European Journal of Human Genetics (2010) 18, 1054–1056

www.nature.com/ejhg

SHORT REPORT

The obesity-associated SNPs in intron 1 of the *FTO* gene affect primary transcript levels

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As shown by genome-wide association studies single-nucleotide polymorphisms (SNPs) within intron 1 of the *FTO* gene are associated with the body mass index and type II diabetes, although the functional significance of these SNPs has remained unclear. Using primer extension assays, we have determined the ratio of allelic *FTO* transcript levels in unspliced heterogeneous nuclear RNA preparations from blood of individuals heterozygous for SNP rs9939609. Allelic expression ratios of the neighboring *RPGRIP1L* gene were investigated in individuals who were heterozygous for SNP rs4784319 and heterozygous or homozygous for rs9939609. In each of five individuals, the *FTO* transcripts containing the A (risk) allele of rs9939609 were more abundant than those with T allele (mean 1.38; 95% confidence interval 1.31–1.44). Similar results were obtained in a fibroblast sample. We also observed skewed allelic expression of the *RPGRIP1L* gene in blood, but skewing was independent of the *FTO* genotype. Our data suggest that increased expression of *FTO* is associated with increased body mass.
European Journal of Human Genetics (2010) **18,** 1054–1056; doi:10.1038/ejhg.2010.71; published online 26 May 2010

Keywords: allelic expression; *FTO*; SNP

INTRODUCTION

Recent genome-wide association studies have revealed a strong association between a block of single-nucleotide polymorphisms (SNPs) in the fat mass and obesity-associated (*FTO*) gene, body mass index (BMI) and other obesity-related traits in children and adults of different populations.[1,2] The obesity-associated SNPs are located in intron 1 of the *FTO* gene, which contains nine exons (Figure 1). Using bioinformatics analysis, Gerken *et al*[3] have proposed that the FTO protein is an Fe(II) and 2-oxoglutarate-dependent oxygenase.[3] Loss-of-function mutation in *FTO* causes severe growth retardation and multiple malformations in homozygotes,[4] whereas loss of one functional copy of this gene seems to be compatible with both lean and obese phenotype.[5] Leanness, postnatal growth retardation and a higher metabolic rate were observed in *Fto* knockout mice[7] and in mice with a missense mutation in exon 6.[8]

The *FTO* gene shares a CpG island with the adjacent *RPGRIP1L* gene, which is transcribed in the opposite direction, suggesting that the two genes are coregulated. The RPGRIP1L protein is located in the cilia and centrosomes.[6] Loss-of-function mutations in *RPGRIP1L* cause Joubert syndrome type 7 or Meckel syndrome type 5. It can be noted that *FTO* and *RPGRIP1L* genes are ubiquitously expressed and show similarity of expression profile both in fetal and adult tissues (data not shown).

As the obesity-associated SNPs in the *FTO* gene are intronic, their functional significance is unclear. It is possible that one of the SNPs is located in a regulatory sequence and that the risk allele increases or decreases the transcription rate, but strong linkage disequilibrium (LD) of these SNPs makes it difficult to identify the functionally relevant SNP. In 2008, Stratigopoulos *et al*[9] reported that the risk allele (A) of SNP rs8050136 preferentially bound to the transcription factor CUTL1 in human fibroblast DNA and that an siRNA knockdown of *CUTL1* by 70% decreased *FTO* and *RPGRIP1L* expression by 90 and 65%, respectively. However, these findings are not consistent with their model in which *FTO* and/or *RPGRIP1L* mediate suppressive effects on energy intake. To fit the physiological model, CUTL1 should preferentially bind to the non-risk allele of rs8050136. Furthermore, several studies have failed to reveal any influence of the *FTO* genotype on total mRNA level of *FTO* or *RPGRIP1L*.[10–12] Recently, an association between intronic variation of the *FTO* gene and transcript levels of the retinoblastoma-like 2 (*RBL2*) gene, which maps 270 kb upstream of *FTO*, was reported.[13] One problem in interpreting the previous findings is that the CCAAT-displacement activity of CUTL1 was implicated in the transcriptional repression of several genes, whereas some CUTL1 isoforms were found to participate in the transcriptional activation.[14] Another problem is that studies on *cis*-regulatory effects on gene transcription in humans are hampered by the fact that the tested individuals unavoidably differ in genetic background, age, life events and environment. To detect subtle differences in transcript levels, very large numbers of individuals would have to be tested. These problems can be circumvented by determining the ratio of allelic transcript levels in heterozygous individuals, in whom each allele serves as an internal control for the other.[15–18] For this approach, only few subjects are needed.

MATERIALS AND METHODS

Study cohort

The study was approved by the ethics committee of the University Hospital Essen. Blood samples and skin biopsies from normal weight individuals (BMI 18.5–25) were obtained after informed consent was given.

Genotyping

Subjects were genotyped by sequence analysis of genomic DNA (gDNA) extracted from whole blood with EZ1 DNA Blood Kit (Qiagen, Hilden,

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Received 14 January 2010; revised 2 March 2010; accepted 31 March 2010; published online 26 May 2010

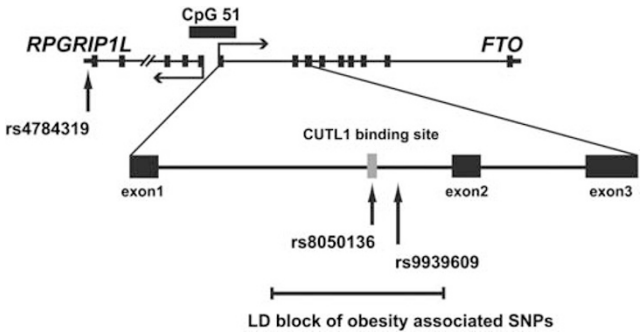

Figure 1 Physical map of the *FTO* and *RPGRIP1L* loci. The *FTO* and *RPGRIP1L* genes are located on the long arm of chromosome 16, share a CpG island with 51 CpG dinucleotides and are transcribed in opposite directions. The obesity-associated SNPs are in strong LD and located within intron 1 of the *FTO* gene. The variants used in this study as well as the CUTL1-binding site identified by Stratigopoulos *et al*[9] are indicated.

Germany). Sequence reactions were carried out with Big Dye Terminators (BigDye Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems, Foster City, CA, USA). Reaction products were analyzed with an ABI 3100 Genetic Analyzer and Sequencing Analysis software (Applied Biosystems).

Preparation of hnRNA and total RNA

For heterogeneous nuclear RNA (hnRNA) extraction, lymphocytes were isolated with Ficoll-Paque PLUS (GE Healthcare, Waukesha, WI, USA) from fresh blood collected in EDTA tubes. Skin fibroblasts were cultured in AmnioMAX+M-C100 medium containing 20% AmnioMAX Supplement (Gibco, Invitrogen, San Diego, CA, USA) at 37°C in a humidified atmosphere containing 5% CO_2. Both lymphocytes and fibroblasts were subjected to hnRNA extraction with the Cytoplasmic & Nuclear RNA Purification Kit (Norgen Biotek Corporation, Thorold, ON, Canada). DNase treatment was carried out in solution followed by cleaning up on spin columns (Qiagen, Hilden, Germany). To minimize loss of hnRNA, all steps were carried out as quickly as possible. Isolated hnRNA was dispensed in several aliquots and frozen in liquid nitrogen. For testing SNP rs4784319, we used total RNA extracted from blood with the PAXgene blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland).

Allelic expression assays

Reverse transcription and primer extension assays were performed with kits from Applied Biosystems. Total RNA from blood was reverse transcribed with random hexamers, whereas cDNA from hnRNA was primed with sequence-specific primers FTO R1 or FTO R2 (all primer sequences and annealing temperatures are given in the Supplementary Table S1). For amplification, the GoTaq DNA Polymerase Kit (Promega, Madison, WI, USA) was used. *FTO* genomic DNA and cDNA were amplified with primers FTO F1 and FTO R1. *RPGRIP1L* genomic DNA was amplified with primers gRP F and gRP R, cDNA was amplified with cRP F and cRP R. SNPs in the primer-binding sites were excluded by sequencing across these regions in all individuals. Samples were heated at 95°C for 2 min, followed by 35 cycles at 95°C for 30 s, 55°C (*FTO* gDNA and cDNA), 60°C (*RPGRIP1L* gDNA) and 62°C (*RPGRIP1L* cDNA) for 30 s, and at 72°C for 40 s, finally at 72°C for 5 min. Using equal amount of amplicons from cDNA and genomic DNA, primer extension assays were carried out with Snapshot R primers and ABI Prism SNaPshot Kit. Reaction conditions were as follows: 96°C for 3 min, 25 cycles at 96°C for 10 s, 53°C (*FTO*, rs9939609) and 41°C (*RPGRIP1L*, rs4784319) for 5 s, and at 60°C for 30 s. The reaction products were analyzed by gel capillary electrophoresis on ABI 3700 DNA Analyzer and the electropherograms were analyzed with the Gene Mapper 4.0 software (Applied Biosystems). Allelic DNA ratios were used to normalize the cDNA ratios. Means and confidence intervals were calculated with JMP7 (SAS, Cary, NC, USA).

RESULTS

To measure the allelic ratios of *FTO* and *RPGRIP1L* transcripts, we developed fluorescence-tagged single-nucleotide primer extension assays. This approach has been successfully used by us and others for detecting skewed and non-skewed allelic expression of many genes (see for example Serre *et al*[18] and Kanber *et al*[19]). For *FTO*, we used unspliced hnRNA because (i) all obesity-associated SNPs are of intronic location and (ii) no expressed polymorphism in LD with these SNPs is known. We chose the clinically associated SNP rs9939609 within intron 1 of *FTO* as a marker (Figure 1), because it was tested in many independent studies of large Caucasian populations. SNP rs9939609 is in complete LD with SNP rs8050136, and the A (risk) allele of rs9939609 is associated with the A allele of rs8050136 (data not shown). For *RPGRIP1L*, we used an expressed SNP located in the 3′ untranslated region (rs4784319) (Figure 1). As SNPs in this gene are not in LD with the obesity-associated *FTO* SNPs and there is no easy way of establishing phase in double heterozygotes, we decided to determine the allelic expression pattern of the *RPGRIP1L* gene in *RPGRIP1L* heterozygous individuals with different *FTO* genotypes. If the obesity-associated *FTO* SNPs affected *RPGRIP1L* expression, we should observe allelic expression imbalance of *RPGRIP1L* in *FTO* heterozygotes, but not in *FTO* homozygotes.

For determining allelic *FTO* transcript levels, we investigated hnRNA from blood of five individuals heterozygous for rs9939609. All assays were performed in sextuplicate. There was no evidence for DNA contamination of the hnRNA samples (Supplementary Figure 1). As shown in Figure 2a, there was very little inter-assay variation of allelic ratios. In each of the five individuals, relative transcript levels were skewed to similar degrees in favor of the A (risk) allele (mean 1.38; 95% confidence interval 1.31–1.44). In skin fibroblasts from individual 18, we observed a ratio of 1.31 (95% confidence interval 1.23–1.39, data not shown).

To investigate the effect of the obesity-associated *FTO* SNPs on allelic *RPGRIP1L* transcript levels, we tested blood RNA of six individuals who were heterozygous for the *RPGRIP1L* SNP rs4784319. Of these, four were heterozygous for the *FTO* SNP rs9939609, one was homozygous for the A (risk) allele of this SNP, and one was homozygous for the T allele. As shown in Figure 2b, we observed similar degrees of skewing in favor of the T allele in all six individuals. These results suggest that *RPGRIP1L* gene expression is not affected by the *FTO* genotype, but by *cis*-regulatory variation, which is in LD with rs4784319.

DISCUSSION

Using single-nucleotide primer extension assays we have determined allelic expression ratios of the *FTO* and *RPGRIP1L* genes. We demonstrate for the first time (i) that the obesity-associated *FTO* SNPs affect *FTO* and not *RPGRIP1L* expression and (ii) that the primary *FTO* transcript made from the risk allele is more abundant than the transcript made from the non-risk allele, at least in blood cells and skin fibroblasts. The observed skewing cannot be due to the presence of genomic DNA in our hnRNA preparations, because (i) there was no evidence for DNA contamination (Supplementary Figure 1) and (ii) allelic PCR products from genomic DNA would be present in equal amounts.

Our observation that allelic expression imbalance of the *RPGRIP1L* gene is not dependent on the *FTO* genotype is consistent with previous studies reporting no association between *RPRGIP1L* variation and obesity.[20] The degree of skewing of allelic *FTO* expression is remarkably similar in our subjects, suggesting that most of the variation in *FTO* expression is due to *cis*-regulatory variation in intron

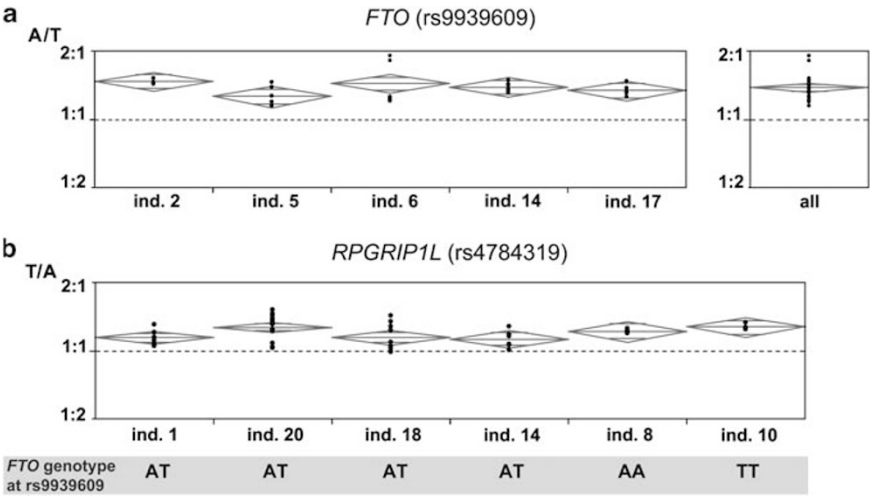

Figure 2 Allelic expression studies. (**a**) Allelic expression of the *FTO* gene in five individuals. The ratio of the two alleles of SNP rs9939609 is skewed in favor of the A (risk) allele in all individuals investigated. Results are displayed as diamonds, in which the horizontal line represents the mean value and the top and bottom of the diamonds represent the 95% confidence interval. (**b**) Allelic expression of the *RPGRIP1L* gene in six individuals with different *FTO* genotypes. In each individual, the ratio of the two alleles of SNP rs4784319 is skewed in favor of the T allele, independently of the *FTO* genotype.

1 of this gene. As the determination of allelic expression with intronic SNPs gives very similar estimates with those obtained with exonic SNPs,[18] we can assume that allelic mRNA levels are also skewed. Higher mRNA levels might translate into higher protein levels, but, of course, we have no proof for this assumption.

One limitation of our study is the fact that we could analyze easily accessible cell types only. Blood cells and fibroblasts are most probably not involved in body weight regulation, but we do not know the cell type through which *FTO* exerts its effect, and this cell type may not be accessible. On the other hand, *FTO* is ubiquitously expressed. Therefore, it not unreasonable to assume that the allelic expression ratios found in blood cells and fibroblasts are similar to those in many other cells.

On the basis of our results, we propose that increased rather than decreased expression of *FTO* is causally evolved in increased BMI measures and obesity-associated traits. This interpretation is consistent with the mouse models, in which decreased levels of *Fto* cause leanness.[7,8] Increased expression of the risk allele as shown here is compatible with a role of CUTL1 in activating *FTO*, as originally suggested by Stratigopoulos *et al*,[9] although other transcriptional activators may have a role. It is tempting to speculate that subtle reduction of *FTO* expression by pharmacological intervention may contribute to the prevention of obesity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank all individuals for donating blood and skin biopsies, physicians for drawing blood, Regina Kubica for technical assistance with fibroblast cultures and the Bundesministerium für Bildung und Forschung (NGFN plus 01GS0820) for financial support.

WEB RESOURCES

The URLs used for this study are as follows: UCSC Human Genome Browser Gateway at http://genome.ucsc.edu/NCBI Single Nucleotide Polymorphism at http://www.ncbi.nlm.nih.gov/projects/SNP/HapMap Genome Browser at http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap3r2_B36/.

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Supplementary Information accompanies the paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)