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Perturbed metabolic profiles associated with muscle weakness seen in adult Ts1Cje mouse model of Down syndrome

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Abstract

Down syndrome (DS) is a genetic condition resulting from a partial or full triplication of human chromosome 21. Besides intellectual disability, DS is frequently associated with hypotonia. Ts1Cje, mouse model of DS, displays the muscle weakness characteristic. The metabolic profiles of the skeletal muscle was characterised using ^{1}H nuclear magnetic resonance spectroscopy and multivariate data analysis. Ts1Cje muscle had significantly decreased levels of glutamine, guanidinoacetate, adenosine monophosphate, and histidine, suggesting perturbation of energy, glutamate, and histidine metabolic pathways. Glycine amidinotransferase/arginine glycine amidinotransferase enzyme-linked immunosorbent assay indicated this mitochondrial enzyme was 74% and 50% lower in Ts1Cje kidney and liver than the wildtype respectively. In conclusion, our findings suggest that perturbed metabolite profiles contribute to muscle weakness in Ts1Cje skeletal muscle.

Key Words: Down syndrome, skeletal muscle, metabolomics

Down syndrome (DS) is a genetic condition resulting from partial or full triplication of human chromosome 21[21,26)]. The extra copy of chromosome 21 alters normal gene expression and eventually leads to a series of clinical manifestations such as cognitive impairment, craniofacial abnormalities,

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and hypotonia. Hypotonia is a condition of low muscle tone often accompanied by a slower response speed together with a reduction in muscular endurance[18].

Overexpression of human chromosome 21 genes in DS patients are known to cause disruption of metabolic pathways that have contributed to pathophysiological features of DS. For example, overexpression of cystathionine β-synthase gene located on chromosome band 21q22.3 has been found to cause perturbation of homocysteine metabolism and eventually lead to generalized alteration of cellular methylation capacity among DS individuals[23]. The effect of metabolic disruption in DS pertaining to the skeletal muscle development and function leading to hypotonia has not been extensively investigated.

Ts65Dn is the the most widely studied mouse model and it exhibits with common DS features including muscle weakness[12]. However, Ts65Dn is trisomic for a subcentromeric region (larger than 5.8 Mb) on MMU17 which is not syntenic to HSA21. Nineteen trisomic genes which are not syntenic to HSA21 is reported[13] and may contribute to other pathologies such as cardiac and gastrointestinal abnormalities[16]. In this study, we employed Ts1Cje mouse model of DS, to identify possible mechanisms involved in DS-associated hypotonia, focusing on metabolic profiles of the Ts1Cje skeletal muscles. Ts1Cje has a partial triplication of chromosome 16 extending from the murine superoxide dismutase gene to the zinc finger protein 295 gene, wherein the extra superoxide dismutase gene is functionally inactivated[27]. The synteny of this segment has homology to the region in human chromosome band 21q22.1 to 22.3. Recent studies reported that Ts1Cje displays reduced grip strength and locomotor activity compared to disomic, wild-type littermates[3].

All experiments that involved animal breeding and handling were approved by the Institutional Animal Care and Use Committee and were performed in accordance with institutional regulations on experimental animals (Reference number: UPM/IACUC/AUP-R003/2014). Sex matched wild-type littlemates were employed as control for this study. All mice were aged from postnatal days 56 to 70. Ts1Cje and disomic mice were generated by mating Ts1Cje males with C57BL/6J females for over 10 generations. Their genotypes were determined by tail genotyping as described previously[17]. All mice were bred under controlled environmental conditions with a 12-h light/12-h dark cycle at 21–23°C and 55% relative humidity. Food and water were available *ad libitum* with a standard pellet diet. All mice were euthanized by cervical dislocation after being anesthetized in a chamber containing 2.5% isoflurane in 100% oxygen.

Gastrocnemius muscle of 11 male mice (disomic wild-type/WT mice, $n = 5$; trisomic Ts1Cje mice, $n = 6$) and snap frozen over liquid nitrogen and stored at −80°C. Precooled ultrapure water (4 mL/g tissue) was added to each tissue sample and homogenized. The resulting supernatant was lyophilized in a freeze drier. The remaining pellet was added with precooled extraction solvent (1 : 1 chloroform: methanol, 4 mL/g tissue) and homogenized. The resulting supernatant was dried by nitrogen gas evaporation and stored at −80°C.

Lyophilized hydrophilic metabolites were resuspended in 580 μL of phosphate buffer containing 100 mM sodium phosphate, 20% deuterium oxide (D_2O; Merck Millipore, Nottingham, UK), and 0.5 mM 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (TSP; Sigma-Aldrich, MO, USA). The lyophilized hydrophobic metabolites were resuspended in 580 μL of chloroform-d containing 0.03% (v/v) tetramethylsilane (TMS; Merck Millipore, Nottingham, UK). An aliquot of 550 μL of the supernatant was transferred subjected to Nuclear Magnetic Resonance (NMR) acquisition using a Bruker AVIII 600-MHz spectrometer (Bruker Biospin, Fallenden, Switzerland) with a 5-mm BB(F)O broadband probe operating at 600.13 MHz (ambient probe temperature, 27°C).

Both unsupervised principal component analysis (PCA) and supervised partial least squares discriminant analysis (PLS-DA) were employed with SIMCA-P 8.0 software (Umetric, Umea, Sweden) to visualize and interpret experimental differences[4,38]. The statistical significance and validity of subsequent results were calculated by performing a permutation test (number of permutations = 1000) on the normalized models. A covariance plot was used to aid interpretation of the significance of each metabolite from the permutation tests[2,31]. The color projected onto the spectrum indicates the correlation of the metabolite peak intensity, discriminating Ts1Cje mice from their corresponding control littermates; blue indicates no significant difference ($P > 0.05$), and red indicates a highly significant difference ($P < 0.01$). The direction and magnitude of the signals relate to the covariation of the metabolites with the classes in the model.

Liver, kidney and gastrocnemius muscle samples dissected from 6 male mice (WT mice, $n = 3$; Ts1Cje mice, $n = 3$) were snap frozen over liquid nitrogen and stored at −80°C for future use. Precooled phosphate buffer containing 1% of protease inhibitor (Merck Millipore, Nottingham, UK) (9 mL/g tissue) was added to each tissue sample, homogenized for 5 min, followed by centrifugation at 5,000 ×g for 5 min at 4°C. The resulting supernatant was used for glycine amidinotransferase (GATM/AGAT) protein quantification.

The levels of GATM present in each samples were determined using double antibody sandwich enzyme-linked immunosorbent assay (ELISA) developemental kit (Wuhan Fine Biological Technology Co., Ltd, Wuhan, China) and those procedures were performed according to the manufacturer's instructions. Significant differences between Ts1Cje and WT controls were determined using a Student's *t*-test.

In the multivariate data analysis, full resolution of complementary ^{1}H NMR spectra data sets for both hydrophilic and hydrophobic metabolite profiles were first analyzed separately using unsupervised PCA to identify any inherent differences within the dataset. The resulting score plot of principle component 1 (PC1) and principle component 2 (PC2) for the hydrophilic metabolite profile (Fig. 1a) showed no discrimination between the two groups, suggesting that the major sources of variation in the data set were not related to genotype but rather, dominated by interindividual variability. However, the PCA of the hydrophobic metabolite profile generated a negative Q^2 value ($Q^2 = -1.03\%$; $R^2 = 30.5\%$), indicating that the model is not predictive at all.

Subsequently, supervised PLS-DA was likewise applied to reveal differences in genotype-related information between the ^{1}H NMR hydrophilic and hydrophobic metabolite profiles of WT and Ts1Cje mouse skeletal muscle. The resulting model for the hydrophilic metabolite profile showed a systemic difference between the two groups ($Q^2 = 20.1\%$; $R^2 = 79\%$), suggesting that there is a significant difference between the hydrophilic metabolic profile of the two genotypes (Fig. 1b). On the other hand, the systemic PLS-DA comparison between the hydrophobic metabolite profile of WT and Ts1Cje mice gave a very low Q^2 value ($Q^2 = 2.5\%$; $R^2 = 41.1\%$), indicating no significant difference between the skeletal muscle hydrophobic metabolic profiles of the two groups.

Later, a permutation test was performed to statistically validate the result of the PLS-DA analysis on the hydrophilic metabolite profile. The results of the permutation test are presented in the covariance plot (Fig. 1c), which shows lower levels of skeletal muscle glutamine, guanidinoacetate (GAA), adenosine-monophosphate (AMP) and histidine in the Ts1Cje samples.

GATM/AGAT is a key enzyme for creatine synthesis by producing GAA from glycine and arginine. GAA, on the other hand, is the immediate biosynthetic precursor of creatine. We anticipated that decreased level of GAA in Ts1Cje mouse skeletal muscle may be caused by enzymatic deficiency in glycine amidinotransferase (GATM/AGAT) in the primary organs of creatine biosynthesis (kidney and liver) and creatine

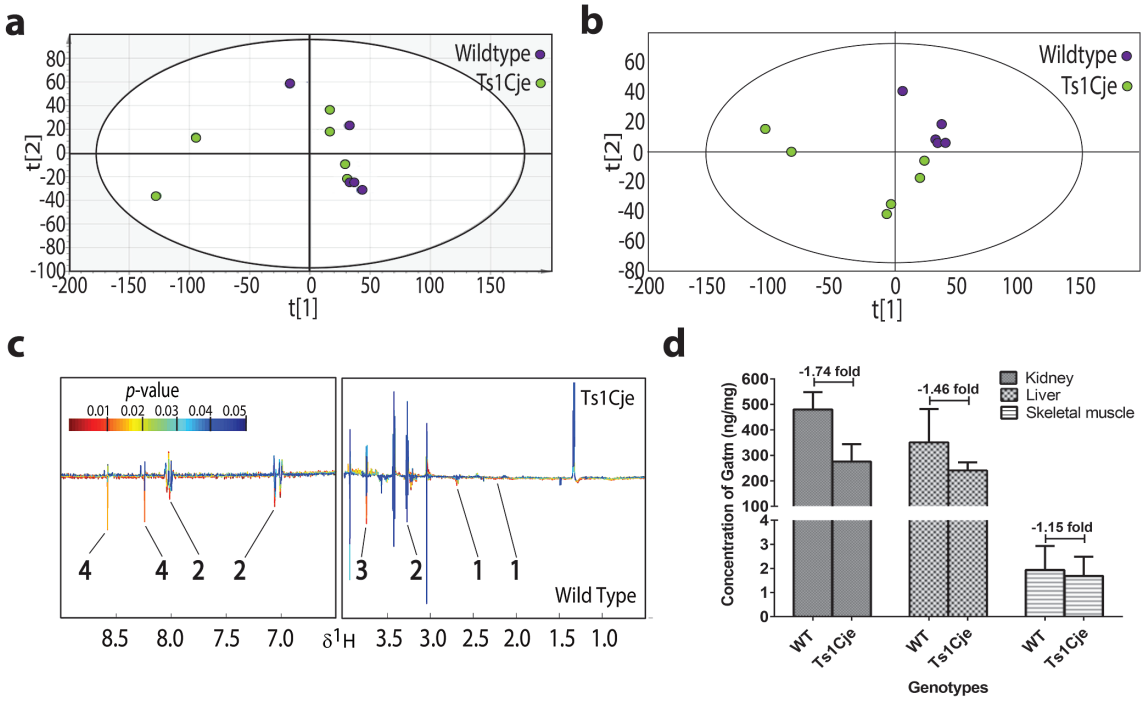

Fig. 1. Metabolic profiling of trisomic Ts1Cje skeletal muscle. (a) PCA score plot for the first two principle components ($Q^2 = 27.1\%$; $R^2 = 48.1\%$) derived from the ^{1}H NMR spectra of skeletal muscle hydrophilic extract of male adult trisomic Ts1Cje (green) and disomic wild-type control (blue) mice. The resulting score plot of PC1 and PC2 for the hydrophilic metabolite profile showed no discrimination between the two groups. **(b)** PLS-DA score plot for the first two principle components ($Q^2 = 20.1\%$; $R^2 = 79\%$) derived from the ^{1}H NMR spectra of skeletal muscle hydrophilic extract of male adult trisomic Ts1Cje (green) and disomic wild-type control (blue) mice. The resulting model showed a systemic difference between the two groups ($Q^2 = 20.1\%$; $R^2 = 79\%$). **(c)** Covariance plot derived from ^{1}H NMR spectra of the skeletal muscle hydrophilic extract of trisomic Ts1Cje and disomic wild-type control mice ($Q^2 = 20.1\%$; $R^2 = 79\%$). The significance of each metabolite from the permutation test is interpreted using the covariance plot, on which the colours projected onto the spectrum indicate the significance of the metabolites (blue indicates no significant difference at $p > 0.05$ confidence levels and red indicates a highly significant difference at $p < 0.001$ regions). The direction and magnitude of signals related to the covariation of the metabolites with the classes in the model. Group comparison was calculated for the groups, trisomic Ts1Cje and disomic wild-type control mice. Key: 1. glutamine; 2. histidine; 3. guanidinoacetate; 4. adenosine mono-phosphate. **(d)** ELISA indicates that the level of GATM/AGAT is lower in tissue homogenates of Ts1Cje as compared to wild-type.

storage site (skeletal muscle). We detected that GATM/AGAT levels in Ts1Cje were 1.70-fold (70%), 1.50-fold (50%) and 1.20-fold (20%) lower than those of WT in kidney, liver and gastrocnemius respectively (kidney: WT = 480.1 ± 68.176 ng/mg, Ts1Cje = 275.52 ± 68.727 ng/mg; liver: WT = 350.74 ± 131.476 ng/mg, Ts1Cje = 240.44 ± 31.911 ng/mg; gastrocnemius: WT = 1.94 ± 0.9947 ng/mg, Ts1Cje = 1.69 ± 0.7993 ng/mg) of total protein (Fig. 1d).

The current ^{1}H-NMR-based metabolomics analysis has shown significant differences in the metabolite composition of skeletal muscle between Ts1Cje mice and their WT littermates, especially with respect to the hydrophilic metabolites. Four hydrophilic metabolites, including GAA, histidine, AMP and glutamine were found to be at lower levels in Ts1Cje mouse skeletal muscle, indicating alterations in energy, glutamate, and histidine metabolic pathways.

Decreased levels of GAA in Ts1Cje mouse skeletal muscle is indicative of an alteration in energy metabolism as GAA is a biosynthetic precursor of creatine, which plays an important role in storage and transmission of phosphate-bound energy in tissues with high energy demands[32]. In arginine-GAA-creatine metabolism, GAA is synthesized by transamidation of the

guanidine group from arginine to glycine via mediation by L-arginine:glycine amidinotransferase (AGAT). Therefore, a decrease in GAA, could be due to a reduction in AGAT activity or a defect in glycine and arginine synthesis pathways. However, Coppus *et al.* previously reported that DS individuals had higher plasma glycine and arginine levels than age-matched healthy controls. Higher plasma glycine and arginine levels among DS individuals may reflect accumulation of these substrates due to deficient AGAT activity[9,10]. Moreover, the AGAT gene knock-out mouse model not only displays reduced grip strength and severe muscle atrophy, it also exhibits major metabolic abnormalities, including impairment of oxidative phosphorylation capacity, increased overall mitochondrial content, increased glycolytic capacity and mitochondrial dysfunction[20]. Interestingly, these abnormalities are similar to those found in various cells and tissues of DS individuals[5,7,35]. Hence, it is tempting to suggest there may be deficiency of AGAT activity in the Ts1Cje mouse that causes a decrease in GAA levels. Thus, its role to development of hypotonia in Ts1Cje mice warrants further investigation.

The reduction in the AMP in Ts1Cje mouse skeletal muscle is another indicator of altered energy metabolism. Dysregulation of energy metabolism resulting from mitochondrial dysfunction has been extensively demonstrated in DS patients and is responsible for many pathologies of DS[1,4,11,19]. The mitochondrion plays a critical role in energy metabolism, generating energy in the form of adenosine triphosphate (ATP) during cellular respiration. During intense exercise, free energy is released by breaking the high-energy phosphoanhydride bonds on ATP to form AMP and a chain of two phosphates, known as a pyrophosphate[19]. Impairment of mitochondrial ATP synthetic machinery is commonly reported in DS. For example, the mitochondrial ATP synthase responsible for ATP synthesis) β-chain protein was found to be significantly reduced in the frontal cortex of DS patients[15]. In addition, a previous study reported that skin fibroblasts of DS individuals demonstrated a 40%–45% decrease in the rate of mitochondrial ATP synthesis[34]. Apart from human subjects, the Ts1Cje mouse model has also displayed a significant decrease in mitochondrial ATP production[29]. Altogether, it is obvious that DS subjects suffer from decreased production of ATP. Therefore, the reduced level of AMP observed in the current study could be a consequence of low ATP production in DS. This finding is consistent with a recent study that reported a significant decrease in the cellular adenine nucleotide pool (ATP, adenosine diphosphate and AMP) in the skin fibroblasts of DS individuals, which suggests impairment of oxidative phosphorylation capacity involving ATPase, adenine nucleotide translocator, and adenylate kinase deficits in DS individuals[34]

The decreased level of histidine in the Ts1Cje mouse skeletal muscle indicates a perturbation in histidine metabolism. Histidine is an essential amino acid in mammals, which is widely recognized as a fairly efficient scavenger of both hydroxyl radicals and non radical toxic oxygen species, especially singlet oxygen[37]. Extensive studies have also demonstrated that the concentration of histidine is closely associated with inflammation and oxidative stress status in the body[30]. On the other hand, it has been long-established that DS individuals have an unusually high level of oxidative stress[6,22]. For example, Valenti *et al*. (2011)[34] reported that mitochondrial superoxide production and oxidative stress in DS fibroblasts were 3 times higher than in normal fibroblasts; at the same time, the mitochondria were morphologically abnormal. By correlating the anti-inflammatory and antioxidant properties of histidine with the high level of oxidative stress in DS, it is postulated that the decrease in histidine levels observed in the current study corresponds to a high oxidative stress in Ts1Cje mouse skeletal muscle.

Glutamine is another metabolite that was found to be decreased in Ts1Cje mouse skeletal muscle. These two amino acid (histidine and glutamine) are members of the glutamate family

of amino acids as they are disposed off through conversion of glutamate. The synthesized glutamate is then used in various metabolic events, including the Krebs cycle, where glutamate undergoes a reversible transamination to become α-ketoglutarate[33]. Therefore, a decrease in the levels of histidine and glutamine will consequently lead to a decrease in the levels of glutamate and α-ketoglutarate or vice versa. Brain samples from DS individuals and Ts2 mice (another DS mouse model) were found to have a significantly lower level of glutamate[14,25]. In the Krebs cycle, isocitrate undergoes oxidative decarboxylation to α-ketoglutarate, catalyzed by isocitrate dehydrogenase, and DS individuals have been shown to have reduced isocitrate dehydrogenase activity[24]. Hence, a decrease in the conversion of isocitrate could lead to a decrease in α-ketoglutarate and eventually cause a decrease in glutamate and glutamine levels. Dysregulation of the Krebs cycle has been documented to contribute to oxidative stress in DS[8]. Moreover, a previous study also found that alternations in the Krebs cycle and glutamate metabolism were associated with high levels of oxidative stress[36]. Connecting these various lines of evidence suggests that the alterations in glutamate metabolism in DS are related to the high levels oxidative stress.

Overall, our data indicate that the perturbed metabolic profile of Ts1Cje mouse skeletal muscle suggesting that it plays a role in DS-associated muscle weakness.

LIST OF ABBREVIATIONS

DS – Down syndrome
NMR – Nuclear Magnetic Resonance
PCA – principal component analysis
PLS-DA – partial least squares discriminant analysis
GATM – glycine amidinotransferase
WT – wild-type
GAA – guanidinoacetate
AMP – adenosine-monophosphate

Conflict of interests

The authors declare that they have no conflict of interest.

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Author contributions

All authors of this research paper have directly participated in the planning, execution, or analysis of this study; CLL, K-HL, and P-SC designed the experiments. C-LL, UB, and MP-YL performed experiments and analyzed the data. IKSY, JS, and RR co-supervised experimentation. CLL, K-HL and P-SC drafted the manuscript.

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