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A synonymous polymorphic variation in *ACADM* exon 11 affects splicing efficiency and may affect fatty acid oxidation

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ABSTRACT

In recent studies combining genome-wide association and tandem-MS based metabolic profiling, a single-nucleotide polymorphism (SNP), rs211718C > T, located far upstream of the MCAD gene (*ACADM*) was found to be associated with serum concentrations of medium-chain acylcarnitines indicating improved beta-oxidation of medium-chain fatty acids. We examined the functional basis for this association and identified linkage between rs211718 and the intragenic synonymous polymorphic variant c.1161A > G in *ACADM* exon 11 (rs1061337). Employing minigene studies we show that the c.1161A allele is associated with exon 11 missplicing, and that the c.1161G allele corrects this missplicing. This may result in production of more full length MCAD protein from the c.1161G allele. Our analysis suggests that the improved splicing of the c.1161G allele is due to changes in the relative binding of splicing regulatory proteins SRSF1 and hnRNP A1. Using publicly available pre-aligned RNA-seq data, we find that the *ACADM* c.1161G allele is expressed at significantly higher levels than the c.1161A allele across different tissues. This supports that c.1161A > G is a functional SNP, which leads to higher MCAD expression, perhaps due to improved splicing.

This study is a proof of principle that synonymous SNPs are not neutral. By changing the binding sites for splicing regulatory proteins they can have significant effects on pre-mRNA splicing and thus protein function. In addition, this study shows that for a sequence variation to have an effect, it might need to change the balance in the relative binding of positive and negative splicing factors.

1. Introduction

Medium-chain acyl-CoA dehydrogenase (MCAD; EC 1.3.8.7) is a key enzyme in the beta-oxidation of medium-chain fatty acids (C4–C12 straight chain) and thus an important player in maintaining energy homeostasis. In recent studies combining genome-wide association and tandem-MS based metabolic profiling, a single-nucleotide polymorphism (SNP), rs211718C > T, located upstream of the MCAD gene (*ACADM*) was found to be associated with the serum concentration of medium-chain acylcarnitines, indicating improved beta-oxidation of medium-chain fatty acids [1,2]. Since this SNP is located far away from the known regulatory elements of *ACADM* it is not likely that it is by itself causative [3]. It may instead be linked to an intragenic SNP, which affects MCAD activity. Interestingly, comparison of the allele frequencies of rs211718C > T and the synonymous SNP in the *ACADM* exon 11, c.1161A > G (rs1061337) [4], shows that they have similar minor allele frequencies. The distance between the rs211718 and c.1161A > G is relatively short (approximately 120,000 bp.) suggesting that the two SNPs are in linkage disequilibrium (LD). Therefore, we examined if rs211718C > T and c.1161A > G are linked, and if the synonymous c.1161A > G variation in *ACADM* could be contributing to the enhanced fatty acid metabolism associated with rs211718T by improving splicing of the *ACADM* pre-mRNA.

Both constitutive and alternative exons are critically dependent on exonic regulatory sequences for correct splicing [5]. Splicing factors bind exonic splicing enhancers (ESEs) or exonic splicing silencers (ESSs) and affect the recognition of splice sites in a positive or negative way. Members of the SR-protein family often bind ESEs promoting recognition of nearby splice sites. hnRNP proteins often bind ESSs suppressing recognition of nearby splice sites. Mutations and SNPs in ESEs or ESSs might thus affect splicing. Synonymous exonic mutations previously assumed to be neutral may in this way have serious effects on protein function potentially leading to malfunction and disease. In *ACADM* exon 5 an ESE element counteracts an ESS for inclusion of this exon. A missense mutation in *ACADM* exon 5 has been shown to disrupt the ESE causing exon skipping, which leads to loss of a functional enzyme. However, we have previously reported that a synonymous SNP inactivates this ESS, thus giving immunity towards disruptive mutations in the ESE [6]. This example perfectly illustrates the principle that synonymous SNPs may not be neutral, and that it is the combination of genetic variation, the haplotype, rather than single mutations that determines the phenotype.

A number of matrices have been designed to enable prediction of ESE and ESS elements and thus the consequences of mutations on

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splicing. However, these matrices are mostly based on computational or SELEX studies [7–11], and they often overestimate the number of functional ESE and ESS elements. In addition, they do not account for the positional effect of these sequences and the competition between positive and negative splicing factors for the same sites in vivo. Thus, to be able to accurately assess the effects of a mutation on RNA splicing, each individual mutation still needs to be analyzed experimentally.

In this study, we have examined if the presumed neutral synonymous SNP, c.1161A > G, in *ACADM* is in linkage disequilibrium with the intergenic SNP (rs211718), and if c.1161A > G affects pre-mRNA splicing and thus may affect expression of full-length MCAD protein.

2. Materials and methods

2.1. Genotyping

Genomic DNA from 82 Danish controls were genotyped for rs211718 and *ACADM* c.1161 by PCR using TEMPase Hotstart Master Mix (Ampliqon) and allele-specific primers MCAD1161GF: 5′-AGG CAATGGATTTAATACAGAATATCCTGCG-3′, MCAD1161AF: 5′-AGGCAA TGGATTTAATACAGAATATCCTGCA-3′, MCAD1161R: 5′-GCTACTAGGG ACATAAAATTTCTAAATAATCAG-3′, rs211718C: 5′-GAAGATGTGAGGA AGAACTTCATGTGAACTCC-3′, rs211718T: 5′-GAAGATGTGAGGAAGA ACTTCATGTGAACTCT-3′, and rs211718R: 5′-GTATTTTATTATGTCCTT TTTGGGGAAGACTTG-3′. Reactions were cycled 30 times with denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. PCR products were analyzed by agarose gel electrophoresis.

2.2. Splicing of ACADM exon 11 minigene

The *ACADM* exon 11 minigene with wild type *ACADM* sequence pMCADEX11WT, harboring c.1161A, and the *ACADM* minigene harboring the c.1194 + 1G > A mutation, pMCAD-IVS11 + 1A, have been described previously [12]. New constructs harboring c.1161G and c.946-2A > C variants were named, pMCADEX11c.1161G and pMCADEX11-IVS10-2A > C, respectively. Chang cells (American Type Culture Collection, No. CCL-13) were transfected with each of the plasmids using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, http://www.roche.com), according to the manufacturer's instructions. Total cellular RNA was isolated (TRIzol Reagent) 48 h post-transfection and 1 μg RNA was reverse-transcribed using an oligo-dT primer (Advantage® RT-for-PCR kit). Using the cDNAs as templates, PCR amplification was performed with an SD6 sense primer 5′-TCTGAGTCACCTGGACAACC-3′ and a SA2 antisense primer 5′-AT CTCAGTGGTATTTGTGAGC-3′, which both anneal to the pSPL3 vector sequence. The products were analyzed by agarose gel electrophoresis and by direct sequencing.

HeLa cells were transfected in duplicates with 2 μg DNA using PolyMag following manufacturer's instructions (OZ Biosciences, http://www.ozbiosciences.com). 48 h post-transfection, total RNA was harvested using Isol-RNA Lysis Reagent following manufacturer's instructions (5 PRIME, http://www.5prime.com). Purified RNA was used as template in first-strand cDNA synthesis using qScript cDNA Supermix following manufacturer's instructions (Quanta Biosciences, http://www.quantabio.com). *ACADM* exon 11 splicing was examined by PCR amplification of the cDNA using TEMPase Hotstart Master Mix (Ampliqon, http://www.ampliqon.com) and using primers SD6 sense primer and SA2 antisense primer. PCR products were analyzed using agarose gel electrophoresis.

2.3. RNA pull-down

Protein pull-down by biotin-coupled RNA oligonucleotides was performed as previously described [6]. RNA oligonucleotides spanning the sequence of *ACADM* exon 11 around c.1161 was used, MCAD1161A: 5′-ACAGAAUAUCCUGUAGAAAAAC-biotin and MCAD1161G: 5′-ACAGA AUAUCCUGUGGAAAAAC-biotin. For immunoblotting antibodies against hnRNP A1 (N15) (sc-10029, Santa Cruz Biotechnology, http://www.scbt.com/) SRSF1 (32-4500, Invitrogen), or SRSF5 (H00006430-M03A, Abnova, http://www.abnova.com/) were used.

2.4. Analysis of RNA-seq

Publicly available RNA-seq alignments were downloaded from Illumina BodyMap 2.0 (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-513/) and Geuvadis RNA sequencing project (http://www.ebi.ac.uk/arrayexpress/experiments/E-GEUV-1/). *ACADM* c.1161A and c.1161G allele reads were counted using SAMtools [13] and a custom perl script. Statistical analysis was performed using R. Linear regression was performed using a fixed interception value of 0.

2.5. Analysis of linkage disequilibrium

We used the publicly available web tool CubeX [14] (http://www.oege.org/software/cubex/) to estimate linkage disequilibrium and haplotype frequencies.

3. Results

3.1. rs211718C > T and c.1161A > G in the ACADM gene are linked

To determine if rs211718C > T and c.1161A > G in the *ACADM* gene are linked, genomic DNA from 82 Danish controls was genotyped by allele-specific PCR (Fig. 1). Primers were designed to be able to distinguish between the two alleles *ACADM* c.1161A and c.1161G and rs211718C and rs211718T, respectively. Amplification of the *ACADM* c.1161 SNP and the rs211718 SNP produced different band sizes, enabling simultaneous genotyping of the c.1161A and the rs211718T allele, and the c.1161G and the rs211718C allele. Forty-one (50%) of the samples were homozygous for rs211718C and *ACADM* c.1161A,

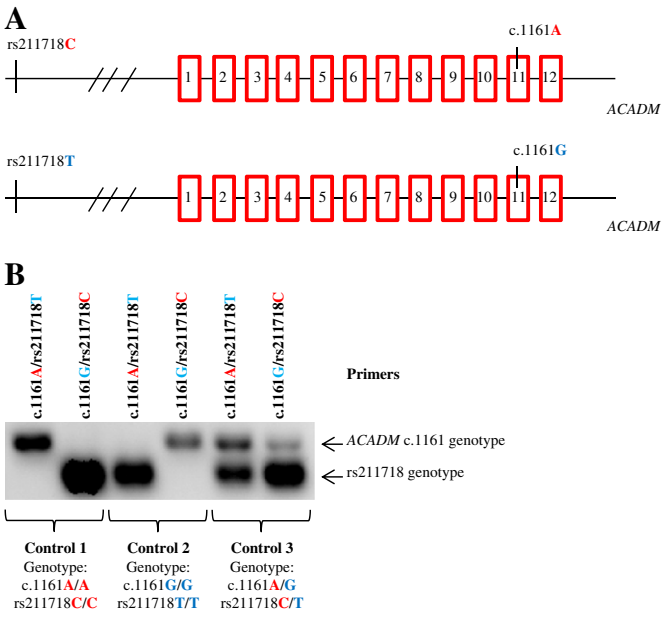

Fig. 1. A. Schematic figure of the phasing of the two variants rs211718C > T and *ACADM* c.1161A > G. Gene structure is not drawn to scale. B. Genotyping of Danish control samples by allele-specific PCR. Amplification with rs211718 specific primers gives a product of 118 bp., while amplification with *ACADM* c.1161 specific primers gives a product of 164 bp. Three genotypes are shown: Control 1: Homozygous c.1161A/A and homozygous rs211718C/C. Control 2: Homozygous c.1161G/G and homozygous rs211718T/T. Control 3: Heterozygous at both loci.