Response to reviewers

To the Editor and anonymous reviewers:

We would like to express our appreciation to the reviewers and the editor for their careful reading of the text and for all the suggestions about our manuscript. We have addressed all the comments made by the two reviewers and updated the manuscript accordingly. Below, we have provided our response to individual points, and provided our reasoning for how we dealt with the issues identified. Thank you and we hope this version is acceptable for publication.

Please note that the original comments are pasted below, with our responses given in blue text. Accordingly, the revised manuscript has all changes highlighted in yellow, to allow a better follow up by the reviewers.

Editor comments:

The manuscript describes a case report of the 2+0 CYP21A2 deletion carrier. The topic is within the scope of the WJCC.

>> We are pleased to hear about the overall positive comment from the editor.

The study is well done, the material is large enough and the methods look reliable.

>> We thank the editor for this positive feedback.

However, the authors should briefly discuss the limitations of the current testing strategy (PCR-based mutation detection methods with sequencing of the entire gene, and multiplex ligation -dependent probe amplification) to detect silent carriers of pathologic genetic disorders

>> We thank the editor for his/her comments and the reviewer's attention to the details here. We have now included discussions about the limitations of the current testing strategy in testing the silent carrier of pathologic genetic disorders. In the discussion, we talk about how the PCR-based mutation detection methods with sequencing of the entire gene and multiplex ligation-dependent probe amplification may influence the results. We have also incorporated relevant discussion from Antao *et al.* into our discussion of the findings from this study. Accordingly, we have added the following paragraph into our revised manuscript: "This report implies that current quantitative copy number variation (CNV) detection methods such as PCR and MLPA have some limitations to detect silent carriers of pathologic genetic disorders, such as gene duplication paired with gene loss on the opposite chromosome or allele (2+0) in this report. MLPA has been regarded as the gold standard for CNV determination. Furthermore, laboratories worldwide commonly rely on MLPA for the diagnosis and research of genetic disorders [18]. Nevertheless, MLPA analysis is limited to analyzing the distribution in the two alleles and has a risk of missed detection of 2/0 carriers. For example, Alías et al. [15] tested 1,562 individuals to determine their SMA carrier status using MLPA, while the exclusive use of such a quantitative detection method of only independent individuals in a given family led to failure in identifying carrier status. In their study, all blood relatives characterized as 2/0 carriers were identified by studying their respective parents, but not by MLPA. The situation is similar for the PCR test method. The protocol is based on long-range PCR amplification with allele-specific primers, followed by DNA sequencing. PCR together with the Sanger sequencing is a robust testing strategy aiming to determine whether a point mutation or indel exists [19]. However, pathologic genetic disorders, such as gene duplication paired with gene loss on the opposite chromosome

or allele (2+0) may be missed using the PCR sequencing testing strategy. In addition, gene CYP21A2 has a duplicated pseudogene called CYP21A1P and they share 98% and 96% sequence homology in exons and in noncoding regions. All the reasons above make the definition of the 2 + 0 carrier in this report more complicated."

The questions raised by the reviewer should be answered

>>Thank you. Our point-by-point responses to each of the reviewers' comments are listed below.

The authors need to provide the CARE Checklist–2016 with page number, and written informed consent provided by the patient.

>>We have uploaded the CARE Checklist–2016 with page number, and written informed consent provided by the patient.

The language classification is Grade C. Please visit the following website for the professional English language editing companies we recommend: https://www.wjgnet.com/bpg/gerinfo/240

>> We apologize for some of the mistakes found on the previous version of the manuscript. We have asked a native English speaker to thoroughly revise this manuscript to improve the quality and we hope the revised manuscript meets the publication requirements.

The authors did not provide the approved grant application form(s). Please upload the approved grant application form(s) or funding agency copy of any approval document(s)

>> Fixed. We have uploaded this file in this new submission.

PMID and DOI numbers are missing in the reference list. Please provide the PubMed numbers and DOI citation numbers to the reference list and list all authors of the references. Please revise throughout.

>> We have added PubMed numbers and DOI citation number to each reference. Additionally, we have checked the author list and made sure that all authors of the references have been listed.

Reviewer 1

Authors should briefly discuss the limitations of the current testing strategy (PCR-based mutation detection methods with sequencing of the entire gene, and multiplex ligation -dependent probe amplification) to detect silent carriers of pathologic genetic disorders, such as gene duplication paired with gene loss on the opposite chromosome or allele (2+0).

>> We agree with Reviewer 1's assessment and, as such, we have included discussions about the limitations of the current testing strategy in testing the silent carrier of pathologic genetic disorders in our revised manuscript. In the discussion, we talked about how the PCR-based mutation detection methods with sequencing of the entire gene and multiplex ligation-dependent probe amplification (MLPA) may influence the results. As the reviewer suggest, we have added the following paragraph in our revised manuscript: "This report implies that current quantitative copy number variation (CNV) detection methods such as PCR and MLPA have some limitations to detect silent carriers of pathologic genetic disorders, such as gene duplication paired with gene loss on the opposite chromosome or allele (2+0) in this report. MLPA has been regarded as the gold standard for CNV determination. Furthermore, laboratories worldwide commonly rely on MLPA for the diagnosis and research of genetic disorders [18]. Nevertheless, MLPA analysis is limited to analyzing the distribution in the two alleles and has a risk of missed detection of 2/0 carriers. For example, Alías et al. [15] tested 1,562 individuals to determine their SMA carrier status using MLPA, while the exclusive use of such a quantitative detection method of only independent individuals in a given family led to failure in identifying carrier status. In their study, all blood relatives characterized as 2/0 carriers were identified by studying their respective parents, but not by MLPA. The situation is similar for the PCR test method. The protocol is based on long-range PCR amplification with allele-specific primers, followed by DNA sequencing. PCR together with the Sanger sequencing is a robust testing strategy aiming to determine whether a point mutation or indel exists [19]. However, pathologic genetic disorders, such as gene duplication paired with gene loss on the opposite chromosome or allele (2+0) may be missed using the PCR sequencing testing strategy. In addition, gene CYP21A2 has a duplicated pseudogene called CYP21A1P and they share 98% and 96% sequence homology in exons and in noncoding regions. All the reasons above make the definition of the 2 + 0 carrier in this report more complicated."

Authors can suggest a group discussion with the family, and the group includes a representative from the laboratory that performed the genetic testing, a genetic counselor, and the primary care provider.

>> We thank Reviewer 1 for this great suggestion. We agree with Reviewer 1 that a group discussion with the family is important and necessary to help the family better get through this hard time and deciding whether or not to get

pregnant in the future. Actually, we have organized such a discussion to let the guardian of the child know about this situation. To better clarify this point, we have added the following paragraph in the discussion in our revised manuscript: “Genetic counseling for the family is important and necessary to help the family better get through this hard time and deciding whether or not to get pregnant in the future. Thus, we organized a group discussion with the family to let the guardian of the child know about this situation. To keep them fully informed, the group includes a representative from the laboratory that performed the genetic testing, a genetic counselor, and the primary care provider. Besides, we also suggest a group discussion with the family in future research if a similar situation occurs.”

Authors can say in the text of the manuscript “a limitation of the genetic testing” instead of “a trap.”

>> We agree with the point being made. We have rephrased this sentence in the revised manuscript as the reviewer suggested.

Authors can say “our report” instead of “our study” in the manuscript, such as in the conclusion of the abstract.

>> We have replaced “our study” with “our report” as requested in our revised manuscript.

Authors can clarify the reference to “this possibility” in the middle of the second paragraph of the discussion section in the manuscript.

>> We thank Reviewer 1 for pointing this out. First, we apologize that we marked the reference in the wrong place in our original manuscript. In fact, Reference [10] should be marked following this sentence “Approximately 1% of the pathogenic variants of CYP21A2 come from new mutations”. Thus, we have remarked the reference in the right place in our revised manuscript. Then, we explain why “this possibility” can be basically ruled out. According to Krone *et al.*, De novo mutations are believed to account for about 1% of CYP21 mutations. In addition, the average new mutation rate of the human genome is very low (about 1.20×10^{-8} per generation), and generally 1-2 are present in the coding region. As a result, the possibility that CYP21 mutations in both the child and the elder brother come from de novo mutations is even below $(1.20 \times 10^{-8})^2$. Considering the two facts that the den novo mutations in exon regions are rarer and low-frequent pathogenic CYP21 mutations come from de novo mutations, this possibility is even much lower. We have updated the corresponding text in the discussion to make this point clearer.

Authors can clarify “detection of the number of copy variants of CYP21A2 gene” in the conclusion section of the discussion.

>> We thank Reviewer 1 for his/her insightful advice. Herein, we intended to convey that previous CYP21A2 carriers have only one copy and were detected using quantitative copy number variation detection methods such as PCR and MLPA. However, this report suggested one CYP21A2 2/0 carrier, complicating carrier diagnosis in CYP21A2. This result suggests that there are some limitations of the current testing strategy to detect silent carriers of pathologic genetic disorders, such as gene duplication paired with gene loss on the opposite chromosome or allele (2+0) as

Reviewer 1 mentioned in the above comments. Thus, only detecting the copy number of variants in CYP21A2 gene with current copy number variation detection methods is not sufficient. We have rewritten the text to make this point clear. Furthermore, we have discussed the limitations of the current test strategy such as PCR and MLPA in our revised manuscript.

Reviewer 2

The study is well done, the material is large enough and the methods look reliable..

>> We thank Reviewer 2 for his/her positive comments.

However the study is based on extensive and very recent literature, gives some new information and this warrants its publication.

>> We thank Reviewer 2 for his/her valuable suggestion. Following Reviewer 2's suggestion, we have discussed more and added two recent literature articles (Matthew *et al*. 2020. and Maremonti *et al.* 2020.) to the reference list to express some new information.