Author's response to reviews

Title: Association Analysis Identifies ZNF750 Regulatory Variants in Psoriasis

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Version: 2 Date: 21 November 2011

Author's response to reviews: see over
Dear Editor,

Thank you for considering our manuscript for publication in BMC Medical Genetics. We are appreciative of the reviewers' generally positive comments and have addressed their concerns in point-by-point fashion below. Thank you for your consideration of this revised manuscript.

Sincerely,

Wilson Liao

**Associate Editor’s comment:**

The most important is the issue of validity of your findings given that multiple variant was tested. The other issue that I like to know, whether published genome-wide association studies on psoriasis showed some evidence for a relation between this gene (or adjacent SNPs) and Psoriasis? If yes, how strong it was? And if not, how then you explain your conclusion when previous GWAs(es) have found no association between ZNF750 and Psoriasis.

**Response:**

Addressing multiple hypothesis testing is an important methodological issue. For our finding that two ZNF750 haplotypes were associated with psoriasis (Table 3), we performed empirical permutation testing via the program Haploview (100,000 permutations) to show that these haplotypes were still significant after accounting for multiple tests (p=0.0311 and p=0.0024). Permutation testing is considered one of the gold standards to control for multiple hypothesis testing (Westfall PH, Young SS, 1993. Resampling-based multiple testing. New York: Wiley). In addition, new to the revised manuscript we have also calculated false discovery rate (FDR) q-values (Benjamini and Hochberg, Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. Roy. Statist. Soc. Ser. B 57 (1995), no. 1, 289–300), now reported in the manuscript text. The q-values for these two haplotypes were q=0.037 and q=0.0077, which again confirm that these two haplotypes are significantly associated with psoriasis. This is now noted in the manuscript text within the Results section, under “Association Testing of Identified Variants.”

For the association testing of ZNF750 rare variant groups (Table 4), the unadjusted p-value of 0.041 for the 5’UTR and promoter variants does not meet a strict Bonferroni-corrected threshold of 0.05/6=0.008 (now noted in the Results). However, given the observation that 10 ZNF750 variants were detected in cases versus only 1 variant in controls, we were prompted to perform functional evaluation of 4 of these rare variants using a luciferase reporter assay, which showed a reduction of promoter activity of all 4 variants in the presence of PMA. Thus, the initial genetic signal was validated functionally.
Regarding GWAS associations in the ZNF750 region, we went back and examined data from a psoriasis GWAS study involving 1348 cases, 1368 controls (Nair RP, et al. Nat Genet 2009, 41(2):199-204). We examined the interval 200 kb upstream to 200 kb downstream of ZNF750 and imputed all HapMap 3/1000 Genome SNPs in this region. This resulted in 1,279 SNPs, none of which had an association p-value less than 1.0 x 10^-3 (this is now noted in the Discussion). We view this as evidence that no common ZNF750 SNPs are causal for psoriasis, which is the result we presented in Table 1.

How does this fit with our detection of two ZNF750 haplotypes associated with psoriasis? The SNP that is driving our significant haplotype associations is rs35156590, a non-coding T/(-) indel that is not present on the GWAS platform and not imputable by HapMap3/1000 Genomes. It is possible that this SNP is tagging a psoriasis variant beyond the 200 kb examined. Thus, we interpret our results to mean that no common ZNF750 SNPs are causal for psoriasis, but that SNPs on 17q in LD with our significant haplotypes could possibly be associated with psoriasis.

With regards to how to interpret our rare variant findings in relation to GWAS, since the GWAS studies to date have assayed only common variants (MAF>5%), they would not be expected to detect the impact of rare variants, which would be more recent mutations. Thus, our finding that ZNF750 promoter variants affect gene expression are consistent with the notion that some of the “missing heritability” in common complex diseases such as psoriasis may reside in rare variants which are not captured by GWAS (Maniolo TA, et al. Nature. 2009 Oct 8;461(7265):747-53).

**Reviewer 1**

We thank Judith Bergboer for reviewing our manuscript and for providing helpful comments.

Major compulsory revision:

1. “Resequencing of candidate genes to find variants associated with complex diseases is a promising approach to identify functional mechanisms that would contribute to disease causation. In case the allele frequencies of these variants are low, large numbers of cases and controls are required. In this study a large number of variants were found but after correction for multiple testing none of the individual variants was found to be significantly associated. In an attempt to investigate if haplotypes of rare variants were associated with disease, six of these were examined and only the 5'- regulatory variants reached borderline significance. Was the p-value corrected for multiple testing? Similarly, how were the empirical p-values of the common haplotypes obtained?”

Response:

As mentioned above, for the haplotype associations, we calculated empirical p-values using permutation testing (100,000 permutations in Haploview software, noted in
Methods section under “Statistical Methods”). This is one method to correct for multiple hypothesis testing. In addition, we have calculated False Discovery Rate (FDR) q-values for these haplotypes and confirmed they remain significant (q=0.037 and q=0.0077). For the 5’ regulatory variants, the p-value of 0.041 does not correct for the 6 groupings tested, but functional testing using luciferase assays indicated that all 4 variants tested affected promoter activity in the presence of PMA. Thus, the initial genetic signal was validated functionally.

2. “In the Results section the segregation analysis is quite extensively shown. The study set up was a case-control study. I don’t see the added value of the segregation analysis, because in complex diseases like psoriasis there are often no clear segregation patterns, and this is also the case in the presented analysis. The data on the (non)segregation of the 233C>T variant with disease are anecdotal and do not contribute to our understanding of disease mechanisms. I would skip the last 4 lines of the Results section.

Response:
We thank the reviewer for this insight. We agree that the segregation analysis (Figure 3) doesn’t add much more to the Results, except to point out that these rare 5’-regulatory variants are not Mendelian (whereas the two previously published ZNF750 familial mutations were autosomal dominant). We have therefore deleted the segregation analysis and Figure 3, and now simply note in the Discussion that these variants did not segregate with disease.

3. In the Methods, section statistical methods, HWE was calculated and the threshold was set at p=0.001. Why?

Response:
The default setting in the software used to calculate HWE was p=0.001; we have revised the threshold to a more standard p=0.05 (all markers passed) and changed this in the manuscript.

4. What do you mean with a biological triplicate? Did you actually use 3 different keratinocyte donors. Or did you perform 3 independent experiments with one donor cell line?

Response:
The promoter luciferase assay was done in three independent experiments. Each experiment was done separately by culturing an aliquot of human primary keratinocytes that were purified from one donor. Cells were seeded in each well of a 6-well plate and were transfected after 24 hours with each reporter construct along with pRL-TK vector containing the Renilla luciferase gene as an indicator for normalization of transfection efficiency. After 24 hours, the cells were stimulated with PMA for the PMA positive set of
experiments. Cells were incubated for an additional 48 hours and then the luciferase activity was measured twice (technical replicate). The firefly luminescence measurements were normalized to Renilla luminescence and presented as relative luciferase units. We repeated these results in three independent experiments. We realized that the last sentence in the “Transfection and Luciferase Assay” of the Method section can be confusing and we changed it accordingly to “All experiments were performed independently three times using primary keratinocytes from a single donor.”

5. In Figure 2, could you please clarify the large differences in SD in the 5’UTR variant vectors and WT luciferase activity?

Response:
Figure 2 shows the luciferase activity of ZNF750 regulatory region in human primary keratinocytes before and after PMA stimulation. The ZNF750 luciferase activity is displayed in units relative to the ZNF750 regulatory wild type (WT) sequence. In addition, we had two controls: the pGL3-Basic, an empty vector, as a negative control, and a TK plasmid contains a tyrosine kinase promoter as a positive control. While the pGL3-Basic was negative, the TK promoter showed four change fold higher promoter activity than the WT sequence (data not shown). For each experiment, we first normalized the transfection efficiency by the Renilla luciferase measurements and then each variant was presented relative to the WT normalized luciferase measurement. The raw SD values were also converted to relative units. Since by default the WT sequence was assigned a value of 1.0 luciferase units, there is no standard deviation for WT. The reason for displaying the WT bar is to demonstrate the reduction in the promoter activity of the variants. We have clarified this in the Methods section by stating “Firefly luminescence was normalized to Renilla luminescence and reported as relative luciferase units compared to wild-type sequence”.

Discretionary revisions:

1. In the Results section the 2 isoforms of ZNF750 mRNA were introduced. The authors checked the presence of isoform A and B in several cell lines, but do not show the data. It would be illuminating to present this data. Moreover, it would also be informative in the PMA stimulations of primary keratinocytes to show which isoform is upregulated by PMA and to consider these two isoforms in the discussion section.

Response:
To characterize the ZNF750 promoter, we performed 5’ Rapid Amplification of cDNA Ends (RACE) for ZNF750 in primary keratinocytes and placenta (as control) and found two 5’ RACE PCR products that were specific to ZNF750 in human primary keratinocytes. The short product represents the mRNA isoform A as in Refseq gi13375990; NM_024702.1 and it abundant in several available mRNA datasets (UCSC genome browser). The long product is rare isoform, previously demonstrated only in cDNA from tongue tumor tissue [GenBank:DA436414] (Kimura et at, Genome Res
2006) represents the mRNA isoform B and includes an additional 500 bp of sequence upstream to exon 1 of mRNA isoform A (Figure 1). Our analysis suggests that both isoforms translate the same ZNF750 protein. The expression levels of isoform A were published in our previous paper (Birnbaum et. al., Nature Genetics 2006) which support that the most abundant variant of ZNF750 transcript corresponds to isoform A and our additional expression analysis basically showed the same information. Therefore, we focused our study on characterizing the ZNF750 regulatory region according to isoform A. Although characterizing the response of isoform B to PMA stimulation is an interesting question, we feel it lies beyond the focus of the current manuscript and thus might be better addressed elsewhere (We are collaborating with a group in Israel to perform further molecular and cellular characterization of ZNF750 which will be published separately).

Minor essential revisions:
1. *Results*, section association testing of identified variants last sentence states $p<0.05$, this should be $p>0.05$.
2. *Table 2*, please check cells Novel_15 and Novel_24 for $F_{case}$ values.

Response:
Thank you, we corrected these in the text as recommended.

**Reviewer 2**

We thank Maartje Aukes for reviewing our manuscript and for providing a helpful suggestion.

Major compulsory revision:

1. “The type, medical status and recruitment of controls used is unclear. How were the cases and controls recruited? The paper now only mentions (section Methods, Patients) where DNA samples were collected (“collected form University of California, SF and Washington University”). Were the controls patients? Have they been screened for psoriasis? Using what methods? Please provide these details.”

Response:
We have now described the cases and controls in more detail. The Methods section now reads: “DNA samples from 716 unrelated Caucasian psoriasis cases, 397 Caucasian healthy adult controls, and 20 additional family members were collected from the University of California, San Francisco and Washington University, St. Louis. Cases were recruited from outpatient dermatology clinics and the diagnosis of psoriasis was confirmed by a board-certified dermatologist. Healthy controls, recruited from the local community, reported no history of autoimmune disease or cancer according to a written screening questionnaire. All subjects gave written informed
consent for study participation in accordance with the institutional review board at their respective institutions.”

We note that, although a written questionnaire might not exclude all control subjects from having psoriasis, the prevalence of psoriasis in the control group would not be expected to exceed the prevalence of psoriasis in the general North American population, approximately 2-3%, which would be unlikely to affect our results. In addition, the presence of psoriasis in the control group would only serve to decrease our power to detect a genetic difference between cases and controls. Thus, our finding of excess rare 5’ regulatory variants in psoriasis cases remains valid.