ORIGINAL ARTICLE

Expression Profiling of Genes Involved in the Development and Function of Skeletal Muscles in Ts1Cje Mouse Model of Down Syndrome

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ABSTRACT

Introduction: Down syndrome (DS) is caused by trisomy of human chromosome 21 (HSA21). Motor dysfunction due to hypotonia has limited labour productivity and have significant effects on socio-economic status in DS individuals. Ts1Cje, a mouse model of DS that exhibits muscle weakness was employed, to investigate the expression profile of selected trisomic and disomic genes involved in skeletal muscle structure and function. **Methods:** Quadriceps and triceps were harvested from the Ts1Cje (C57BL/6) postnatal day 60-70 mice and corresponding wild-type littermates. Total RNA extracted from these tissues was subjected for quantitative expression profiling of three trisomic genes (*Itsn1, Synj1* and *Rcan1*) involved in neurotransmission and six disomic genes (*Lamc1, Leprel1, Myl6b, Msn, Pgm5* and *Tmod1) essential fo*r maintenance of muscle structure and function. Real-time quantitative PCR method was used for the profiling. **Results:** Differential gene expression in DS is reflected by 1.5-fold or more increase in the level of expression as predicted by the gene dosage imbalance hypothesis. The analysis showed no significant changes in the expression level of trisomic genes (*Itsn1, Synj1* and *Rcan1*). On contrary, disomic genes, *Leprel1* and *Pgm5*, were upregulated for more than 1.5-fold in DS quadriceps whereas *Lamc1, Myl6b* and *Pgm5* were upregulated for more than 1.5 fold in DS triceps as compared to the wild-type group. **Conclusions:** Our findings suggest that the dysregulation of *Lamc1, Leprel1, Myl6b* and *Pgm5* genes is associated to muscle weakness seen in Ts1Cje and may play a role in molecular pathogenesis of muscle weakness in DS.

Keywords: Muscle weakness, Down syndrome, Gene expression, Neurotransmission, Skeletal muscle

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INTRODUCTION

Down syndrome (DS) is a chromosomal disorder caused by trisomy of human chromosome 21 (HSA21) (1). The prevalence of the disorder is 1 in 800 to 1,000 live births across different ethnic groups (2) and 1 in 959 live births in Malaysia (3). DS is characterised with over 70 phenotypes including intellectual disability, cardiac defect (1, 4-5) and hypotonia (weak muscle tone) (6,7). DS individuals suffer with limitation in labour productivity and economic self-dependency, which eventually affect the socio-economic statue of such individual and the community at large. Effort to study the pathogenesis of hypotonia in DS individuals is limited due to the lack of comprehensive investigations at molecular and cellular levels.

Different mouse models for DS have been produced genetically to provide a better understanding on various phenotypes associated with the disorder (8,9). The HSA21 shared a conserved synteny with orthologous regions of three mouse chromosomes, MMU10, MMU16 and MMU17 (10). Ts1Cje, a DS mouse model that carries a shorter region of MMU16 with ~85 genes (*Sod1-Znf295*) synteny to those located on HSA21 (8) was employed in the present study. Triplication of these genes have been associated with features seen in DS individuals (11).

Various hypotheses have been proposed to correlate the genotype and phenotype seen in DS individuals. It is believed that either the entire HSA21 is causing a

global genomic imbalance or specific regions in HSA21 are causing multitude of DS traits via their respective genotype-phenotypes contribution (12). Two most acceptable hypotheses are (i) gene dosage imbalance hypothesis, and (ii) amplified developmental instability hypothesis. The gene dosage imbalance hypothesis suggests that there is an average of 50% higher expression of HSA21 genes in DS samples as compared to the euploid samples (13). This hypothesis is supported by previous transcriptomic studies on the brain and neural progenitor cells isolated from Ts1Cje (14-15). On the other hand, the amplified developmental instability hypothesis postulated that the dosage imbalance of HSA21 genes leads to a general disruption of genomic regulation and expression and further upsets the cellular homeostasis (16).

Ts1Cje mice have profound motor dysfunction with weak muscle strength, poor balance and impaired motor coordination compared with their control littermates (17). Hence, we further dissected the molecular mechanisms that underlie muscle weakness in DS. In this study, three trisomic genes; namely, intersectin1 (*Itsn1*), synaptojanin1 (*Synj1*) and regulator of calcineurin (*Rcan1*) which are involved in neuromuscular transmission were selected for evaluation in the skeletal muscles of Ts1Cje mouse model. These genes are located on the Down syndrome critical region (DSCR) of HSA21 (1) and upregulation of these genes have been shown to cause motor defects by affecting the neuromuscular junction activities and synaptic morphology leading to motor impairment in *Drosophila* (18). To date, the expression profile of these genes has not been reported in Ts1Cje skeletal muscles.

Skeletal muscle is a key player in motor movement and effective motor performance, which depends on its structural and functional integrity. Six disomic genes namely, laminin gamma-1 (*Lamc1*), leprecan-like protein-1(*Leprel1*), myosin light chain 6B (*Myl6b*), Moesin (*Msn*), tropomodulin-1(*Tmod1*) and phosphoglucomutase-5 (*Pgm5*) are known to play essential roles in the maintenance of skeletal muscle structure and function. To the best of our knowledge, the expression profile of these genes has yet to be reported in skeletal muscles of Ts1Cje mouse model and therefore were selected for the study. We hypothesised that global genomic imbalance due to trisomy 21 may dysregulate the expression of genes involved in the maintenance of structural function of the skeletal muscles.

METHODS

Ethical approval, animal breeding, genotyping, and husbandry

Two groups of mice, the Ts1Cje (n = 9) and wild-type (WT) (C57BL/6) (n = 8) at postnatal day 60-70 were used in the study. The Ts1Cje males were mated with the WT C57BL/6 females. Polymerase chain reaction (PCR) technique was used to genotype genomic DNA obtained from mouse tail biopsy. The amplification of the *Grik1* gene alone or both *Grik1* and *Neo* genes indicate WT and Ts1Cje genotypes, respectively (8). Mice were kept in the individually ventilated cages at Genetics and Regenerative Medicine Research Centre (GRMRC), Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM). All mice were housed under controlled temperatures with a 12:12 hour light:dark cycle. The mice were given unlimited access to standard animal feed (Altromin 1324, Germany) and clean water *ad libitum*. This study was approved by the Institutional Animal Care and Use Committee (IACUC), UPM (Reference number: UPM/IACUC/AUP-R030/2015). Animals handling was performed in accordance with IACUC guidelines.

Sample collection

The mice were deeply anaesthetised with inhalation of 4% (v/v) isoflurane to eliminate perception of pain prior to cervical dislocation. The mice were disinfected with 70% ethanol, pinned on dissecting board and the entire skin of the limbs was incised. Quadriceps and triceps which are the largest skeletal muscles of hindlimb and forelimb, respectively, were exposed, excised and collected. Each muscle tissue was rinsed with chilled 1X phosphate buffered saline (PBS) followed by snapped frozen on dry ice and stored at -80°C for RNA isolation.

RNA isolation and cDNA synthesis

Genomic-free total RNA from the skeletal muscle was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen) according to manufacturer's protocol. The concentration, purity and integrity of the extracted RNA were assessed by using the NanoVueTM Plus Spectrophotometer (GE HealthCare), agarose gel electrophoresis and Agilent 2100 Bioanalyzer (Agilent). The RNA sample with A260/280 ratio values ranging from 1.9-2.1 were considered of high purity. RNA Integrity Number (RIN) above 5 was defined as good quality total RNA and RIN above 8 as perfect quality total RNA (scale of 1 to 10, 1 = lowest and 10 = highest) suitable for RT-qPCR analysis (19). First strand of cDNA was synthesized from good quality total RNA using the SuperScript™ III Reverse Transcriptase Kit (Invitrogen), according to manufacturer's protocol, under an RNAse-free environment.

Primer Design and RT-qPCR

Primers were designed using ProbeFinder version 2.45 at the Universal ProbeLibrary (UPL) Assay Design Centre (Roche Applied Science; www.lifescience.roche.com). Relevant UPL probe was selected for each primer. For the selection of the primers, the following parameters were considered; length between 19-23bp, GC % between 45-58, Tm between 55-60 °C and amplicon size between 69-143 bp. All primers were synthesised by 1st BASE (Malaysia) except for *Itsn, Syjn* and *Rcan1*

(Bioneer, Korea). All primers used are tabulated in Table I. A total volume of 10µl of final reaction was prepared for the real-time quantitative PCR (RT-qPCR) containing 1X LightCycler® 480 (LC480) Probe Master Mix (Roche Diagnostics, USA), 0.25µM each of forward and reverse primers, 0.1µM of an appropriate UPL probe (Roche Diagnostics, USA), PCR grade water (Roche Diagnostics, USA) and finally 1µl of diluted (0.2X) synthesised cDNA. The RT-qPCR was performed using LightCycler® 480 Real Time PCR System instrument (Roche Diagnostics, USA). Briefly, the RT-qPCR consists of three different steps; pre-incubation, amplification and cooling step. The samples were pre-incubated (denaturation) at 95°C for 10 minutes followed by amplification for 45 cycles at 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 1 second and a final step (cooling) was at 40°C for 30 seconds.

For each primer set used in the RT-qPCR, a 4-5 data-point standard curve was constructed using serially diluted (0.2X) pooled cDNA samples. The standard curve was used to determine the efficiency and reproducibility of each RT-qPCR assay. Three housekeeping genes; phosphoglycerate kinase 1 (*Pgk1*), proteasome subunit beta type 2 (*Psmb2*) and hydroxymethylbilane synthase (*Hmbs*) were used for data normalisation. An assay was considered successful when the PCR efficiency was between 85-110% with $r^2 > 0.985$ and a minimum of two successful assays for housekeeping genes in each experimental batch. Relative quantification by standard curve method was employed to compare the level of expression of selected genes (20-21). The data obtained was analysed using GraphPad Prism v6 (GraphPad Software) statistical programme and the results are expressed as mean ± SEM. Two tailed unpaired *t*-test was used to compare the $\log_2$ normalised expression of each gene between the Ts1Cje and the WT mice. Genes with expression fold change value of >1.5 or <-1.5 between the two groups are considered differentially expressed according to the gene dosage imbalance hypothesis. The differences are considered statistically significant when *p*-value<0.05.

RESULTS

RNA quality and integrity

All RNA samples were having A260/280 nm absorbance ratios between 1.9 and 2.1 indicating a high purity extracts. In addition, agarose gel electrophoresis of the RNA samples indicated the presence of both 28S and 18S bands representing good RNA integrity (Fig. 1A). Agilent 2100 Bioanalyzer analyses confirmed all extracted RNA samples were of high integrity with RIN between 7.8-9.1 (Fig. 1B).

Table I: List of all the primers used in this study

Name	Pr.	L	Tm	%GC	Sequence (5′→3′)	Size (nt)	UPL Probe ID
Lamc1	F	21	59	52	gaggtgaatggtatgctgagg	130	#16
	R	19	59	58	cattgagctcagcctcctg		
Leprel1	F	22	60	50	ttacactggaccctctttaccg	69	#96
	R	22	60	45	gatccaagatggcaatcacttc		
Msn	F	20	59	55	ggaagagctgatggagaagc	109	#68
	R	20	59	50	cttccgttcctgctcaagtt		
Myl6b	F	20	60	50	caacggctgcatcaactatg	103	#19
	R	20	55	55	cttgcctctcaagcggatac		
Pgm5	F	19	60	58	tcacgaccaggaaccacag	138	#99
	R	20	59	50	atctctcggagtgctctgct		
Tmod1	F	21	59	52	cgtggagtccaacttcatctc	120	#33
	R	20	60	45	actttgttgcccaaaggttg		
Itsn1	F	20	59	50	accagttctcgcctgatgat	139	#18
	R	19	60	58	aggcggaccacaatctctc		
Synj1	F	19	60	53	ctcctgacagccaaagcaa	143	#18
	R	20	59	55	ggcttcagtggttctggaag		
Rcan1	F	20	59	50	cccgacaaacagttcctcat	70	#52
	R	23	60	48	gcatgcagttcatacttctctcc		
Pgk1	F	18	60	61	tacctgctggctggatgg	65	#108
	R	20	60	50	cacagcctcggcatatttct		
Psmb2	F	20	59	55	gagggcagtggagcttctta	71	#25
	R	20	60	50	aggtgggcagattcaagatg		
Hmbs	F	20	59	45	aaagttccccaacctggaat	96	#35
	R	20	60	50	ccaggacaatggcactgaat		

Pr. = Primer; F=Forward; R=Reversed; L= Length (nt); Tm= Melting temperature (°C)

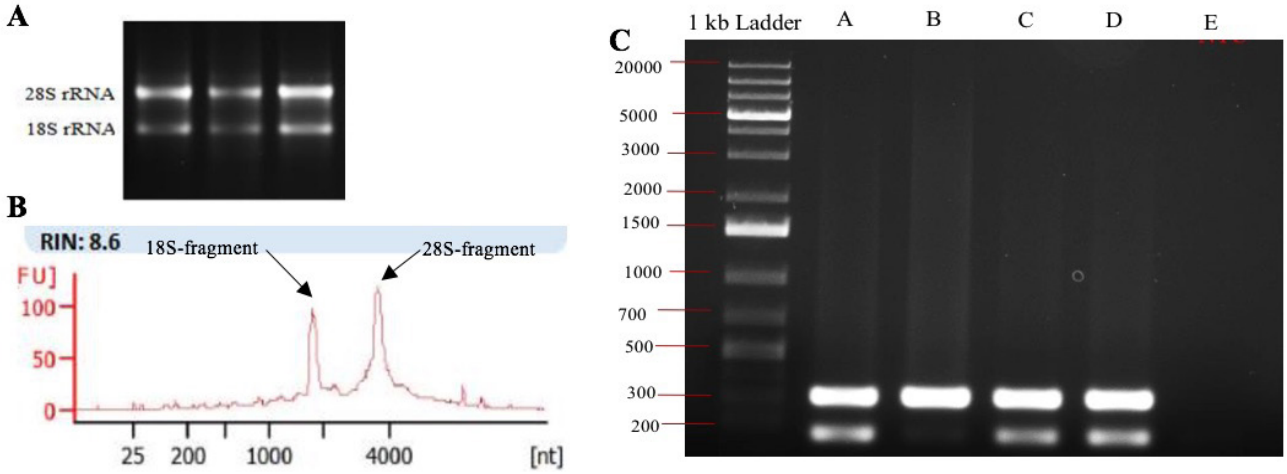

Figure 1: Integrity and quality of total RNA and DNA genotyping. (A) Gel electrophoresis of representative good quality total RNA samples show intact 28S rRNA bands that are approximately twice as intense as the 18S rRNA bands. (B) Representative electropherogram generated by Agilent 2100 Bioanalyser shows the presence of distinctive 18S and 28S peaks indicating high RIN score of 8.6. (C) Gel electrophoresis of PCR amplicons from DNA genotyping analysis. Ts1Cje mice is represented by two bands, *Grik1* (333 bp) and *Neo* (150 bp) (lanes A, C and D) while only *Grik1* was amplified in wildtype sample (lane B). There is no PCR amplification in lane E which served as the negative control (NTC)

Genotyping

The PCR products were subjected to gel electrophoresis for genotyping purpose. The *Grik1* gene lies in chromosome 16 and served as internal control. PCR amplified the *Grik1* gene in WT mice only, while both *Grik1* and *Neo* genes were amplified in the Ts1Cje mouse (Fig. 1C).

Gene expression profile

The expression profiles of three trisomic genes (*Itsn1, Synj1* and *Rcan1*) and six disomic genes (*Lamc1, Leprel1, Myl6b, Msn, Pgm5* and *Tmod1*) were performed. For comparison, a fold change (FC) of ± 1.5 was considered differentially expressed and reported as upregulation or downregulation, respectively. RT-qPCR result showed no differences in the expression of *Itsn1, Synj1*, and *Rcan1* between the Ts1Cje and WT groups for both quadriceps and triceps (Fig. 2A-C and Table II). The FC analysis of all the trisomic genes in both quadriceps and triceps showed very small differences in the level of expression between the Ts1Cje and the WT mice (Table 2) with FC values were relatively close to 1.

No significant differences were observed in the expression level of all selected disomic genes between Ts1Cje and WT groups (Table II, Fig. 2D-I). *Lamc1, Myl6b* and *Pgm5* expression were upregulated for approximately 1.7-, 1.6- and 2.2-fold, respectively, in triceps of Ts1Cje mice as compared with the WT group (Fig. 2D, F & H, Table II). There was more than 1.5-fold upregulation in the *Leprel1* and *Pgm5* expression level in the quadriceps of the Ts1Cje mice as compared to the WT with approximately 1.70- and 3.50-fold, respectively (Fig.2E & H, Table II).

DISCUSSION

For several years, efforts have been made by scientists to understand the relationship between the increased gene dosage and phenotypic appearances such as hypotonia in DS individuals. However, there is no clear understanding of how trisomy 21 causes muscle weakness in DS individuals. It is known that, motor performance is a collective function of the nervous and skeletal systems. A defective muscular system affects the motor function of an individual even in the presence of functional nervous system and vice versa. Thus, in an attempt toward understanding the molecular basis of the muscle weakness in DS, the expression profile of nine protein coding genes in Ts1Cje skeletal muscles was evaluated. The genes of interest included three trisomic genes (*Itsn1, Synj1* and *Rcan1*) that are involved in neuromuscular transmission and other six disomic genes (*Lamc1, Leprel1, Myl6b, Msn, Tmod1,* and *Pgm5*) that are known to play essential roles in structural and functional stability of skeletal muscle.

It is believed that, the presence of an extra copy of chromosome 21 in DS individuals would result in 50% increment in the expression level of all the trisomic genes compared to the disomic genes. *Rcan1, Itsn1* and *Synj1* are trisomic genes located within the triplicated region of *Mmu16* of the Ts1Cje mice. Surprisingly, their expression level in the quadriceps and triceps of Ts1Cje mice was nearly similar to that of the WT mice. Using microarray technique and FC analysis, the expression level of *Rcan1* and *Itsn1* but not *Synj1* was significantly greater than 1.5 in the soleus of another DS mouse model, Ts65Dn (22). Similarly, another study revealed

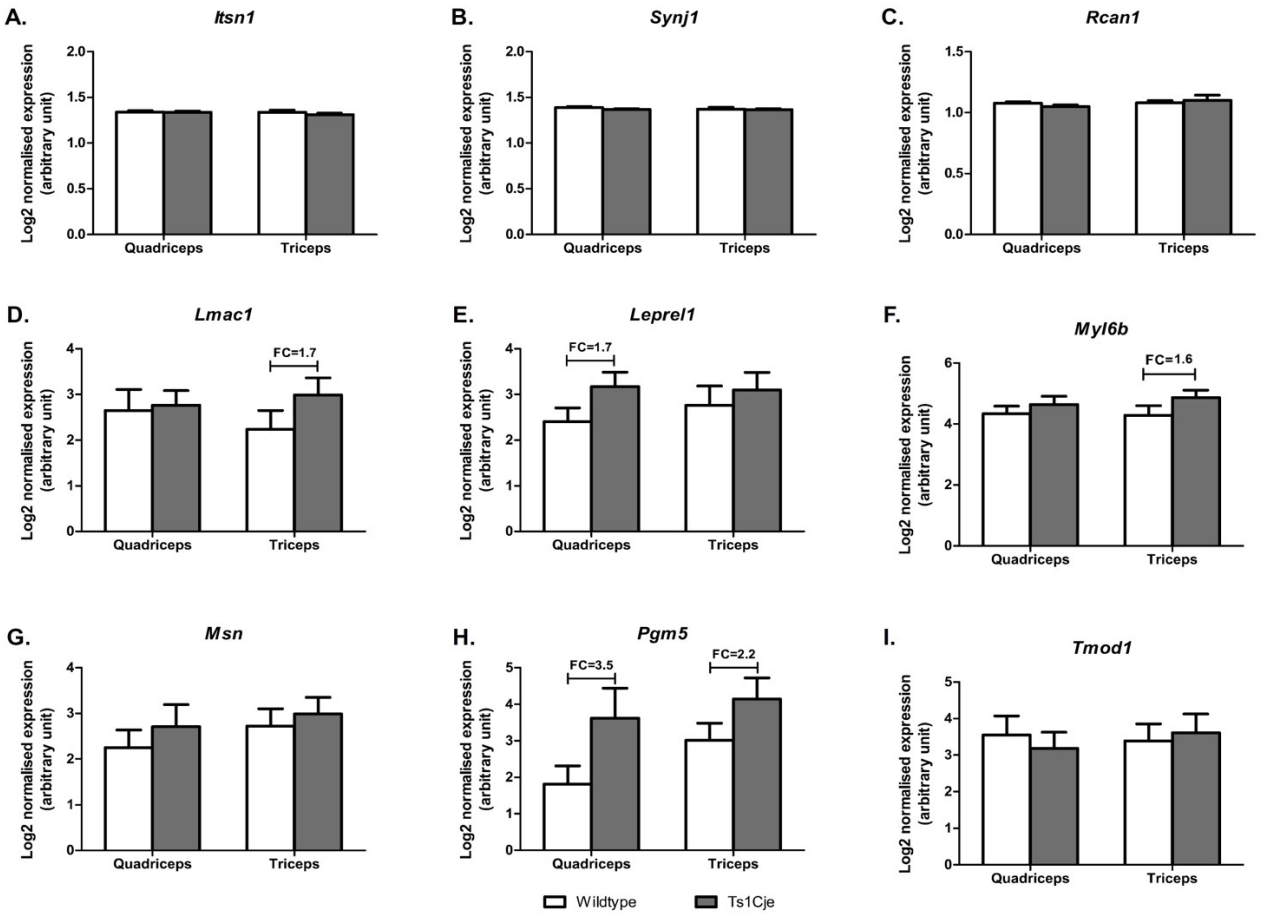

Figure 2: Relative quantitation of gene expression by standard curve method. The bar chart represents the Log2 normalised expression (against *Pgk1, Psmb2* and *Hmbs* housekeeping genes) of selected trisomic and disomic genes for Ts1Cje (n=9) and WT groups (n=8). Values are presented as mean±SEM. Relative gene expression of quadriceps and triceps of Ts1Cje is expressed as fold change (FC) against the WT group.

Table II: Expression profile of the selected genes

Gene group	Gene Name & symbol	Quadriceps				Triceps			
		Ts1Cje (mean ± SEM)	WT (mean ± SEM)	Fold change	P value	Ts1Cje (mean ± SEM)	WT (mean ± SEM)	Fold change	P value
Trisomic genes	Intersectin (*Itsn1*)	1.337 ± 0.013	1.340 ± 0.012	0.9976	0.848	1.311 ± 0.016	1.336 ± 0.024	0.9832	0.407
	Synaptojanin (*Synj1*)	1.369 ± 0.007	1.387 ± 0.012	0.9875	0.199	1.365 ± 0.012	1.373 ± 0.019	0.9943	0.721
	Regulator of calcineurin (*Rcan1*)	1.049 ± 0.014	1.077 ± 0.013	0.9809	0.162	1.100 ± 0.041	1.080 ± 0.016	1.0142	0.652
Disomic genes	Laminin gamma 1 (*Lamc1*)	2.765 ± 0.322	2.650 ± 0.459	1.3134	0.837	2.990 ± 0.371	2.233 ± 0.417	**1.6889** *	0.194
	Leprecan-like 1 (*Leprel1*)	3.169 ± 0.314	2.405 ± 0.301	**1.6990** *	0.101	3.100 ± 0.382	2.764 ± 0.417	1.2630	0.559
	Myosin light polypeptide 6B (*Myl6b*)	4.715 ± 0.300	4.444 ± 0.329	1.2066	0.557	4.970 ± 0.253	4.283 ± 0.321	**1.610** *	0.115
	Moesin (*Msn*)	2.707 ± 0.485	2.253 ± 0.383	1.3690	0.482	2.991 ± 0.358	2.721 ± 0.380	1.2066	0.611
	Phosphoglucomutase 5 (*Pgm5*)	3.623 ± 0.811	1.814 ± 0.500	**3.5016** *	0.086	4.140 ±0.580	3.010 ± 0.465	**2.1886** *	0.156
	Tropomodulin 1 (*Tmod1*)	3.185 ± 0.443	3.547 ± 0.522	0.7778	0.602	3.610 ± 0.515	3.391 ± 0.459	1.164	0.757

* Indicates a fold change of ≥ 1.5 upregulated

an upregulation of *Itsn1* and *Synj1* in an unspecified skeletal muscle in Ts65Dn mice using RT-qPCR (23).

There are two major differences that distinguish the current study from the previously reported studies including the choice of DS mouse model and the type of the muscle used. Most of the studies that characterised gene expression profiles in the skeletal muscle of DS mouse and their relation to muscle functionality was performed in the Ts65Dn model. Both Ts1Cje and Ts65Dn mouse models shared a segment of triplicated region of *Mmu16* but it is important to take into consideration that the genetic make-up of these two models for DS differs (24,25). Ts65Dn model has partial trisomy of *Mmu16* with approximately 132 known trisomic genes (*App-Znf295*) synteny to Hsa21 (26). The major setback of this model includes 19 trisomic genes which are not syntenic to HSA21 (15) and these differences in the genetic materials have significantly influenced the phenotypic features observed in these two models for DS and perhaps affecting on the level at which certain genes are expressed. In general, there are limited studies that characterise gene expression profile in Ts1Cje mice skeletal muscle especially in quadriceps and triceps suggesting the current findings as novel and the differentially expressed genes may serve as candidates to further understand the underlying mechanism of muscle weakness.

Six disomic genes namely (*Lamc1, Leprel1, Myl6b, Msn, Tmod1* and *Pgm5*) which are known to play essential roles in the skeletal muscle structure and function were selected in this study. *Lamc1* is a major non-collagenous component of basement membrane and is essential for normal cardiac and skeletal muscle formation during embryonic development (27). *Lamc1* null mouse embryos failed to develop basement membrane leading to increased deposition of extracellular matrix between adjacent cells, thus, interfering with electrical signal propagation (28). *Leprel1* is a gene of the Leprecan family of proteoglycans that is highly expressed in most tissues including the sarcoplasm of skeletal muscle (29) and mutated *Leprel1* resulted in abnormal collagen in the sclera (30). *Myl6b* is an ATPase cellular motor protein expressed in both smooth and skeletal muscle (31) and its mutation leads to decreased force of contraction and velocity of shortening in smooth muscles (32). *Msn*, together with Ezrin and Radixin formed ERM protein family which are the membrane cytoskeleton proteins. ERM are essential in regulating structure and function of plasma membrane (33). Disruption in epithelial cells morphology was observed in cells lacking *Msn* (34). *Tmod1* is essential for striated muscle development and function as capping protein (35). *Tmod1*-null embryos showed normal skeletal muscle development but with cardiac defects (36,37). *Pgm5* is a cytoskeletal protein which forms a part of the various cell-matrix adheres junctions in muscle cell. Lack of *Pgm5* has resulted in failure in membrane attachment and myofibril assembly as well as significant reduction in myofibril number (38). The present study shows that the expression of the disomic genes of interest in Ts1Cje mice differs from that of the WT mice. Fold change analysis revealed higher expression of three disomic genes (*Lamc1, Leprel1* and *Pgm5*) in Ts1Cje mice relative to WT mice in the selected skeletal muscles. The observation suggests that they may exert certain level of influences on the neurotransmission, structure and function of the skeletal muscle in Ts1Cje mice.

There were limited studies on the skeletal muscle function of the mouse models for DS. To the best of our knowledge, this is the first study investigating the expression profiles of selected trisomic and disomic genes in the targeted muscle types of Ts1Cje mice. To further determine the causative genes for muscle weakness in Ts1Cje mouse model, global transcriptomic analysis of the genes in the skeletal muscles of the Ts1Cje is highly recommended, hence, provide a broader view of the molecular basis that may lead to muscle weakness as well as targets for future therapy.

CONCLUSIONS

In this study, *Lamc1, Leprel1, Myl6b* and *Pgm5*, which were implicated in structural and functional stability of the skeletal muscle have higher levels of expression in Ts1Cje skeletal muscle as compared to wild-type group. The data provide a rationale for further investigation of their biological significance particularly in the molecular pathogenesis of muscle weakness in DS individuals.

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