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Mitochondria Single Nucleotide Variation across Six Blood Cell Types

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Abstract

It has been shown that heteroplasmic mitochondrial DNA variants can be tissue specific. However, whether mitochondrial DNA variants are specific by blood cell types has not been investigated. Motivated by this question and using mitochondria sequences extracted from RNAseq data from six distinct blood cell types (neutrophil, monocyte, myeloid dendritic, natural killer, T and B), we thoroughly compared SNPs and heteroplasmies among these cell types. Each cell type from each subject was sequenced at four time points used as biological replicates. We found that mitochondria content is low in neutrophil compared to the other five blood cell types. Subsequent analysis on the other five blood cell types showed that at the SNP level, there was no discrepancy. At the heteroplasmy level, we observed good concordances among all blood cell types. However, the allele frequencies of the heteroplasmy differed between blood cell types for certain heteroplasmic sites. Furthermore, we identified five tri-allelic sites (1610, 2617, 8303, 12146, 13710) that are likely caused by RNA editing. Three out of these five sites are located at the ninth position of tRNA genes, and are likely resulting from post-transcriptional methylation.

Introduction

The human mitochondria genome is a double stranded circular DNA molecule, consisting of 16,569 nucleotides. It contains 13 protein coding genes, 22 transfer RNAs (tRNAs), and two ribosomal RNAs. Mitochondrial DNA (mtDNA) is maternally inherited, thus its genome is haploid. However, many sites in mitochondria DNA are heteroplasmic (Payne et al., 2013), containing two alleles. Mitochondrial DNA variants have been linked to various diseases and important biological processes, including cancer (Bai et al., 2007; Canter et al., 2005; Chen, 2012; Herrmann et al., 2003; Modica-Napolitano and Singh, 2004; Petrosillo et al., 2005), aging (Kann et al., 1998; Smigrodzki and Khan, 2005; Sondheimer et al., 2011), and coronary disease (Griffiths, 2012; Jia et al., 2013).

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Previous studies on mtDNA variants typically focused on single source mitochondria from blood or occasionally tissue, and it was commonly assumed that mitochondria heteroplasmy variants were unique among unrelated individuals. However, this assumption was disputed when Samuels et al. reported that multiple unrelated individuals share tissue-specific positive selections of mitochondria variants (Samuels et al., 2013b), an observation that was later confirmed (Li et al., 2015). The authors identified four heteroplasmic somatic mutations in liver and kidney tissues that recurred in multiple subjects. Since liver and kidney tissue arise from the endoderm and mesoderm germ layers, respectively, it is unlikely that the four identified heteroplasmies share a common developmental origin. While tissues remain the preferred source for detecting somatic mutations, blood is the preferred source in genetic and medical testing due to its easy accessibility. Yet, whether or not mitochondria variants are blood cell type specific remains unexamined. Therefore, we thoroughly examined mitochondria variants (SNP and heteroplasmy) and their consistencies across six blood cell types.

It has been demonstrated that mitochondria sequences can be data mined in high throughput sequencing data (Picardi and Pesole, 2012; Samuels et al., 2013a; Ye et al., 2014). The abundance of mitochondria in the cell insures the indirect sequencing of mtDNA in high throughput sequencing data. A recent study demonstrated that mtDNA single nucleotide variants (SNVs) can be detected using RNAseq data (Zhang et al., 2015). Using these findings as a foundation, we performed a study to examine mtDNA single nucleotide variant consistency across six different blood cell types plus PMBC using RNAseq data. One limitation with identifying variants using RNAseq data is the difficulty of distinguishing true variants in the DNA from RNA editing, the post transcriptional modification of RNA nucleotides from their DNA encoded sequence. The most common type of editing is the modification of adenosine to inosine, then the interpretation of inosine as guanosine by cellular machinery. Although mtDNA sequences have not been as thoroughly studied as the nuclear genome, a few RNA editing sites within mitochondria have been documented. In a study by Hodgkinson et al., an analysis of 708 individuals revealed three potential RNA editing sites (C295T, G2129A, and G6691A) (Hodgkinson et al., 2014). These three RNA editing sites were later confirmed by Zhang et al. with three additional RNA editing candidates (C296T, G5746A, and T5878C) (Zhang et al., 2015). Furthermore, Hodgkinson et al. identified 13 mitochondria sites as multi-allelic (containing more than two alleles in one individual) (Hodgkinson et al., 2014). However, since RNA was the data source, it is likely the identification of these sites was the result of systematic sequencing errors or chemical modifications of the RNA.

Methods

RNAseq data were obtained from our previous study on immune responses after influenza vaccination (GSE64655). The original study contained two healthy subjects who were given flu vaccines. Blood was drawn at day 0, 1, 2, and 7 post-vaccination. Six blood cell types were extracted: neutrophil, monocyte, dendritic cells (mDC), natural killer (NK), T cells and B cells. In addition, peripheral blood mononuclear cells (PBMC) which is a mixture of all blood cell types except neutrophil were also extracted. RNAseq was performed for each blood cell type plus PBMC at each time point for the two subjects (total N = 56). The

detailed description of cell extraction and sequencing is available in the original study (Hoek et al., 2015).

We processed the RNAseq data using the best practice recommendation from Genome Analysis Tool Kit developed by the Broad Institute (DePristo et al., 2011). Multi-stage quality controls (Guo et al., 2013c) were performed on the data to ensure data integrity using QC3 (Guo et al., 2014). Only bases with quality score > 20 and depth > 20 were used for further analysis. Mitochondrial DNA variants including SNPs and heteroplasmy were inferred using MitoSeek (Guo et al., 2013b). Cluster and heatmap analyses were carried out using Heatmap3 (Zhao et al., 2014). In the cluster analysis, we coded SNPs as categorical variables based on their genotype, and we used the allele frequency of the minor allele for the heteroplasmic sites. The revised Cambridge Reference Sequence (rCRS) (Andrews et al., 1999) was used as the mitochondria reference. T-tests were used to assess the difference between heteroplasmy levels between the different blood cell types. Results with FDR < 0.01 were considered significant. We also obtained haplogroup information for each sample by checking the SNP identified against the phylotree.org's mtDNA phylogeny tree (van Oven and Kayser, 2009).

Previous studies have shown mitochondrial SNVs identified from RNAseq data to be subjected to higher false positive rates than exome sequencing data (Zhang et al., 2015). To overcome this limitation, we treated the four time intervals of the mitochondria sequences as biological repeats. Only SNVs detected from all four time points are considered true. We hypothesized that none of the mitochondria variants were caused by the flu vaccination. To test this hypothesis, we used class correlation across the four time points to measure the SNP and heteroplasmy consistency. We considered a mitochondrial DNA position to be a SNP if over 90% of the reads were different from the reference. Positions were considered heteroplasmic if 1% to 50% of the reads support alternative alleles.

Results

Our quality control showed that the percent of reads from RNAseq data mapped to neutrophil was 4–6 times lower compared to the other five blood cell types for both subjects (Figure 1). This suggested that the mitochondria content is low in neutrophil cells, and the variants detected at low coverage in neutrophil cells were not reliable. Thus, we excluded neutrophil samples and focused the rest analyses on the other five types of blood cell plus PBMC.

After quality control, we obtained high-quality mitochondrial DNA sequences (Figure S1). On average, for all 48 samples (excluding the neutrophil samples), we obtained a median depth of 6,002 (range: 4,061–7,246). The average coverage of the mitochondria genome was 99.92% (range: 99.51% – 99.99%). The coverage is computed as the number of mitochondria positions with depth coverage > 20 divided by the length of the rCRS (16,569). The median mapping quality was 60 (range: 60–60). The median number of mapped reads was 2,996,390 (range: 1,685,868 – 5,682,697).

First, we computed the SNP and heteroplasmy consistency across the four time points. For the SNPs, we observed no discrepancy between any two time points for any blood cell types. For heteroplasmy, high consistency was also observed among the time points. The average intra-class correlation (ICC) for heteroplasmy was 0.90 (range 0.82–0.95) (Table 1). These results support our hypothesis that no mitochondrial DNA variant was introduced by receiving the flu vaccination. Thus, the blood samples extracted from the four time points can be treated as biological replicates.

Overall, we identified 33 SNPs in subject 1 and 13 SNPs in subject 2 (Table S1). The mean allele frequency for the major allele of the SNP is over 99% of the total reads. The haplogroup analyses showed that subject1 is of haplogroup I5c1 and subject2 is of haplogroup H1a1. The haplogroup results match the subjects' European ancestry. Cluster analysis on SNPs separated the two subjects (Figure S2). Perfect consistencies were observed for the SNPs detected among the different blood cell types within each subject (Figure S3).

The number of heteroplasmies detected depends on the applied detection threshold. Using the detection threshold of minor allele frequency > 1% with 24 samples per subject, we identified 11 and 10 sites with strong evidence for minor alleles for subject1 and subject2, respectively (Figure 2). The complete allele frequency data can be seen in Table S2 and S3. The allele frequency of the minor allele ranges from 1% to 48% of the total reads. Overall, the consistency of frequencies for the minor allele was similar among the blood cell types (Figure 3). After FDR correction in subject1, two heteroplasmic sites (1120 and 1405) were identified as significantly higher in B cells as compared to the other cell types (Figure 4). Also, we identified another six sites in the mitochondrially encoded 12S RNA (RNR1) that have significantly higher heteroplasmy levels in B and T cells as compared to the other four cell types (Figure 5).

Using 1% as the detection threshold for heteroplasmy, we identified five sites that are tri-allelic (Table 2). Three of the sites (G1610AT, A8303GT, and A13710GT) are within tRNAs (TV, TK, and TH, respectively), and all of them occurred at the 9th position of a tRNA. One likely explanation is that these extra alleles in tRNAs are the results of post transcriptional methylation (Machnicka et al., 2013; Mercer et al., 2011; Sanchez et al., 2011) (Figure 6). The site 2,617 resides in the 947th position of ribosomal RNA RNR2. The site 13,710 resides in the 1374th position of the gene ND5 as a non-synonymous mutation. Both sites 2,617 and 13,710 have been previously described as canonical RNA editing events (Bar-Yaacov et al., 2013) and both sites have been described as multi-allelic sites (Hodgkinson et al., 2014). Cluster analysis showed that unlike SNPs, using the heteroplasmic allele frequency cannot clearly distinguish the two subjects (Figure 7A), indicating that the clusters were convoluted by RNA editing events that were in common between the two individuals. After removing the putative editing events, the two subjects can be separated based on heteroplasmy levels (Figure 7B). This suggests that RNA editing events are more universal than are true heteroplasmic events. Below the detection threshold of 1%, many sites share multiple alleles from multiple samples (Table S4, S5). However, at such a low level, it is difficult to distinguish real heteroplasmy and/or RNA editing from noise.

Discussion

Using the unique RNAseq data set collected from two individuals and six different blood cell types, we conducted a thorough mitochondrial DNA variants study. We found that neutrophil cells had relatively lesser mitochondria content compared to other blood cell types. The lower mitochondria content in neutrophil did not allow us to accurately identify mitochondria variants. Thus, we focused our remaining analyses on the other five blood cell types plus PBMC. At the SNP level, all five blood cell types produced the same results. At the heteroplasmy level, no discrepancies were observed above the detection threshold. Any minor inconsistencies were most likely contributed by errors from sequencing and limitations of using RNA as the data source.

The biggest obstacle of this study is overcoming the higher false positive rate as compared to SNV detection using DNA based sequencing data (Han et al., 2014; Zhang et al., 2015). The higher false positive rates are caused by random errors introduced from reverse transcription of RNA to cDNA, PCR amplification, and additional complications from alignment due to the complexity of the RNA structure, such as splicing and polyadenylation. We effectively overcame these limitations by measuring SNV in four time points representative of technical repeats for each blood cell type, by only considered signals detected in all four repeats.

RNA as the data source did create minor inconveniences for data analysis, but simultaneously enabled us to detect RNA editing events. Unlike the random errors mentioned above, RNA editing events follow inherent and repeatable patterns. Five candidate RNA editing sites passed our filters. Three of the five were in the 9th position of a tRNA, which is a 1-methylguanosine. The primary structure of tRNA is usually 73 to 93 nucleotides long, with the secondary structure in a cloverleaf shape. The 9th position is the connecting point between the acceptor stem and D-arm. The evidence suggests that these 9th position modifications of the tRNA were post transcriptional methylation. The other two sites, 2,617 and 13,710, are also known RNA editing sites in mitochondria. Below the detection thresholds, we identified many sites that were tri-allelic. The allele frequencies of the third alleles for these sites were low. Thus, it is difficult to distinguish the true signal from noise (Guo et al., 2013a). High depth mitochondria targeted sequencing is required to identify the true tri-allelic sites. There are two possible explanations for observing tri-allelic sites: the post transcriptional modification of RNA and the fact that heteroplasmies are not limited to a single fixed allele. It has been reported that tri-allelic sites in the nuclear genome result from copy number amplification. Typically, there are 100 to 10000 copies of mitochondria in a human cell. Thus, the chance of seeing a third or fourth allele in a single individual is not improbable. With high throughput sequencing technology, the sightings of tri-allelic sites in mitochondria of a single individual is not uncommon (Hodgkinson and Eyre-Walker, 2010; Hodgkinson et al., 2014).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Neutrophil has lower mitochondria content compared to other blood cell types.
- The consistencies were good for heteroplasmy and SNP across different blood cell types.
- RNA editing can cause mitochondrial heteroplasmies appear multi-allelic in RNAseq data.
- Post transcriptional methylation tends to happen at the 9th position of tRNA.

Figure 1. Percent of RNAseq reads mapped to mitochondria reference. Neutrophil has substantially less reads which implies that neutrophil has less mitochondria content.

Figure 2.

A. Circos plot of the allele frequencies of the minor allele for subject1. B. Circos plot of the allele frequencies of the minor allele for subject2. Each circle represent a blood cell type. From outer to inner, the order of the blood cell types are: B, dendritic, monocyte, NK, PBMC, T.

Figure 3.

Distance measured as Spearman's correlation coefficient between any two blood cell types. Two detection thresholds were used for heteroplasmy: 1% and 5%. The overall correlations are high among the five blood cell types plus PBMC. A. Subject1. B. Subject2.

Figure 4. Two heteroplasmic sites were identified as significantly higher in B cell compared to the other five blood cell types after adjusting for FDR.

Figure 5. We identified six heteroplasmic sites in RNR2 with higher allele frequencies for heteroplasmy for T and B cells compared to the other four cell types. This phenomenon is observed in both subjects. A. Subject1. B. Subject2.

Figure 6.

Secondary structure of tRNA is in a cloverleaf shape. The 9th position is the post transcriptional methylation site 1-methylguanosine. It is not surprising to observed multiple allele at this position in RNAseq data.

Figure 7.

A. Cluster analysis using allele frequencies of the minor allele, two subjects cannot be clearly distinguished. B. Cluster analysis using allele frequencies of the second allele after filtering out putative RNA editing sites, two subjects can be clearly separated.

Table 1

Heteroplasmy level consistency across blood cell types

Subject	celltype	ICC(95% CI)	p value
Subject1	B	0.94(0.92,0.95)	2.64E-232
Subject1	DENDRITIC	0.85(0.81,0.88)	4.33E-156
Subject1	MONOCYTE	0.93(0.91,0.94)	1.04E-223
Subject1	NK	0.89(0.86,0.91)	1.14E-174
Subject1	PBMC	0.95(0.93,0.96)	9.15E-174
Subject1	T	0.89(0.87,0.91)	2.70E-227
Subject2	B	0.91(0.89,0.93)	1.89E-252
Subject2	DENDRITIC	0.89(0.87,0.91)	2.77E-237
Subject2	MONOCYTE	0.93(0.91,0.95)	3.41E-268
Subject2	NK	0.82(0.79,0.86)	4.78E-164
Subject2	PBMC	0.88(0.84,0.91)	9.07E-114
Subject2	T	0.88(0.86,0.9)	1.04E-253

ICC = intra class correlation

CI = confidence interval

Table 2

Tri-allelic positions in mitochondria

POS	Alleles Detected in Subject 1	Alleles Detected in Subject 2	Gene	Description	Starting	Ending	Position in gene
1610	ATG	ATG	TV	tRNA Valine	1602	1670	9
2617	ATG	ATG	RNR2	16S Ribosomal RNA	1671	3229	947
8303	ATG	ATG	TK	tRNA Lysine	8295	8364	9
12146	ATG	ATG	TH	tRNA Histidine	12138	12206	9
13710	ATG	ATG	ND5	NADH dehydrogenase subunit 5	12337	14148	1374