

Protein levels of genes encoded on chromosome 21 in fetal Down syndrome brain: Challenging the gene dosage effect hypothesis (Part II)

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Summary. Down syndrome (DS) is the most common genetic cause of mental retardation. To explain the impact of extra chromosome 21 in the pathology of DS, gene dosage effect hypothesis has been proposed, but several investigators including our group have challenged this hypothesis. Although analysis of the sequence of chromosome 21 has been essentially completed, the molecular and biochemical mechanisms underlying the pathology are still unknown. We therefore investigated expression levels of six proteins encoded on chromosome 21 (HACS1, DYRK1A, α A-crystallin, FTCD, GARS-AIRS-GART, and CBS) in fetal cerebral cortex from DS and controls at 18–19 weeks of gestational age using Western blot analysis. Protein expression of HACS1 was significantly and remarkably decreased in DS, and the expression levels of five proteins were comparable between DS and controls suggesting that the gene dosage effect hypothesis is not sufficient to fully explain the DS phenotype. We are continuing to quantify proteins whose genes are encoded on chromosome 21 in order to provide a better understanding of the pathobiochemistry of DS at the protein level.

Keywords: Chromosome 21 – Down syndrome – HACS1 – DYRK1A – α A-crystallin – FTCD – GARS-AIRS-GART – CBS

Abbreviations: DS, Down syndrome; HACS1, hematopoietic adapter containing Src homology 3 domain and sterile α motifs; DYRK1A, dual specificity tyrosine phosphorylated and regulated kinase; α A-crystallin, alpha crystallin subunit A; FTCD, formiminotransferase cyclodeaminase; GARS-AIRS-GART, glycylamide ribonucleotide synthetase-aminoimidazole ribonucleotide synthetase-glycinamide ribonucleotide formyltransferase; CBS, cystathionine β -synthase; NSE, neuron specific enolase; GFAP, glial fibrillary acidic protein

Introduction

Down syndrome (DS), trisomy 21, is the most frequent chromosomal aberration syndrome and genetic cause of mental retardation. In addition to mental retardation, individuals with DS show a series of neuropathological features, including a reduction of brain size, abnormal neuronal migration, differentiation and abnormal dendritic arborization. However, how three copies of normal genes on chromosome 21 segment can lead to complex metabolic and developmental aberration is still unknown. To explain the impact of an extra chromosome 21 on the pathology of DS, several hypotheses have been proposed. One of these is “gene dosage effects”. This hypothesis holds that dosage imbalance of a specific individual gene or small group of genes from chromosome 21 is responsible for specific individual DS traits. However, the gene dosage effect hypothesis has been challenged with several studies suggesting that not all genes present in chromosome 21 are overexpressed in DS brain (Greber-Platzter et al., 1999; Engidawork et al., 2001a).

The essentially complete sequence of chromosome 21 provides a rich source of data for exploring chromosome functional organization and will accelerate discoveries concerning the disease mechanisms of DS. One important research goal is now to determine

which of the chromosome 21 genes are contributing to the development of the DS phenotypes and which are not. Although a large series of proteins have been found to be deranged in adult DS brain (probably confounded by Alzheimer's disease that regularly occurs from the fourth decade), information about proteins on chromosome 21 in developing DS brain is limited so far.

Here we evaluated expression levels of six proteins encoded on chromosome 21-hematopoietic adapter containing Src homology 3 domain and sterile α motifs (HACS1; 21q11.2), dual specificity tyrosine phosphorylated and regulated kinase (DYRK1A; 21q22.13), alpha crystallin subunit A (α A-crystallin; 21q22.3), formiminotransferase cyclodeaminase (FTCD; 21q22.3), glycylamide ribonucleotide synthetase-aminoimidazole ribonucleotide synthetase-glycylamide ribonucleotide formyltransferase (GARS-AIRS-GART; 21q22.11), and cystathionine β -synthase (CBS; 21q22.3) (Fig. 1) – in fetal brains from DS and controls at the early second trimester.

Materials and methods

Fetal brain samples

Fetal brain tissues (cerebral cortex) of DS (4 females with 18–19 weeks of gestational age) and controls (4 females with 18–19 weeks of gestational age) used in this study. Brain samples were obtained from Dr. Mara Dierssen (Medical and Molecular Genetics Center-IRO, Hospital Duran i Reynals, Barcelona, Spain) and Dr. Joan Carles Ferreres (Department of Pathology UDIAT-CD, Corporació Sanitària Parc Taulí, Sabadell, Barcelona, Spain). All samples had a postmortem time of less than 6 hours, were stored at -70°C and the freezing chain was never interrupted until use.

Antibodies

The details of the preparation and characterization of antibodies have been described previously: HACS1 (Claudio et al., 2001); GARS-AIRS-GART (Brodsky et al., 1997); CBS (Chen et al., 1999). Polyclonal rabbit antibody was developed from C-terminal peptides of α A-crystallin (VSREEKPSSAPSS) and no cross-reaction with α B-crystallin observed. Five antibodies for DYRK1A (Santa Cruz, USA), FTCD (Sigma, USA), neuron specific enolase (NSE; Chemicon, UK), glial fibrillary acidic protein (GFAP; Chemicon, UK), and actin (Sigma, USA), were purchased.

Western blotting

Fetal brain tissues ground under liquid nitrogen were homogenized in lysis buffer containing protease inhibitor cocktail tablet (Rache, Germany) at 4°C and centrifuged at $8,000 \times g$ for 10 minutes. The BCA protein assay kit (Pierce, USA) was applied to determine the concentration of protein in the supernatant. Samples ($10\mu\text{g}$) were mixed with the sample buffer (100mM Tris-HCl, 2% SDS, 1% 2-mercaptoethanol, 2% glycerol, 0.01% bromophenol blue, pH 7.6), incubated at 95°C for 15 minutes and loaded onto a ExcelGel SDS

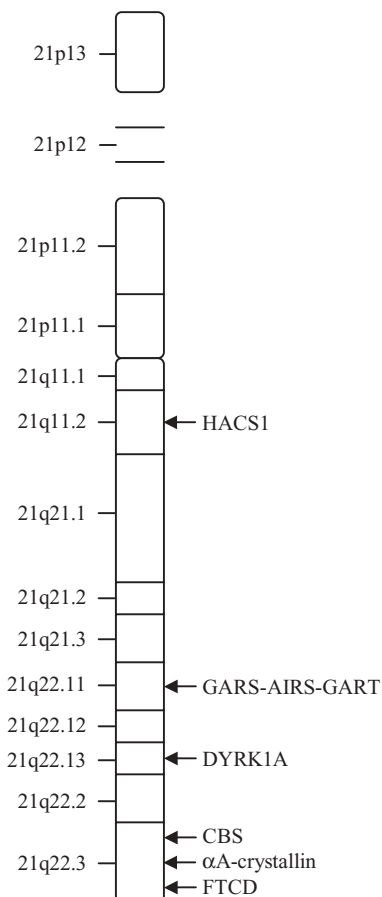


Fig. 1. Giemsa banding (G-bands) of human chromosome 21. Arrows indicate six proteins, products of genes encoded on chromosome 21, used in this study

homogenous gel (Amersham Pharmacia Biotech, Sweden). Electrophoresis was performed with Multiphor II Electrophoresis System (Amersham Pharmacia Biotech). Proteins separated on the gel were transferred onto PVDF membrane (Millipore, USA) and membranes were incubated in blocking buffer (10mM Tris-HCl, pH 7.5, 150mM NaCl, 0.1% Tween 20 and 2% non-fat dry milk). Membranes were incubated for 2 hours at room temperature with diluted primary antibodies (1:200 for DYRK1A (G-19); 1:500 for HACS1 and FTCD; 1:1,000 for α A-crystallin and actin; 1:3,000 for NSE; 1:5,000 for GARS-AIRS-GART, CBS, and GFAP). After 3 times washing for 15 minutes with blocking buffer, membranes were probed with secondary antibodies coupled to horseradish peroxidase (Southern Biotechnology Associates, Inc., USA) for 1 hour. Membranes were washed 3 times for 15 minutes and developed with the Western LightningTM chemiluminescence reagents (PerkinElmer Life Sciences, Inc., USA).

Statistics

The density of immunoreactive bands was measured by RFLPscan version 2.1 software program (Scanalytics, USA). Between group differences were calculated by non-parametric Mann-Whitney U test using GraphPad Instat2 program and the level of significance was considered at $P < 0.05$.

Results

We evaluated the expression level of six proteins encoded on chromosome 21 (HACS1, DYRK1A, α A-crystallin, FTCD, GARS-AIRS-GART, and CBS, Fig. 1) in fetal brains with DS compared to controls by Western blot analysis (Fig. 2). Three proteins, NSE, GFAP, and actin were used as reference proteins for neurons, astrocytes, and total cells, respectively, and none of them showed the different expression levels in DS compared to controls. The anti-DYRK1A antibody recognized one major band and one minor band, whose molecular masses were approximately 200kDa and 80kDa, respectively. We detected two bands of 110kDa and 50kDa with anti-GARS-AIRS-GART antibody. Two bands of 60kDa and 45kDa were also observed with anti-CBS antibody. Interestingly, HACS1 was significantly decreased in fetal DS and this decrease was even more pronounced when related to actin level (Fig. 3). However, the density of immunoreactive bands of five proteins except for HACS1 was comparable between DS and controls. When levels of five proteins were normalized with those of actin, no difference was observed between two groups as shown in Fig. 3.

Discussion

HACS1 encodes a protein containing two domains, SH3 and SAM, which are protein interaction motifs and are predominantly seen in signaling molecules, adaptors, and scaffold proteins. The presence of SH3 and SAM in HACS1 and its mainly cytosolic localization have suggested that it may act as a cytoplasmic adaptor to mediate a signaling pathway (Claudio et al., 2001). Recently, a predicted protein (termed Nash1) with a nuclear localization signal, SAM and SH3 was also mapped to chromosome 21q11.1 (Uchida et al., 2001), but the function and expression level in DS brain for Nash1 remain to be tested. Based on the considerable decrease of HACS1 protein expression in fetal DS brain, HACS1 could play a critical role in the development of fetal DS brain. However, many questions remain to be answered. We are currently studying the consequence of the decreased HACS1, including binding partner protein, SH3 – signal pathway and are about to generate a knock-out mouse for this gene.

DYRK1A, the human homolog of the *Drosophila minibrain* (mnB) gene, maps to the so-called DS

critical region of human chromosome 21. DYRK1A encodes a serine-threonine kinase, which is expressed during neuroblast proliferation in *Drosophila*. DYRK1A has been reported to have a predominantly nuclear location (Becker et al., 1998). It is therefore likely that DYRK1A phosphorylates nuclear substrates such as the transcription factor Forkhead in rhabdomyosarcoma that has been implicated in the control of gene expression by insulin, as well as the regulation of apoptosis by survival factors. Several studies have been performed to identify the biological function of DYRK1A. Mutant mnB flies have reduced optic lobes and central brain, and show learning deficits and hypoactivity (Heisenberg et al., 1985). Transgenic mice with a yeast artificial chromosome containing DYRK1A gene exhibited some alterations analogous to those found in DS pathology, such as memory deficits (Reeves et al., 1995). In addition, transgenic mice overexpressing DYRK1A have shown neurodevelopmental delay, motor abnormalities and cognitive deficits suggesting a causative role of DYRK1A in mental retardation and in motor anomalies of DS (Altafaj et al., 2001). Interestingly, DYRK1A mRNA overexpression has been reported in fetal DS brain and Ts65Dn mice (Guimera et al., 1999). In our study, the anti-DYRK1A antibody recognized one major band and one minor band, whose molecular masses were approximately 200kDa and 80kDa, respectively. Human DYRK1A is a protein of 763 amino acids with a calculated molecular weight of 84.552kDa. Other groups have also reported size inconsistency and multiple bands with DYRK1A in mouse and rat tissues (Rahmani et al., 1998; Okui et al., 1999). We can not explain the inconsistency in size and presence of two bands, but same migration pattern of the 200kDa and 80kDa proteins was observed when nuclear extracts from HeLa cells were used as a reference (data not shown), and statistics did not show any difference between DS and controls for either major band or minor band. Considering that its closet relative, the protein kinase MNB of *Drosophila*, is presumably involved in postembryonic neurogenesis, a signaling pathway regulated by brain DYRK1A protein could normally function before the birth of individuals with DS.

Alpha-crystallin is a major lens protein and belongs to the small heat-shock protein family of molecular chaperones. It is a polymeric complex of two ~20kDa subunits (α A and α B). The expression of α -crystallin in a variety of tissues outside the lens and its ability to

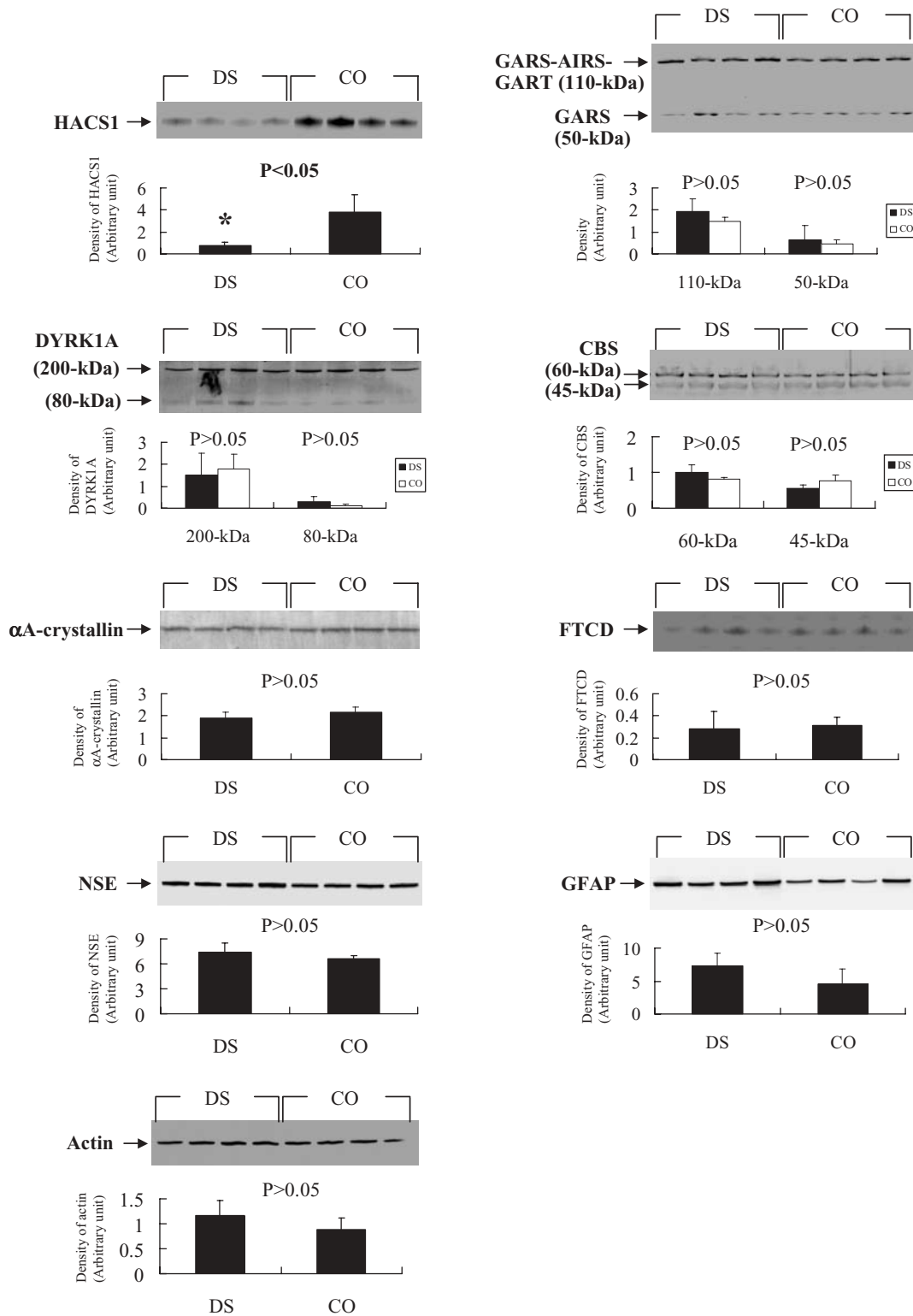


Fig. 2. Western blot analysis for six proteins whose proteins are encoded on chromosome 21 in cerebral cortex from fetal brain with DS and controls. Denatured proteins (10 μ g) were loaded, separated on a homogeneous gel and transferred onto PVDF membrane. As described in "Materials and methods", the membranes were incubated with primary and secondary antibodies, and immunoreactive bands (HACS1, 49.5 kDa; DYRK1A, 200 and 80 kDa; α A-crystallin, 25 kDa; FTCD, 50 kDa; GARS-AIRS-GART, 110 and 50 kDa; CBS, 60 kDa and 45 kDa; NSE, 45 kDa; GFAP, 48 kDa; actin, 42 kDa) were detected using chemiluminescence reagents. The density of detected bands was measured and calculated by non-parametric Mann-Whitney U test, and the level of significance was considered at $P < 0.05$

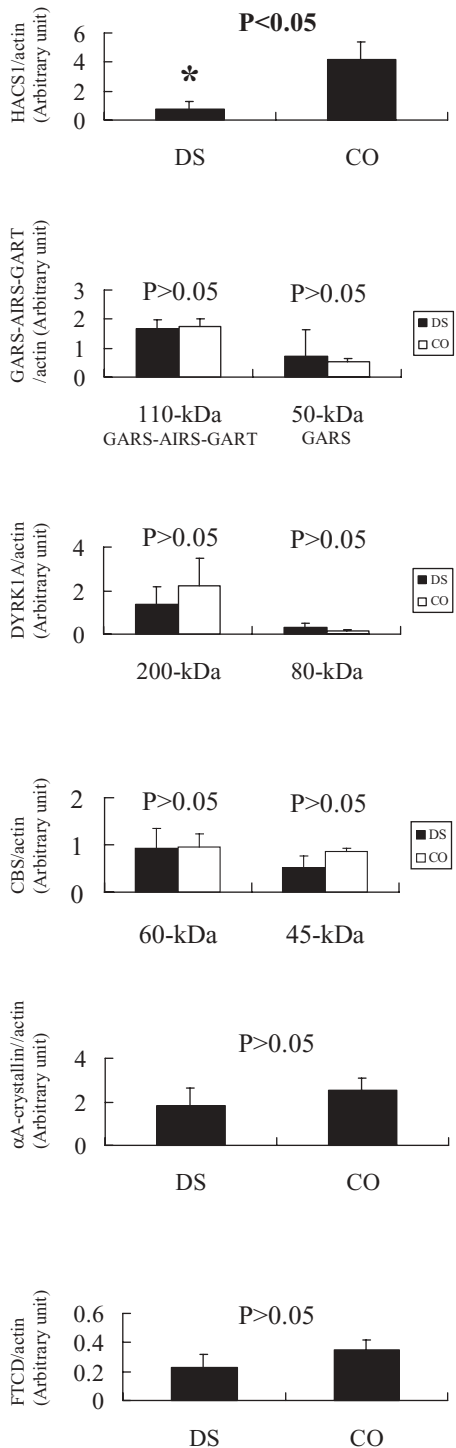


Fig. 3. Expression levels of six proteins normalized with those of actin. The density of immunoreactive band for each protein was normalized with that of actin, which was used as a reference protein

prevent thermal aggregation of protein in a manner similar to molecular chaperones suggest that it has general cellular functions over and above its role in light refraction (Horwitz, 2000). Several studies

indicate that the expression of α A- or α B-crystallin enhances the resistance of cells to a variety of stress conditions and both of crystallin subunits have the ability to protect cells from apoptosis induced by cytokines, protein kinase C inhibitor staurosporine, and ultraviolet A radiation (Andley et al., 2000). The ability of α -crystallin to prevent apoptosis may be linked to an inhibition of caspase activity (Kamradt et al., 2001). The unchanged levels of caspase-3 expression with unaltered levels of apoptosis-related proteins has been observed in fetal brain suggesting that enhanced apoptosis may not be apparent in the brain development of DS (Engidawork et al., 2001b). Interestingly, in the nervous system of α A-crystallin transgenic mice, α A-crystallin protein expression appeared in specific cells (astrocytes and Schwann cells), and α A-crystallin transgenic mice develop a peripheral and central neuropathy primarily affecting spinal cord areas at the dorsal side, dorsal root and sciatic nerve (De Rijk et al., 2000). Taken together the unaltered expression levels of chaperon proteins including α B-crystallin in fetal DS brain (Yoo et al., 2001), the comparable expression of α A-crystallin in fetal DS brain could suggest that the polymeric complex, α -crystallin, could function normally during the development of fetal DS brain.

FTCD has predominantly a metabolic function, catalyzing two sequential reactions in the histidine degradation pathway. The FT domain of this bifunctional intermediate metabolism enzyme first transfers the formimino group of formiminoglutamate to tetrahydrofolate (THF) to produce formimino-THF and glutamate, and then the CD domain catalyzes the cyclization of the folate intermediate to produce methenyl-THF and releasing ammonia (for review see Shane and Stokstad, 1984). In addition, FTCD has been shown to be identical to the 58K Golgi protein, initially proposed to link Golgi membranes to the microtubule cytoskeleton. Recent study indicated that FTCD binds to polyglutamated residues preferentially found in brain tubulin and does not interact with tubulin from other sources (Bashour and Bloom, 1998). Based upon reports that the metabolism involved in the breakdown of histidine occurs in only a few tissues such as liver and kidney (Fowler, 2001), and that brain was shown to contain very low levels of FTCD (consistent with our present results, Bashour and Bloom, 1998), FTCD might have, in addition to its enzyme activity, a second physiological function in mediating interaction of Golgi-derived membranes

with microtubules. FTCD also participates in the interaction of the Golgi complex with the vimentin intermediate filament cytoskeleton suggesting that it might be a candidate protein integrating the Golgi compartment with the intermediate filament cytoskeleton (Gao and Sztul, 2001). Here we observed comparable protein expression levels of FTCD between fetal DS and controls suggesting that FTCD may function normally in histidine degradation pathway and the Golgi dynamics during the development of fetal DS brain. Furthermore, methenyl-THF, the functional product of FTCD, can be produced and used in both purine synthesis and homocystein metabolism which are regulated by other chromosome 21-encoded proteins, GARS-AIRS-GART and CBS, respectively (see below).

Purine biosynthesis plays an essential role in a number of different cellular processes. Purine nucleotides are precursors for RNA and DNA, coenzymes, energy transfer molecules and regulatory factors. In human, a trifunctional protein with GARS, AIRS and GART enzymatic activities can catalyze the second, third and fifth steps of *de novo* purine biosynthesis, which is necessary for the conversion of phosphoribosyl pyrophosphate to inosine monophosphate. This gene encodes not only the trifunctional protein GARS-AIRS-GART of 110kDa, but also a monofunctional protein GARS of 50kDa. The expression of both GARS and GARS-AIRS-GART proteins are regulated during development of the human cerebellum. These proteins are expressed at high levels during normal prenatal cerebellum development, while becoming undetectable in this tissue shortly after birth. In contrast, GARS and GARS-AIRS-GART proteins continue to be expressed during the postnatal development of the cerebellum in DS (Brodsky et al., 1997). In our studies, we also detected both GARS and GARS-AIRS-GART proteins in cerebral cortex of fetal brain and expression levels of both proteins were unchanged in fetal DS brain relative to controls. These results imply that both GARS and GARS-AIRS-GART proteins may play a normal role in the regulation of purine biosynthesis in prenatal stage of fetal DS brain.

The first enzyme of the cysteine biosynthetic pathway, CBS, catalyzes the condensation of homocysteine with serine forming cystathionine. CBS deficiency causing plasma homocysteine accumulation (leading to homocystinuria) also results in S-adenosylhomocysteine accumulation. S-adenosyl-

homocysteine is a potent inhibitor of the various methyltransferases. When methylation is inhibited, synthesis of creatine, sarcosine, lecithin and methylation of protein and nucleic acids are affected. Homocysteine administration during avian embryonic development has been shown to induce congenital defects of the heart and neural tube (Rosenquist et al., 1996). In contrast, in cases of homocystinuria due to CBS deficiency, there is no evidence of neural tube or cardiac dysmorphogenesis at birth (Watanabe et al., 1995). Recently, the child with trisomy 21 had a five-fold increase in cystathionine level relative to normal children (Al-Gazali et al., 2001), consistent with overexpression of CBS. In addition, elevated CBS expression in DS results in low plasma homocysteine compared with non-DS individuals (Pogribna et al., 2001). Human CBS gene encodes native enzyme of 63kDa subunit for homotetramer and this protein is partially cleaved to yield 45kDa active core by sequential trypsin proteolysis (Kery et al., 1998; Dong et al., 1997). In the present study, we detected two bands of 60kDa and 45kDa with anti-CBS antibody and immunoreactivity for both bands was comparable between fetal DS brain and controls. In consist with this result, same band pattern was observed when Western blot analysis was done with yeast strain expressing human CBS protein (Shan et al., 2001). Considering the study that neurological and cardiovascular abnormalities develop several weeks after birth in human homocystinuria patients (Watanabe et al., 1995), our finding may support the hypothesis that the biological consequences of homocysteine accumulation would start to appear after birth.

We, herein, report the decreased levels of HACs1 and unaltered expression of five proteins of chromosome 21 in fetal DS brain, and these results do not support the gene dosage effect hypothesis. Now we are in process of quantifying all gene products of chromosome 21. These studies may provide the basis for better understanding of the pathobiochemistry of DS, and could reveal target molecules for therapeutic interventions.

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