Author's response to reviews

Title: A comprehensive evaluation of the role of genetic variation in follicular lymphoma survival

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Author's response to reviews: see over
BMC Medical Genomics

Dear Editor,

We hereby resubmit the manuscript “A comprehensive evaluation of the role of genetic variation in follicular lymphoma survival”.

We thank the reviewers and editors for excellent comments and the opportunity to address the comments and revise the manuscript. Below please find our specific responses.

Most sincerely,

Fredrik Baecklund, on the behalf of all authors
Stockholm, 9 June 2014
Reviewer: Niels Weinhold

Major Compulsory Revision

Methods:

1. How did the authors calculate the first three principal components using Eigenstrat? Did they use the complete SNP set or a pruned one including only unlinked SNPs?

We apologize for being unclear with regard to this issue. In both SCALE and UCSF we used a pruned SNP set with unlinked SNPs only. In the UCSF cohort we did multidimensional scaling in PLINK, while we used Eigenstrat in R in SCALE. The different methods used were due to different preferences of the primary analysts in SCALE and UCSF, respectively, but should not have impacted on the results, since the methods are similar (Wang, BMC Proceedings 2009, reference 48 in the revised manuscript). The methods used are now described in the Methods section (page 10, rows 4-6).

2. The authors adjusted the GWAS for age at diagnosis and population stratification. Analyses for candidate gene associations were additionally adjusted for sex. Why didn’t the authors adjust for sex in the GWAS although the data was available? If sex was not associated with the frequency of identified candidate SNPs they should at least mention that. What about FLIPI adjustment in GWAS?

We agree that adjusting for different variables (i.e. sex) in the two parts of the analyses is inconsistent, although sex was not a strong confounder in the candidate gene study.

To improve consistency, and in recognition of this comment, we added sex as a covariate in the regression model in both the SCALE and UCSF GWAS. Overall, the results changed very little, as illustrated in SCALE by the graph below. In the revised manuscript, we present the results additionally adjusted for sex.

Adjusting for FLIPI in the combined GWAS was not possible, since clinical information was not available for the UCSF cohort.
Results:

3. Was there any association of the promising candidate at 17q24 with established prognostic factors like performance status or lactate dehydrogenase levels?

Data on performance status and lactate dehydrogenase levels were available in the Swedish SCALE cohort. In this cohort, there was no evidence of an association of rs10491178 with performance status (ECOG categorized into <2 and ≥2, Fisher’s exact test p=0.15) or lactate dehydrogenase levels (Fisher’s exact test p=0.40). Nor was there any association with FLIPI risk groups (Fisher’s exact test p=0.27). We have added a brief comment in the Results section about the lack of correlation with these factors (page 13, rows 10-13).

4. Was there any association between the promising candidate at 17q24 and lymphoma progression in the Swedish cohort?

We investigated this but found no evidence of an association with the top variant at 17q24 and lymphoma progression (HR= 1.02, 95% CI 0.63-1.65). In the revised manuscript, we have added this information in the Results section (page 13, rows 16-17) and discuss differences in the definition of these outcomes in the Discussion section (page 20, rows 9-17). In response to reviewer 2, comment 5, about potential associations with overall survival, we have also added a supplementary table.
5. Fifty-four of the SNPs were nominally significantly associated with lymphoma-specific death in the UCSF cohort... How many of them showed the same direction of effect in comparison to the SCALE cohort?

We now performed a full meta-analysis of the two GWAS rendering this question less relevant to the revised manuscript. In the previous combined analysis, of the 54 SNPs nominally associated with lymphoma-specific death in UCSF 39 (72%) showed the same direction of effect in SCALE.

6. Results of the candidate SNP study are difficult to follow. A) The authors should not write that in their study the opposite allele showed the opposite effect. They should use the same order of alleles. B) If there were large differences between published results and their own data that should be described in the results part and not only in discussion as this was a confirmation study, e.g. rs18001131 in MTHFR. C) In addition to that they should mention in the results part which sets they used for validation and for which association (death/progression). Rs2466571 in CD46 is an example for a SNP showing an effect for progression but not for lymphoma-specific death. This could be just by chance but nevertheless there might be SNPs being associated with progression/event free survival but not overall survival. D) If the authors want to validate published results they should use the same outcome variable as used in previous studies. In addition to that they could present data for lymphoma specific death and discuss differences or comparable results. E) Finally, the authors should calculate the power of their study to demonstrate a relationship between published SNPs and outcome, at the 5% threshold.

We thank the reviewer for excellent suggestions!

A) We now use the same order of alleles for rs2466571 (CD46) in our study as in the previously published study (revised in Results section page 14, row 12-13 and Discussion section page 17, row 11-15 + Table 4). The two SNPs in IL8 (rs4073 and rs2227307) have been reported in two previous studies with different order of alleles in each, however, and we adhere to one of the studies (Cerhan et al 2007, ref no 7 in manuscript).

B) We added information in the Results section of results pointing in different directions between the current and previous studies (rs1801131 and rs2069762, page 14, rows 10-12).

C) We have now clarified in the Results section which cohort that was used for analysis of time to lymphoma-specific death and progression, respectively (page 14, rows 5-6). We now also state which outcome measure that was
used in the previously published study(-ies) when we compare the previous results with our own findings (page 14).

D) We agree with the reviewer that the same outcome measure should be used when the aim is to validate previous findings. Our aim was rather to use previously published studies of SNPs and any outcomes in FL as a basis for identification of SNPs with potential prognostic impact, and then test these specifically for the association with lymphoma progression and lymphoma-specific death. Therefore, in the revised manuscript version, we have clarified this aim and do not use the word validation, but rather investigation of previously associated SNPs. Revisions along these lines have been made in the abstract (page 3, row 12) and throughout the manuscript (page 5, row 23-; page 9, rows 6-9; page 10, row 20; page 10, rows 21-23; page 12, row 24: page 16, rows 19-21).

E) We performed power calculations using R (survSNP package) with the results presented as graphs below. We added a note about this in the Methods (page 11, row 10-11) and Results sections (page 15, rows 7-12), and a comment in the Discussion section (page 19, rows 17-19). The graphs are also included in the supplementary material as Supplementary Figure 6 (and displayed below). The MAFs in the previous studies ranged from 0.09 to 0.49 (median 0.24) and the HRs from approximately 1.5 and 3.5, hence those were the numbers we used as a basis for the power calculations.
7. How many SNPs which were associated with FL risk were tested for an association with survival in this study, 5 (page 8) or 6 (page 13)?

We apologize for the mix-up of numbers. Six SNPs have been associated with FL risk, of which one (rs6457327) also was associated with FL prognosis in two previous studies (Berglund 2011, Wrench 2011). The number 5 has been changed to 6 on page 9 (original manuscript page 8).

8. Figure 1: SNPs were treated as continuous variables but in the plots only data for wildtype and variant are presented (dominant model).

We agree that it is not ideal to use different models in the same study and thank the reviewer for pointing this out. We have removed these plots in the revised version, since they add little extra information.

9. Table 3: The results in this table should be reduced to the best SNP per linkage block. Complete results might be presented in supplementary tables.

We thank the reviewer for pointing this out. We modified Table 3 accordingly and added a table with the complete top results in the supplement (Supplementary table X).

Discussion:
10. A) The authors should discuss the results of the GWAS and the validation analyses in separate paragraphs. B) Furthermore if they think that imputation might have caused misclassification of genotypes they should check some cases using another method, e.g. sequencing, if possible.

   A) We have made changes in the discussion section accordingly.
   B) In fact, misclassification of genotypes due to the imputation is unlikely, since we used strict thresholds (genotypes with probabilities >0.9, SNPs with information scores >0.8 and call rates >0.9). Hence, we removed this sentence from the discussion section.

11. ...we found further support of a role for [...] and two SNPs in IL8 [...] in FL progression. -> In the original studies IL8 SNPs were associated with OS! The authors should not mix outcome variables.

   We have re-worded this section to clarify that different outcome measures were used in the present study and in the previous investigations of the two IL8 variants (page 18, rows 4-5).

Discretionary Revisions

12. In case of the confirmation study the authors could perform meta analyses with published and their own data and present the results.

   We decided not to go forward with a meta-analysis since somewhat different outcome measures were used in the previous studies (event-free or overall survival) and the present study (time to progression and lymphoma-specific death).

Reviewer: Federico Canzian

Major Compulsory Revisions

1. My main criticism is that only the top 1000 SNPs from the SCALE study are used for replication in the UCSF study. Since both series of patients have been genotyped with arrays suitable for GWAS analysis, why not doing a full-scale genome-wide meta-analysis? This would not rehire a huge amount of additional work, and might yield additional interesting hits. The two series of patients have not been genotyped with the same array, but through imputation it is perfectly feasible to merge the datasets. If the authors choose not to do this work, I would like to see at least a compelling explanation for their reasons.

   In recognition of this comment, we performed a full meta-analysis of SCALE and UCSF GWAS data and revised the manuscript. Of the 298,702 variants in SCALE...
and 319,693 in UCSF, 295,134 variants were present in both cohorts. No strong hits emerged in addition to those previously identified. Among the 48 top hits in the UCSF study ($p \leq 10^{-5}$), 7 SNPs were not genotyped in SCALE, of which 6 could be imputed with high confidence (information score $>0.8$). Pooling of the HRs for these 6 SNPs yielded weak associations ($p > 10^{-4}$).

2. A point connected to the previous, is that through imputation the authors can somehow compensate the rather small scale of the arrays used for genotyping, which are not anymore state-of-the-art. This could lead to a better coverage of the genome.

Our samples have been genotyped on the older versions of the Illumina arrays, consisting of ~300,000 SNPs across the genome which is much fewer than the latest state-of-the-art arrays. While imputation will help to improve the coverage of the genome beyond these genotyped SNPs, any additional variants that can be accurately imputed in our dataset will already have been well tagged by our genotyped SNPs. In theory, an imputation would mostly serve to increase the number of overlapping SNPs for comparison between the two datasets if genotyped on different platforms, and also to fine-map regions of interest. However, in view of the near-complete overlap between the SCALE and UCSF arrays, not much more can be achieved. Therefore, we believe that a genome-wide imputation will add little value to our discovery scan.

Minor Essential Revisions

3. Why sex was not used as adjustment variable in the GWAS analysis?

See also response to Reviewer 1, comment 2. Sex has now been included in the multivariate model in the GWAS analysis.

4. It is not clear why adjustment with FLIPI 5 categories or first-line rituximab has been performed only for the analyses of validation of previous candidate SNPs and not also for the main GWAS analysis. Given the possible role of the ABC transporters where the top hit is located, it is important to check whether the observed association is independent of treatment. Although table 2 shows that FLIPI is available only for SCALE cases and rituximab only for the Swedish ones, these adjustments should be done at least for the analysis of the top hits.

We have performed complementary analyses of the top hits of the GWAS among the patients where covariate data was available (SCALE for FLIPI and SCALE Sweden for rituximab) as suggested by the reviewer.

A. The estimated HRs for rs11932201 and rs2250066 were virtually unchanged in SCALE ($n=373$) after adding FLIPI to the model. The HR for rs10491178
was slightly strengthened while it was slightly weakened for rs3131729 with additional adjustment for FLIPI.

<table>
<thead>
<tr>
<th>Chr</th>
<th>SNP</th>
<th>age + PC + sex</th>
<th>+ FLIPI</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HR (95% CI)</td>
<td>p</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>17</td>
<td>rs10491178</td>
<td>3.10 (1.97; 4.89)</td>
<td>1.12E-06</td>
<td>3.77 (2.34; 6.09)</td>
</tr>
<tr>
<td>1</td>
<td>rs3131729</td>
<td>2.58 (1.77; 3.76)</td>
<td>9.24E-07</td>
<td>2.34 (1.60; 3.43)</td>
</tr>
<tr>
<td>4</td>
<td>rs11932201</td>
<td>2.08 (1.47; 2.94)</td>
<td>3.97E-05</td>
<td>2.05 (1.46; 2.98)</td>
</tr>
<tr>
<td>19</td>
<td>rs2250066</td>
<td>2.08 (1.45; 2.97)</td>
<td>5.75E-05</td>
<td>2.00 (1.39; 2.88)</td>
</tr>
</tbody>
</table>

B. The estimated HRs for the top hits were virtually unchanged in the Swedish cohort (n=231) after adding first-line rituximab to the model (see table below). Also, the top hits were not significantly associated with rituximab first line in the Swedish cohort (Fisher’s Exact test p value>0.05).

<table>
<thead>
<tr>
<th>Chr</th>
<th>SNP</th>
<th>age + PC + sex</th>
<th>+ first-line rituximab</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HR (95% CI)</td>
<td>p</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>17</td>
<td>rs10491178</td>
<td>3.32 (1.88; 5.84)</td>
<td>3.38E-05</td>
<td>3.20 (1.79; 5.69)</td>
</tr>
<tr>
<td>1</td>
<td>rs3131729</td>
<td>2.55 (1.57; 4.13)</td>
<td>1.51E-04</td>
<td>2.62 (1.61; 4.27)</td>
</tr>
<tr>
<td>4</td>
<td>rs11932201</td>
<td>2.03 (1.30; 3.19)</td>
<td>1.86E-03</td>
<td>2.05 (1.46; 2.98)</td>
</tr>
<tr>
<td>19</td>
<td>rs2250066</td>
<td>2.51 (1.55; 4.06)</td>
<td>1.71E-04</td>
<td>2.50 (1.54; 4.06)</td>
</tr>
</tbody>
</table>

These results are now briefly noted in the Results section in the revised manuscript (page 13, rows 12-14).

5. The authors argue that progression-free survival and lymphoma-specific survival are superior to overall survival (OS). On the other hand, they acknowledge that lymphoma-specific survival may miss deaths due in part to progression. In addition, given how complex is often death certification, it is entirely possible that some deaths were attributed to other causes, but lymphoma was at least an underlying cause of death. Thus, I think there would be value in exploring also OS as an end-point.

We agree with the reviewer that OS could be of value to explore. However, at large, we wanted to keep the investigation focused on lymphoma-specific death as we believe that this analysis best answered our hypothesis of a potential association between genetic variation and a dismal clinical course of lymphoma. Also, analyzing the entire discovery dataset as well as candidate genes for overall survival would
greatly increase the number of results and tests performed adding to concerns of multiple testing.

Still, in recognition of this valuable comment, we performed additional analyses for the top SNPs and overall survival in the SCALE cohort with the following results (adjusting for age at diagnosis, the first three principal components and sex). A more complete version of this table has been added to the revised manuscript as Supplementary Table 4, and a brief note has also been added to the Results section (page 13, rows 16-17).

<table>
<thead>
<tr>
<th>Chr</th>
<th>SNP</th>
<th>SCALE (N=373)</th>
<th>SCALE Sweden (N=231)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lymphoma-specific death</td>
<td>All-cause death</td>
<td>Lymphoma progression</td>
</tr>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>p</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>17</td>
<td>rs10491178</td>
<td>3.10 (1.97; 4.89)</td>
<td>1.12E-06</td>
</tr>
<tr>
<td>1</td>
<td>rs3131729</td>
<td>2.58 (1.77; 3.76)</td>
<td>9.24E-07</td>
</tr>
<tr>
<td>4</td>
<td>rs11932201</td>
<td>2.08 (1.47; 2.94)</td>
<td>3.97E-05</td>
</tr>
<tr>
<td>19</td>
<td>rs2250066</td>
<td>2.08 (1.45; 2.97)</td>
<td>5.75E-05</td>
</tr>
</tbody>
</table>

6. Since the top SNPs are located inside a gene which is also a good candidate from the biological point of view, it would be interesting to use bioinformatics tools (e.g. RegulomeDB, eQTL analysis) to check if these SNPs (or other SNPs in high LD) have a possible function.

We thank the reviewer for this suggestion! We used the RegulomeDB to explore the top SNPs as well as SNPs in strong LD \((r^2 \geq 0.8)\) with these in the 1000 Genomes CEU population. There was some data to support a putative functional role for six of the ten top SNPs, of which rs10491178 (chr 17), rs11932201 (chr 4) and rs2250066 (chr 19) had RegulomeDB scores of 5, indicating that they may affect transcription factor binding or are located in a DNAse hypersensitivity site. There was no data for the SNP rs3131729 (chr 1). The lowest RegulomeDB score (i.e. strongest evidence of being in a regulatory site) among our tested SNPs was found at the SNP rs113464685 in ABCA10 (score 3b) that was in LD with rs10491178. We imputed the genotype for this SNP, using the same method as described in the manuscript, and ran the Cox model for lymphoma-specific death for rs113464685 in SCALE. The estimates were in the same range as for rs10491178 (HR = 3.10 (1.97; 4.89), p = 1.12 x10^-6).
We have added a description of these complementary analyses in the Methods (page 10, rows 15-18), Results (page 13, rows 18-23) and Discussion (page 16, rows 15-23).

7. In table 3 and supplementary table 1, commas should be replaced by points

We have corrected the typos.

Discretionary Revisions

8. The authors mention that power of their study to validate associations previously reported in the literature may be limited. In this respect it may be useful to add to the discussion a statement about statistical power to replicate them, taking into account the frequency of the SNPs and the HRs observed in the previous studies.

We thank the reviewer for this suggestion. Please see the estimated power for different MAFs and HRs in SCALE and the Swedish SCALE cohort presented as graphs above (Reviewer 1, comment 6). We added a statement in the Discussion section on this matter (page 19, row 17-19).