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Functional characterisation of *ADIPOQ* variants using individuals recruited by genotype

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ABSTRACT

Four non-coding GWAS variants in or near the *ADIPOQ* gene (rs17300539, rs17366653, rs3821799 and rs56354395) together explain 4% of the variation in circulating adiponectin. The functional basis for this is unknown.

We tested the effect of these variants on *ADIPOQ* transcription, splicing and stability respectively in adipose tissue samples from participants recruited by rs17366653 genotype.

Transcripts carrying rs17300539 demonstrated a 17% increase in expression ($p = 0.001$). Variant rs17366653 was associated with disruption of *ADIPOQ* splicing leading to a 7 fold increase in levels of a non-functional transcript ($p = 0.002$). Transcripts carrying rs56354395 demonstrated a 59% decrease in expression ($p = <0.0001$). No effects of rs3821799 genotype on expression was observed.

Association between variation in the *ADIPOQ* gene and serum adiponectin may arise from effects on mRNA transcription, splicing or stability. These studies illustrate the utility of recruit-by-genotype studies in relevant human tissues in functional interpretation of GWAS signals.

1. Introduction

Despite the progress made by genome-wide association studies (GWAS) in identifying common variants associated with complex disease, definition of the functional effects of such variants has proven very difficult since most reside in non-coding parts of the genome (Manolio et al., 2008). The location of much disease-associated variation suggests that many of the effects may be mediated by disruption of regulatory elements involved in moderating aspects of gene expression. Effects may also be context driven or tissue specific (Vafiadis et al., 1996, 1997), and thus only detectable in tissues of interest which may not be readily accessible for study.

ADIPOQ encodes adiponectin, a protein that can exist in monomeric or multimeric forms, and is expressed almost exclusively from the adipose tissue, and from adipocytes in bone marrow and placenta (Scherer et al., 1995; Viengchareun et al., 2002; van Stijn et al., 2015). Adiponectin has been studied extensively as a potentially important insulin sensitising hormone (Dridi and Taouis, 2009), although work on the precise mechanics of its role in insulin resistance have produced conflicting results with inconsistent effects reported in different species and in vitro (Halleux et al., 2001; de Oliveira et al., 2011; Hozumi et al., 2010). Variants in *ADIPOQ* have been reported to be associated with metabolic alterations such as type 2 diabetes in some populations (Tsai et al., 2014; Liao et al., 2012; Ramya et al., 2013), although data from other populations has been less clear (Siitonen et al., 2011) and Mendelian randomisation studies indicate that the relationship between adiponectin and type 2 diabetes or insulin resistance may not be causal (Yaghootkar et al., 2013). Apart from this, it is a regulator of energy homeostasis and ligand of adiponectin receptors in the human hypothalamus (Kos et al., 2007).

Here we sought to assess the functional effects of 4 SNPs within the *ADIPOQ* gene (rs17300539; rs17366653, rs3821799 and rs56354395) that together explain 4% of the variation in serum adiponectin levels (Yaghootkar et al., 2013). Potential mechanisms of action were defined on the basis of bioinformatics analyses and we then assessed the effect of each variant on *ADIPOQ* mRNA transcription, splicing or stability in adipose tissue samples from participants accessed through a recruit by genotype approach. The

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advantage of our approach was the ability to study common variants with relatively large effects on a common phenotype, the knowledge of the target gene, and the availability of relevant human tissues from individuals carrying the variants. Discovery of effects of common variants on the expression and regulation of genes involved in human diseases is an important prerequisite to understanding the molecular basis of GWAS associations.

2. Materials and methods

2.1. Selection of variants for study

We selected the variants for study from a recent deep sequencing effort, which identified 7 SNPs which together explained 6% of the variation in adiponectin levels in Europeans (rs17366653, rs17366743, rs1354091, rs3774261 [a proxy for rs56354395], rs3821799, rs16848727, and rs1868146) (Warren et al., 2012). 4 variants (rs17366653, rs17300539, rs3821799 and rs56354395) explained 4% of the variance in adiponectin levels. Including the other 3 SNPs in our analysis did not increase the proportion of variance explained. These variants, rs17300539, rs17366653, rs3821799 and rs56354395, ranged in minor allele frequency from 0.005 to 0.50 in 1000 Genomes data (Table 1). rs17300539, rs3821799 and rs56354395 are inherited in the same linkage disequilibrium block (supplementary figure 1), rs17366653 is independently inherited since it is very rare. All of these variants lie in regions of the *ADIPOQ* gene that may be involved in the control of the amount or nature of gene expression. No systematic study of their potential functional effects has previously been made and experimentally validated.

2.2. Bioinformatic assessment of variants associated with serum adiponectin levels

Evidence of potential promoter, enhancer, insulator or repressor activities for each variant was accessed from ENCODE/Broad gene regulation data accessed through the University of Santa Cruz Genome Browser (http://genome.ucsc.edu/). Assessment of the strength of conserved splicing signals was assessed using the Berkeley Drosophila Genome Project BDGP splice site (NNSplice) predictor web interface (http://www.fruitfly.org/seq_tools/splice.html). Potential Exon and intron splicing enhancer and silencer binding sites were assessed by ESEfinder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home). Potential candidate long non-coding RNAs (lncRNAs) for *ADIPOQ* were identified using the UCSC Genome Browser (http://genome.ucsc.edu/). Potential microRNA (miRNA) binding sites in the region of rs56354395 were identified using RegRNA (http://regrna2.mbc.nctu.edu.tw/). We screened for the presence of stability/instability elements such as A-rich elements (AREs) and GU rich elements (Barreau et al., 2005) and zip code elements involved in localisation of mRNAs (Mili and Macara, 2009). Potential binding sites for 77 RNA binding proteins were assessed using Transterm (http://mrna.otago.ac.nz/) (Jacobs et al., 2009). Changes to mRNA secondary structure related to genotype at rs56354395 were assessed using the MFOLD web interface (http://mfold.rna.albany.edu/?q=mfold) (Zuker, 2003).

2.3. Recruit by genotype study and adipose tissue sample collection

Genotype data from over 5000 participants in the Exeter 10000 study were available from previous analysis (unpublished data, T. Frayling). 4 female and 2 male participants heterozygous for the low frequency minor allele of rs17366653 and 6 age- sex- and BMI-matched major allele homozygous controls were approached to provide adipose tissue biopsies. Clinical characteristics of participants can be found in Table 2.

8 additional adipose tissue samples from female subjects homozygous or heterozygous for rs17300539 were sourced from an ongoing Exeter tissue bank initiative. These samples were also used for basic mRNA, lncRNA and miRNA expression profiling. Characteristics of these samples are found in Table 3.

A pea-sized sample of abdominal fat was obtained from each participant under local anesthetic and was flash frozen in liquid nitrogen immediately after excision and stored at −80 °C. Liver and Kidney RNA samples for assessment of miRNA and lncRNA expression were commercially obtained (AMS Biosciences, Albany, USA). Blood RNA samples were sourced from the Exeter 10000 cohort. Ethics approval was provided by the Exeter CRF Tissue Bank Steering Committee and informed consent was obtained from all participants. All experiments were carried out according to the declaration of Helsinki.

2.4. mRNA, lncRNA and miRNA extraction

Messenger RNAs (mRNAs), long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) were extracted from adipose tissue samples using TRI Reagent® technology according to manufacturer's instructions (Life Technologies, Foster City, USA). The small amount of contaminating genomic DNA present in the samples was amplified by Whole Genome Amplification (REPLI-g® Mini Kit, Qiagen, Paisley, UK) to provide material for confirmation of genotype at the variants of interest by Sanger sequencing. All samples were then DNase treated to remove contaminating genomic DNA prior to analysis according to manufacturer's instructions (TURBO DNA-free™ Kit, Life Technologies, Foster City, USA).

2.5. Reverse transcription

100 ng total RNA was reverse transcribed for each sample in a total volume of 20 μl using the SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies, Foster City, USA) according to the manufacturer's instructions. Reverse transcription for miRNA analysis was

Table 1
Bioinformatic prediction of the effects of serum adiponectin-associated genetic variation on *ADIPOQ* gene regulation. The bioinformatically-predicted consequences of genetic variation within the *ADIPOQ* gene on adiponectin transcription, mRNA processing and non-coding RNA regulation are given here. MAF refers to minor allele frequency. UCSC refs to the Santa Cruz Genome Browser interface. Evidence included suggestion of DNAse1 hypersensitivity sites (open chromatin), CpG islands (regulation by DNA methylation), ChIP (chromatin immunoprecipitation) interaction (Transcription factor or other protein binding), FAIRE (Formaldehyde Assisted Isolation of Regulatory Elements) (open chromatin), histone modification data (open chromatin), chromatin segmentation data (promoters, enhancers, insulators), and ORegAnno data (promoters, enhancers, insulators).

SNP	Effect allele	Predicted effect	Global MAF	Source
rs17300539	G	Disruption of transcription?	0.03	UCSC (http://genome.ucsc.edu/)
rs17366653	C	Disruption of pre-mRNA splicing?	0.005	NN Splice (www.fruitfly.org/seq_tools/**splice**.html)
rs3821799	C	Disruption of lncRNA regulation?	0.46	UCSC (http://genome.ucsc.edu/)
rs56354395	Insertion of 'A'	Disruption of miRNA regulation?	0.50	RegRNA 2.0 (http://regrna2.mbc.nctu.edu.tw/)

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