Author's response to reviews

Title: Association between a rare SNP in the second intron of human Agouti related protein gene and increased BMI

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Author's response to reviews: see over
To Editors of BMC Medical Genetics

Regarding manuscript ID 1852391747232845

Thank you for your repeated evaluation of our manuscript and the valuable comments from the referees.

We have performed additional experiments and included additional data in paper as requested by some referees. In particular we included substantial number of additional samples and performed genotyping of other SNP identified in our sequencing experiments.

We enclose a revised manuscript and a detailed list of the changes made, and response to each of the comments raised by the referees. Inclusion of new date resulted in some minor changes that are not directly requested by referees, but are necessary to adequately describe methods, results and analysis.

We hope that with these changes the manuscript will now be acceptable for publication in BMC Medical Genetics.

Best regards,

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Janis Klovins Ph.D.
Changes in manuscript caused by inclusion of additional data:

1. Due to additional work needed for experiments, analysis and drafting the paper Ineta Kalnina is pointed as a first author of the manuscript.

2. Due to the inclusion of additional samples all quantitative values obtained as a result of the new analysis have been changed throughout the paper and tables.

   We also introduce the following changes in Results and discussion: “The estimated minor allele frequency of rs11575892 was 0.015 and no significant departure from Hardy-Weinberg equilibrium was observed (p=1.0). When tested for non-genetic factors, we found that hypertension, angina pectoris, heart failure, myocardial infarction, type 2 diabetes (T2DM) and dyslipidemia. were significantly (p<0.05) associated with increased logBMI. We did not find significant difference in logBMI between females and males (p=0.34).”

3. Due to the inclusion of additional SNP in the analysis we introduce the following changes in the Methods section: “Our sample size provided 80% power (at α=0.05) to detect difference between rs11575892 genotypes in logBMI of >0.040 assuming minor allele frequency of 0.015 and to detect difference between rs5030980 genotypes in logBMI of >0.028 assuming minor allele frequency of 0.044. PLINK 1.00 software (http://pngu.mgh.harvard.edu/purcell/plink/) [19] was used to perform Hardy-Weinberg test, LD calculations, haplotype based association and ……”

4. We add following sentence in acknowledgment section: “We acknowledge Genome Database of Latvian Population, Latvian Biomedical Research and Study Center for providing data and DNA samples.”
Referee #1:

We thank the referee for the critical comment.

Answer to the following comment:

Referee's comment: “I understand and appreciate the difficulty of studying rare SNPs, coupled with the limited size of cohorts available. However, given the rare nature of the SNP being studied, I still really believe that a bigger study is required before any proper conclusions can be made.”

Answer: We agree that disadvantage of present study is small sample size and include additional 439 samples in study. In order to avoid stratification of study group we included the samples collected by the same research group using the same questionnaires and protocols. Similarly we applied the same selection criteria. Power calculations indicated that new sample size decrease the difference of logBMI mean value that can be detected between genotypes of rs11575892 with 80% power from 0.053 to 0.04. We observed change in mean logBMI of 0.045 (from adjusted values) in our study.

We include following changes in the Abstract: “We further screened rs11575892 in a selected group of 1135 and rs5030980 in group of 789 participants from the Genome Database of Latvian Population and Latvian State Research Program Database.”

We include the following changes in the Methods: “The study was based on data and samples from the Genome Database of Latvian Population (Biobank 1), the disease based biobank comprising 1173 subjects and 439 subjects from population based Latvian State Research Program Database (Biobank 2). Both biobanks were collected by using the same protocols, questionnaires and sample treatment techniques.”; and “Genotyping of the rs11575892 in all other samples and genotyping of rs5030980 was performed using pre-designed TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, California, USA) on ABI 7500 Real-Time PCR system (Applied Biosystems) according to the supplier’s recommendations.”

The following change is included in the Results and discussion section: “The rs11575892 had not previously been associated with any phenotype and we tested the association between this SNP and BMI in a cohort of 1135 individuals from the biobank collections that were available to us.”
Referee #2:

We thank the Referee for the critical comments and careful inspection of the results.

Answers to the following comments:

1. Referee’s comment: “Three SNPs have been detected in this sample, and the authors choose to study the less variable of them for which no variant homozygote was observed. Why they did not analyzed also one of the two other SNP that are in complete linkage disequilibrium with each other?”
   It would be important to analyze SNPs that have been reported previously to be associated with body composition such as Ala67Thr. Did the authors detect this relatively frequent variant in their sample of 95 patients?” and “But why not present these results or at least made a statement on them in the Discussion?”

1. Answer: Due to request from at least two referees to present additional data on rs5030980, we decided to genotype this SNP in samples that were available from previous genotyping and in all additionally included samples.

We include following changes in the Abstract: “and investigate whether the previously reported SNP rs5030980 and the rs11575892, a SNP that so far has not been studied with respect to obesity is associated with increased body mass index (BMI).” and “We further screened rs11575892 in a selected group of 1135 and rs5030980 in group of 789 patients from the Genome Database of Latvian Population and Latvian State Research Program Database.” and “No association was found between rs5030980 and BMI”

We include following changes in the Methods: “rs5030980 was genotyped subsequently in total of 789 samples comprising 350 samples from Biobank 1 and all 439 samples included in study from Biobank 2. The 348 samples from initial selection were not available for genotyping of rs5030980.” and “Our sample size provided 80% power (at α=0.05) to detect difference between rs11575892 genotypes in logBMI of >0.040 assuming minor allele frequency of 0.015 and to detect difference between rs5030980 genotypes in logBMI of >0.028 assuming minor allele frequency of 0.044.”

The following text is included in the Results and discussion section: “In order to test previously reported associations of rs5030980 with decreased body fatness we subsequently performed genotyping in available samples (n=789) that were used for genotyping of rs1157892. The estimated minor allele frequency of rs11575892 was 0.044 and we did not observe significant departure from Hardy-Weinberg equilibrium (p=1.0). Pairwise LD analysis showed absence of LD between both SNPs (D’=1.00, r^2=0.001). No association of rs5030980 with BMI was found neither in GLM or logistic regression analysis (Table 2.). We did not find any difference in mean values between GG and GA genotypes as opposite to previous report [16]. We found only
one AA genotype carrier in our study group with slightly increased BMI value (29.58 kg/m$^2$). This is in disagreement with results of Marks et.al where carriers of AA displayed significantly lower BMI [17]. Relatively low frequency of this SNP however permits to compare these effects with sufficient power. Haplotype analysis did not return significant difference in BMI values between three possible haplotypes (data not shown).”

2. Referee’s comment: “The association of a minor allele with body composition phenotypes, here a lower fatness, was reported earlier, as the association with higher values of obesity for a major allele. Both indicated a similar effect of AGRP on body composition. What is the point of the authors on their "new" association? They cannot state that this is an association of the T allele since they did not test yet TT homozygotes. Also, they cannot claim at an association of a minor allele at this point but only at an effect of heterozygotes. We would also like to point that Referee #3 agrees on the fact that this is the first time when SNP in AGRP is associated with increased BMI.” and “Not true since the paper of Argyropoulos.”

2. Answer We replace previous statement in the Abstract with following: “This study presents an association of rare allele of AGRP polymorphism in heterozygous state with increased BMI”

3. Referee’s comment: “In Methods, it makes no sense to use an ANOVA knowing the importance on BMI of covariate effects such as age and gender.” and “This did not give more information but false information. This should be skipped. Significance of co-variates could be tested in the ANCOVA.”

3. Answer: We remove the results of unadjusted analysis from Table 2. and introduce the following changes in Results and discussion: “…we adjusted association of rs11575892 with BMI using general linear model including only those factors and covariate that showed significant association with BMI (Table 2).” and “Correrted empirical P values generated by permutation test were significant (p=0.0016) in multivariate linear regression analysis. We also used median BMI (27.34 kg/m$^2$) as a cut off value for overweight threshold in a categorical analysis. A significant difference in genotype distribution between the normal and the overweight groups was found in logistic regression analysis adjusting for age and sex (p=0.011) (Table 2). “
Referee #3:

We are encouraged that the referee states that: “The manuscript has been adequately revised”
Referee #4:

We thank the referee for the comments.

Answers to the following comments:

1. Referee’s comment: “As it has been noted by more than one reviewer, additional genotyping of at least one SNP detected by resequencing would be desirable in the studied sample.”

1. Answer: Due to request from at least two referees to present additional data on rs5030980, we decided to genotype this SNP in samples that were available from previous genotyping and in all additionally included samples.

We include following changes in the Abstract: “and investigate whether the previously reported SNP rs5030980 and the rs11575892, a SNP that so far has not been studied with respect to obesity is associated with increased body mass index (BMI).” and “We further screened rs11575892 in a selected group of 1135 and rs5030980 in group of 789 patients from the Genome Database of Latvian Population and Latvian State Research Program Database.” and “No association was found between rs5030980 and BMI.”

We include following changes in the Methods: “rs5030980 was genotyped subsequently in total of 789 samples comprising 350 samples from Biobank 1 and all 439 samples included in study from Biobank 2. The 348 samples from initial selection were not available for genotyping of rs5030980.” and “Our sample size provided 80% power (at α=0.05) to detect difference between rs11575892 genotypes in logBMI of >0.040 assuming minor allele frequency of 0.015 and to detect difference between rs5030980 genotypes in logBMI of >0.028 assuming minor allele frequency of 0.044.”

The following text is included in the Results and discussion section: “In order to test previously reported associations of rs5030980 with decreased body fatness we subsequently performed genotyping in available samples (n=789) that were used for genotyping of rs1157892. The estimated minor allele frequency of rs11575892 was 0.044 and we did not observe significant departure from Hardy-Weinberg equilibrium (p=1.0). Pairwise LD analysis showed absence of LD between both SNPs (D'=1.00, r^2=0.001). No association of rs5030980 with BMI was found neither in GLM or logistic regression analysis (Table 2.). We did not find any difference in mean values between GG and GA genotypes as opposite to previous report [16]. We found only one AA genotype carrier in our study group with slightly increased BMI value (29.58 kg/m^2). This is in disagreement with results of Marks et.al where carriers of AA displayed significantly lower BMI [17]. Relatively low frequency of this SNP however permits to compare these effects with sufficient power.
Haplotype analysis did not return significant difference in BMI values between three possible haplotypes (data not shown).”

2. **Referee’s comment:** “Replication genotyping of the rare detected variant rs11575892 in additional sample still should be considered.”

2. **Answer:** We agree in general that replication of results in additional population would be the best indicator of real association. Unfortunately, at the present time we do not have access to the equally designed study group from different population. However in order to increase statistical power we included additional 439 samples in study and our calculation shows significantly increased power. (see answer to referee 1.)

3. **Referee’s comment:** “Table 1: under “Values” header reader can find summary statistics of different kind. This column is unclear, needs to be completed”

3. **Answer:** We have changed the Table 1 by introducing the separate columns for categorical and quantitative data.

3. **Referee’s comment:** “Table 2 still remains difficult for understanding. This table needs more work and has to be self explanatory. It’s not a good practice to present means and standard errors from one statistical test and p-values from another. Statistical tests can be stated in the header. Legend of this table is too long, which makes the table unreadable. Genotype counts are needed in this table.”

3. **Answer:** We agree to the Referee comment and change the Table 2 to make it more readable. In addition results from genotyping of rs5030980 are included in this table. We have also removed the results of unadjusted statistical analysis as requested by referee 2.

4. **Referee’s comment:** “Answer to the reviewer 2, comment 7, seems to be unsatisfactory, since the unnecessary speculation still remains”

4. **Answer:** We agree to the Referee’s comment and remove the comment regarding the diagnostic markers.