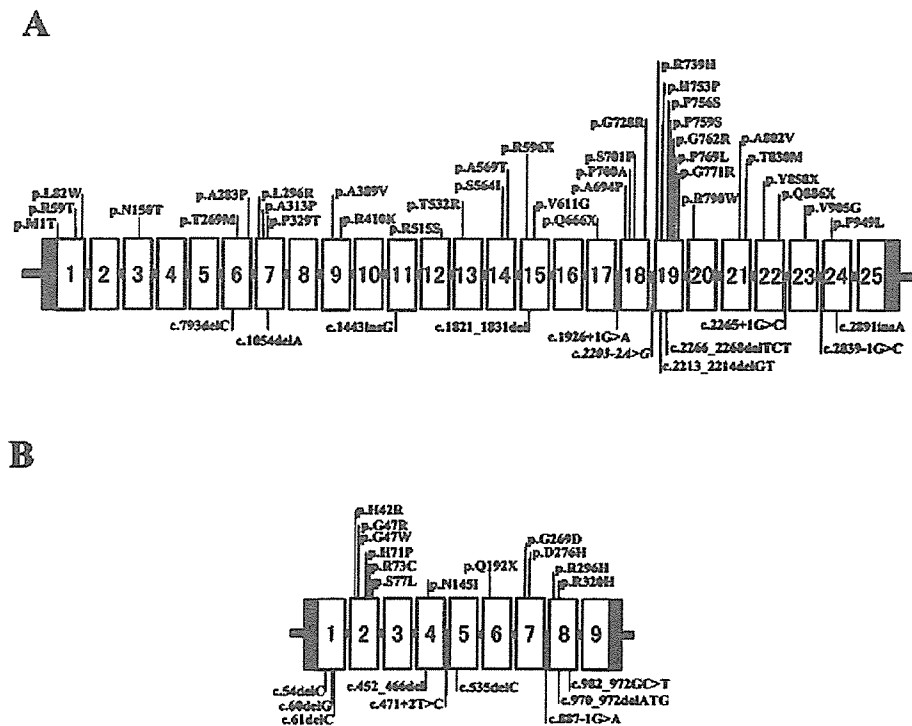


FIGURE 2. NKH mutations identified in this and previous studies. The *GLDC* (A) and *AMT* (B) exons are indicated by open boxes, and noncoding regions are shaded. Missense and nonsense mutations are shown above the exon boxes, and deletions/insertions and mutations of splicing errors are indicated below the exon boxes.

TABLE 1. Patients With GLDC or AMT Mutations

Family number	Ethnicity	Age of onset	Consanguinity	CSF Gly (mM)	CSF/serum Gly ratio	Gene	Mutation <sup>a</sup>		Reference
							Mutation 1	Mutation 2	
Neonatal type									
P14	Caucasian	Day 1	No	296	0.25	GLDC	Exon 1 deletion	c.2714T>G	This study
P21	Caucasian	Day 1	Yes	71	0.24	AMT	c.230C>T	c.230C>T	This study
P26	Caucasian	Day 1	Yes	86	0.27	AMT	c.125A>G	c.125A>G	Kure et al. [1998a]
P29	Caucasian	Day 1	No	43	0.04	GLDC	c.793delC	ND	This study
P31	Caucasian	Day 1	No	196	0.26	AMT	c.471+21>C	c.887G>A	This study
P32	Caucasian	Day 1	No	32	0.20	GLDC	Exon 1 deletion	c.1786C>T	This study
P36	Caucasian	Day 1	No	220	0.18	GLDC	Exon 1 deletion	Exon 1 deletion	This study
P39	Caucasian	Day 1	No	187	0.13	GLDC	c.1595C>G	c.1832T>G	This study
P48	Caucasian	Day 1	No	198	0.20	GLDC	c.2665+1G>C	ND	This study
P49	Caucasian	Day 1	No	213	0.17	GLDC	c.2203-2A>G	ND	This study
P5	Caucasian	Day 1	Yes	202	0.23	GLDC	c.1996C>T	c.1996C>T	This study
P6	Oriental	Day 1	No	240	0.18	GLDC	Exon 1 deletion	Exon 1 deletion	Ohya et al. [1991]
P19	Oriental	Day 1	No	160	0.09	GLDC	c.245T>G	c.1821_1831del	Kure et al. [2004]
P25	Oriental	Day 1	No	264	0.30	GLDC	c.449A>C	c.2368C>T	Kure et al. [2004]
P74	Oriental	Day 1	No	74	0.34	AMT	c.54delC	c.826G>C	Kure et al. [1998b]
P86	Oriental	Day 1	No	117	0.12	GLDC	c.2311G>A	ND	This study
P93	Oriental	Day 1	No	177	0.20	GLDC	c.2213_2214delGT	ND	This study
P115	Oriental	Day 1	No	130	0.13	GLDC	c.2105C>T	ND	This study
P124	Oriental	Day 1	No	145	0.11	AMT	c.147delG	c.970_972delATG	This study
P30	Caucasian	Day 2	No	300	0.11	GLDC	c.255+1G>A	c.806C>T	This study
P44	Caucasian	Day 2	No	121	0.19	AMT	c.60delG	c.471+21>C	This study
P46	Caucasian	Day 2	No	98	0.11	GLDC	c.806C>T	c.255+1G>A	This study
P47	Caucasian	Day 2	No	155	0.13	GLDC	c.2293C>T	c.1705G>A	This study
P61	Caucasian	Day 2	No	78	0.17	GLDC	c.2519T>A	ND	This study
P75	Caucasian	Day 2	No	186	0.16	AMT	c.982_972GC>T	c.452_466del	This study
P3	Oriental	Day 2	No	167	0.10	GLDC	c.212A>C	c.217C>T	This study
P4	Oriental	Day 2	No	387	0.28	GLDC	c.2306C>T	c.2846C>T	This study
P7	Oriental	Day 2	No	164	0.10	GLDC	c.2258A>C	c.2839-1G>C	This study
P8	Oriental	Day 2	No	209	0.31	GLDC	c.2080G>C	ND	Kure et al. [1991]
P10	Oriental	Day 2	No	517	0.20	GLDC	c.887T>G	ND	This study
P77	Oriental	Day 2	No	148	0.12	GLDC	c.449A>C	c.1926+1G>A	This study
P91	Oriental	Day 2	No	132	0.08	AMT	c.61delC	c.535delC	This study
P69	Oriental	Day 3	No	220	0.12	GLDC	c.2311G>A	ND	This study
P70	Oriental	Day 3	No	68	0.19	GLDC	Exon 1 deletion	ND	This study
P120	Oriental	Day 3	No	324	0.19	GLDC	c.2574T>G	ND	This study
P23	Oriental	Day 4	No	83	0.14	GLDC	c.2182G>C	ND	This study
P104	Caucasian	Day 4	No	240	0.26	GLDC	c.1166C>T	c.1443insG	This study
P15	Black	Day 5	No	333	0.08	GLDC	Exon 1 deletion	c.2891insA	This study
P34	Caucasian	Day 6	No	174	0.08	AMT	c.139G>T	ND	This study
P76	Caucasian	Day 6	No	215	0.18	AMT	c.136G>A	c.230C>T	This study
P72	Black	Day 7	No	117	0.20	GLDC	c.2665+1G>C	c.176G>C	This study
Infantile type									
P50	Caucasian	3 months	No	200	0.25	GLDC	Exon 1 deletion	c.2311G>A	This study
P78	Caucasian	6 months	No	46	0.06	GLDC	c.2216G>A	c.2216G>A	Dinopoulos et al. [2005]
P12	Caucasian	12 months	No	41	0.04	GLDC	c.2216G>A	ND	Christodoulou et al. [1993]
P107	Caucasian	Unclear <sup>b</sup>	No	150	0.09	GLDC	c.1166C>T	c.1166C>T	Dinopoulos et al. [2005]
P108	Caucasian	Unclear <sup>b</sup>	No	55	0.05	GLDC	c.1166C>T	c.1166C>T	Dinopoulos et al. [2005]

<sup>a</sup>DNA mutation numbering is based on cDNA sequence; +1 corresponds to the A of the ATG translation initiation codon. GLDC: NM\_000170; AMT: NM\_00481.

<sup>b</sup>Developmental delay in infantile period.

ND, not detected.

TABLE 2. *GLDC* Mutations\*

Mutation	Location	Consequence of mutation	No. of families	Novel mutation	Evolutionary conservation				
					Human	Rat	Chicken	Pea	<i>E.coli</i>
<b>Missense mutations</b>									
c.176G>C	Exon 1	p.R59T	1	Yes	Arg	Arg	Arg	Ser	Ser
c.245T>G	Exon 1	p.L82W	1	No	Lue	Lue	Val	Val	Val
c.449A>C	Exon 3	p.N150T	2	No	Asn	Asn	Asn	Asn	Asn
c.806C>T	Exon 6	p.T269M	2	Yes	Thr	Thr	Thr	Thr	Thr
c.887T>G	Exon 7	p.L296R	1	Yes	Leu	Leu	Leu	Leu	Ile
c.1166C>T	Exon 9	p.A389V	3	No	Ala	Ala	Ala	Ala	Ala
c.1595C>G	Exon 13	p.T532R	1	Yes	Thr	Thr	Thr	His	Thr
c.1705G>A	Exon 14	p.A569T	1	Yes	Ala	Ala	Ala	Met	Ile
c.1832T>G	Exon 15	p.V611G	1	Yes	Val	Val	Ile	Phe	Val
c.2080G>C	Exon 18	p.A694P	1	Yes	Ala	Ala	Ala	Ala	Cys
c.2105C>T	Exon 18	p.S701F	1	Yes	Ser	Ser	Ser	Ser	Ser
c.2182G>C	Exon 18	p.G728R	1	Yes	Gly	Gly	Gly	Gly	Gly
c.2216G>A	Exon 19	p.R739H	2	No	Arg	Arg	Arg	Ser	Ser
c.2258A>C	Exon 19	p.H753P	1	Yes	His	His	His	His	His
c.2293C>T	Exon 19	p.P765S	1	Yes	Pro	Pro	Pro	Pro	Pro
c.2306C>T	Exon 19	p.P769L	1	Yes	Pro	Pro	Pro	Pro	Pro
c.2311G>A	Exon 19	p.G771R	3	Yes	Gly	Gly	Gly	Gly	Gly
c.2368C>T	Exon 20	p.R790W	1	No	Arg	Arg	–	–	–
c.2519T>A	Exon 21	p.M840K	1	Yes	Met	Met	Met	Met	Ile
c.2714T>G	Exon 23	p.V905G	1	Yes	Val	Val	Ile	Ile	Val
c.2846C>T	Exon 24	p.P949L	1	Yes	Pro	Pro	Pro	Pro	Pro
<b>Nonsense mutations</b>									
c.1786C>T	Exon 15	p.R596X	1	Yes					
c.1996C>T	Exon 17	p.Q666X	1	Yes					
c.2574T>G	Exon 22	p.Y858X	1	Yes					
<b>Deletions and insertions</b>									
Exon 1 deletion	Exon 1	Deletion including exon 1	6	No	A recurrent mutation				
c.793delC	Exon 6	Deletion of first letter of 265 Gln codon	1	Yes	FS after codon 264/TRM at codon 296				
c.1821_1831del	Exon 15	11-bp deletion started at third letter of 607 Gly codon	1	No	FS after codon 607/TRM at codon 669				
c.1443insG	Exon 11	Insertion of G after third letter of 481 Leu codon	1	Yes	FS at codon 482/TRM at codon 491				
c.2213_2214delGT	Exon 19	Deletion of GT in 738 Cys codon	1	Yes	FS after codon 738/TRM at codon 745				
c.2266_2268deITTC	Exon 19	Deletion of TTC in 756 Phe codon	1	No	No FS/TRM at codon 1019				
c.2891insA	Exon 24	Insertion of A after second letter of 964 Tyr codon	1	Yes	No FS/TRM at codon 964				
<b>Aberrant splicing</b>									
c.255+1G>A	Intron 2	Disruption of splicing donor site, gt>at	1	Yes					
c.1926+1G>A	Intron 17	Disruption of splicing donor site, gt>at	1	Yes					
c.2203-2A>G	Intron 18	Disruption of splicing acceptor site, ag>gg	1	Yes					
c.2665+1G>C	Intron 22	Disruption of splicing donor site, gt>ct	2	Yes					
c.2839-1G>C	Intron 23	Disruption of splicing acceptor site, ag>cg	1	Yes					

\*DNA mutation numbering is based on cDNA sequence: +1 corresponds to the A of the ATG translation initiation codon. *GLDC*: NM\_000170. FS, frameshift; TRM, termination codon.

observed in CSF glycine levels between 42 mutation-positive and 14 mutation-negative individuals. *GLDC* mutations were detected in five (83%) of the six patients with infantile NKH. No mutations were found in *GLDC*, *AMT*, or *GCSH* in any of seven patients with late-onset NKH. NKH mutations were highly heterogeneous. Only nine of the 47 mutation-positive individuals were homo-

zygous for a single mutation (three individuals from consanguineous families (P21, P26, and P59), and five homozygotes of recurrent mutations (P36, P5, P78, P107, and P108)). Patient P32 was homozygous for a private mutation (p.R596X), even though there was no evidence that he was the product of a consanguineous marriage. No *GCSH* mutations were identified in this study.



TABLE 3. AMT Mutations\*

Mutation	Location	Sequence alternation	No. of families	Novel mutation	Evolutionary Conservation				
					Human	Rat	Chicken	Pea	<i>E.coli</i>
<b>Missense mutations</b>									
c.125A>G	Exon 2	p.H42R	1	No	His	His	His	His	His
c.136G>A	Exon 2	p.G47R	1	Yes	Gly	Gly	Gly	Gly	Ala
c.139G>T	Exon 2	p.G47W	1	Yes	Gly	Gly	Gly	Gly	Ala
c.212A>C	Exon 2	p.H71P	1	Yes	His	His	His	Asn	Ala
c.217C>T	Exon 2	p.R73C	1	Yes	Arg	Arg	Arg	Arg	Arg
c.230C>T	Exon 2	p.S77L	1	Yes	Ser	Ser	Ser	Ser	Gly
c.826G>C	Exon 7	p.D276H	1	No	Asp	Asp	Asp	Asp	Glu
c.887G>A	Exon 8	p.R296H	1	No	Arg	Arg	Lys	Arg	Lys
<b>Deletions and insertion</b>									
c.54delC	Exon 1	Deletion of third letter of 18Phe codon	1	No	FS at codon 20/TRM at codon 95				
c.60delG	Exon 1	Deletion of third letter of 20Pro codon	1	Yes	FS at codon 21/TRM at codon 95				
c.61delC	Exon 1	Deletion of first letter of 21Ala codon	1	Yes	FS at codon 21/TRM at codon 95				
c.147delG	Exon 2	Deletion of third letter of 49Met codon	1	Yes	FS at codon 50/TRM at codon 95				
c.452_466del	Exon 4	Deletion start at second letter of 151Lys codon	1	Yes	No FS/TRM at codon 398				
c.535delC	Exon 5	Deletion of first letter of 179Leu codon	1	Yes	FS at codon 179/TRM at codon 180				
c.970_972delATG	Exon 8	Deletion of 320Met codon	1	Yes	No FS/TRM at codon 482				
c.982_972GC>T	Exon 8	GC in 328Ala codon was substituted byT	1	Yes	FS at codon 328/TRM at codon 337				
<b>Aberrant splicing</b>									
c.471+2T>C	Intron 4	Disruption of splicing donor site, gt>gc	2	Yes					

\*DNA mutation numbering is based on cDNA sequence: +1 corresponds to the A of the ATG translation initiation codon. AMT: NM\_00481. FS, frameshift; TRM, termination codon.

### GLDC Mutations

Deletion of exon 1 was detected in six patients (four Caucasian, one oriental, and one black). In this study we identified 36 GLDC mutations, 28 of which were novel (Table 2). None of the identified mutations were detected in 100 control alleles. There were 21 missense mutations, three nonsense mutations, seven deletion/insertion mutations, and five splicing mutations Table 3. For all of the 21 missense mutations the substituted amino acids are conserved in rats, and 13 of the 21 amino acids are conserved from humans to *E. coli*. Such a high degree of evolutionary conservation presumably reflects the functional importance of each of these amino acids. Seven mutations were found in multiple individuals with no apparent relationship. A missense mutation p.N150T was identified in oriental families, while p.T269M, p.A389V, p.R739H, and c.2665+1G>C were found in Caucasian families. Sequencing analysis of the GLDC gene revealed the presence of several polymorphisms, which were also found in control subjects (Table 4). Five of the polymorphisms were found in at least 10% of the control alleles tested. Three of them were found within exons (exon 1: c.249G>A; exon 4, c.501G>A; and exon 25, c.3070C>G (3' noncoding region)). Two of them were located in introns (intron 9, c.1262+36A>G; and intron 19, c.2203-6insA). The c.2203-6insA polymorphism was located at the intron 19/exon 20 boundary, and substitutes the ttaaaaaaaatcacg/GAAGAAA (A8 allele) to the ttaaaaaaaatcacg/GAA GAAA (A9 allele). Other polymorphisms that were less frequently observed were c.438G>A (p.T146T) in exon 3, c.1261+36A>G (intron 9), and c.1261+52G>A (intron 9).

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### AMT Mutations

A total of 17 mutations (including 13 novel mutations) were identified, including eight missense mutations, eight deletion/insertion mutations, and one splicing mutation. The evolutionary conservation of each mutated amino acid is shown in Table 3. All of these amino acids are conserved among humans, rats, and chickens, and six of eight amino acids are also conserved in peas, which suggests that they are functionally important. All of the deletion/insertion mutations generated a profoundly truncated AMT polypeptide. Two polymorphic sites were observed in two AMT exons: exon 2, c.327T>C; and exon 8, c.1083G>A.

### Characterization of the GLDC Exon 1 Deletion

To define the boundaries of the exon 1 deletions, eight STSs were designed: seven STSs at the region 150 kb upstream of GLDC, and one STS in intron 2 of GLDC (Fig. 1; Supplementary Table S1). Two additional published STSs (D9S281 and RH92434) and GLDC exons 1–5 were also used for the deletion mapping. We amplified a total of 15 STSs using genomic DNA as the templates obtained from Patients P5 and P36, who were homozygous for the GLDC exon 1 deletion. In Patient P5, STSs 2–8 and exons 1–3 were not amplified, indicating that the 80–100 kb was homozygously deleted. In Patient P36, STSs 6–8, and exons 1–2 failed to be amplified, demonstrating a 35–45 kb homozygous deletion. Both deletions did not extend to a known gene 5' adjacent to GLDC: jumonji domain containing protein2C (*JMJD2C*). *Alu* repeats in this region were identified using the UCSD Genome Browser (Fig. 1).

TABLE 4. Haplotype Analysis of the *GLDC* Allele With the Exon 1 Deletion

Patients	Ethnicity	Allele	Polymorphic site			
			Exon 4 c.501G>A <sup>a</sup>	Intron 9 c.1262+36A>G	Intron 19 c.2203–6insA	Exon 25 (3' noncoding region) c.3070C>G
P5	Oriental	Allele 1	G	A	A8	C
		Allele 2	G	A	A8	C
P14	Caucasian	Allele 1	G	A	A8	C
P36	Caucasian	Allele 1	G	A	A8	C
		Allele 2	G	A	A9	C
P50	Caucasian	Allele 1	A	G	A9	C

<sup>a</sup>DNA mutation numbering is based on cDNA sequence: +1 corresponds to the A of the ATG translation initiation codon. *GLDC*: NM\_000170.

### Haplotype Analysis of the *GLDC* Mutations

The identification of five polymorphisms within *GLDC* allowed us to determine the haplotypes of the mutant alleles with the exon 1 deletion. Haplotypes of the deletion allele were identified in four families (P5, P14, P36, and P50), but could not be determined in two families (P15 and P86; Table 4). Four mutant alleles shared the same haplotype (G, A, A8, C), with the genotypes of four polymorphic sites being written as follows: (c.501G>A, c.1232+36A>G, c.2203-6ins A, c3070C>G). Two additional haplotypes, (G, A, A9, C) and (A, G, A9, C) were also found, suggesting multiple origins of the exon 1 deletion.

### Mutation Spectra of the *GLDC* and *AMT* Genes

The *GLDC* mutations detected in this study, as well as those from previous reports, are illustrated in Fig. 2A. The most striking feature of the mutation distribution is the clustering of the missense mutations in exon 19. Seven of the 32 missense mutations (22%) were identified in exon 19. The distribution of the *AMT* mutations is shown in Fig. 2B. No obvious clustering of the mutations was found in this gene.

## DISCUSSION

We undertook an extensive screening for *GLDC*, *AMT*, and *GCSH* mutations in a cohort of patients with NKH, and the results reveal a comprehensive picture of the mutation spectrum of NKH to date. In this study, 36 *GLDC* mutations and 17 *AMT* mutations were detected, including 28 novel *GLDC* and 13 novel *AMT* mutations. In total, 75% of neonatal and 83% of infantile patients were positive for *GLDC* or *AMT* mutations. Mutations in NKH patients were highly heterogeneous. Patients were found to be compound heterozygotes in 38 of 47 mutation-positive cases (81%). This is in sharp contrast with findings in countries with a high incidence of NKH, such as Finland [von Wendt et al., 1979] and Israel [Korman and Gutman, 2002]. In those countries there are common mutations and many homozygotes for each common mutation. A *GLDC* missense mutation (p.564I) has been identified in Finland [Kure et al., 1992], and a *GLDC* missense mutation (p.M1 T) and *AMT* missense mutation (p.H42R) have been found in Israel [Boneh et al., 2005; Flusser et al., 2005].

In 16 of 36 families (44%) with *GLDC* mutations, we were able to identify a mutation in only one of two mutant alleles despite extensive mutational screening of *GLDC*, *AMT*, and *GCSH*. Mutations may present in promoter regions or introns of the *GLDC* gene, or large deletions or duplications may exist. Deletion of *GLDC* exon 1 was detected in eight of the 36 families (22%) with *GLDC* mutations. The deletion was found in Caucasian,

oriental, and black patients. Subsequent haplotype analysis suggested multiple origins of the deletion. There is a high-density repeat region of *Alu* motifs in the 5' upstream region and the introns of *GLDC*, which are reported to trigger a homologous recombination between two *Alu* motifs and cause a large deletion (e.g., the C1-inhibitor gene, *C1-INH* [Stoppa-Lyonnet et al., 1991]; lysyl hydroxylase gene, *PLOD1* [Pousi et al., 1994]; and a MutL mismatch repair gene, *hMLH1* [Mauillon et al., 1996]). We therefore speculate that the deletions of *GLDC* exon 1 are caused by homologous recombination between *Alu* repeats. Recently, Sellner et al. [2005] reported a deletion of *GLDC* exons 2–15 that was flanked by *Alu* motifs. *Alu*-mediated homologous deletion may occur not only in the 5' region of *GLDC*, but also in other regions of *GLDC*.

No mutations were detected in seven patients with late-onset NKH, despite intensive screening of the entire coding regions of *GLDC*, *AMT*, and *GCSH*, which suggests that gene(s) other than *GCS* genes may be responsible for late-onset NKH. Several reports have described hyperglycinemic patients with no evidence of neurological symptoms until 1 year of age [Bank and Morrow, 1972; Steiman et al., 1979; Lane et al., 1991, 1998; Wiltshire et al., 2000; Ellaway et al., 2001]. The diagnosis of NKH was not confirmed by mutational analysis in any of those patients. Patients with atypical GE tend to have a relatively modest elevation of serum and CSF glycine concentrations, which may be also caused by other genetic disorders or therapeutic agents [Korman and Gutman, 2002]. Vanishing white matter disease is a type of leukoencephalopathy with characteristic MRI findings that is commonly associated with mild elevation of CSF glycine concentration [van der Knaap et al., 1999]. This disorder might have been classified as a late-onset NKH if the responsible genes (*EIF2B5* and *EIF2B2*) had not been identified [Leegwater et al., 2001]. For a more accurate diagnosis and better understanding of late-onset NKH patients, genetic characterization is of paramount importance. There are several other proteins that are functionally related to the GCS reaction. Lipoylation of H-protein is catalyzed by lipoyltransferase. The gene encoding the enzyme would be a good candidate for NKH [Fujiwara et al., 1999]. Two types of glycine transporters, GlyT1 and GlyT2, have been identified [Zafra et al., 1997]. GlyT2 is located only in the brain stem and spinal cord, while GlyT1 is distributed in the brain, kidney, and liver, and the latter transporter may have a role in maintaining the glycine level in both CSF and plasma. Thus, the GlyT1 gene is another good candidate gene for NKH.

A mutation in at least one of two alleles was identified in 47 of 62 patients with neonatal or infantile type NKH, 36 of whom (77%) had *GLDC* mutations. The dominance of the *GLDC* mutations over *AMT* mutations is in agreement with previous

enzymatic studies [Tada and Hayasaka, 1987; Toone et al., 2000]. No GCSH mutations were identified in any of the three clinical subtypes of NKH, suggesting that GCSH mutations are extremely rare in NKH. We recently identified a heterozygous splicing mutation in GCSH in a Japanese family with a peculiar type of NKH—transient type NKH [Kure et al., 2002]. Transient NKH is indistinguishable from neonatal NKH in terms of the onset of disease, but serum and CSF glycine are normalized within 2–8 weeks [Luder et al., 1989; Schiffmann et al., 1989]. A girl with atypical NKH was reported to have low enzymatic activity of the H-protein [Hiraga et al., 1981]. Unlike other patients with NKH, this patient showed progressive deterioration and extensive spongy degeneration of white matter with marked gliosis on postmortem examination [Trauner et al., 1981]. It may be that GCSH mutations will be found in patients with atypical NKH rather than the more readily recognized clinical form of NKH.

Seven of 32 GLDC missense mutations were clustered in exon 19 (Fig. 2). Glycine decarboxylase consists of 1,020 amino acids, and exon 19 encodes 37 of 1,020 amino acids (3.6%), that is, 22% of the GLDC missense mutations were clustered in only 3.6% of the protein-coding region. Two octapeptides encoded in exon 19—His749 to Phe756 (HLNLHKTF) and Pro759 to Gly766 (PHGGGGPG)—are perfectly conserved in humans, chickens, peas, and *E. coli*. Crystal structure analysis revealed that His749, Asn751, His753, Lys754, and His760 (underlined) formed an active-site pocket of the GLDC enzyme [Nakai et al., 2005], and the cofactor of GLDC, pyridoxal phosphate, was covalently bound to Lys754 [Fujiwara et al., 1987]. Amino acid changes in this conserved region frequently abolish GLDC enzyme activity, which may be a possible explanation for the high incidence of GLDC missense mutations in this region.

Patients P107 and P108 were homozygous for the p.A389V mutation, while patient P104 was a compound heterozygote of the p.A389V mutation and a null mutation, c.1443insG (Table 1). The p.A389V mutation had approximately 8% residual activity in the COS7 expression analysis [Dinopoulos et al., 2005]. Patients P107 and P108 did not present with comas or seizures in the neonatal period, but exhibited developmental delay and abnormal behaviors that developed with age. In contrast, Patient P104 had a typical presentation of the neonatal form of NKH, but subsequently the course of the disease was less severe. The presence of the pA389V mutant allele, which allows some residual enzyme activity, may explain this milder phenotype of classic NKH. The clinical course of P104 resembled those of P6 and P19, who were expected to have 5–8% residual activity by the *in vitro* expression analysis [Kure et al., 2004]. These results suggest that only a few-percent difference in GCS residual activity dramatically alters the clinical picture, such as age of onset and prognosis. A previous enzymatic study of patients with neonatal and infantile NKH supports this suggestion [Hayasaka et al., 1987]. GCS activities ranged from 0 to 0.7 nmoles of CO<sub>2</sub> formed/mg protein/hr in the neonatal type, while it ranged from 0.7 to 1.4 in the infantile type (for which the control range was 3.9–5.2). However, an exception was observed. Both P69 and P50 were compound heterozygotes of GLDC exon 1 deletion and p.G771R. Patient P69 manifested typical symptoms on the second day of life, whereas Patient P50 did not develop symptoms until 3 months of age. Thus far, residual activity is a major determinant of the clinical course, but it is probably modified by environmental factors and/or genotypes of other than the GCS genes.

Elucidation of the responsible gene(s) for late-onset NKH, and screening for deletions in all GLDC exons are needed to establish

a more complete picture of the genetic background and develop the genotype–phenotype relationships in NKH.

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**Research Report**
**De novo and salvage pathways of DNA synthesis in primary cultured neural stem cells**
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**ABSTRACT**

We studied the de novo and salvage pathways of DNA synthesis in sphere-forming neural stem cells obtained from mouse embryos by a neurosphere method. The former pathway needs folic acid (FA) for nucleotide biosynthesis, while the latter requires deoxyribonucleosides (dNS). We examined the proliferative activity of sphere-forming cells in E14.5 embryos by counting the number of spheres formed in media that lacked FA and/or dNS. Proliferation failure and apoptosis occurred in a deficient medium lacking of both FA and dNS. Spheres formed in the deficient medium supplemented with dNS, without FA, did not produce neuron, but rather only seem to generate astrocytes and oligodendrocytes when plated under differentiation condition in culture. On the other hand, a subpopulation of cultured cells formed spheres in the deficient medium supplemented with FA alone in an appropriate concentration, and did possess the self-renewing and multipotential characteristics of neural stem cells. Spheres formed in the media containing low dose Azathioprine and methotrexate, inhibitors of de novo DNA synthesis, were selectively prevented from producing neurons even in the presence of FA. These results suggested that activating de novo DNA synthesis was needed for neural stem cells to proliferate with multipotentiality.

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## Abbreviations:

FA, folic acid  
 dNS, deoxyribonucleosides  
 dNTPs, deoxynucleoside triphosphates  
 dAdo, 2'-deoxyadenosine  
 dCyd, 2'-deoxycytidine  
 dGuo, 2'-deoxyguanosine  
 Thy, thymidine  
 AZP, azathioprine  
 MTX, methotrexate  
 NTD, neural tube defect  
 DIV, days in vitro  
 GFAP, glial fibrillary acidic protein

## 1. Introduction

Normal CNS development involves the sequential differentiation of multipotent stem cells. Alteration in the number of stem cells or their proliferative capability has major effects on the appropriate development of the nervous system (Sommer and Rao, 2002). One of the essential steps for the proliferation of stem cells is DNA synthesis. DNA precursors, i.e., nucleotides, are synthesized through two metabolic pathways referred to as de novo pathway and salvage pathway (Fig. 1). In the de novo pathway, deoxynucleoside triphosphates (dNTPs) are generated in multiple steps from 5-phosphoribosyl-1-pyrophosphate, glutamine, glycine, aspartate, and folic acid (FA). Since animal cells cannot synthesize FA, FA supplementation into their culture media is required for activating de novo DNA synthesis (Murray et

al., 1999). In the salvage pathway, dNTPs are produced in single steps from exogenous and endogenous deoxyribonucleosides (dNS). The de novo pathway is predominant in normal differentiated tissues, whereas the salvage pathway is activated in rapidly proliferating tissues, including fetal and regenerative tissues and cancer cells (Hatse et al., 1999). Previous studies functionally evaluated the DNA synthesis in erythroblasts using culture media deficient of FA and/or dNS, and that indicated that erythroblasts underwent apoptosis during S phase in the lack of both FA and dNS (James et al., 1994; Koury and Horne, 1994; Koury et al., 2000). To date, the DNA synthesis pathway employed in neural stem cells remains uninvestigated.

Reynolds and Weiss (1996) reported that neural stem cells could be established from rodent brains by the neurosphere culture method. Using the neurosphere method, single

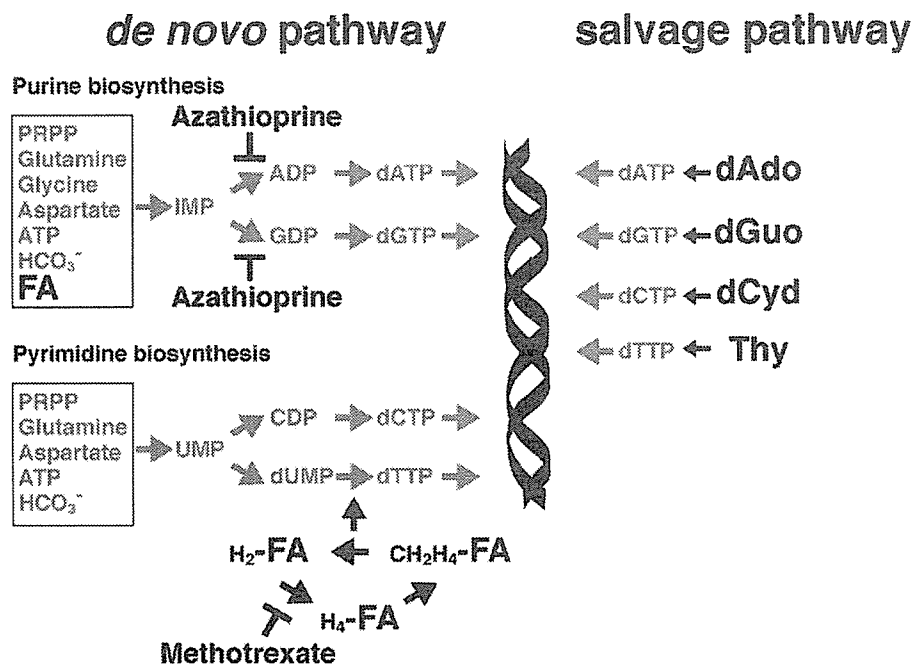


Fig. 1 – De novo and salvage biosynthesis of DNA. DNA is synthesized through two pathways: the de novo pathway (left panel) and salvage pathway (right panel). Abbreviations: IMP, inosinic acid; UMP, uridine monophosphate; dAdo, 2'-deoxyadenosine; dCyd, 2'-deoxycytidine; dGuo, 2'-deoxyguanosine; Thy, thymidine; H<sub>2</sub>-FA, dihydrofolate; H<sub>4</sub>-FA, tetrahydrofolate; CH<sub>2</sub>H<sub>4</sub>-FA, N<sup>5</sup>,N<sup>10</sup>-methylenetetrahydrofolate.

neural stem cells can be selectively expanded in a chemically defined medium containing epidermal growth factor (EGF), fibroblast growth factor 2 (FGF-2), or both, giving rise to floating spheroid cell aggregates called neurospheres. Since D-MEM/F-12, the commonly used medium for the neurosphere culture, contains both FA and two kinds of dNS, namely, thymidine (Thy) and hypoxanthine, the pathway of DNA synthesis that operates in the proliferation of neural stem cells remains unknown.

In this report, we performed the neurosphere method by using a custom-prepared D-MEM/F-12 medium that lacked FA and/or dNS for the functional evaluation of DNA synthesis pathways in embryonic sphere-forming neural stem cells. We examined the efficiency of the sphere formation and characterized the multipotency of the neural stem cells by *in vitro* differentiation analysis. Specific inhibitors of *de novo* DNA synthesis were used for the further characterization of sphere-forming cells. These approaches enabled us, for the first time, to perform the functional evaluation of DNA synthesis in neural stem cells.

## 2. Results

### 2.1. Sphere formation derived from the E14.5 mouse striata in FA+/dNS- or FA-/dNS+ media

To determine the DNA synthesis pathway utilized by embryonic neural stem cells for proliferation, we cultured neurospheres derived from the E14.5 mouse striata in D-MEM/F-12 medium lacking the precursors of DNA synthesis, defined as a deficient medium. We supplemented the deficient medium with varying amounts of FA, as a precursor for the *de novo* pathway, or dNS (equimolar addition of dAdo, dGuo, dCyd, and Thy), as precursors for the salvage pathway. In the deficient medium, designated as FA-/dNS- medium, no sphere was formed, and the condensation of cultured cells and the fragmentation of nuclei, which are characteristic of apoptosis, were observed. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) showed increased TUNEL-positive cells (mean  $\pm$  SEM,  $56.7\% \pm 1.5\%$ ;  $n = 4$ ) in the deficient medium when compared with those in the deficient medium containing  $10 \mu\text{M}$  FA and  $50 \mu\text{M}$  dNS ( $29.2\% \pm 1.1\%$ ,  $n = 5$ ,  $P < 0.01$ ) (data not shown). These results suggested that cultured cells did not proliferate and underwent apoptosis in the medium without the precursors of DNA synthesis. In the medium containing only FA, maximum number of spheres was formed with  $10 \mu\text{M}$  FA (Fig. 2A). In the absence of FA, peak sphere formation efficiency with each dNS was observed at a concentration of  $50 \mu\text{M}$  (Fig. 2B). We defined the culture condition of  $10 \mu\text{M}$  FA without dNS as FA+/dNS-. Similarly, the condition in which the culture was supplemented with  $50 \mu\text{M}$  of each dNS without FA was designated as FA-/dNS+.

### 2.2. Sphere-forming activity in the presence of various combinations of dNS

Eukaryotes generally have converting enzymes among purine and pyrimidine deoxyribonucleosides (Murray et al., 1999). To

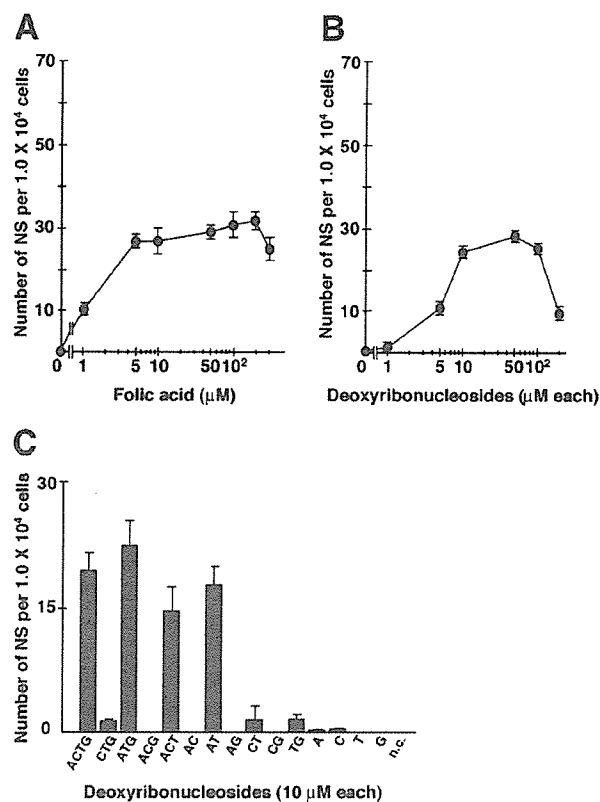


Fig. 2 – (A and B) Dose–response curves of sphere formation cultured for 7 DIV in the deficient medium supplemented with FA (A) or dNS (B). During this developmental period, spheres can be formed in the presence of FA or dNS in an appropriate concentration. (C) The number of spheres cultured for 7 DIV in the deficient medium supplemented with 16 different combinations of deoxyribonucleosides, each at a concentration of  $10 \mu\text{M}$ . Graphs show mean  $\pm$  SEM number of spheres per  $1 \times 10^4$  cells prepared from the E14.5 mouse striata. Abbreviations: A, 2'-deoxyadenosine; G, 2'-deoxycytidine; C, 2'-deoxyguanosine; T, thymidine.

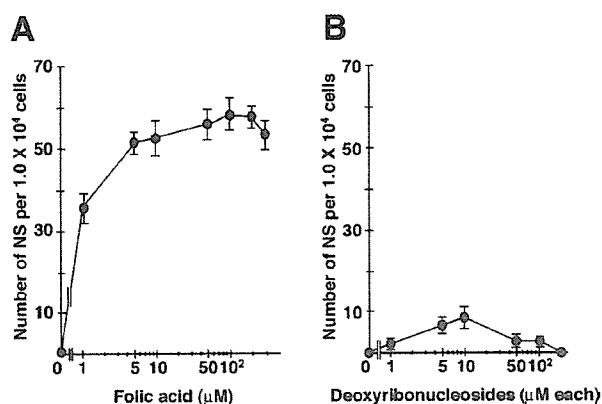
determine the precursors of the salvage pathway that are required for sphere formation, we tested 16 different combinations of  $10 \mu\text{M}$  dNS. As shown in Fig. 2C, the combination of dAdo with Thy yielded maximum sphere-forming efficiency and additional supplementation with other dNSs had little effect on the efficiency.

### 2.3. Developmental-stage specific changes of sphere-forming activities under the FA+/dNS- and FA-/dNS+ conditions

To examine the developmental changes of sphere-forming activities under the FA+/dNS- and FA-/dNS+ conditions, we performed the neurosphere cultures derived from the E12.5 and E16.5 mice striata in the deficient medium supplemented with varying amounts of FA or dNS (Fig. 3). In the cultures derived from the E16.5 striata, nearly the same number of spheres was formed with appropriate concentrations of FA and dNS. However, in the culture derived from



## E12.5



## E16.5

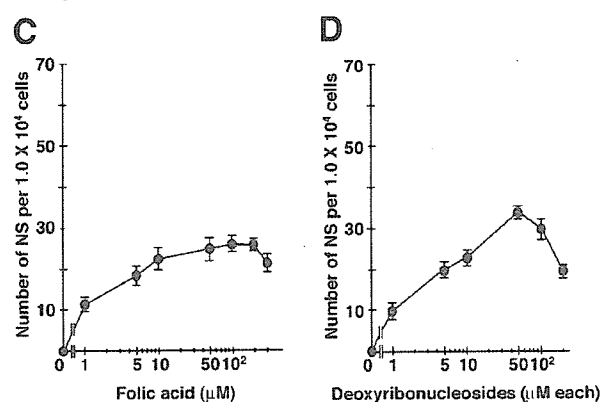


Fig. 3 – Developmental change of dose–response curve of sphere formation, cultured for 7 DIV in the deficient medium supplemented with FA (A and C) or dNS (B and D). Graphs show mean  $\pm$  SEM number of spheres per  $1 \times 10^4$  cells prepared from E12.5 (A and B) and E16.5 (C and D) mice striata.

E12.5, only a few spheres were formed under the FA-/dNS+ condition.

#### 2.4. Morphological and immunochemical characterizations of the formed spheres in the FA+/dNS- or FA-/dNS+ media

Spheres formed under the FA-/dNS+ condition morphologically differed from those formed under the FA+/dNS- condition (Figs. 4A, B). After 7 DIV, the FA+/dNS- spheres were large, smooth, and spherical in shape, while the FA-/dNS+ spheres were small and irregular in shape. The FA+/dNS- spheres were  $77.0 \pm 3.8 \mu\text{m}$  ( $n = 66$ ) in diameter, larger than the FA-/dNS+ spheres ( $49.8 \pm 2.5 \mu\text{m}$ ,  $n = 78$ ,  $P < 0.01$ ). It is unlikely that both spheres were derived from ciliated cells by their morphological characteristics (Chiasson et al., 1999; Laywell et al., 2000). Immunochemical characterization showed that both spheres were immunopositive for Nestin (a neural progenitor marker), GFAP (an astrocyte marker), and O4 (an oligodendrocyte marker) (Figs. 4C, D, G–J). However, tubulin  $\beta$  III (a neuron

marker) positive cells were observed only in the FA+/dNS- spheres (Fig. 4E), and not in the FA-/dNS+ spheres (Fig. 4F), suggesting that cells in the FA-/dNS+ spheres barely differentiated into neurons.

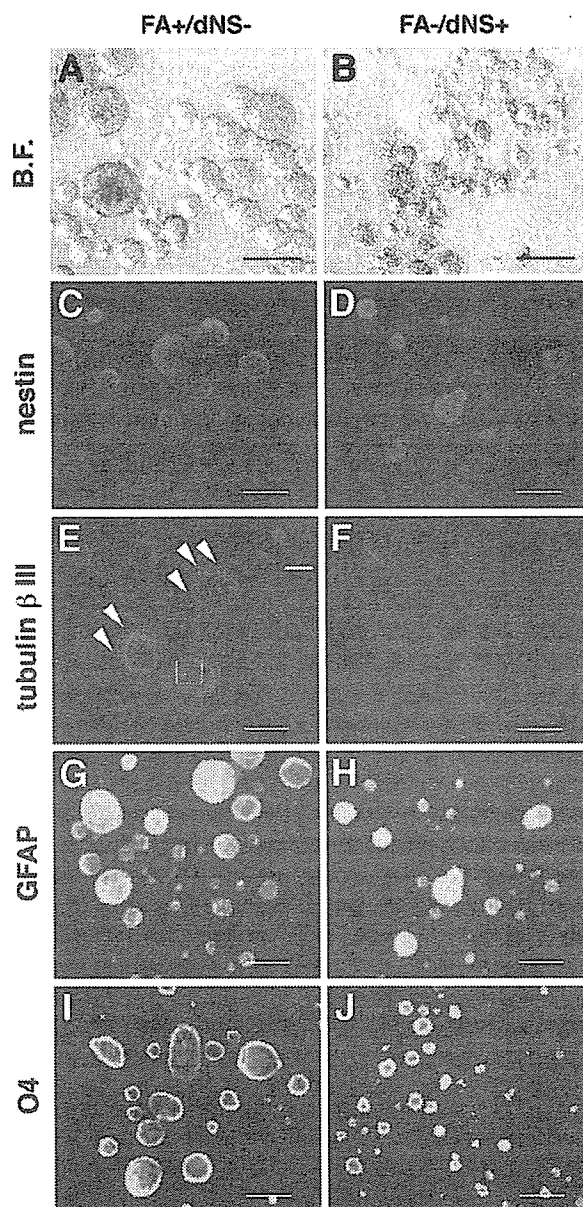


Fig. 4 – FA-/dNS+ spheres contain astrocytes and oligodendrocytes, but not neurons. (A and B) Phase-contrast images of live floating spheres under the FA+/dNS condition (A) and FA-/dNS+ condition (B). (C–J) Immunofluorescence of chemical markers for neural progenitors (nestin, red; C and D), neurons (tubulin  $\beta$  III, red; E and F), astrocytes (GFAP, green; G and H), oligodendrocytes (O4, green; I and J), and nucleus (DAPI, blue; C–J) derived from the FA+/dNS- spheres (C, E, G, and I) and FA-/dNS+ spheres (D, F, H, and J). White arrowheads indicate tubulin  $\beta$  III-positive neurons. Inset in panel E is high magnification of a white box. Scale bars: A–J, 200  $\mu\text{m}$ ; Inset, 20  $\mu\text{m}$ . Abbreviations: B.F., bright field; GFAP, glial fibrillary acidic protein.

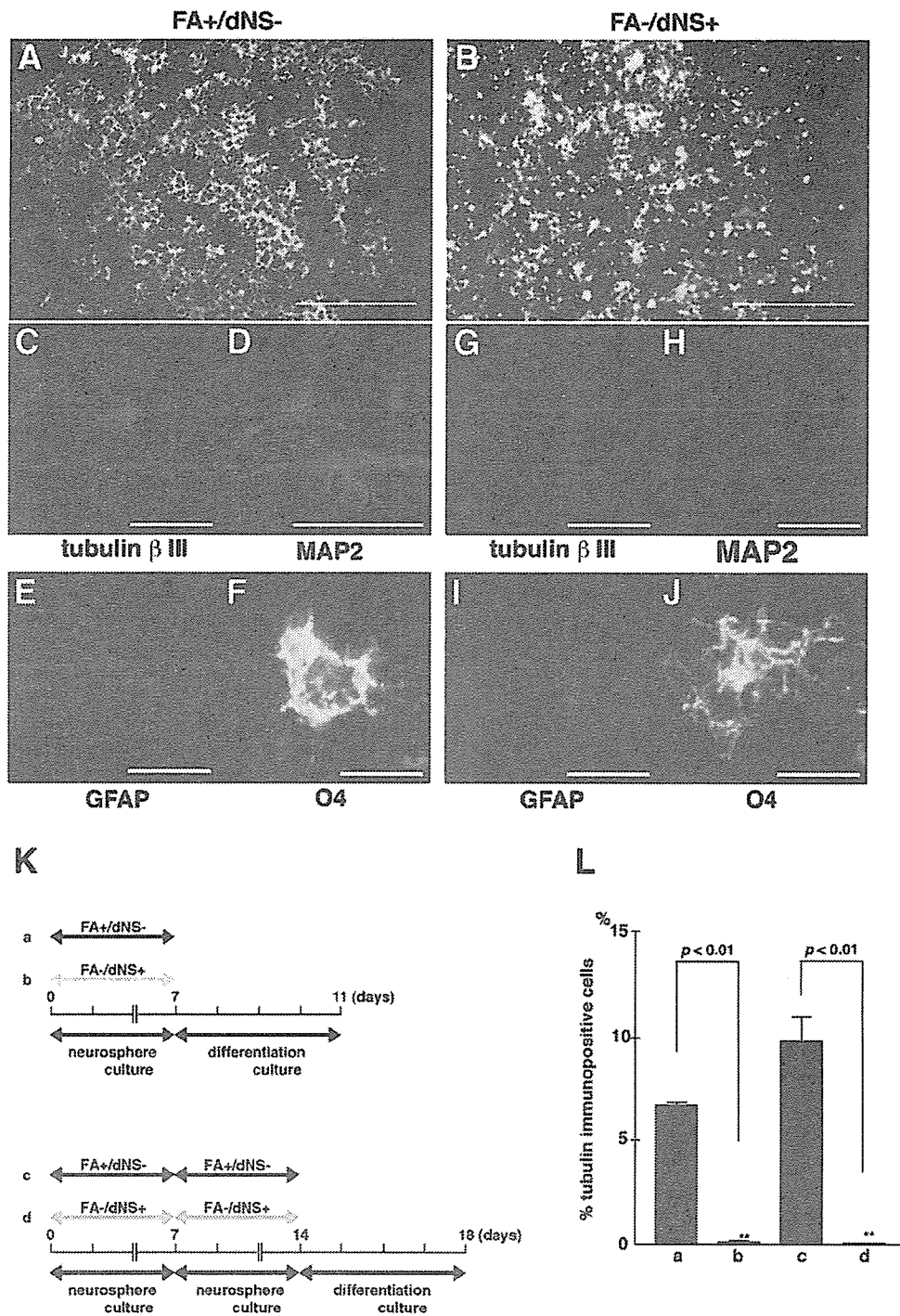


Fig. 5 - Tri-colored immunofluorescence of markers for neurons (tubulin  $\beta$  III, red), astrocytes (GFAP, blue), and oligodendrocytes (O4, green) shows that the FA+/dNS- clonal spheres gave rise to different cells expressing markers of all three neural cell types, as depicted in a triple-labeled survey (A), and the details of individual cells of each type (C-F). The FA-/dNS+ spheres gave rise to astrocytes and oligodendrocytes, but not neurons, as shown in a triple-labeled survey (B), which also depicted the details of individual cells (G-J). Scale bars: A, B, 200  $\mu$ m; C-J, 20  $\mu$ m. (K) Experimental protocol. The 7-DIV primary culture in the FA-/dNS+ (a) and FA+/dNS- (b) condition was placed under the differentiation condition for 4 DIV (total 11 DIV). (c), The 7-DIV primary culture in the FA+/dNS- medium was placed under the FA+/dNS- condition for 7 DIV (total 14 DIV) and, then, the cultured cells were placed under the differentiation condition for 4 DIV (total 18 DIV). (d) Likewise, the FA-/dNS+ primary culture was placed under the FA-/dNS+ condition and, then, under the differentiation condition. (L) Graph showing mean  $\pm$  SEM percentage of tubulin  $\beta$  III-immunopositive neurons of the total number of cells per field derived from each protocol.

\*\*Significant difference between the two groups ( $P < 0.01$ ).

### 2.5. *In vitro* differentiation analysis of sphere-initiating cells in the FA+/dNS- or FA-/dNS+ media

To examine the multipotency of spheres under the FA+/dNS- and FA-/dNS+ conditions, we transferred and placed isolated spheres under the differentiation condition. After 4 DIV of differentiation, triple-labeled immunocytochemistry for tubulin  $\beta$  III, GFAP, and O4 revealed that cells with the morphological and antigenic characteristics of neurons, astrocytes, and oligodendrocytes, respectively, were generated from the FA+/dNS- spheres (Figs. 5A, C–F). In contrast, the FA-/dNS+ spheres yielded astrocytes and oligodendrocytes, but neither tubulin  $\beta$  III nor MAP2 positive neurons (Figs. 5B, G–I). The percentage of tubulin  $\beta$  III positive neurons that differentiated from the FA-/dNS+ primary spheres was  $0.067\% \pm 0.009\%$  ( $n = 4$ ) significantly lower than that from the FA+/dNS- primary spheres ( $6.618\% \pm 0.188\%$ ,  $n = 5$ ,  $P < 0.01$ ), as well as the percentage of tubulin  $\beta$  III positive neurons from secondary spheres (FA-/dNS+,  $0.035\% \pm 0.004\%$  vs. FA+/dNS-,  $9.81\% \pm 1.516\%$ ,  $n = 4$ ,  $P < 0.01$ ) (Fig. 5J). According to previous studies, the percentage of neurons derived from the neurosphere culture under the regular condition was 5%–10% (Laeng et al., 2004; Palmer et al., 1999). These results suggested that spheres formed under the FA+/dNS- condition, but not under the FA-/dNS+ condition, coincided with the definition of a neurosphere, self-renewal capability, and multipotency (Seaberg and van der Kooy, 2002).

### 2.6. Effects of inhibitors of *de novo* DNA synthesis on *in vitro* differentiation

We hypothesized that the disturbance of *de novo* DNA synthesis in the sphere-forming neural stem cells resulted in the decrease of neurons. To test the hypothesis, we added *de novo* synthesis inhibitors to the FA+/dNS+ medium. AZP blocks the conversion of inosinate to adenylyl succinate, which is the key reaction in the formation of purine adenylylate; additionally, it also blocks an important step in the production of the purine base guanylate (Belgi and Friedmann, 2002) (Fig. 1). MTX is a competitive inhibitor of dihydrofolate reductase (EC 1.5.1.3), which generates tetrahydrofolate from dihydrofolate. As a result, MTX interferes with the conversion of deoxyuridylate to thymidylate in the synthesis of DNA (Olsen, 1991) (Fig. 1). Both AZP and MTX inhibited sphere formation in a dose-dependent manner (Figs. 6A–C). The inhibition was most evident under the FA+/NS- condition. These results suggested that lower doses of AZP and MTX (e.g.,  $<1 \mu\text{M}$ ) selectively inhibited the *de novo* DNA synthesis in sphere-forming cells. We transferred the spheres obtained under these conditions and placed them under the differentiation condition (Fig. 6D). After 4 DIV, the percentage of tubulin  $\beta$  III positive neurons derived from the FA+/dNS+ spheres significantly ( $n = 4$ ,  $P < 0.01$ ) decreased in the presence of AZP or MTX (Fig. 6E). Figs. 6F–I show representative fields of the culture under the FA+/dNS+ condition (Fig. 6F), FA-/dNS+ condition (Fig. 6G), FA+/dNS+ with  $1 \mu\text{M}$  AZP condition (Fig. 6H), and FA+/dNS+ with  $0.1 \mu\text{M}$  MTX condition (Fig. 6I). The number of astrocytes did not significantly differ among these conditions (data not shown).

## 3. Discussion

We performed the neurosphere method by using modified media that lacked FA or dNS and added specific inhibitors of DNA synthesis. Cultured cells barely proliferated and underwent apoptosis under the FA-/dNS- condition. Although sphere formation was observed, the spheres formed under the FA-/dNS+ condition poorly produced neurons. Since extracellular nucleosides exert various effects on cell differentiation, apoptosis, mitogenesis, and stimulators of cytokine release in the nervous system (Neary et al., 1996), the decreased percentage of neurons derived from the FA-/dNS+ spheres could be attributed to the effects of extracellular nucleosides. However, there was no significant difference ( $P > 0.05$ ) between the percentage of neurons derived from FA+/dNS+ spheres ( $7.662 \pm 1.672\%$ ,  $n = 4$ ) (Fig. 6E) and FA+/NS- spheres ( $6.618\% \pm 0.188\%$ ,  $n = 5$ ) (Fig. 5J). Addition of inhibitors of *de novo* DNA synthesis, namely, AZP and MTX, resulted in poor neuronal differentiation even under the FA+/dNS+ condition. Taken together, low percentage of neurons derived from spheres formed under the FA-/dNS+ condition is not due to the extracellular effects of dNS, but due to the proliferation failure of sphere-forming neural stem cells induced by inactivation of the *de novo* DNA synthesis pathway.

The neurosphere cultures established from the E12.5 mouse proliferated to a lesser extent than those obtained from the E14.5 or E16.5 mouse under the FA-/dNS+ condition, while they proliferated to an equivalent extent under the FA+/dNS- condition. As shown in the differentiation assay, FA-/dNS+ spheres were likely to consist of cells committed to the glial lineage (Seaberg and van der Kooy, 2002). This result may be in line with the fact that neurogenesis precedes gliogenesis (Qian et al., 2000). To clarify the mechanism responsible for the developmental change in the DNA synthesis pathway, we compared the mRNA expression of thymidine kinase (EC. 2.7.1.21), deoxycytidine kinase (EC. 2.7.1.74), and adenosine kinase (EC. 2.7.1.45)—key enzymes for the salvage pathway (Karbownik et al., 2003)—and that of thymidylate synthetase (EC. 2.1.1.45)—a key enzyme for the *de novo* pathway (Chu et al., 2003)—in the E11.5 and E16.5 mouse neuroepithelium. There was no detectable change in semi-quantitative RT-PCR (data not shown). The activities of several key enzymes involved in DNA synthesis were previously reported using developmental brain tissues (Hyndman and Zamenhof, 1978; Suleiman and Spector, 1982; Sung, 1971). These reports suggested that the specific activities of both the DNA synthesis pathways equally decreased in proportion with maturation. Since there is a discrepancy between the mRNA levels and enzymatic activities, the activities of the key enzymes involved in the DNA synthesis pathways may also be regulated translationally and/or posttranslationally (Ayu-sawa et al., 1986; Sherley and Kelly, 1988).

FA is a cofactor in one-carbon metabolism, and it promotes the remethylation of homocysteine. Folate deficiency allows the accumulation of intracellular homocysteine, a potentially neurotoxic amino acid that can induce DNA strand break, thereby triggering apoptosis (Mattson

and Shea, 2003). FA deficiency and elevated homocysteine levels endanger postmitotic neurons in neurodegenerative disease model mice (Duan et al., 2002; Kruman et al., 2002). To test the effect of homocysteine on sphere-forming neural stem cells, we performed a neurosphere assay in the FA+/dNS+ medium supplemented with 0.1–1000  $\mu\text{M}$  homocysteine. However, no toxic effect was observed with any concentration of homocysteine. Additionally, no inhibitory effect of homocysteine on neuronal differentiation was observed in the *in vitro* differentiation assay (unpublished data). These results suggest that the high concentration of homocysteine due to FA deficiency cannot explain the proliferation failure of neural stem cells observed in our study.

De novo DNA synthesis inhibitors, such as MTX and AZP, are reported to induce selective malformation of the rhombencephalic and telencephalic brain regions in rat embryo cultures (Schmid, 1984) and neural tube defects (NTD) in rabbits (Lloyd et al., 1999). In humans, NTD can be prevented by FA supplementation in the periconceptual period (Czeizel and Dudas, 1992; Daly et al., 1995). Although FA deficiency does not cause NTDs in normal mice (Heid et al., 1992) or in cultured rat embryos (Cockroft, 1991), Fleming and Copp (1998) identified a mouse model of NTD, namely, homozygous Splotch (Pax3) mouse embryos, in which exogenous FA and thymidine prevent NTDs in culture. This observation suggested that the disturbance of DNA synthesis was involved in the pathogenesis of NTDs.

Our observation may explain the vulnerability of embryonic brain to FA deficiency. Craciunescu et al. (2004) reported that FA deficiency decreased progenitor cell proliferation and increased apoptosis in the fetal mouse brain (Craciunescu et al., 2004). In this study, we showed that the activity of de novo DNA synthesis is necessary for primary cultured neural stem cells to proliferate with multipotency. Glial cells can proliferate under the activation of either de novo or salvage DNA synthesis pathway whereas neuronal cells cannot be generated under the activation of salvage pathway alone. Neural tube defect can be prevented by administration of FA during the periconceptual period, which may result from activation of de novo DNA synthesis and differentiation to neurons from neural stem cells. Further studies are necessary to elucidate a role of de novo DNA synthesis pathway in differentiation from neural stem cells into neurons in both embryonic and adult central nervous system.

## 4. Experimental procedures

### 4.1. Neurosphere culture

Neurosphere cultures were prepared as described previously (Reynolds and Weiss, 1996). In brief, striata were removed from E12.5, E14.5, and E16.5 C57BL/6 mice (Japan SLC, Hamamatsu, Japan) embryos and were mechanically dissociated. In place of the complete D-MEM/F-12 medium (Invitrogen, San Diego, CA), which contains 6  $\mu\text{M}$  FA, 1.5  $\mu\text{M}$  Thy, and 1.5  $\mu\text{M}$  hypoxanthine, the cells were cultured in a custom-prepared deficient D-MEM/F-12 medium lacking FA, Thy, and hypoxanthine in order to stress the availability of precursors for both the *de novo* pathway (folate derivatives) and salvage pathway (Thy, hypoxanthine) of DNA synthesis. The cells were resuspended in the deficient D-MEM/F-12 medium supplemented with N-2 supplement (Invitrogen) (progesterone, 20 nM; apo-transferrine, 100  $\mu\text{M}$ ; sodium selenite, 30 nM; insulin, 25  $\mu\text{g}/\text{ml}$ ), 20 ng/ml human recombinant (hr-) basic FGF-2 (R&D Systems, Minneapolis, MN), 20 ng/ml hr-EGF (Upstate Biotechnology, Lake Placid, NY), and various concentrations of FA and dNS as described. FA was purchased from Takeda Pharmaceutical Co. Ltd. (Tokyo, Japan) and prepared as a 15 mg/ml stock solution before it was added to the culture at a final concentration of 1.0–400  $\mu\text{M}$ . 2'-deoxyadenosine (dAdo), 2'-deoxycytidine (dCyd), 2'-deoxyguanosine (dGuo), Thy, azathioprine (AZP), and methotrexate (MTX) were purchased from Sigma (St. Louis, MO) and prepared as a 20 mM stock solution in water. Viable single cells at a density of  $1.0 \times 10^5$  cells/ml were seeded into 75-cm<sup>2</sup> tissue culture flasks and incubated at 37 °C in an atmosphere of 5% CO<sub>2</sub> and treated with 20 ng/ml basic fibroblast growth factor (bFGF) every other day. Secondary neurosphere cultures were prepared by mechanical dissociation of 7 days *in vitro* (DIV) primary neurospheres into single cells and resuspended at a density of  $2.0 \times 10^4$  cells/ml in a fresh culture medium. For neurosphere counting, viable single cells at a density of  $1.0 \times 10^4$  cells/ml were seeded into uncoated 96-well microplates. It has been demonstrated previously that culturing cells at this density will result in clonal neurosphere colonies, as form in single-cell cultures, and that neurospheres do not arise as a result of cell aggregation at the cell culture densities used here (Seaberg and van der Kooy, 2002; Tropepe et al., 2000). To assess their differentiation potential, 7-DIV spheres were plated onto poly-L-ornithine-coated chamber slides (Nunc, Naperville, IL) in D-MEM/F-12 supplemented with N-2 supplement and 10% fetal bovine serum (differentiation condition) and cultured for 4 DIV before fixation for immunocytochemistry. For neuron counting, 7 DIV spheres were mechanically dissociated, and viable cells were resuspended at a density of  $5.0 \times 10^4$  cells/ml and plated onto the precoated chamber slides under the differentiation condition. Cell numbers and viability were assessed by trypan blue dye exclusion by using a hemocytometer.

**Fig. 6 – Inhibitors of de novo DNA synthesis decrease tubulin  $\beta$  III-immunopositive neurons in the neurosphere culture. (A)** Experimental protocol. Neurospheres were cultured under the FA+/dNS-, FA-/dNS+, and FA+/dNS+ conditions supplemented with AZP or MTX for 7 DIV. **(B and C)** Dose–response curves of sphere formation, cultured for 7 DIV under each condition supplemented with AZP **(B)** and MTX **(C)**. Among the three conditions, the FA+/dNS- condition was the most sensitive to the inhibitors. Graphs show mean  $\pm$  SEM number of spheres per  $1 \times 10^4$  cells. **(D)** Experimental protocol. Spheres formed under the conditions, as described above, were placed under the common differentiation condition. **(E)** Quantitative analysis of the effects of de novo inhibitors. Graph shows mean  $\pm$  SEM percentage of tubulin  $\beta$  III-immunopositive neurons of the total number of cells per field derived from the spheres under the FA+/dNS+ condition or under the FA+/dNS+ condition with inhibitors. \*\*Significant difference compared with the FA+/dNS+ condition ( $P < 0.01$ ). **(F–I)** Double immunocytochemistry for neurons (tubulin  $\beta$  III, red) and astrocytes (GFAP, green). Representative fields derived from the spheres under the FA+/dNS+ conditions **(F)**, FA-/dNS+ **(G)**, FA+/dNS+ with 1  $\mu\text{M}$  AZP **(H)**, and FA+/dNS+ with 0.1  $\mu\text{M}$  MTX **(I)** conditions are shown. White arrowheads indicate tubulin  $\beta$  III-positive neurons. Scar bars: F–I, 50  $\mu\text{M}$ .