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Genetic Variation in *PARL* Influences Mitochondrial Content

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Abstract

Given their involvement in processes necessary for life, mitochondrial damage and subsequent dysfunction can lead to a wide range of human diseases. Previous studies of both animal models and humans have suggested that *PARL*, presenilins-associated rhomboid-like protein, is a key regulator of mitochondrial integrity and function, and plays a role in cellular apoptosis. As a surrogate measure of mitochondrial integrity, we previously measured mitochondrial content in a Caucasian population consisting of large extended pedigrees, with results highlighting a substantial genetic component to this trait. To assess the influence of variation in the *PARL* gene on mitochondrial content, we re-sequenced 6.5kb of the gene, identifying 16 SNPs and genotyped these in 1,031 of these Caucasian individuals, distributed across 162 families. Statistical genetic analysis revealed that one promoter variant, T-191C, exhibited significant effects (after correction for multiple testing) on mitochondrial content levels. Comparison of the transcription factor binding characteristics of the T-191C promoter SNP by EMSA indicates preferential binding of nuclear factors to the T allele, suggesting functional variation in *PARL* expression. These results suggest that genetic variation within *PARL* influences mitochondrial abundance and integrity.

Keywords

mitochondrial DNA; association; mitochondrial function/dysfunction; genotyping; sequencing

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Introduction

The mitochondria genome (mtDNA) is vital for maintaining mitochondrial function and meeting the energy needs of the body. The mitochondrial genome encodes genes for the biochemical reactions of respiration, and specific molecules involved in protein synthesis. Given the complexity and importance of these organelles in many vital processes essential to life, one would surmise there are many such mechanisms whose interruption would lead to mitochondrial dysfunction. One such influence, variation in mitochondrial abundance, has been shown to contribute to disease susceptibility (Antonetti et al. 1995; Yamada et al. 2006; Kim et al. 2005). The abundance and integrity of mitochondrial DNA is essential for maintaining normal mitochondrial function (Chen and Butow 2005). It is clearly evident that mitochondrial dysfunction contributes to a variety of human disorders ranging from diabetes, cancer, obesity, multiple sclerosis, several psychiatric disorders and a wide range of age-related disorders (Weissig et al. 2004; Wallace 2005; Dakubo et al. 2006; Begriche et al. 2006; Dutta et al. 2006; Fattal et al. 2006).

In the past, mitochondrial DNA content has usually been determined using tissue biopsy samples. Sukhorukov et al (2000) reported that lymphocyte mtDNA content quantification was comparable to that from skeletal muscle biopsies in mitochondrial pathologies (Sukhorukov et al. 2000). Bai et al (2004) investigated mitochondrial content in muscle and blood samples of individuals suspected of having a mitochondrial disorder. Results of their study showed that mitochondrial content was significantly higher in muscle tissue than in blood. However, regardless of the tissue type, patients with abnormal mtDNA levels (either depletion or proliferation) had significant clinical manifestations characteristic of mitochondrial disease in addition to abnormal respiratory enzymes and mitochondrial cytopathies (Bai et al. 2004). Using peripheral blood lymphocytes, Song et al (2001) showed that mtDNA content was lower in offspring of type 2 diabetic patients when compared to age, sex and BMI matched controls without a family history of disease. mtDNA content was also correlated with insulin sensitivity, indicating that this phenotypic measure might be an early genetic marker for diabetes development. Animal studies have also shown a decrease in mtDNA in diabetes susceptible rat and mouse models (Song et al. 2001). Results from our previous study of mitochondrial content, a surrogate measure of mitochondrial integrity, suggested a substantial genetic component to variation in mitochondrial abundance (Curran et al. 2007), although our linkage analyses did not detect evidence of potential QTL effects at the 3q27 location of *PARL*.

Using differential gene expression information from diseased and healthy skeletal muscle of *Psammomys obesus*, we identified a mitochondrial intramembrane protease known as presenilins-associated rhomboid-like protein (*PARL*) (Walder et al. 2005). There have been many recent studies highlighting a key role for rhomboid protease genes in maintaining mitochondrial integrity (McQuibban et al. 2003; Sesaki et al. 2003; Herlan et al. 2003). Additionally, several of these studies have looked at the role of *PARL* in mitochondrial morphology and apoptosis. *PARL*, together with an interacting gene *OPA1*, are required for maintaining the structure of the inner mitochondrial membrane folds, termed cristae. Both of these genes are essential regulators of cristae remodelling, a process that must occur for cytochrome c to be released from the mitochondria during apoptosis (Cipolat et al. 2006; Frezza et al. 2006; Gottlieb 2006; Pellegrini and Scorrano 2007).

The human *PARL* gene consists of ten exons, encodes a 379 amino acid protein and is located on chromosome 3q27. The human *PARL* protein is localized to the mitochondrial inner membrane. Given recent studies, *PARL* appears to be an essential component in the network of genes involved in maintaining mitochondrial integrity. The main aim of this study was to investigate the influence of genetic variation in *PARL* on mitochondrial function.

Methods

Human Subject Selection

All human samples and phenotypic data were utilized from the Metabolic Risk Complications of Obesity Genes project, as previously described (Kissebah et al. 2000). These families were recruited from the TOPS (Take Off Pounds Sensibly, Inc.) membership. The sample consisted of 1,031 individuals from 169 families predominantly of European ancestry and residing in the United States. There were 272 males and 759 females in this sample with a mean age of 47.2 years. All research protocols were approved by the Institutional Review Board of the Medical College of Wisconsin. For variant identification, the complete *PARL* gene was sequenced in 42 Caucasian individuals, consisting of healthy as well as obese and/or diabetic individuals that represented extremes of the phenotypic measures, to ensure most of the genetic variation was captured. This number of individuals in our resequencing set provided us with a 90% likelihood of identifying genetic variants with frequencies as low as 0.025.

Mitochondrial Content Quantitation

The mitochondrial content quantitation has been described in detail elsewhere (Curran et al. 2007). Human nuclear and mitochondrial DNA samples were assayed in the same reaction plates with a CEPH DNA standard and real-time PCR performed using the 7900HT Sequence Detection System (Applied Biosystems, Foster City CA).

Primer Design and Sequencing

A 6.5 kb region encompassing the putative promoter, all exons, 3'UTR and intronic sequences identified as conserved between human and mouse was sequenced. Repetitive DNA was identified using RepeatMasker (www.repeatmasker.org) and primers designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Sequencing PCRs were performed as previously described (Curran et al. 2005).

Genotyping

Genotyping was carried out on the Sequenom MassARRAY system (Sequenom, San Diego CA) using standard protocols as described by the manufacturer. A detailed description of the procedure has been previously described (Curran et al. 2005; Buetow et al. 2001).

Statistical Genetic Methods

Allele Frequency Estimation—Maximum likelihood techniques that account for pedigree structure were used to estimate allele frequencies for each *PARL* variant using SOLAR (Almasy and Blangero 1998). Similarly, SOLAR was used to perform tests of Hardy-Weinberg equilibrium allowing for non-independence amongst pedigree members.

Measured Genotype Association Analysis—The association model that we have employed represents a simple extension of the classical variance component model described more fully elsewhere (Blangero et al. 2005; Boerwinkle et al. 1986). For a candidate locus with m polymorphic nucleotide sites, define a variate s_i for the i -th SNP that takes the values of 0, 1, and 2 for the marker genotypes AA, Aa, and aa, respectively where a refers to the minor allele. Using this framework, we model the phenotype, p , as a linear combination of fixed effects and random variables, $p = \mu + \sum \alpha_i s_i + \sum \beta_i x_i + \sum q_k + g + e$, where μ is the trait mean, the α_i are fixed-effect regression coefficients on the measured genotype (s_i), the β_i are fixed-effect regression coefficients for any measured covariates (x_i), and the q_k , g , and e are random effects representing other QTLs, residual genetic effects, and random environmental effects respectively. For an additive model of gene action, the parameter α measures one half of the displacement between the contrasting homozygous means. Estimation of the various fixed

effects and variance components associated with the random effects can be performed using standard maximum likelihood methods. Note that this approach takes into account non-independence amongst family members. All analyses were performed using the computer package, SOLAR (Blangero et al. 2005; Almasy and Blangero 1998).

To eliminate the potential for hidden population stratification that the standard fixed effect association approach is susceptible to, we also utilized the QTD approach to association testing developed by Abecasis et al (Abecasis et al. 2002) and implemented in SOLAR (Blangero et al. 2005). While this approach exhibits substantially decreased power than measured genotype analysis (Havill et al. 2005), it maintains the appropriate significance levels under the null hypothesis even in the presence of population stratification. It also allows a direct test of stratification for each marker which we use (when this test is non-significant) to justify use of the more powerful measured genotype analysis (Blangero et al. 2005).

Correction for Multiple Testing—In order to correct for the effect of multiple testing for a given phenotype, we estimated the effective number of SNPs using the method of Li and Ji (Li and Ji 2005) which employs a modification of an earlier approach by Nyholt (Nyholt 2004). After obtaining the effective number of SNPs (N_{em}), we employed a modified Bonferroni procedure to identify a target alpha level ($0.05/N_{em}$) that would maintain an overall significance level of 0.05 or less.

Multivariate Association Analysis—Because marginal analyses of single SNPs can miss important functional signals, we also employed multivariate analysis to detect associations between *PARL* sequence variation and the focal phenotype. This was done using a forward-selection regression-type process to identify the N_{em} variants that provide the best overall prediction of phenotype levels. The resulting test identifies a vector of SNPs constrained by the effective number of SNPs that provide the best association with the phenotype. This approach generates an overall gene-centric omnibus test defined by twice the difference in likelihoods between a null model in which no SNPs are considered versus the best model in which the simultaneous effects of N_{em} SNPs are estimated. The resultant test is distributed as a chi-square variate with N_{em} degrees of freedom.

Imputation of Missing Genotypes—Multivariate methods generally require that individuals with missing data be excluded from the analysis, which can result in a significant decrease in sample size. To minimize this issue, we employ likelihood-based imputation with the MERLIN computer package (Abecasis et al. 2002), which uses an adaptation of the method by Burdick et al (Burdick et al. 2006) to rapidly impute SNP data in pedigrees. For this study of *PARL* sequence variation, our average genotyping call rate was 95.9%. Thus, our rate of missing genotypes was approximately 4%. These missing genotypes were imputed as described.

Functional Analysis Methods

Electrophoretic mobility shift assay (EMSA)—Complementary 5' biotinylated oligonucleotides representing both allelic forms of the T-191C *PARL* promoter SNP were obtained commercially (Operon Biotechnologies, Huntsville AL). The sequences of the oligonucleotide probes used were:

PARL191T: 5'GAGCCAGCTGAGCAGTGGGAGGGGAAGCGGT3';

PARL191C: 5'GAGCCAGCTGAGCAGCGGGAGGGGAAGCGGT3'.

Nuclear extract preparation, binding reactions and gel analysis and detection were as described previously (Goring et al. 2007).

Results

Variant Identification

A total of 6.5 kbp encompassing the promoter, 3'UTR, all exons and conserved intronic regions was sequenced in 42 individuals. We identified 16 variants (as outlined in Table 1), two of which were novel (not listed in any public databases). These 16 polymorphisms were then genotyped in a sample of 1,031 individuals from 169 families. The frequency of the minor alleles varied from less than 0.003 to 0.50.

Linkage Disequilibrium

The 16 identified SNPs were in varying degrees of disequilibrium. Figure 1 shows the overall pattern of linkage disequilibrium as measured by the squared correlation (r^2) among genotypes. This analysis utilized all genotyped individuals, which is appropriate when trying to assess redundant statistical information used in association analysis. Overall, the linkage disequilibrium across *PARL* was substantial. Using the eigenstructure method of Li and Ji (Li and Ji 2005), we estimated that these 16 SNPs behave statistically like 8 effectively independent SNPs. However, because the C21967T SNP is very rare (minor allele frequency 0.003), we also excluded it from the association analyses. After this exclusion, we reran the eigenstructure analysis and determined that there were 7 effectively independent SNPs out of a total of 15. Thus, in our subsequent statistical tests for association, we require a target nominal significance level of $0.05/7 = 0.0073$ to obtain an experiment-wide significance level of 0.05.

Heritability of Mitochondrial Content

Using variance component-based analysis quantitative genetic methods implemented in SOLAR, we estimated the heritability of mitochondrial content to be 0.523 ($P = 2.0 \times 10^{-28}$) in our Caucasian sample (previously described in Curran et al. 2007). All analyses allowed for the effects of sex and age. These results provide the necessary prior evidence that genetic factors influence the phenotypic variability of mitochondrial content.

Association of *PARL* Genetic Variants with Mitochondrial Content

Marginal association analysis was performed using a measured genotype approach in SOLAR. Several covariates (including sex, age, and smoking status) were simultaneously adjusted for to increase the genetic signal-to-noise ratio. A direct test of stratification, implemented in SOLAR (Blangero et al. 2005), was used to test for hidden population stratification prior to analysis. None of the SNPs showed statistical evidence of stratification, allowing us to safely employ the more powerful measured genotype procedure to assess association. Table 2 shows the results of these association analyses. One polymorphism, the promoter variant T-191C showed the strongest association with mitochondrial content ($P = 0.0014$) which is significant even after correction for multiple tests. The rarer C allele of this variant leads to a 0.62 difference in standard deviation units between contrasting homozygotes and accounts for about 1.7% of the phenotypic variance in mitochondrial content. A second promoter variant, G-197C (which exhibits high correlation with T-191C variant due to linkage disequilibrium) also showed a significant association ($P = 0.0034$) with mitochondrial content levels. Two other variants (C318T and C44055G) showing linkage disequilibrium with T-191C also exhibited nominal evidence for association ($P = 0.0141$ and $P = 0.0469$ respectively).

We also performed multivariate association analysis with all *PARL* sequence variants. Given that there is evidence for only seven independent dimensions of SNP variation, we performed a forward-stepping measured genotype analysis that was limited to a maximum of seven SNP covariates. This global test automatically accounts for the inherent multiple testing. Using this omnibus gene-centric test, we found that sequence variation in *PARL* has a significant influence

on mitochondrial content ($\chi^2_7 = 25.0$, $P = 0.00076$). This multivariate test only modestly increased the percentage of explained phenotypic variance to 2.1% over that seen for the T-191C variant alone (1.7%). It is noteworthy that this omnibus test remains significant even if we assume that the 15 SNPs are completely independent. Examination of the estimated α parameters revealed that three of the SNPs (T-191C, A448C, and G17094A) show independent significant influences suggesting the presence of multiple potential independent functional variants within *PARL*. Overall the multivariate tests provide strong evidence that *PARL* variation is likely to influence mitochondrial content.

Functional Analysis of the T-191C Promoter Polymorphism

Results of our association analysis suggested that the T-191C promoter polymorphism was directly functional or in high linkage disequilibrium with a functional variant that was not detected in our resequencing. We therefore decided to directly test the functionality of the T-191C promoter variant in relation to its effect on *PARL* gene expression.

To test whether the -191 nucleotide variation was involved in transcriptional variation, EMSA was performed by comparing sequences that differed only at the T-191C nucleotide. The results indicated that the -191 SNP did affect binding of nuclear factors, with a major complex preferentially binding to the -191T allele (Figure 2). This complex is absent in the -191C sample and an alternative complex forms. Bioinformatic analysis using P-Match (Chekmenov et al. 2005; Wingender et al. 2000) revealed that the -191T allele is embedded in a consensus Sp1 binding site (Kadonaga et al. 1987) (TRANSFAC 6.0 Core similarity = 0.957, matrix similarity = 0.967) whereas the -191C SNP ablates the core sequence. These results suggest that the -191 SNP contributes to allele-specific differences in *PARL* expression.

Discussion

The mitochondria are the major site of eukaryotic cellular respiration and energy metabolism. It is well established though that the mitochondria are involved in many other processes including cell signaling, cell growth and apoptosis, lipid metabolism and free radical production (Wallace 2005; Wallace 1999). The mitochondria have their own genome, approximately 16.5kb in size and packed into mt-nucleoids. The genome encodes 22 RNA molecules and 13 proteins that are essential to cellular energy production. These 13 proteins along with some 70+ proteins encoded in the nucleus and transported to the mitochondria are involved in the oxidative phosphorylation process. Thousands of other nuclear proteins form the mitochondrial proteome, and are involved in such processes as replication, repair, transcription and translation (Schapira 2006). Mitochondrial DNA (mtDNA) abundance and integrity is very important for maintaining normal mitochondrial function (Chen and Butow 2005). Mutations in the mitochondrial genome contribute to a variety of human diseases including diabetes, cancers and several diseases of aging (Weissig et al. 2004; Wallace 2005; Dakubo et al. 2006; Begriche et al. 2006; Dutta et al. 2006; Fattal et al. 2006). Results from our previous study of mitochondrial content, a surrogate measure of mitochondrial integrity, suggested a substantial genetic component to variation in mitochondrial abundance (Curran et al. 2007).

Given the prior evidence for a role for *PARL* in mitochondrial integrity and function, we analyzed sequence variation within this gene and mitochondrial content levels. Results from this study confirm *PARL* as a candidate gene influencing mitochondrial abundance. In our resequencing effort, we identified 15 variants of sufficient frequency across the gene. We measured mitochondrial content as a surrogate of mitochondrial integrity and performed association analyses with *PARL* sequence variation. Of these variants, the promoter SNP T-191C showed the strongest association with mitochondrial content; with this variant alone accounting for almost 2% of the phenotypic variation observed. This is substantial, given the average phenotypic variance generally explained by SNPs in complex traits. A further three

SNPs, in varying degrees of disequilibrium with T-191C, also showed association, indicating that sequence variation within *PARL* appears to influence mitochondrial content. Multivariate analysis was performed to assess the global effect of *PARL* sequence variation on mitochondrial content; with results showing that sequence variation in *PARL* has a significant influence on mitochondrial content ($P = 0.00076$).

Functional analysis revealed that the presence of the common -191T SNP introduces an Sp1 transcription factor binding site into the *PARL* promoter. This binding site creates a transcription domain that could upregulate *PARL* expression when the T allele is present. Sp1 is a member of the Kruppel-like transcription factor family, is ubiquitously expressed and appears to regulate cell growth and differentiation (Kaczynski et al. 2003). If the differential binding seen in EMSA is in fact due to Sp1, given the ubiquitous distribution of this factor (and a similar distribution of mitochondria), it is likely that the functional allelic differences in *PARL* expression due to the T-191C variation is manifest in all tissue types.

PARL has previously been shown as playing a significant role in maintaining mitochondrial function and integrity, in several different species. In this paper, we have determined that *PARL* genetic variation appears to directly influence mitochondrial content and have identified a promoter variant influencing *PARL* expression levels. Overall, our results support a significant role for *PARL* genetic variation influencing mitochondrial integrity.

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Fig 1.
Pattern of linkage disequilibrium within the *PARL* gene. The intensity of red color within a block indicates the magnitude of the squared correlation (ρ^2) between SNP alleles (or genotypes)

Fig. 2. Functional analysis of the T-191C SNP

EMSA analysis of the region surrounding the PARL T-191C using Jurkat lymphocyte nuclear extracts. Biotin-labeled double-stranded PARL191T (lanes 1 and 3) and PARL191C (lanes 2 and 4) oligonucleotides were incubated either without (lanes 1 and 2) or with (lanes 3 and 4) nuclear extract and analysed on a 6% non-denaturing polyacrylamide gel. Comparison of -191T (lane 3) with -191C (lane 4) sequences showed a major lower complex that binds to both alleles. A second major upper complex binding to -191T is absent in the -191C sample and a different complex forms (indicated by the arrowhead).

Table 1*PARL* genetic variation identified

SNP ID	Location	dbSNP ID	SNP Sequence	Frequency of Minor Variant
G-309A	Promoter	rs3792587	CGCCTGGTAT[G/A]TGCCGTTACT	0.479
G-197C	Promoter	rs3792588	GGAGCCAGCT[G/C]AGCAGTGGGA	0.113
T-191C	Promoter	rs3792589	AGCTGAGCAG[T/C]GGGAGGGGAA	0.111
C318T	Intron 1		GCCCAGCCCC[C/T]CGCGAGCCTG	0.123
T384C	Intron 1	rs12054027	TAAGCTGAGG[T/C]TTCATTGCGT	0.480
A448C	Intron 1	rs6803120	TGAAGTGAAG[A/C]AGTTAGTACA	0.275
G16630A	Intron 1	rs3749446	GAATTTTGTG[A/G]CTTTGAATTC	0.364
T16752C	Intron 1	rs953419	TGCAAGACGC[T/C]ACTGTGTTTC	0.177
G17094A	Intron 2	rs1402000	GGGATCAAAA[G/A]GAGCTGGGCT	0.384
C21967T	Intron 3		CTCTTATGTG[C/T]GAGGCATTTT	0.003
T40359G	Intron 4	rs3811725	GCTGATACTT[T/G]AAAAGCATTC	0.496
C42440T	Exon 6	rs13091	CATTCAGTCA[C/T]TTCTCCTTAT	0.456
C44055G	Intron 6	rs12636826	CCGGTAGGCA[C/G]AGGTTGCAGT	0.158
C44233G	Exon 7	rs3732581	TGTCAGTTAC[C/G]TGGGTAAAGT	0.473
T50902G	Intron 7	rs2305666	TGAAGCTTTG[T/G]GCTCACCTAG	0.173
A55670G	3' UTR	rs263003	CATAAAAGTC[A/G]AGAGTATCCC	0.294

Table 2

Association between *PARL* variants and mitochondrial content levels in 1,086 individuals (Results obtained from measured genotype analysis using an additive model)

SNP ID	Location	dbSNP ID	Association with Mitochondrial Content (p-value)
G-309A	Promoter	rs3792587	0.8538
G-197C	Promoter	rs3792588	0.0034
T-191C	Promoter	rs3792589	0.0014
C318T	Intron 1		0.0141
T384C	Intron 1	rs12054027	0.5925
A448C	Intron 1	rs6803120	0.0641
G16630A	Intron 1	rs3749446	0.1270
T16752C	Intron 1	rs953419	0.2371
G17094A	Intron 2	rs1402000	0.1922
T40359G	Intron 4	rs3811725	0.7588
C42440T	Exon 6	rs13091	0.9493
C44055G	Intron 6	rs12636826	0.0466
C44233G	Exon 7	rs3732581	0.0701
T50902G	Intron 7	rs2305666	0.3560
A55670G	3' UTR	rs263003	0.1243