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Analysis of Heteroplasmic Variants in the Cardiac Mitochondrial Genome of Individuals with Down Syndrome

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ABSTRACT: Individuals with Down syndrome (DS, trisomy 21) exhibit a pro-oxidative cellular environment as well as mitochondrial dysfunction. Increased oxidative stress may damage the mitochondrial DNA (mtDNA). The coexistence of mtDNA variants in a cell or tissue (i.e., heteroplasmy) may contribute to mitochondrial dysfunction. Given the evidence on mitochondrial dysfunction and the relatively high incidence of multiorganic disorders associated with DS, we hypothesized that cardiac tissue from subjects with DS may exhibit higher frequencies of mtDNA variants in comparison to cardiac tissue from donors without DS. This study documents the analysis of mtDNA variants in heart tissue samples from donors with ($n = 12$) and without DS ($n = 33$) using massively parallel sequencing. Contrary to the original hypothesis, the study's findings suggest that the cardiac mitochondrial genomes from individuals with and without DS exhibit many similarities in terms of (1) total number of mtDNA variants per sample, (2) the frequency of mtDNA variants, (3) the type of mtDNA variants, and (4) the patterns of distribution of mtDNA variants. In both groups of samples, the mtDNA control region showed significantly more heteroplasmic variants in comparison to the number of variants in protein- and RNA-coding genes ($P < 1.00 \times 10^{-4}$, ANOVA).

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KEY WORDS: Down syndrome; trisomy 21; heteroplasmy; cardiac; mitochondrial variants; massively parallel sequencing

Introduction

Mitochondria are organelles critical for various cellular functions including the synthesis of ATP by oxidative phosphorylation, maintenance of cellular calcium homeostasis, and detoxification of

reactive oxygen species (ROS) generated from cellular respiration [Nicholls, 2002]. Each mitochondrion contains multiple copies of mitochondrial DNA (mtDNA), and there are hundreds to thousands of mtDNA copies per cell [Ye et al., 2014]. Human mtDNA is a double-stranded circle of 16,569 base pairs. The mitochondrial genome contains a noncoding control region (CR), 13 genes encoding for proteins of the oxidative phosphorylation system, 22 tRNA genes, and two rRNAs genes. Damage to mtDNA can result in mitochondrial dysfunction. Mitochondrial dysfunction contributes to a number of metabolic and degenerative diseases, and is also involved in cancer and ageing (Wallace, 2005). Metabolically active tissues such as heart and brain are especially susceptible to the bioenergetic deficits resulting from mitochondrial dysfunction (Wallace and Chalkia, 2013).

Individuals with Down syndrome (DS, trisomy 21) exhibit mitochondrial dysfunction (Arbuzova et al., 2002; Ogawa et al., 2002; Roat et al., 2007; Infantino et al., 2011; Coskun and Busciglio, 2012). Mitochondrial dysfunction has been associated with the etiology of early-onset dementia in patients with DS. Higher frequency of mutations in mtDNA, specifically in the CR, and decreased mtDNA copy numbers have been observed in brains of patients with DS and dementia in comparison to age-matched controls (Coskun, et al., 2010). The exact molecular events linking variation in mtDNA sequence to disease are not yet fully characterized (Dai, et al., 2012). Some individuals with DS display signs and symptoms of premature ageing in specific body systems including the endocrine, musculoskeletal, immunological, and neurological systems. The cardiovascular system seems to be less affected by ageing in the DS setting, for example, the incidence of hypertension and ischemic cardiac disease is relatively low in adults with DS (Zigman, 2013). However, patients with acute leukemia and DS may be more susceptible to the cardiotoxic effects of anticancer anthracycline drugs (Hefti and Blanco, 2016). A recent study suggests that in myocardial tissue from individuals with DS, the expression of certain anthracycline metabolizing enzymes (i.e., AKR7A2) and mtDNA content are important variables in determining the cardiac synthesis of toxic drug metabolites (Hoefer, et al., 2016). Increases in the frequency of specific mtDNA variants (i.e., heteroplasmy) in cardiac tissue may contribute to the development of cardiac dysfunction that does occur in some cases with DS (Balli, et al., 2016; Vetrano, et al., 2016). Recently, we documented a nonsignificant 30% decrease in the levels of cardiac mtDNA and a nonsignificant 30% increase in the frequency of the "common" mtDNA[4977] deletion in heart tissue samples from donors with DS ($n = 11$) in comparison to samples from donors without DS ($n = 31$). We noted that the trend toward decreased mtDNA content in DS hearts seemed to be independent from the donor's age (Hefti, et al., 2016). Thus, and given both the evidence

on mitochondrial dysfunction and the relatively high incidence of multiorganic disorders associated with DS, we hypothesized that cardiac tissue from subjects with DS may exhibit higher numbers and frequencies of mtDNA variants in comparison to cardiac tissue from donors without DS (Hwang and Jea, 2013; Glasson, et al., 2014; Head, et al., 2015). With the exception of data on mtDNA heteroplasmy derived from brain tissue from subjects with DS and a report on tissular heteroplasmy from Naue et al. (2015), there is a noticeable paucity of reports describing the extent of mtDNA heteroplasmy in most tissues (Coskun and Busciglio, 2012; Naue, et al., 2015). This is especially true with regards to cardiac tissue from subjects with DS.

Massively parallel sequencing is a robust, high-throughput technique that allows the accurate identification of heteroplasmic mtDNA variants. This method was pioneered by Tang and Huang (2010) for identifying mitochondrial heteroplasmy in 2010. The analysis of mtDNA variants by massively parallel sequencing will contribute to expand our understanding of the molecular bases underlying mitochondrial dysfunction in various clinical settings, including DS. In this study, massively parallel sequencing was applied to the analysis and annotation of heteroplasmic mtDNA variants in a collection of heart tissue samples from donors with and without DS. These data provide the first insights into the extent of heteroplasmic mtDNA variation in the cardiac tissue of individuals with DS.

Materials and Methods

Heart Samples

The Institutional Review Board at the University at Buffalo approved this research. Heart samples from donors with ($n = 12$) and without DS ($n = 33$) were procured from The National Disease Research Interchange, The Cooperative Human Tissue Network, and the National Institute of Child Health and Human Development Brain and Tissue Bank. The postmortem to tissue recovery interval was ≤10 hr. Samples (0.50–20 g, myocardium, left ventricle only) were frozen immediately after recovery and stored in liquid nitrogen until further processing. The main demographics from donors with and without DS are summarized in Suppl. Table S1. DS status (yes/no) and relevant diagnoses (when available) were obtained from anonymous medical histories. Heart samples were processed following standardized procedures to isolate total cardiac DNA as described (Hefti, et al., 2016). Age matched groups ($n = 10$ for each group) were utilized for some comparisons to minimize the potential confounding effect of age on mtDNA variation (mean age$_{NDS}$ = 46.50 years, SD = 22.40 vs. mean age$_{DS}$ = 46.6, SD = 21.83, $P = 0.99$, Student's t-test) (Sevini, et al., 2014; Naue, et al., 2015).

Cardiac mtDNA Amplification

The entire mitochondrial genome was amplified in two fragments using high-fidelity long-distance PCR (LD-PCR) with Takara LA (Clontech, CA) as described by Tang and Huang (2010). Cardiac mtDNA fragment 1 (9,289 bp in length) was amplified with the following primers: forward 5′-AACCAAACCCCAAAGA CACC-3′, reverse 5′-GCCAATAATGACGTGAAAGTCC-3′. Cardiac mtDNA fragment 2 (7,626 bp in length) was amplified with the following primers: forward 5′-TCCCACTCCT AAACACATCC-3′, reverse 5′-TTTATGGGGTGATGTGAGCC-3′. The LD-PCR amplification conditions were as follows: 25 cycles at 94°C for 25 sec and 68°C for 16 min. PCR amplification products were analyzed by electrophore-

sis on agarose gels and purified with suitable kits (Bio Basic, Ontario, CA). DNA concentrations were determined with the PicoGreen Assay (Invitrogen, Waltham, MA).

Massively Parallel Sequencing

cDNA libraries were prepared with Nextera sample preparation kits (Illumina, San Diego, CA). The cDNA libraries were quantified using a PicoGreen assay (Invitrogen) and Kapa qPCR library quantification kit (Kapa Biosystems, Wilmington, MA). Size and quality of cDNA libraries were confirmed using an Agilent Bioanalyzer 2100 (Santa Clara, California) DNA high-sensitivity chip. The cDNA libraries were then pooled based on qPCR values. cDNA products were sequenced in rapid mode on an Illumina HiSeq 2500 DNA sequencing system at the University at Buffalo Genomics and Bioinformatics Core Facility (Buffalo, NY). The sequencing data were uploaded to GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The accession number for the data is SRP074312.

Sequencing Output Analysis

Paired sequence reads were demultiplexed using Illumina's bcl2fastq version 1.8.4. Reads were then mapped to the mitochondrial genome NC_012920.1 (version GI: 251831106) utilizing the CLC Genomics Workbench version 8.0.1 alignment tool (Venlo, The Netherlands). After mapping, reads underwent variant calling using CLC Genomics Workbench (v8.0.1) requiring a minimum coverage of 1,000X and a minimum frequency of 1.00×10^{-6}. An average of 7.50 million reads were mapped for each sample. Imported reference data from dbSNP was used to identify mitochondrial variants, which were compiled into Microsoft Excel spreadsheets for further analysis. Data on mtDNA variants were submitted to the MITOMAP database (www.mitomap.org). Diagnostic single-nucleotide polymorphisms (SNPs) available on the MITOMAP database were utilized to identify mtDNA haplogroups (Suppl. Table S1). Distributions and frequencies of heteroplasmic mtDNA variants were mapped on a circular plot using the Circlize package in R.

Data Analysis

The mean number of heteroplasmic mtDNA variants present in a locus was obtained from the average number of individual heteroplasmic variants present in the specified location from all samples within the groups being considered. The frequency represents the percentage of mitochondrial genomes that contain a specific variant. Mean frequency values present in a locus were obtained from the average frequency of all heteroplasmic mtDNA variants present in the specified location from all samples within the group. Frequency values were used to classify mtDNA variants after the application of a conservative 2% error rate (He, et al., 2010). mtDNA variants exhibiting frequency values between 2% and 98% range were considered heteroplasmic. The D'Agostino and Pearson omnibus tests were used to ascertain the normality of datasets. Two-tailed Student's t-test was used to compare the means of normally distributed datasets, and the Mann-Whitney U test was used to compare the means of non-normally distributed datasets. Correlation analyses were performed with Pearson's product-moment test. Analysis of variance (ANOVA) with post-hoc Turkey's test was used to compare mean number of variants and mean frequency. In all cases, differences between means were considered to be significant at $P < 0.050$.
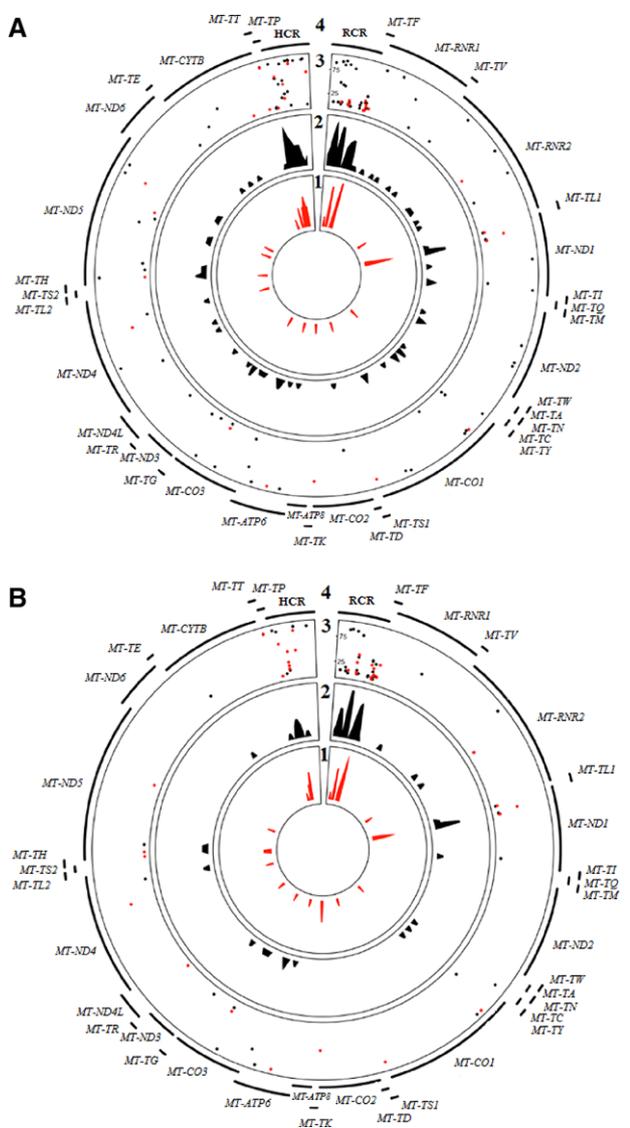
Figure 1. **A**: Heteroplasmic mtDNA variants in cardiac tissue from donors with and without DS. Rings 1 and 2 show the density of mtDNA variants in tissue samples from donors with (red, *n* = 12) and without DS (black, *n* = 33). Ring 3 shows the frequency of the corresponding variants. The closer the circle is to the outer region of ring 3, the higher the frequency of the variant. Ring 4 indicates the genes or regions of the mitochondrial genome. **B**: Map of heteroplasmic mtDNA variant density and frequency in heart samples from age-matched groups of donors with and without DS (*n* = 10 for both groups).

Results

Cardiac mtDNA Variants in Samples from Donors with and Without DS

Figure 1A shows the distribution and frequency of heteroplasmic mtDNA variants in heart samples from donors with (red, *n* = 12) and without (black, *n* = 33) DS. The CR and the *MT-ND1* gene showed relative increases in the density of mtDNA variants in comparisons to other loci in the mitochondrial genome (Fig. 1A). The frequency values associated with the heteroplasmic variants in the CR and the *MT-ND1* gene were highly variable and ranged from 2.03% to 95.96% for *MT-ND1* and 2.01% to 97.80% for the CR.

There were 415 heteroplasmic mtDNA variants in the group of heart samples from subjects without DS (12.57 heteroplasmic variants per sample). According to the NCBI dbSNP database, 89 of the variants (21.44%) have been previously identified; however, these variants have not yet been associated to a particular disease state. Of the remaining mtDNA variants, 323 (77.83%) have not yet been previously identified, and three (0.72%) were identified as pathogenic or possibly pathogenic by NCBI dbSNP and MITO-MAP databases. Specifically, the single-nucleotide variants (SNVs) rs200145866:T>C (Ile165Thr), rs1599988:T>C (Tyr304His), and rs28357980:A>G (Asn150Asp) have been associated with the pathogenesis of Leber's optic neuropathy (Johns and Berman, 1991; Yu-Wai-Man and Chinnery, 1993; Howell, et al., 1995). The frequency values of these potentially pathogenic mtDNA variants in cardiac tissue were 97.25%, 95.97%, and 96.71%. There were 73 nonsynonymous SNVs in the group of samples from donors without DS (mean = 2.21, SD = 1.41 nonsynonymous variants per sample).

Figure 1A compiles the distribution and frequency values of mtDNA variants in 12 cardiac mitochondrial genomes from donors with DS. The CR of mitochondrial genomes from individuals with DS showed a relative increase in the density of variants in comparison to the other mtDNA regions (Fig. 1A). There was an area of increased mtDNA variant density in *MT-ND1*, similar to the NDS group. In general, heteroplasmic mtDNA variants were more prevalent in the CR and protein coding regions.

There were 127 mtDNA variants in the group of heart tissue samples from donors with DS (10.6 variants per sample). Twenty-nine variants (22.83%) were previously identified and are present in the NCBI dbSNP database. These variants have not yet been clinically associated with any disease state. Ninety-eight mtDNA variants (77.16%) are currently unidentified and are not present in the NCBI dbSNP database. There were no pathogenic or potentially pathogenic mtDNA variants in the group of cardiac mitochondrial genomes from individuals with DS. There were 25 nonsynonymous heteroplasmic mtDNA variants, with an average of 2.08 (SD = 2.28) nonsynonymous variants per sample in donors with DS.

Cardiac mtDNA Variants in Samples from Age-Matched Donors with and Without DS

Aging affects mtDNA heteroplasmy in tissues (Sevini, et al., 2014; Naue, et al., 2015). Thus, we compared cardiac heteroplasmic mtDNA variants in a subset of age-matched samples to control for this potential confounder. Figure 1B shows the distribution and frequency of heteroplasmic mtDNA variants in age-matched heart samples from donors with (red) and without (black) DS. The distribution of variants and corresponding frequency values across the mitochondrial genomes from donors with and without DS were similar (Fig. 1A and B).

Overall, the number of mtDNA variants per gene were similar between age-matched samples from donors with and without DS (Suppl. Table S2; Table 1). In age-matched samples from donors with DS, there was a trend toward a decrease in the mean frequency (freq%) of mtDNA variants in the hypervariable control region (HCR) versus the regulatory control region (RCR) (mean freq%$_{DS-HCR}$ = 11.54, SD = 18.62 vs. mean freq%$_{DS-RCR}$ = 30.45, SD = 7.32, P = 0.070, Mann-Whitney U test). This trend was not apparent in the group of age-matched samples from donors without DS (mean freq%$_{Non-DS-HCR}$ = 35.97, SD = 42.94 vs. mean freq%$_{Non-DS-RCR}$ = 30.33, SD = 7.28, P = 0.54, Mann-Whitney U test). Samples from donors with and without DS also showed similar

Table 1. Comparison of Number and Frequency of Heteroplasmic mtDNA Variants in Control Region, Protein Coding Region, and RNA Coding Region in Heart Samples from Age-Matched Donors with and Without DS ($n = 10$ Samples for Both Groups)

		Down syndrome(mean ± SD)	Non-Down syndrome(mean ± SD)	P value[a]
Control region variants	Mean variant number	7.20 ± 3.85	8.60 ± 2.46	0.40
	Mean variant frequency (freq%)	31.06 ± 7.23	33.62 ± 10.38	0.54
Protein coding variants	Mean variant number	2.30 ± 1.55	3.20 ± 1.40	0.11
	Mean variant frequency (freq%)	25.68 ± 30.62	18.61 ± 17.61	0.68
RNA coding variants	Mean variant number	0.80 ± 0.92	0.80 ± 0.63	0.77
	Mean variant frequency (freq%)	16.17 ± 30.01	16.95 ± 30.91	0.85

[a]Student's t-test used for comparisons of normally distributed data; Mann-Whitney U test used for non-normally distributed data.
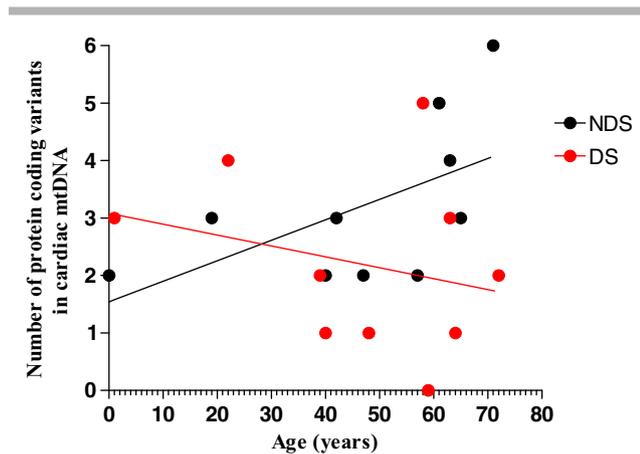

Figure 2. Linear regression analysis of the number of protein coding variants in mtDNA versus age in heart samples from age-matched donors with and without DS ($n = 10$ samples for both groups, non-DS = 0.57, $P = 0.080$ vs. $r_{DS} = -0.27$, $P = 0.46$, Pearson's Product-Moment correlation). Each symbol represents the total number of protein coding variants per sample.

distributions of mtDNA variants along the binding motifs for essential mitochondrial transcription factors (Suppl. Table S3).

There was a trend toward a positive correlation between the total number of protein coding mtDNA variants and age in the group of age-matched samples from donors without DS ($r = 0.57$, $P = 0.080$, Pearson's Product-Moment correlation). The trend was not evident in the group of age-matched samples from donors with DS ($r = -0.27$, $P = 0.46$, Pearson's Product-Moment correlation; Fig. 2). There were no significant correlations between either the number or frequency of heteroplasmic mtDNA variants and age in both age-matched groups (Table 2).

There were no significant differences in RNA or protein coding nonsynonymous, synonymous, or SNV number or frequency between the age-matched groups (Table 3). There were no statistically significant differences in the number of RNA or protein coding insertions and deletions (INDELs) between the age-matched groups (Table 3). The coding INDELs observed in both groups ranged from 1 to 9 bp in length.

The mean number of mtDNA variants in the CR, protein, and RNA coding regions were significantly different in both groups of age-matched samples ($ANOVA_{Non-DS}$ and $ANOVA_{DS}$, $P < 1.00 \times 10^{-4}$; Fig. 3). The number of heteroplasmic mtDNA variants located in RNA coding regions was lower than the number of variants in protein coding and CR, respectively (Fig. 3). In terms of protein coding mtDNA variants, both groups of samples showed a preponderance of nonsynonymous over synonymous variants (non-DS: 84.38%;

DS: 78.26%). The number of nonsynonymous variants per sample was similar between both groups (Table 3).

Discussion

Massively parallel sequencing allows the examination of tissular mtDNA variants with a very high depth of coverage ($>1,000\times$). With the exception of data on mtDNA variants derived from brain tissue from subjects with DS, there is a noticeable paucity of reports describing the extent of mtDNA heteroplasmy in most tissues, including cardiac tissue, from subjects with DS. This is noteworthy because several lines of evidence suggest that widespread mitochondrial dysfunction contributes to many of the pathophysiological alterations associated with DS (Coskun and Busciglio, 2012; Pagano and Castello, 2012). Despite these pathophysiological alterations, the current life expectancy for individuals with DS has reached the sixth decade of life with some individuals ($\sim10\%$) living up to 70 years of age; this increase is attributed to improved medical care for those with DS (Brown, et al., 2001; Head, et al., 2015). In this study, massively parallel sequencing was applied to the analysis of mtDNA heteroplasmy in a collection of heart tissue samples representative of the current life span for persons with DS. For comparison, mtDNA heteroplasmy was analyzed in a group of samples from subjects without DS, which included an age-matched subgroup.

Our initial hypothesis postulated that the cardiac mitochondrial genomes from subjects with and without DS would show differences in the number and/or distribution of mtDNA variants. However, the study's main findings suggest that the cardiac mitochondrial genomes from individuals with and without DS exhibit many similarities in terms of (1) total number of mtDNA variants per sample, (2) the frequency of mtDNA variants, (3) the type of mtDNA variants, and (4) the patterns of distribution of mtDNA variants (Fig. 1; Suppl. Table S2). At first glance, these findings are surprising given that DS status has been associated with increased intracellular generation of damaging ROS combined with less effective mtDNA repair mechanisms (Druzhyna, et al., 1998; Roat, et al., 2007). However, studies that considered noncardiac cells (e.g., DS astrocytes and lymphocytes) showed that trisomic samples displayed an "adaptive response" to the chronic increase in oxidative stress. This "adaptive response" results in an overall decrease in mitochondrial function in order to minimize the production of ROS and reduce further damage to the mtDNA (Perluigi and Butterfield, 2012; Helguera, et al., 2013). It is possible that this "adaptive response" is present in the myocardial tissue of individuals with DS through the entire lifespan. The result would be the preservation of the cardiac mitochondrial genome, as manifested by levels of mtDNA variation comparable to the non-DS setting, at the expense of relative decreases in mitochondrial function. Further comparative studies in cardiac tissue samples and/or cellular models (e.g., induced pluripotent stem cell derived cardiomyocytes) would be needed to determine whether the

Table 2. Correlation Analysis of Heteroplasmic mtDNA Variant Number and Frequency with Age in Age-Matched Donors with and Without DS ($n = 10$ Samples for Both Groups)*

		Down syndrome		Non-Down syndrome	
		r	P value	r	P value
Control region variants versus age	Mean variant number	0.44	0.20	0.25	0.48
	Mean variant frequency (freq%)	−0.15	0.68	−0.016	0.96
Protein coding variants versus age	Mean variant number	−0.27	0.46	0.57	0.080
	Mean variant frequency (freq%)	0.10	0.79	0.16	0.66
RNA coding variants versus age	Mean variant number	0.32	0.37	0.042	0.48
	Mean variant frequency (freq%)	−0.77	0.072	0.20	0.70

*Pearson's Product-Moment correlation analysis.

Table 3. Comparison of SNVs, Coding INDELs, and Nonsynonymous Heteroplasmic mtDNA Variants in Heart Samples from Age-Matched Donors with and Without DS ($n = 10$ Samples for Both Groups)

		Down syndrome(mean ± SD)	Non-Down syndrome(mean ± SD)	P value[a]
Single-nucleotide variants (SNVs)[b]	Mean variant number	6.00 ± 2.94	7.60 ± 3.50	0.25
	Mean variant frequency (freq%)	24.50 ± 17.90	32.54 ± 21.33	0.32
Coding insertions and deletions (INDELs)	Mean variant number	0.70 ± 1.34	1.20 ± 1.03	0.17
	Mean variant frequency (freq%)	6.19 ± 15.11	15.74 ± 21.56	0.12
Nonsynonymous SNVs	Mean variant number	1.30 ± 1.25	2.10 ± 1.37	0.18
	Mean variant frequency (freq%)	8.37 ± 11.74	12.92 ± 15.44	0.40

[a]Student's t-test used for comparisons of normally distributed data; Mann-Whitney U test used for non-normally distributed data.
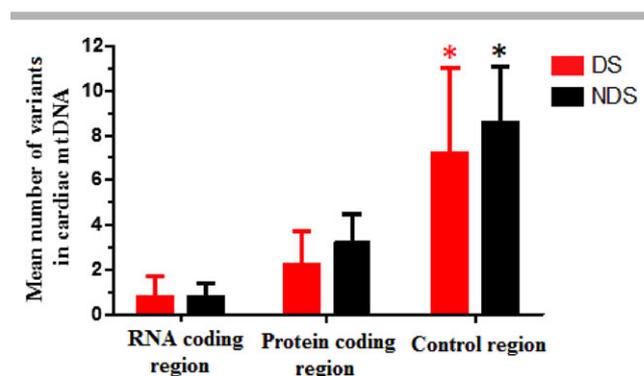[b]Total SNV in coding and noncoding regions.

Figure 3. Mean number of heteroplasmic mtDNA variants (protein coding, RNA coding, and control regions) in heart samples from age-matched donors with and without DS ($n = 10$ samples for both groups). Each bar represents the mean ± SD. *$P < 0.050$ (one-way ANOVA with post-hoc Turkey test).

control of cardiac mtDNA heteroplasmy in the trisomic setting is associated with compensatory changes in mitochondrial function.

This study documented relatively high numbers of mtDNA variants in cardiac tissue (Tables 1 and 2). Cardiac tissue is mostly composed of postmitotic cells, and the majority of the cardiomyocytes in an individual will not be replaced during a normal lifespan (Bergmann, et al., 2009). Thus, the low turnover of cardiac tissue may result in the accumulation and propagation of certain heteroplasmic mtDNA variants (Taylor and Turnbull, 2005; Tuppen, et al., 2010). Heart tissue samples from donors with and without DS showed a wide range of heteroplasmy (i.e., >2%–<98%), and mtDNA variants with frequency values near the upper limit may be in fact homoplasmic mtDNA variants. In general, there was good agreement between the donor's ethnicity as listed in the available clinical reports (i.e., "white" or European American and "black" or African American) and corresponding mtDNA haplogroups from

Africa and Europe (Suppl. Table S1). However, in the absence of paired samples of maternal DNA, it was not possible to: (1) estimate the fraction of haplotype associated variants, and (2) estimate the fractions of germline and "de novo" mtDNA variants (He, et al., 2010).

In both groups of samples, the variants with the highest degree of heteroplasmy tended to concentrate in the CR and the *MT-ND1* gene. The CR is critical for transcription and regulation of the mitochondrial genome. The CR is the most variable region in the mitochondrial genome. Naue et al. (2015) examined mtDNA variants in the CR in nine different tissues from 100 individuals, and found similar numbers of mitochondrial point heteroplasmies in heart and brain, whereas skeletal muscle and liver exhibited the highest number of heteroplasmies. Variation in the CR may affect mtDNA copy number (i.e., mtDNA content) (Coskun, et al., 2010). In a previous study involving this set of heart samples, we documented a nonsignificant 30% ($P = 0.65$) decrease in mtDNA content in samples with DS in comparison to samples without DS (Hefti, et al., 2016). Coskun et al. (2010) examined the frequency of mtDNA CR mutations in samples from cerebral frontal cortices from age-matched subjects with and without DS, and found that mean mutation frequencies were similar between both groups. In agreement, heart tissue samples from subjects with and without DS showed similar levels of heteroplasmy when considering the entire CR as well as distinct regulatory elements within the CR that are involved in the replication of mtDNA (Fig. 1A and B; Suppl. Table S3).

Age-matched groups of cardiac samples from donors with and without DS displayed similar distribution and mean frequency values of heteroplasmic mtDNA variants in RNA and protein coding genes (Fig. 1B). Both groups displayed reduced numbers of RNA variants, as well as reduced numbers of protein coding variants in comparison to the number of variants in the CR (Fig. 3). The majority of the protein coding variants in both groups were nonsynonymous, therefore, potentially damaging. In general, increased numbers and frequencies of mtDNA variants in protein coding and

tRNA coding regions are more likely to be pathogenic compared with other mtDNA regions (Ye, et al., 2014). This pattern of regional variation in cardiac mtDNA is consistent with the recent conclusions made by Ye et al. (2014) after analyzing massively parallel sequencing data from the 1000 Genomes Project. The authors proposed that a purifying selection process may reduce the prevalence and accumulation of potentially pathogenic mtDNA variants in specific mitochondrial loci such as tRNAs and protein coding genes to avoid the propagation of mitochondrial dysfunction (Ye, et al., 2014).

There are limitations in the present study. The sample size was limited due to the scarcity of good quality cardiac tissue samples from donors with DS; this common problem impacts research on many of the issues concerning the pathobiology of DS (Oster-Granite, et al., 2011). Thus, it was not possible to detect relatively subtle differences in mtDNA frequencies (Suppl. Table S4). The lack of matched maternal tissue samples precluded determining whether the cardiac mtDNA variants were either inherited or acquired (i.e., somatic) mtDNA variants. Thus, the observed frequency values may not fully reflect the impact of DS status on somatic mtDNA variants. Furthermore, the reliance on cadaveric tissue samples from donors with various clinical histories makes it almost impossible to control for potential clinical confounders. Furthermore, the resulting cross-sectional nature of the comparisons should be considered when evaluating the potential impact of age and/or other putative covariables on heteroplasmic mtDNA frequencies (Sevini, et al., 2014). Although massively parallel sequencing is a powerful method for the analysis of mtDNA variants in human tissues, it often cannot detect large deletions in mtDNA. For example, the "common" mtDNA 4,977 bp deletion cannot be reliably detected by massively parallel sequencing because of its relatively low frequency in cardiac tissue ($DS_{\%\text{ frequency mtDNA}}{}^{4977}{}_{\text{deletion}}$: 0.0086 ± 0.0166, non-$DS_{\%\text{ frequency mtDNA}}{}^{4977}{}_{\text{deletion}}$: 0.0066 ± 0.0124; $P = 0.514$) (Tang and Huang, 2010; Hefti, et al., 2016).

The results from this study suggest similar distribution of heteroplasmic mtDNA variants in hearts from individuals with and without DS. The data presented in this study offer a glimpse into the patterns of heteroplasmic mtDNA variants in cardiac tissue from donors with DS. These data may advance our understanding of tissular mtDNA variation and its potential relation to the complex pathobiology of DS.

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