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ORIGINAL ARTICLE

No Evidence for Mutations that Deregulate GARS–AIRS–GART Protein Levels in Children with Down Syndrome

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Abstract *GARS–AIRS–GART* is crucial in studies of Down syndrome (DS)-related mental retardation due to its chromosomal location (21q22.1), involvement in de novo purine biosynthesis and over-expression in fetal DS brain postmortem samples. *GARS–AIRS–GART* regions important for structure–function were screened for mutations that might alter protein levels in DS patients. Mutation screening relied on multiplex/singleplex PCR-based amplification of genomic targets followed by amplicon size determination/fingerprinting. Serum protein samples were resolved by SDS-PAGE and immunoblotted with a GARS–AIRS–GART monoclonal antibody. No variation in amplicon size/fingerprints was observed in regions encoding the ATP-binding, active site residues of GARS, the structurally important glycine-rich loops of AIRS, substrate-binding, flexible and folate-binding loops of GART or the poly-adenylation signal sequences. The de novo occurrence or inheritance of large insertion/deletion/rearrangement-type mutations is therefore excluded. Immunoblots show presence of GARS–AIRS–GART protein in all patient samples, with no change in expression levels with respect to either sex or developmental age.

Keywords Down syndrome-related mental retardation · GARS–AIRS–GART · Mutation screen · Immunoblot · Survival

Introduction

Down syndrome (DS), resulting from trisomy 21, is the most common genetic cause of mental retardation (MR), a complex and variable phenotype, characterized by developmental delay and cognitive impairment [1]. Human glycinamide ribonucleotide synthetase (*GARS*)–aminoimidazole ribonucleotide synthetase (*AIRS*)–glycinamide ribonucleotide transformylase (*GART*) is an important candidate gene in studies of DS-related MR by virtue of localization to chromosome 21q22.1 [2, 3], over-expression in fetal postmortem DS cerebellum [4] and biochemical function in de novo purine biosynthesis [5]. *GARS–AIRS–GART* spans ~38 kb with 22 exons [6] and the cDNA has been cloned by functional complementation in *Escherichia coli* [7]. The gene harbors two polyadenylation signal sequences in intron 11 which participate in alternative splicing leading to the generation of monofunctional *GARS* and tri-functional *GARS–AIRS–GART* transcripts [8]. Purines play vital roles in embryonic development [9, 10] and nervous system function [11]. Aberrant purine metabolism has also been reported in DS [12, 13].

We wondered if DS-related MR arises, in part, from aberrant *GARS–AIRS–GART* function due to mutation. Mutations could directly alter function or indirectly affect susceptibility to MR. As illustrated in Fig. 1, the inheritance of

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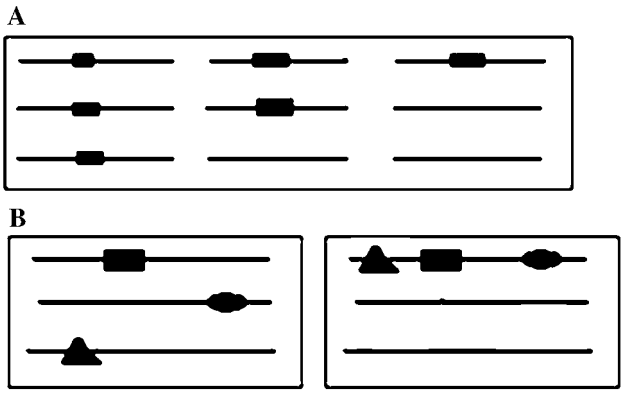

Fig. 1 Possible dosage effect of a mutation being present in single or multiple copies on a trisomic chromosome. The *solid black rectangle* represents mutation. Combination of mutations (represented by *solid black rectangle*, *triangle* and *oval*) in different regions of *GARS–AIRS–GART* may disrupt epistatic interactions

single/multiple copies of a mutation gives rise to a dosage effect; however, a combination of mutations in different regions of the *GARS–AIRS–GART* gene that encodes distinct enzymatic functions may disrupt epistatic interactions and account for the range of phenotypic severity of DS-related MR. Interestingly, the inheritance of 1–4 copies of mutations in the α-globin chain disrupts interactions in the $\alpha_2\beta_2$ complex of haemoglobin leading to the observed range of severity of β-thalassemia [14]. Accordingly, *GARS–AIRS–GART* regions previously identified as being crucial for alternative splicing [8], structure–function or regulation of gene expression [15] were prioritized for screening of mutations that could potentially alter protein levels in DS patients. We used multiplex/singleplex PCR-based target amplification followed by amplicon size determination and fingerprinting. This approach has been successful in the screening of mutations in the *dmd* [16], *CFTR* [17] and α-globin genes [14]. Patient serum protein samples were subjected to immunoblotting followed by enhanced chemiluminescence to assess change(s) in GARS–AIRS–GART protein levels.

Materials and Methods

Subject Ascertainment and Diagnostic Procedures

The sample included 76 DS patient families (51 trios, 25 duos) and 8 singletons recruited from the Out-patients Department of MRIH following clinical and psychological evaluation. All patients met diagnostic criteria as per the DSM-IV-TR [18]. Detailed clinical history, developmental milestones and socioeconomic variables were recorded by means of a structured questionnaire. The sample comprised of 84 DS patients having mean age of 6.79 ± 4.56 years. For DS cases below 5 years of age, the developmental delay was evaluated by functional assessment; in other children with DS, the degree of MR was assessed by means of Weschler's IQ testing procedure [19]. A sample of ~5 ml peripheral blood was collected from each individual for genetic and biochemical analyses after securing written informed consent from the guardians and prior approval for the study from the Institutional Ethics Committee of Manovikas Kendra.

Molecular Screen for Mutations

Genomic DNA was isolated from whole blood lymphocytes as described previously [20]. All reactions were performed in the DNA engine Thermal Cycler (MJ Research PTC 0200). Primers were designed with Primer3 software (http://hpc.ilri.cgiar.org/cgi-bin/primer3_www.cgi); forward primers for intron 11A and 11B were as reported previously [8]. Primer sequences and amplification conditions are detailed in Table 1 and amplification procedures were standardized as described previously [21, 22]. PCR products were resolved by poly-acrylamide gel (16%) electrophoresis [45 V constant voltage for ~17 hours]. Size discrimination of bands and estimation of band intensity values was performed with Quantity One software (BioRad, CA). The average background intensity value (ABIV) for each gel, obtained from intensities recorded at 3 randomly selected regions of the gel without band, was subtracted from recorded intensity values to obtain specific band intensities. The signal saturates at 255 intensity units.

Size and sequence of the amplification products of exons 5, 6, 9 (encoding ATP-binding and Active site residues of GARS) were further confirmed by DNA sequence analysis of all 84 DS patient samples, in Applied Biosystems 3130 Genetic analyzer using Big Dye, v 3.1 chemistry and analysis was done using Sequencing Analysis Software, v 5.2 (Applied Biosystems) and Chromas v 2.33.

Quantification of Serum GARS–AIRS–GART and GARS Protein Levels

Protein levels were estimated by immunoblot assay of serum protein samples from 71 DS cases [39 males and 32 females with mean age 6.4 ± 3.9 years]. The serum was isolated by centrifugation (3,000 rpm, 10 min at 4°C) of plasma and the Bradford assay was used to estimate protein concentrations [23]. Protein samples were dissolved in reducing Laemmli buffer containing [62.5 mM Tris–Cl (pH 6.8), 100 mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue and 10% glycerol] [23] by boiling for 10 min. Equal amounts (10 μg) of protein were resolved by 10% SDS-PAGE at 20 mA constant current for 2 hours followed by wet transfer to nitrocellulose membrane (NC; Schleicher & Schuell, Keene, N.H.) at 110 volts (constant voltage) for $2\frac{1}{2}$ hours at 4°C. The blots were placed in

Table 1 Primer sequences and cycling conditions for multiplex and singleplex PCR reactions

	Site	Primer sequences	Annealing temperature and duration	
MULTIPLEX SET I	Ex-5	F-5′- TGGTTTATTGGATGTTGACGA-3′ R-5′-CAGAAGACCCAGCCTACCTG-3′	61.3°C for 30 secs	initial denaturation at 95°C (5 mins), 30 cycles of denaturation at 95°C (45 secs), annealing, elongation at 72°C (30 secs), final elongation at 72°C (10 mins)
	Ex-6	F-5′-AATCAGGAGAAAGCCTTTGG-3′ R-5′-CACTACAAAAGCCAAAAAGATGAA-3′		
	Ex-9	F-5′-CATCATAGATTTTCTTTCTCTCTTTTT-3′ R-5′-GATATTTTTACCCACTTGGCACTC-3′		
	Ex-15	F-5′-TTCTTGTCTTATCCCACCAG-3′ R-5′-GCATTTCCAAGCAGGACAGT-3′		
	Int-11A	F-5′-CTTCATGATAGCGTAAGTTTGG-3′ R-5′-AGGATTTCAGACTGGCCTTG-3′		
MULTIPLEX SET II	Ex-13	F-5′-TCCTCTCCAACAGGCTGTAAA-3′ R-5′-GGCCTTTATTCACAAAGGTGTC-3′	62°C for 30 secs	
	Ex-18	F-5′-GTTTGAAATGAAGGTTCCCC-3′ R-5′-GGAAGCCACATCTCACCTGT-3′		
	Ex-19	F-5′-CTGTTTTTCTCCCTTCTCAG-3′ R-5′-CACAGCAAAGATATTTTCCC-3′		
	Int-11B	F-5′-ACTGAAGATGAGAATACTGGTC-3′ R-5′-TCAAAACACCCACATTTCCA-3′		
SINGLEPLEX SET	Ex-12	F-5′-AATTTTTCCCTGACGTTTAGGTG-3′ R-5′-GATCACTGACCTGATCTGGAA-3′	62°C for 30 secs	
	Ex-20	F-5′-TCATTGAAAAGGGTTGTGCTT-3′ R-5′-ACAAAGGGGCCAGAAAGAAT-3′	61.4°C for 30 secs	
	Ex-21	F-5′-GGATTTTTAAATTCTGACCACTATGC-3′ R-5′-GACATACTCACAGCTACAAA-3′	50°C for 30 secs	
	Ex-22	F-5′-CTTTTGCTTACTTTTCCAG-3′ R-5′-TTGTTTTTAGTGAGTCTC-3′	52.3°C for 30 secs	

buffer containing 5% non-fat milk in 0.1 M PBS (pH 7.4) and 0.2% Tween-20, overnight at 4°C. Blots were incubated with 1:2000 dilution of GARS–AIRS–GART monoclonal antibody [Abnova (Taiwan) Corp.] for 2 hours at room temperature (RT) followed by extensive washing with buffer [0.1 M PBS, 0.2% Tween 20; 6 min × 5]. Subsequently blots were incubated with 1:5000 dilution of Horse Radish Peroxidase-conjugated secondary goat-anti-mouse antibody (IgG) [Bangalore Genei, India] for 2 hours at RT. Washing was repeated as before and the antigen was detected using the enhanced chemiluminescence (ECL, Amersham) reagent. Control immunoblot with anti-cyclophilin monoclonal antibody was prepared after stripping the previous blot with buffer [62.5 mM Tris–Cl (pH 6.8), 100 mM 2-mercaptoethanol and 2% SDS] and re-probing with 1:500 dilution of Cyclophilin monoclonal antibody. The films (XBT-5, Kodak) were subjected to densitometric scanning followed by ratiometric analysis of band intensities corresponding to the GARS–AIRS–GART and GARS proteins. Statistical tests (student *t* test and one way ANOVA) were performed using SigmaPlot 10 software (Cranes software International Limited, India).

Results

We screened 11 out of 22 exons and 2 introns of the *GARS–AIRS–GART* gene for mutations in either singleplex or multiplex reactions. The exons coding for ATP-binding and active-site residues of *GARS* (exons 5, 6, 9) were co-amplified with the exon 15 encoding for the 3rd glycine-rich loop of *AIRS* [15] and a poly-A signal sequence within Intron 11 (Intron-11A) [8] in Multiplex Set-I reactions along with singleplex controls (Fig. 2a). Exons 13, 18, 19 that include the 2nd glycine rich loop of *AIRS* and the substrate binding loop of GART, respectively, [15] were co-amplified with the Poly-(A) signal sequence located within Intron 11B [8] in Multiplex Set-II reactions (Fig. 2b). The singleplex controls for both multiplex sets (Figs. 2a, b) confirm amplicon size and region-specific co-amplification. Exons 12, 20, 21, 22 encoding 1st glycine-rich loop of *AIRS*, flexible- and folate-binding loops of *GART*, respectively [15] were amplified in individual singleplex reactions (Fig. 2c). Neither singletons nor DS patient family samples revealed differences in amplicon size or fingerprints. The results exclude de novo occurrence and/or inheritance of large insertion/deletion/rearrangement-type of mutations. Estimation of band intensity values for both multiplex PCR amplicons and corresponding singleplex controls, as detailed in Supplementary Table 1, indicate that all PCR fragments were amplified without bias for target sequence complexity (Supplementary figures S1–S6). Amplicon fingerprint comparisons rule out allelic loss due to selective/non-selective amplification.

Furthermore, we confirmed size and sequences of exons 5, 6 and 9 by direct sequencing as described in methods.

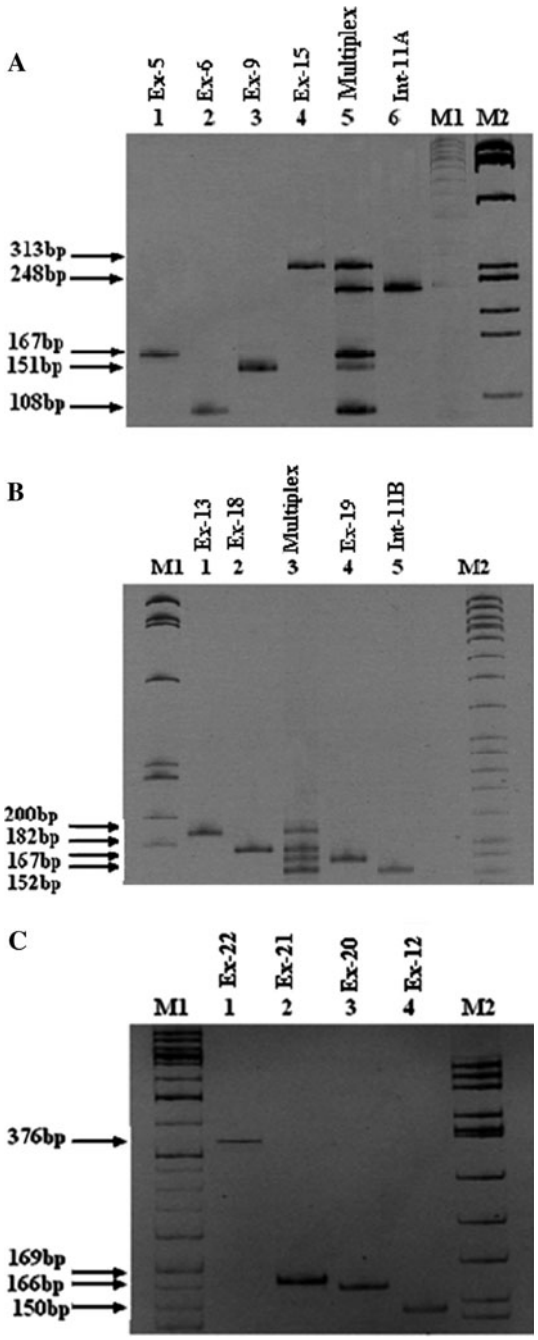

Fig. 2 **a** Multiplex Set-I with corresponding singleplex controls for amplicon size determination, amplicon fingerprint and amplification efficiency as described in "Methods". *Lanes* M1 and M2 represent migration of DNA molecular weight markers 20 bp DNA ladder and ΦX174DNA/*Hae*III digest. **b** Multiplex Set-II with corresponding singleplex controls for amplicon size determination, amplicon fingerprint and amplification efficiency as described in "Methods". *Lanes* M1 and M2 represent migration of DNA molecular weight markers ΦX174DNA/*Hae*III digest ladder and 20 bp DNA. **c** Singleplex target amplification under conditions as described in methods. *Lanes* M1 and M2 represent migration of DNA molecular weight markers 20 bp DNA ladder and ΦX174DNA/*Hae*III digest

Exon 5 encodes ATP-binding amino acid residues, exon 6 encodes ATP-binding residues and exon 9 encodes ATP-binding residues and active site residue 296P. The results exclude the occurence of small in/del and/or point mutations in these functionally important exonic regions. Representative chromatograms along with corresponding BLAST results are provided as supplementary data (Supplementary figures S7–S10).

Immunoblotting reveals the presence of both full-length GARS–AIRS–GART (~110 kDa band) and GARS (~55 kDa band) proteins [4] that are derived from alternatively spliced transcripts [7, 8] (Fig 3a). Both proteins are detectable in all DS patient samples tested. Relative band intensity ratios (110 kDa:55 kDa) plotted as a function of patient sex and age (Fig. 4), indicates that the mean ratio ranges from 1.10–1.30. The significant difference ($p = 0.037$) of relative band intensity ratios between male and female probands in the 8.5–10.5 years age group (Fig. 4) is merely due to small and unequal sample size (Suppl. Table 2). The gene for human peptidyl-prolyl isomerase-A (cyclophilin) is located on chromosome 7 that is disomic in all DS individuals. Control immunoblots with anti-cyclophilin monoclonal antibody demonstrated similar band intensities in all lanes suggesting that an equal amount of total protein was loaded in each lane (Fig. 3b).

Discussion

Primary screening of *GARS–AIRS–GART* regions involved in alternate splicing, structure function or gene regulation does not reveal de novo occurrence or the inheritance of large insertion/deletion/rearrangement mutations or point mutations that could potentially impair function by changing protein levels in DS patients. Mutation detection depends on the effect of natural selection on the variant allele in a given population [24] and purifying selection may eliminate mutations that grossly alter GARS–AIRS–GART protein levels or function. It is possible that the small size of exons screened (size range 108–376 bp) may preclude mutation detection by the multiplex PCR method. This is unlikely since in Duchenne Muscular Dystrophy, 6 exons (size range 82–186 bp) of the *dystrophin* gene harbour deletions that could be detected using the same technique [16].

The evolutionary conservation of de novo purine biosynthetic enzymes [25] and their compartmentalization into purinosomes [26] suggests that they are less prone to mutations. Biochemical assays have indicated a delicate interplay between de novo and salvage pathway of purine biosynthesis in the 2–4 cell stage [9]. Mutant CHO cell lines isolable by media supplementation with hypoxanthine [4], harbor G/A transitions resulting in non-synonymous changes at amino acid positions 75 and 684 of GARS and

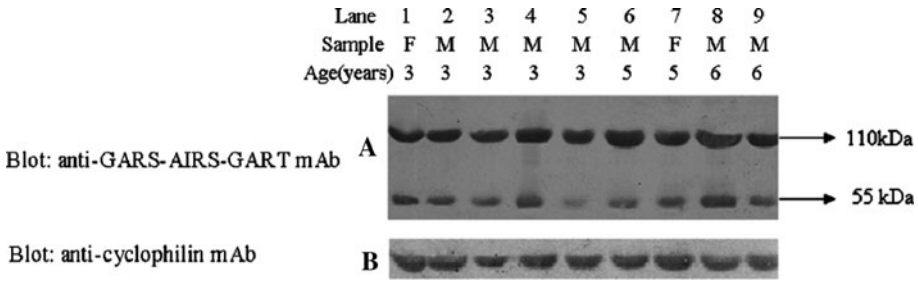

Fig. 3 **a** Western blot of GARS–AIRS–GART and GARS proteins detected in serum protein samples of male (M) and female (F) DS patients **b** Control immunoblot of cyclophilin protein

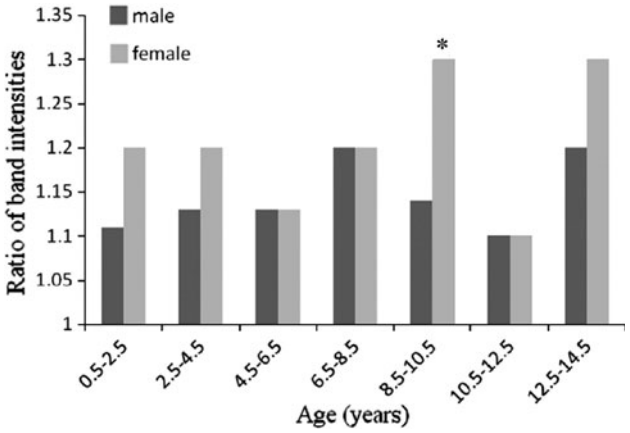

Fig. 4 Mean band intensity ratio of GARS–AIRS–GART to GARS protein as a function of age. The *asterisk* denotes significant difference ($p = 0.037$, C.I.95%) between male [6] and female [3] samples as revealed by student's t test

AIRS, respectively, with abolished enzyme activities but no change in protein stability [27]. This raises the possibility that GARS–AIRS–GART may be critical for survival rather than DS-related MR.

Our study shows for the first time that there is no difference in GARS–AIRS–GART protein levels with respect to either sex or developmental age of children with DS. The immunoblot results (Fig. 3) indicate that the tri-functional GARS–AIRS–GART and mono-functional GARS proteins are both detectable in all samples examined. The *GARS–AIRS–GART* gene undergoes alternate splicing to generate two transcript isoforms [8]. We have previously determined that the 3′-UTR of the shorter transcript forms an energetically stable secondary structure [$\Delta G = -103.7$ kcal/mol] [15] that may regulate translation of the 55 KDa GARS protein. The variable intensity of the 55 kDa band may arise from a post-transcriptional effect.

The de novo pathway is crucial in early human development [5], deregulation of which may cause embryonic lethality. Indeed, a large proportion of DS fetuses die before birth [1]. However, immunoblot analysis revealed two bands corresponding to tri-functional GARS–AIRS–GART and monofunctional GARS in the fetal postmortem DS cerebral cortex samples and control fetuses of 18–19 weeks gestational age [28] suggesting unaltered expression levels of these proteins in DS and controls [28, 29]. Analysis of fetal post-mortem DS cerebellum samples reveals that GARS–AIRS–GART fusion protein expression decreases from the 21st week of gestation to 49 days post natal, whereas, the GARS protein is constitutively expressed [4]. Their study did not address the expression of GARS–AIRS–GART protein in other brain regions.

Microarray studies of heart tissue from trisomy-21 fetus with mean gestational age 16.8 weeks when compared to age-matched controls reveals a 3.02-fold over-expression of GARS–AIRS–GART [30]. In Ts65Dn mice that exhibit segmental trisomy for genes on human chromosome 21, only four tissues (cerebral cortex, heart, testis and liver) show the 1.5-fold overexpression rule with regard to gene expression [31]. These observations may reflect tissue- and development stage-specific regulation of gene expression.

All genes on chromosome 21 (HSA 21) could be regarded as candidates with respect to the study of DS-related MR, since MR is present to varying degree in all DS patients. The DS critical region (DSCR) contains an estimated ~33 of ~300 genes on chromosome 21 [32, 33], of which only a subset may be involved in nervous system development. A high-resolution analysis of human segmental trisomies has indicated that more than one critical region on HSA21 is responsible for the DS-related MR phenotype [34]. Accordingly, aberrant function of additional genes on HSA21 may also contribute to DS-related MR.

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