nature portfolio

Peer Review File

High incidence and geographic distribution of cleft palate in Finland are associated with the *IRF6* gene

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
I have reviewed the manuscript previously and can confirm that the paper has further improved. All my prior concerns have been properly addressed. I only have some minor suggestions remaining, which all relate to some wording in the Discussion section. However, given that these comments are rather minor, they don't compromize the overall great work.

- Discussion, line 652: "which explains the high incidence rate of CP in Finland". I agree that rs570516915 is likely part of the explanation, but I am not so sure whether this supports such an absolute statement. I suggest to weaken this statement.
- Discussion, line 671: "disrupts". Similar to the previous comment, "disrupts" seems very binary. Given that the functional effect is not absolute, I again suggest to weaken this statement.
- Discussion, line 681/682: If the authors state "to date" and then add specific numbers of how many pathogenic mutations are known, then the reference should not be 7 years old (as reference 63 is).

Reviewer #4

(Remarks to the Author)
Overall the revised manuscript addresses most of my previous concerns. However the updated figure 5 does not seem to compare allele states at the indicated snp position as in the previous revision. The authors claim in their response letter to perform QPCR with consistent masses of DNA yet still reflect values as a percent of input and include IgG values. Either the figure is badly mislabelled or there is not a clear difference in these epigenetic/TF binding based on allele state and QPCR. Figure 5 d is indicative in a bias based on the given snp allele but there are many reasons Sanger sequencing of CHIP-PCR amplified DNA might be biased. Are there any other positions nearby and might be present elsewhere in the traces that have different allele states but are captured equally relative to input? This would help demonstrate that this difference is specific to this site.

Reviewer #5

(Remarks to the Author)
The study identified a low frequency SNP, rs570516915, which is enriched in Finnish population, is strongly associated with non-syndromic CP. The enrichment of this SNP can explain the higher prevelence of isolated CP than CL/P in Finland. The authors showed that the risk allele disrupts a binding site of IRF6 within the IRF6 enhancer MCS-9.7, potentially disrupt the autoregulation of IRF6 expression.

The finding is very exciting as it exemplifies how the enrichment of a genetic variance can contribute to the prevelence of a phenotype/disease in a certain population, and the model for how the risk allele of rs570516915 may contribute to CP is logical, plausible, and supported by the wet lab results.

The data analyses are robust, the in vitro experiments are well designed, and the results were significant.

The only weakness of the study is that the in vivo reporter assay results are less convincing. As mentioned by other reviewers, due to the randomness of genomic integration, the mouse transgenic reporter assay is bond to be highly variable and difficult to pick up small to moderate quantitative changes. Although Wilcoxon Signed Ranked test reached a statistical

significance of P<0.05, I still have concerns about whether the results are reproducible. Moreover, no difference in the signal from secondary palate was found, which could be due to technical reasons, such as how fast the substrate can reach the palatal shelves.

Despite the relatively weak evidence from the mouse transgenic reporter assay, the in vitro studies, especially the study using iPSCs derived oral epithelial cells, provided strong evidence to support the model. The authors have also properly addressed reviewers' comments.

Version 1:

Reviewer comments:

Reviewer #6

(Remarks to the Author)
The researchers have addressed all previous issues sufficiently.

Point-by-point response to reviews (3rd revision)

We thank the reviewers for their comments. Below, we include the reviewer comments in which changes in the manuscript were requested, in boldface, and our responses, in regular typeface.

Reviewer #2

I have reviewed the manuscript previously and can confirm that the paper has further improved. All my prior concerns have been properly addressed. I only have some minor suggestions remaining, which all relate to some wording in the Discussion section. However, given that these comments are rather minor, they don't compromise the overall great work.

1. **Discussion, line 652: "which explains the high incidence rate of CP in Finland". I agree that rs570516915 is likely part of the explanation, but I am not so sure whether this supports such an absolute statement. I suggest to weaken this statement.**

   It is the aggregate effect of three independent SNPs, not rs570516915 alone, which all have the highest allele frequency in Finland, that we think contributes to the high incidence rate of CP in Finland. We have weakened the statement, now the sentence reads as: *"Here we have shown an association between three independent SNPs and CP in Finland. Their high allele frequency, compared to other populations, and their relatively strong effect size help to account for the high incidence of CP in Finland."*

2. **Discussion, line 671: "disrupts". Similar to the previous comment, "disrupts" seems very binary. Given that the functional effect is not absolute, I again suggest to weaken this statement**.

   To weaken the statements with "disrupt", we substituted the word "disrupt" in multiple places with "alter" where we mention changes within the IRF6 transcription factor binding motif, with "diminish" where we describe changes in the regulatory activity of the IRF6 enhancer, with "hinder" regarding binding of the transcription

factor IRF6 to its enhancer, and with “impair” or “perturb” in the context of autoregulation.

3. **Discussion, line 681/682: If the authors state, "to date" and then add specific numbers of how many pathogenic mutations are known, then the reference should not be 7 years old (as reference 63 is).**
   We removed this paragraph altogether, including reference 63, as this information was redundant for the Discussion.

Reviewer #4

Overall, the revised manuscript addresses most of my previous concerns. However, the updated figure 5 does not seem to compare allele states at the indicated SNP position as in the previous revision. The authors claim in their response letter to perform qPCR with consistent masses of DNA yet still reflect values as a percent of input and include IgG values. Either the figure is badly mislabelled or there is not a clear difference in these epigenetic/TF binding based on allele state and QPCR. Figure 5 d is indicative in a bias based on the given snp allele but there are many reasons Sanger sequencing of CHIP-PCR amplified DNA might be biased. Are there any other positions nearby and might be present elsewhere in the traces that have different allele states but are captured equally relative to input? This would help demonstrate that this difference is specific to this site.

We have closely followed the analysis method suggested by this reviewer, i.e., from Solomon et al., 2021, which uses consistent masses of DNA for the PCR template and reflects ChIP’ed DNA values as a percent of input. As is standard in the field, although not performed by Solomon et al., 2021, we also included IgG as a negative control to reflect non-specific pull down by antibodies.

To check for allele-specific binding, in an earlier revision of the manuscript we compared the amount of chromatin pulled-down by anti-IRF6 antibody in separate

precipitation reactions conducted in clones of the GMSMK cell line that were homozygous for the risk or the non-risk allele. In the last revision, we improved the experiment in two ways. We switched to induced oral epithelium cells, which express higher levels of *IRF6* than do GMSMK, and we used cells engineered to be heterozygous at the SNP of interest. This allows the antibody to bind either allele in the same precipitation reaction, avoiding the possible differences between precipitation reactions. To grossly quantify the amount of each allele pulled down, we sequenced the PCR product and evaluated the height of the chromatogram peaks representing each allele in the three replicates (Fig. 5d and Supplementary Fig. 12). These peaks were of consistent height in the input DNA, arguing against any bias of the PCR reaction for one allele of the other, whereas the peak for the non-risk allele was much higher than that of the risk-allele in the chromatin pulled down by anti-IRF6 or anti-H3K27Ac antibodies. Although we cannot readily state the absolute ratio of binding efficiencies, we contend this single-reaction chromatin immunoprecipitation approach is the best way to show a preference of a transcription factor for an allele.

As suggested by the reviewer, to address concerns about potential bias of Sanger sequencing of ChIP-PCR amplified DNA, in the current revision we have included an additional peak of IRF6 binding, i.e., identified in a published IRF6 ChIP-seq experiment in keratinocytes and confirmed by ChIP-qPCR by us in induced oral epithelium cells. Within this locus is a heterozygous SNP that does not lie within a predicted IRF6 binding site. The ratio of the height of the peaks representing the two alleles of this SNP is equivalent in the input DNA and in the chromatin precipitated by anti-IRF6, supporting the absence of bias in Sanger sequencing of ChIP-PCR amplified DNA (Supplementary Figure 13). To describe these new results in the current revision we have added the following text: “By contrast, no allele bias was detected in chromatin precipitated by anti-IRF6 at a heterozygous SNP present underneath a separate peak of IRF6 binding but not within a predicted IRF6 binding site (Supplementary Fig. 13a-c).” We have also added labels to the panels in Fig 5b-d to that the cells are heterozygous.

Reviewer #5

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We removed the results of the Wilcoxon Signed Ranked test and replaced the text with the following statement: “Therefore, the results based on the transient transgenic embryo assay were inconclusive. Experiments with F1 embryos from multiple transgenic lines may be needed to detect the subtle effects of a common variant on the activity of this enhancer.”

Despite the relatively weak evidence from the mouse transgenic reporter assay, the in vitro studies, especially the study using iPSCs derived oral epithelial cells, provided strong evidence to support the model. The authors have also properly addressed reviewers' comments.

We have included the source data file for all the bar charts/dot plots presented in the manuscript.