Dear Dr Le,

Thank you for taking the time to review our manuscript entitled "CT60 genotype does not affect CTLA-4 isoform expression despite association to T1D and AITD in northern Sweden”. After carefully considering the comments from all three referees, we have amended our manuscript accordingly. We feel that we have addressed all points raised by the reviewers, and we hope that you will reconsider our manuscript for publication in BMC Medical Genetics. Please find below a summary of our amendments in relation to each reviewer's comments.

Yours sincerely,

Dan Holmberg

Referee 1:

Major Compulsory Revisions

1. The major concern is on the genetic association studies. It is not acceptable to combine two different diseases in one category. The reason for combining T1D and AITD is because of the small sample size. The authors should increase the sample size for both diseases if they wish to present the association data.

Reply:

The study undertaken here is based on several reports suggesting that the contribution of CTLA-4 to susceptibility to both T1D and AITD is mediated by the same genetic variants. This includes our own studies of familial cases of T1D and AITD affecting different members of the same family and allowing us to obtain evidence in favour of linkage to the CTLA-4 locus under a model in which both types of autoimmune disease were set as affected (Einarsdottir et al. Eur. J. Hum Gen. 11:81-84, 2003). We believe these data justified our study design in which T1D and AITD cases were all considered as affected. Since this was not made totally clear in the manuscript we have now modified the Background section to include "In line with this notion we previously analyzed a large pedigree from northern Sweden in which multiple individuals suffered T1D and AITD. Applying a model in which all T1D and AITD patients were considered affected, evidence in form of linkage to the MHC and the CTLA-4 loci was obtained [5]."
2. The explanation of higher T1D frequency in Sweden by the higher CTLA4 genotype frequency is not really justified. This is just a correlation.

Reply:
We agree with the reviewer that the higher CTLA-4 genotype frequency would not explain the higher T1D frequency in Sweden. We do retain, however, that the observed higher frequency of the disease associated variants of CTLA-4 in the northern Swedish population is interesting and as we had phrased it in the manuscript "might contribute to the high incidence...". This statement we still believe is justified by the data.

3. Presentation of the association data in Table 1 is very misleading. Indeed, the so-called "reference" genotype is different between cases and controls.

Reply:
We agree with the criticism of the reviewer and have now revised Table 1 accordingly.

Referee 2:
Major Compulsory Revisions

1. The authors use logistic regression for association analysis. This is unusual as a regular chi-square analysis of counts in contingency tables is more commonly used for single-marker analysis. With contingency/chi-square analysis the findings seem less significant. The risk therefore exists that the logistic regression analysis has inflated the significance.

Reply:
We agree with the reviewer that regular chi-square analysis of counts in contingency tables is more commonly used for single-marker analysis. However, logistic regression calculations should give basically the same values as chi-square analysis when no correction for confounders is made. We have now performed the calculation using chi-square analysis as well and the results were marginally less significant with the logistic regression method used in the manuscript:

**Chi-square**

MH30: CG vs. CC p= 0.011 OR 2.07 (1.17<OR<3.65)

GG vs. CC p= 0.0014 OR 2.467 (1.40<OR<4.35)

CT60: AG vs. AA p= 0.006 OR 2.20 (1.23<OR<3.91)

GG vs. AA p= 0.0002 OR 2.82 (1.58<OR<5.05)

JO31: GT vs. TT p= 0.008 OR 2.04 (1.20<OR<3.48)

GG vs. TT p= 0.0005 OR 2.51 (1.47<OR<4.30)

**Logistic regression**

MH30:CG vs. CC p = 0.01 OR 2.07 (1.18-3.65)
GG vs. CC p = 0.002 OR 2.46 (1.40-4.35)
CT60: AG vs. AA p = 0.008 OR 2.20 (1.23-3.91)
GG vs. AA p = 0.0005 OR 2.83 (1.58-5.05)
JO31: GT vs. TT p = 0.009 OR 2.04 (1.20-3.48)
GG vs. TT p = 0.0008 OR 2.51 (1.47-4.30)

If so requested by the reviewer, the results from the Logistic regression could be exchanged for the results of the Chi-square in Table 1.

The authors claim that heterozygous individuals have a greater risk compared to non-carriers. This is not at all obvious from Table 1 where the percentage of heterozygosity is very close to 47% for the three SNPs in both cases and controls. GG homozygous genotypes are consistently increased in cases with around 7 to 9%. This seems to conflict with the statement that there is no increased risk for homozygous compared to heterozygous.

Reply:
Since Table 1 is now revised the greater risk of heterozygous individuals compared to non-carriers is more apparent. The p-value for MH30, CT60 and JO31 when heterozygous individuals are compared to non-carriers is 0.01, 0.008 and 0.009 respectively (Table 1).

P-values for the comparison between homozygous individuals and heterozygous carriers have been added to the Results section and the text has been modified as follows: "However, we did not observe any increased risk in homozygous individuals compared to heterozygous carriers (MH30 p= 0.26, CT60 p= 0.11 and JO31 p= 0.17) allowing for both dominant and multiplicative modes of inheritance in our AITD/T1D case-control material".

2. Table 1 is a bit confusing as it is unclear to what precisely the OR’s and P value relate.

Reply:
We agree with the criticism of the reviewer and have now revised Table 1 accordingly.

3. The genotype percentages, when calculated from the counts on the basis of the full number of controls (n=865) and cases (n=253) are incorrect. This suggests that not all controls and cases yielded suitable genotype information. It is therefore important that the genotyping success rate is given for each SNP in cases and controls separately. This should be inserted as an extra column in Table 1. For MH30, for instance, 777 controls gave genotype information out of a total 865 used. This implicates a genotyping success rate of 89.9%.

Reply:
In accordance with the suggestion of the reviewer, the genotyping success rate has been calculated for each SNP in cases and controls and the following sentence has been added to the methods section "A genotyping success rate from 89.9% to 99.5% (Table 1) was obtained for the SNPs under investigation". The genotype success rates were added to Table 1.
4. Since the authors have not implemented any correction method for multiple comparisons, they should clearly state the single-hypothesis replication analysis they aim to perform on the basis of the previously published work by Ueda and others.

Reply:

In accordance with the suggestion of the reviewer we have added to the Abstract that "The aims of our study were to replicate the association previously published by Ueda et al. of polymorphisms in the CTLA-4 region to T1D and AITD and to determine whether the CT60 polymorphism affects the expression level ratio of sCTLA-4/flCTLA-4 in our population" and to the Background section that "Based on this background, we set out to investigate the previously published [8] association of three polymorphisms in the CTLA-4 region to T1D and AITD in the population of northern Sweden and subsequently investigate the expression of flCTLA-4, sCTLA-4 and ICOS based on the CT60 genotype".

Minor Essential Revisions

The title and the abstract of the manuscript should be changed to reflect that the analysis refers to AITD anc T1D only. The current title is a bit misleading as there are many more autoimmune diseases than only AITD and T1D.

Reply:

The title is now changed to: CT60 genotype does not affect CTLA-4 isoform expression despite association to T1D and AITD in northern Sweden. Moreover, we have consistently exchanged "autoimmune diseases" to "T1D and AITD" all through the text of the manuscript.

Discretionary Revisions

1. The authors have pooled AITD and T1D patients. It might be interesting to evaluate the association in the separate patient cohorts as well as in the pooled one.

Reply:

As discussed above (Reviewer #1, p1) the study undertaken here was conducted under a model in which both types of autoimmune disease were set as affected. Dividing the diseases into two different cohorts and publishing these results could in this context be considered as multiple testing. We do, however, find significant association to all three SNPs when analyzing the AITD and T1D patients as separate cohorts (for AITD: p=0.033 for MH30; p=0.031 for CT60, p=0.033 for JO31; for T1D: p=0.026 for MH30; p=0.003 for CT60 and p=0.009 for JO31)

Referee 3:

Minor Essential Revisions

Abstract: please specify the number of AITD and T1D cases for clarity what type of autoimmune disease this study uses.

Reply:

The number of AITD and T1D cases is now added to the Abstract and the text modified to read "Three SNPs were genotyped in 253 cases (104 AITD cases and 149 T1D cases) and 865 ethnically matched
controls."

Abstract: add the fact that mRNA was measured in CD4+ cells.

Reply:

The fact that mRNA from CD4+ cells was used to measure expression of CTLA-4 isoforms is now added to the abstract and the text has been revised to read: "Blood from 23 healthy individuals was used to quantify mRNA expression of CTLA-4 isoforms in CD4+ cells using real-time PCR"

Results section: "The analysis of serum sCTLA-4 protein levels". I know from experience that the kits available for measuring serum levels are at least questionable. This is illustrated by the figure 3, in which only one healthy subject shows measurable serum level. Is this expected? Or is this a sign that the kit does not detect serum CTLA4? The kit detects no doubt the CTLA4-Ig protein, but we and others have failed to detect any positive subject with a variety of kits. I suggest that the authors discuss the probability that the kits fails to detect any serum level CTLA4.

Reply:

To clarify this issue, we have revised the text in the Method section t to read: "Detection of sCTLA-4 serum levels" and "Sandwich immunoassay for sCTLA-4 was developed in our laboratory and performed using Gyrolab Bioaffy platform (Gyros AB, Uppsala, Sweden)".

In the Discussion section the following sentences has been added: "We have developed a sensitive sandwich immunoassay using the Gyrolab Bioaffy platform technology to detect and quantify sCTLA4 in serum samples. This technique has several advantages compared with standard ELISA assay. First, the standardization of liquid input into the detection system reduced dramatically variations within samples as well as intra- and infra-assay variation. Further, the possibility to visualize binding peaks as a histogram from the Gyros platform improved the optimisation of the assay and decreases background levels and finally the Gyros matrix technology and the particular sensitive luminescence read-out has significantly improved the detection limit of the assay compared to commercial ELISA kit (data not shown)".

We have also altered Figure 3 to more clearly illustrate the detection of low levels of serum CTLA-4 in the different disease status and genotypes.

4. Discussion: I would appreciate some more (immunological) discussion of the consequences of using different cell-types for the mRNA analysis.

Reply:

The following sentences have been added to the discussion paragraph: "The studies mentioned above have used different populations of cells to measure mRNA expression of CTLA-4 isoforms. This might influence the result, since CD4+ CD25+ T cells constitutively express CTLA-4, while CD4+ CD25- T cells display a much lower expression of CTLA-4 [20]. Besides the difference in expression between CD4+ CD25+ and CD4+CD25- cells, CTLA-4 is expressed by a variety of other cell types including activated CD8+ T cells, activated B cells and both single and double positive thymocytes [22, 23]".