Author’s response to reviews

Title: Deleterious Genetic Variants in Ciliopathy Genes Increase Risk of Ritodrine-induced Cardiac and Pulmonary Side Effects

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Author’s response to reviews:
Overview of Changes:

Deleterious Genetic Variants in Ciliopathy Genes
Increase Risk of Ritodrine-induced Cardiac and Pulmonary Side Effects

We greatly appreciate the reviewers’ valuable comments, which have helped to improve the quality of our manuscript. We are hereby submitting a revised manuscript that includes changes according to the reviewers’ suggestions. Our point-by-point responses to their comments appear below, with additions to the manuscript marked in blue.

Revision on comments of the reviewer 1
General comment: This manuscript describes an exploratory study to understand genetic factors that may predispose to severe adverse drug reactions (ADRs) with the drug ritodrine, a short-
acting β2 adrenoreceptor agonist used to prevent premature labour. The analysis focused on 13 women who suffered serious cardiac and pulmonary side effects. Whole exome analysis was applied and non-synonymous substitution variants were evaluated as possible contributing factors to these ADRs.

This is an interesting cohort, and exome analysis is a reasonable approach to seek strongly penetrant genetic factors that may underlie side effects. That said, discovering variants and proving they are relevant to the ADR will be challenging in a small cohort such as this.

Major issues:
Comment 1. Presumably the patients are of Korean ethnicity? This important detail is not specified.

[Response to the comment]
We thank the reviewer for these comments. We have corrected and improved our manuscript in numerous ways.
We agree with the reviewer that it is necessary to specify the ethnicity of the patients, as it is an important patient characteristic. We have done this by adding a more elaborate description of the patients to the revised manuscript.

[Correction in the revised manuscript]
(In the Methods section, 2nd paragraph)
Thirteen Korean pregnant women were analyzed using next-generation sequencing technology. … For the control group, we selected exomes of 30 healthy Korean subjects … Although the subjects had never been exposed to ritodrine, the exomes were sequenced using the same platform as the cases to minimize platform-specific biases. A total of 43 Koreans were recruited for WES analysis.

Comment 2. 1000G data is used for calling and filtering variants. How well represented would Korean variants be in this dataset? By discarding detected variants not represented in the 1000G
data is there not a risk that important ethnic-specific variants may have been lost? There should be some clarification and discussion around these issues.

[Response to the comment]
We thank the reviewer for this insightful comment. The 1000 Genomes Project (1KGP) database containing fully phased genomes of 2,504 subjects is one of the finest human variant catalogues from five populations, including African, American, East Asian, European, and South Asian populations. As Korean genome data are not available, this study considered genome data from genetically similar populations such as Chinese (Han Chinese in Beijing, China, n = 103) and Japanese (Japanese in Tokyo, Japan, n = 104) genomes [ref 1]. In Fig. 1, we excluded 30,706 variants that are not presented in 1KGP. Among them, 24,493 (80%) of variants are not present in the Genome Aggregation Database (gnomAD), which contains genomic variants from ~140 K individuals, including 9,435 East Asian individuals (EAS). In this study, we hypothesized that these variants were likely false positives [ref 2], and were thus excluded from the downstream analysis. Additionally, 16,208 (66%) of the highly likely false positives appeared as singletons in this cohort, implying that those singletons would not be enriched among cases (The figure included in the supplementary file).

The remaining variants (6,213, 20%) were reported in gnomAD; the distribution of the deleterious variants in shown below (The figure included in the supplementary file).

We found that 778 (12.5%) variants were predicted to be deleterious by SIFT. Among the deleterious variants, 36 (4.6%) were present in EAS, with more than 1% of AF (allele frequency) in gnomAD. We probably missed these EAS-specific deleterious variants that may have been associated with ritodrine side effects in this study.

Among the excluded variants, we believe that the fraction of erroneous calls is much greater than that of the potential true positives. To improve the use of terms in Fig. 1, we have rephrased “Variants found in the 1000 Genomes Project” as “AF (variant) ≥ 1/5008” in Fig. 1 in the revised manuscript. We have provided a more elaborate description in the revised manuscript and modified Fig. 1 for clarification.
Among the 13 cases, a total of 558,091 variants were detected from 117,633 loci (Fig. 1). Initially, we included 86,927 loci with allele frequencies (AFs) ≥ 1/5,008, as reported in the 1000 Genomes Project (T1GP; n = 2,504), phase 3, with the assumption that variants in a highly curated public database would be less likely to contain errors [21, 22]. Next …

Comment 3. For the rare-variant association tests (line 136-149) what are the implications of the use of multiple gene lists (particularly in terms of multiple testing correction), and to what degree are these gene lists correlated? It seems unnecessarily complex to use multiple gene sets for this analysis.

[Response to the comment]
We sincerely thank the reviewer for the constructive criticism and valuable comments, which were helpful when revising the manuscript. Initially, we conducted rare-variant association tests for ciliopathy genes and their subsets. The Joubert syndrome (JBTS) and Meckel-Gruber syndrome (MKS) combined gene (JBTMKS) list from Invitae was significant via SKAT (Pburden = 0.1, PSKAT = 0.03, and PSKAT-O = 0.05). Additionally, we obtained genes from Genetic Home Reference (GHR) for each syndrome: JBTS and MKS, which were subsets of JBTMKS (see figure below). We detected a higher significance level with JBTS for three difference rare-variant association tests (Pburden = 0.02, PSKAT = 0.01, and PSKAT-O = 0.02). Moreover, the significance levels were replicated and robustly sustained with JBTS-related genes (n = 22) from Wikipedia; however, we excluded this gene list as recommended by another reviewer. On the other hand, MKS-related genes were not statistically significant (Pburden = 1, PSKAT = 0.26, and PSKAT-O = 0.42). Therefore, we showed that rare variants in some cases were associated with JBTS-related genes.

According to the reviewer’s suggestion, we have clarified this analysis by simplifying the multiple gene lists for rare variant association testing. Moreover, Bonferroni corrected p-values were obtained for multiple testing correction, and marginal significance was detected by SKAT (Bonferroni corrected P = 0.05).

(The figure included in the supplementary file)
… ciliopathies (n = 303) [25]. In addition, we downloaded genes known to be involved in
ciliopathy (n = 102) and Joubert and Meckel-Gruber syndromes (JBTMKS, n = 30, Supplementary Table S2) [26]. As the genes for Joubert syndrome (JBTS, n = 11) and Meckel-
Gruber syndrome (MKS, n = 8) were grouped together in the JBTMKS category, we obtained

genes …

The SKAT rare variant association test between the case (n = 13) and control (n = 30) groups showed marginal significance for JBTMKS after Bonferroni correction (P = 0.1054, Table 3) from the Invitae. Since JBTMKS harbour both JBTS- and MKS-related genes, we further evaluated the association signals for JBTS and MKS separately using GHR. Only JBTS genes showed a marginal trend toward significance, whereas MKS genes did not. This result suggests that genetic variations identified in patients with serious ritodrine-induced cardiac and pulmonary side effects may be associated with JBTS. Table 4 presents the rare …

Comment 4. The comment on line 167 about the 28 identified genes "with deleterious variants" should be edited to indicate that these were "variants predicted deleterious by SIFT". Were these mutations confirmed by a resequencing method?

[Response to the comment]
We thank the reviewer for inviting us to clarify this important point. To verify variants calls, we selected 32 SNVs that were predicted to be deleterious by bioinformatics algorithms among 82 SNVs in the 28 significant genes (Table 2). Fluidigm™ SNV genotyping assays were used to confirm and validate all 32 SNVs from 11 cases. The revised manuscript includes a better description that reflects this comment.

[Correction in the revised manuscript]
(In the Results section, 1st paragraph)
In summary, we identified 28 genes SIFT-predicted deleterious variants that were statistically significantly associated with ritodrine-induced cardiac and pulmonary side effects (Table 2). Additionally, all 32 variants that were predicted to be deleterious by either SIFT or CADD, among the 82 variants in the 28 significant genes, were successfully replicated using Fluidigm™ genotyping assays in 11 cases with sufficient DNA.

(In the Methods section, 4th paragraph)

Validation with genotyping assay

We validated 11 cases with side effects (out of the 13 cases with sufficient DNA) using an array-based high throughput method for 32 variants in 20 genes. In the single nucleotide polymorphism (SNP) type assay, 40 ng of genomic DNA flanking the SNP of interest was amplified by polymerase chain reaction (PCR) with a specific target amplification primer set. PCR was performed as described in the manufacturer’s instructions (Fluidigm, San Francisco, CA, USA). After amplification, the SNP type assay reaction was carried out according to the manufacturer’s instructions. SNP analysis was performed using Fluidigm SNP Genotyping Analysis software (ver. 4.0.1).

Comment 5. A collection of rare variants were found to be overrepresented in the cases, in ciliopathy-related genes as well as drug metabolising genes. Given the difficulties in obtaining a replication cohort for this type of study, and the lack of functional knowledge around the mechanisms of action of this drug or its metabolism, these variants can only be regarded as candidates that are associated with the adverse reactions. The authors acknowledge this in line 242, indicating that the findings are speculative, but their conclusions (line 271 onwards) are phrased inappropriately strongly when claiming that they "identified rare deleterious variants affecting ritodrine-induced … side effects". This should be rephrased as "rare predicted deleterious variants associated with…". The final sentence (line 275-276) should focus on the need for replication studies to clarify the validity of these variants.

[Response to the comment]

We thank the reviewer for bringing this critical point to our attention. Here, we identified rare predicted deleterious variants that were significantly associated with the ritodrine-induced
serious cardiac and pulmonary side effects. Due to the limited number of cases, such rare variants provide a set of candidate genes that require further analyses. We agree that validation of the rare variants in a larger cohort is required, and we have described this point more clearly in the revised manuscript.

[Correction in the revised manuscript]
(In the Conclusions section)
Using WES, this study identified rare deleterious variants associated with ritodrine-induced serious cardiac and pulmonary side effects in Korean preterm labour subjects. Most importantly, rare variants on ciliopathy genes were demonstrated to be significantly associated with JBTS. Asian-specific rare and common variants related to the pharmacokinetics of ritodrine may elicit serious cardiac and pulmonary side effects. Further studies are needed to validate the rare variants in a larger cohort for replication, and to elucidate the role of these variants in the molecular mechanisms of the side effects.

Minor issues:
Comment 1. Abstract conclusions (Line 23-24) should be softened to read "may be associated with" rather than "are associated with".

[Response to the comment]
We sincerely thank the reviewer for this valuable comment. According to which we have modified the conclusions in the Abstract in the revised manuscript.

[Correction in the revised manuscript]
(In the Abstract)
Conclusions: Ritodrine-induced cardiac and pulmonary side effects may be associated with deleterious genetic variants in ciliary and pharmacokinetic genes.

Comment 2. What is the prevalence of the side effects observed in this study?

[Response to the comment]
Thank you for your comment. According to a Korean multicentre study, which included Ewha Womans University, and Konkuk University Medical Center, 62% of patients receiving uterine contraction inhibitors were given ritodrine as the first-line tocolytic treatment. Side effects were found in 13.1% of these patients [ref 3]. Based on this finding, we speculate that our cohort would demonstrate a similar frequency of side effects, although the prevalence cannot be investigated in this study. Accordingly, we have added this point to the Background sections.

[Correction in the revised manuscript]
(In the Background, 2nd paragraph)
In Korea, 13.1% of patients receiving ritodrine experienced side effects [2].

Comment 3. In line 51 this statement is unclear "… the mechanism of ritodrine …"; does this refer to mechanism of therapeutic action, or mechanism underlying side effects?

[Response to the comment]
We thank the reviewer for this helpful comment. We have revised the statement in the revised manuscript indicating that the molecular mechanisms underlying exonic deleterious variants in cases with ritodrine-induced side effects remain unclear.

[Correction in the revised manuscript]
(In the Background section, 3rd paragraph)
However, genetic polymorphisms that lead to ritodrine-induced cardiac and pulmonary side effects have not yet been identified, and the molecular mechanisms underlying the adverse effects of ritodrine remain unclear.

Comment 4. Were the healthy controls drug exposed (line 67)? Why did the control group include males?

[Response to the comment]
We are grateful to the reviewer for correctly pointing out that our control group included males. We had exomes from only 30 healthy Korean individuals, from in-house data that were sequenced with Ion Proton (Thermo Fisher); these exomes were used to sequence the case group
to minimize the platform-specific differences between the two groups. Although we were aware that there might be physiological differences between males, females and controls who had never been exposed to ritodrine, we believed that minimizing the platform-specific biases was the most important aspect in sequencing data analysis for reliable study results. Additionally, there was no significant difference (P = 0.89) in the number of variants: 41,587 ± 1,810 and 41,689 ± 2,008 for males (n = 19) and females (n = 11), respectively. To address these potential shortfalls, we are collecting control samples from individuals who were exposed to ritodrine without side effects, and from healthy pregnant women who were not exposed to ritodrine for further analysis.

[Correction in the revised manuscript]
(In the Methods section, 2nd paragraph)
… (http://www.snubi.org/). Although the subjects had never been exposed to ritodrine, the exomes were sequenced using the same platform as the cases to minimize platform-specific biases. A total of 43 Koreans were recruited for WES analysis.

(In the Results section, 1st paragraph)
… respectively. Additionally, we confirmed that there was no significant difference in the number of variants between males and females in controls (P = 0.89). Next, …

Comment 5. Need a specific comment on lack of replication of CACNA1C involvement in cardiac side effects as described by Baek et al. (2017).

[Response to the comment]
We thank the reviewer for this insightful comment. Baek et al. (2017) selected five SNPs of the CACNA1C with more than 20% of AF in EAS, and used genotyping assays rather than high-throughput technology. Among the five SNPs, three are exhibited in intron regions that are not able to annotate SIFT scores. For the two remaining SNPs, we annotated SIFT scores and found that both have a score of 1, which was predicted to be benign according to the SIFT. However, we only included variants with a SIFT < 0.7 as an input of the geometric mean for G score calculation (Methods). As a result, none of the five SNVs was considered as an input of the CACNA1C’s G score calculation, which did not quite reach conventional levels of statistical
significance in our analysis. We provide a more elaborate description in the revised manuscript for clarification.

[Correction in the revised manuscript]
(In the Discussion section, 2nd paragraph)
… ritodrine treatment of PTB. An association between rs10774053 in CACNA1C and ritodrine side effects was reported recently [10]; however, this genetic association was not replicated in this study as no significant difference (P = 0.1962) was detected in CACNA1C; G score (mean ± SD) between the case and the control groups (0.28 ± 0.2 and 0.35 ± 0.3, respectively). However, they selected five SNPs in CACNA1C, including three and two variants in intron and exon regions, respectively. The two exonic variants are predicted to be benign according to SIFT, and a recessive model was applied for statistical tests.


[Response to the comment]
We thank the reviewer for this comment. We removed the italics from the sentence in the revised manuscript.

[Correction in the revised manuscript]
(In the Discussion section, 1st paragraph)
Little is known about the physiological mechanism of ritodrine; ADRB2 is the only known target.

Comment 7. It would be useful for the authors to indicate their views on the validity of the prior CACNA1C association observed for ritodrine side effects, given that they did not observe CACNA1C variants in this study.

[Response to the comment]
We thank the reviewer for this insightful comment. We considered 19,729 coding genes, including CACNA1C, for the analysis steps (Fig. 1). However, CACNA1C did not reach the
FDR corrected P value of 0.1. As we mentioned in the Comment 4 above, the significant SNP reported in the previous work was not used in our analysis due to having a SIFT score of 1. We added the statistical result and G scores of CACNA1C in the revised manuscript.

[Correction in the revised manuscript]

(In the Discussion section, 2nd paragraph)

An association between rs10774053 in CACNA1C and ritodrine side effects was reported recently [10]; however, this genetic association was not replicated in this study as no significant difference (P = 0.1962) was detected in CACNA1C; G score (mean ± SD) between the case and the control groups (0.28 ± 0.2 and 0.35 ± 0.3, respectively). However…

Revision on comments of the reviewer 2

Comment 1: My major concern regarding the paper is related to the sample size and the p-value threshold employed. Namely, in WES studies, the acceptable p-value should be much lower than 0.05 (at least 0.000001, even lower for rare variations (European Journal of Human Genetics (2016) 24, 1202-1205), while the sample size of the study, if small, runs a risk of single marker association testing being underpowered, especially in detecting rare variants (BMC Genetics (2017) 18:14). If the study is underpowered and acceptable p value threshold is set too high, the conclusions might not be valid.

[Response to the comment]

We thank the reviewer for bringing this critical point to our attention. To identify rare and/or low frequency variants that have moderate to high effect sizes, sampling individuals with extreme phenotypes is particularly important, as those variants will be more highly expressed among them [ref 4,5]. A more recent study confirmed that the statistical power of association was greater in a group of extreme-selected samples than in a group of random samples [ref 6]. In our study, we selected cases with extreme phenotypes that probably have associations with rare and/or low frequency variants predicted to be deleterious. Severe adverse cardiac and pulmonary side effects of ritodrine are rare but extreme phenotypes in the clinical setting. Although we sequenced a limited number of exomes, our analysis had increased statistical power due to preferential selection of affected individuals.
Comment 2. The cases are women, but controls are both men and women: is there any data on sex-related effect of examined variations?

[Response to the comment]
We thank the reviewer for this comment. To minimize the platform-specific sequencing biases, we selected all exomes, including those from males and females generated by Ion Proton (Thermo Fisher) from in-house data. We conducted several diagnostic tests to determine whether there were significant differences in variant distributions between cases and controls, and between males and females in the control group. The distribution of the number of variants between cases and controls showed no significant difference (P = 0.81). Furthermore, we found no significant difference in the variant distributions between males and females in the control group (P=0.89). As our analysis was conducted based on G scores, we additionally checked the distribution of G scores in individual controls; no outliers were detected (see figure below). Throughout the analytical steps, no significant factors or outliers were observed between males and females in the control group. We have clarified this point by adding a more detailed description to the revised manuscript.

(The figure included in the supplementary file)

[Correction in the revised manuscript]
(In the Results section, 1st paragraph)
… . Based on the GATK Best Practices guidelines, we modified a variant-calling pipeline to function with Ion Proton data and obtained an average of 41,819 ± 1,976 [mean ± standard deviation (SD)] and 41,661 ± 1,848 variants from the case and control groups (P = 0.81), respectively. Additionally, we confirmed that there was no significant difference in the number of variants between males and females in controls (P = 0.89). Next …

Comment 3. Additional discussion in regard to observed difference in distribution of variants between cases and controls, related to biological/pharmacological plausibility, is needed.

[Response to the comment]
We thank the reviewer for this insightful comment. Through a literature review, we found additional evidence of polymorphisms in CYP1A1 and their clinical impact, including substrate specificity and disease risk. However, further studies are required to investigate the physiological effects of mutations that cause the side effects induced by ritodrine.

[Correction in the revised manuscript]
(In the Discussion section, 3rd paragraph)
… in this study. An association of CYP1A1 polymorphisms with an increase in CYP1A1 activity has been confirmed by functional studies [37, 38]. In particular, the catalytic activity in oestrogen metabolism was significantly higher in those with rs1048943 than wild-type [39]. The tocolytic effect of ritodrine was enhanced by adding natural progesterone in pregnant women [40]. A more recent study revealed the loss of a transcription factor binding site at Sp7 due to rs1048943 at exon 7 of CYP1A1; this variant was predicted to be deleterious by SIFT and CADD [41]. We also identified rare variants in drug metabolism-related genes with a relatively higher frequency in Asian subjects, despite the small sample size. Therefore, overrepresented and/or significantly higher deleterious variants in drug metabolism genes may also increase the risk of ritodrine-induced side effects, such as pulmonary embolism, in the Korean population.


[Response to the comment]
We thank the reviewer for bringing this critical point to our attention. As suggested by the reviewer, the revised manuscript does not include the Wikipedia; this change is reflected in the Methods and Results sections.

[Correction in the revised manuscript]
(In the Methods section, 9th paragraph)
Since genes for Joubert syndrome (JBTS) and Meckel-Gruber syndrome (MKS) were combined in the JBTMKS category together, we obtained genes for each syndrome from two different sources: Genetics Home Reference [27, 28] and Wikipedia [29, 30].

In addition, we downloaded genes known to be involved in ciliopathy (n = 102) and Joubert and Meckel-Gruber syndromes (JBTMKS, n = 30, Supplementary Table S2) [26]. As the genes for Joubert syndrome (JBTS, n = 11) and Meckel-Gruber syndrome (MKS, n = 8) were grouped together in the JBTMKS category, we obtained genes for each syndrome from the Genetics Home Reference (GHR) [27, 28].

References


This revised manuscript was proofread by professional native-English-speaking scientific editors [Textcheck, Hong Kong]. For a certificate, please see:
http://www.textcheck.com/certificate/index/2V11KX