Peer-Review Record

Rare variants in the *FBN1* gene are associated with sporadic dilated cardiomyopathy in a Chinese Han population

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by Dongyang Wu, Yang Sun, Chenze Li, Lei Xiao, Jiaqi Dai, Yanghui Chen, Peng Chen, Hong Wang, Bo Yu, Haoran Wei, Rui Li, Xiuli Song, Ting Yu, Leming Shi, Dao Wen Wang

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Reviewer 2: Anonymous

Round 1

Reviewer 1 Report

The authors report identification of 17 variants of the *FBN1* gene in patients with dilated cardiomyopathy (DCM), which were distributed across the *FBN1* protein. Those who carried the *FBN1* variants showed a trend toward worse clinical outcomes. The authors concluded that *FBN1* gene is a risk gene for developing DCM.

Comments

1. The findings as presented could not support the conclusion of the study. The authors need to include a matching control group and compare the population frequency of the pathogenic and likely pathogenic variants in the *FBN1* genes in the cases with DCM and in controls. The authors have included a group of 414 individuals as controls, however, the characteristics of these individuals are missing. It is unclear whether they were healthy individuals that were matched to the controls. Statistical evidence of enrichment of the pathogenic and likely pathogenic variants in the cases should be presented to support conclusions of the study.

2. It is peculiar that the authors already have published whole exome sequencing data in their populations but evidently this gene was not included in the report or was not found to have a significant enrichment of the pathogenic or likely pathogenic variants in the DCM cases. If *FBN1* was included in the previous studies, then the data in the present study should be expanded focused on the genotype-phenotype correlation.

3. Missense variants in the *FBN1* gene are also found in the general population per GenomeAD. They are rare and given the smaller sample size of the controls as compared to the DCM cases, the a priori chance of detecting them in a smaller sample size control is low. The sample size of the control, if possible, should be expanded.

4. Specific criteria used to annotate pathogenic and likely pathogenic variants should be described.

5. Pathogenic variants and likely pathogenic variants identified in other genes by exome sequencing in this patient population should be included.

6. The phenotype in those with compound variants (2 or more variants) should be analyzed and compared to those with one variant. Gene burden analysis would be valuable.

7. Phenotypic data (Table 1) is inadequate and too general. For example, the category of "arrhythmia" is not very informative. All phenotypic data should be expanded and biomarkers of heart failure should be included.

8. Echocardiographic data should be provided and assessed for the effects of the *FBN1* variants (carries vs. non-carriers DCM cases).

9. Given that *FBN1* is a known gene for Marfan and thoracic aortic aneurysm, phenotypic data with regards to Marfan syndrome/aneurysm should be discussed in the paper. Did any of these patients had a known associated phenotype with the *FBN1* gene?

10. Those with a family history of DCM should be analyzed separately (additional analysis) and the relatedness of the family members should be considered in the data analysis.

11. Data should not be over-interpreted to state that was an association between the *FBN1* gene variants and the clinical outcomes. The p value is 0.4, which is non-significant.

Author Response

The authors report identification of 17 variants of the *FBN1* gene in patients with dilated cardiomyopathy (DCM), which were distributed across the *FBN1* protein. Those who carried the *FBN1* variants showed a trend toward worse clinical outcomes. The authors concluded that *FBN1* gene is a risk gene for developing DCM.

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1. The findings as presented could not support the conclusion of the study. The authors need to include a matching control group and compare the population frequency of the pathogenic and likely pathogenic variants in the *FBN1* genes in the cases with DCM and in controls. The authors have included a group of 414 individuals as controls, however, the characteristics of these individuals are missing. It is unclear whether they were healthy individuals that were matched to the controls. Statistical evidence of enrichment of the

pathogenic and likely pathogenic variants in the cases should be presented to support conclusions of the study.

We appreciate your thoughtful comments and agree that a well-matched control group is essential for the validity of our conclusions.

In response to your suggestion, we have now provided additional details about the control group in the revised manuscript. The control individuals (n=414) were confirmed to have no history of dilated cardiomyopathy (DCM) or other cardiovascular diseases. They were selected to be ethnically matched to the DCM group. We acknowledge that some of the controls have controlled hypertension, which is considered a systemic disease rather than a specific cardiovascular disease.

The detailed characteristics of these control individuals, including age and sex, along with other baseline clinical features, are now provided in **Table S1** in the revised manuscript.

Furthermore, to address your concern about the comparison of the frequency of pathogenic and likely pathogenic variants in *FBN1* between the DCM cases and controls, we performed a gene-based association test using SKAT-O, the Optimal Unified Approach for Rare-Variant Association Testing. **(PMID: 22863193)** This model is specifically applicable to small-sample case-control whole-exome sequencing studies and has shown superior average power across a range of simulated datasets. **(PMID: 25906071)** In the SKAT-O model, we adjusted for confounding factors such as sex and age at enrollment. After excluding individuals carrying pathogenic or likely pathogenic variants in other DCM-associated genes, the SKAT-O test showed a significant enrichment of rare deleterious variants in *FBN1* in DCM patients compared to controls (17/1041 vs 1/414, $P_{\text{SKAT-O}} = 0.006375$). This supports our conclusion that *FBN1* variants are associated with DCM.

We hope these revisions adequately address your concern and provide robust support for our conclusions.

2. It is peculiar that the authors already have published whole exome sequencing data in their populations but evidently this gene was not included in the report or was not found to have a significant enrichment of the pathogenic or likely pathogenic variants in the DCM cases. If *FBN1* was included in the previous studies, then the data in the present study should be expanded focused on the genotype-phenotype correlation.

Thank you for your insightful comment.

In our previous work, we did conduct whole exome sequencing across our populations, but the focus of those studies was different. In the present study, we chose to specifically examine the *FBN1* gene due to emerging evidence suggesting its potential involvement in DCM.

As for your suggestion on genotype-phenotype correlation, we appreciate this constructive idea. In the revised manuscript, we have now expanded our analyses to explore potential

genotype-phenotype correlations in patients carrying *FBN1* variants. Specifically, after our gene-based association analysis demonstrated a significant enrichment of rare deleterious variants of *FBN1* in DCM compared to controls, we conducted a correlation analysis between genotype and phenotype. We found that, compared to non-carriers, DCM patients carrying rare deleterious *FBN1* variants had a significantly higher incidence of atrial fibrillation and arrhythmias. **(Table 1)** This difference remained even when compared to carriers of positive mutations in other DCM-associated genes, suggesting that rare deleterious *FBN1* variants may be involved in the occurrence of arrhythmic events in DCM patients. **(Table S7)** These detailed descriptions have been added to the Results section of our revised manuscript.

We believe these additional analyses strengthen our study and provide valuable insights into the role of *FBN1* in DCM.

3. Missense variants in the *FBN1* gene are also found in the general population per GenomeAD. They are rare and given the smaller sample size of the controls as compared to the DCM cases, the a priori chance of detecting them in a smaller sample size control is low. The sample size of the control, if possible, should be expanded.

Thank you for your comment. We agree that rare variants, including missense variants in the *FBN1* gene, can also be found in the general population as per GenomeAD. We also understand that our smaller control sample size compared to the DCM cases may limit the power to detect such rare variants.

However, we would like to clarify that our study design was based on the principles of rare variant association tests, which account for the rarity and potential large effects of such variants. Our aim was to compare the burden of rare deleterious variants between cases and controls rather than to identify individual variants associated with DCM.

We recognize that a larger control sample size would have been ideal and would certainly increase the statistical power of our study. Unfortunately, due to constraints, we are currently unable to expand the sample size of our control group. We do, however, appreciate your suggestion and agree that future studies with larger control groups would be beneficial to further validate our findings. This limitation has been acknowledged in the revised manuscript.

4. Specific criteria used to annotate pathogenic and likely pathogenic variants should be described.

Thank you for your valuable comment. We recognize the importance of clearly outlining the criteria used to annotate pathogenic and likely pathogenic variants in our study.

In the revised manuscript, we have provided a detailed description of the variant annotation and deleteriousness evaluation process in the "**Bioinformatics analysis and variants deleteriousness evaluation**" section of the **Methods**. This process includes the following steps:

(1). After the joint call was made for the study population, we used VCFtools to document the final variant in a variant call format (VCF). Variants with a read depth less than 20 or a missing rate exceeding 20% across the entire cohort were considered missing and subsequently discarded.

(2). ANNOVAR was utilized to annotate the remaining variants. We focused on rare variants, defined as those with a minor allele frequency (MAF) < 0.001 in East Asian populations, based on public databases.

(3). We paid special attention to the rare variants of 44 DCM-related genes evaluated by ClinGen, as well as rare *FBN1* variants to probe their potential association with DCM.

(4). Missense and truncating variants evaluated as pathogenic or likely pathogenic under the American College of Medical Genetics and Genomics (ACMG) guidelines were included in subsequent analyses.

(5). We also used REVEL and VEST3, in silico functional prediction methods, for deleteriousness prediction of *FBN1* missense variants predicted as variants of uncertain significance (VUS) by the ACMG. To cross-verify the deleteriousness of variants, we employed two additional prediction tools, namely CADD and MutationTaster.

(6). Based on the deleteriousness predictions, we set thresholds for the REVEL score (≥ 0.4) and VEST3 score (≥ 0.5). For cross-validation, a CADD-phred-like score of >20 and a prediction of 'D' from MutationTaster were considered indicative of a deleterious prediction.

(7). Finally, *FBN1* missense variants predicted as deleterious by the software tools, along with those *FBN1* variants predicted as pathogenic or likely pathogenic by the ACMG, were classified as rare deleterious variants in *FBN1* discovered in our cohort.

The entire process is also summarized in **Figure S1**, which presents a simplified flowchart of the *FBN1* rare deleterious variants selection procedure.

We hope that this detailed response, coupled with the additional information in our revised manuscript, sufficiently addresses your comment.

5. Pathogenic variants and likely pathogenic variants identified in other genes by exome sequencing in this patient population should be included.

We appreciate your thoughtful comment and understand your concern about the potential impact of pathogenic and likely pathogenic variants in other DCM-associated genes on our study.

In our study, we primarily focused on the potential association of *FBN1* with DCM, while also considering 44 DCM-associated genes evaluated by ClinGen. (**PMID: 33983834**)

Although *FBN1* is not included in these 44 genes, we believe it is crucial to investigate its potential association with DCM.

We agree with your suggestion that variants in other DCM-associated genes could potentially influence our study results. Therefore, we have adopted a rigorous approach to account for this. Specifically, carriers of pathogenic or likely pathogenic variants in other DCM-associated genes, as classified by ACMG, were excluded from our *FBN1* rare variants' gene-based association test. This approach has been clearly outlined in the methods section of our revised manuscript.

We trust that this approach mitigates the potential impact of variants in other DCM-associated genes on our study and provides a clearer focus on the role of *FBN1* rare variants in DCM. We appreciate your valuable input, which has helped us to improve the clarity of our study.

6. The phenotype in those with compound variants (2 or more variants) should be analyzed and compared to those with one variant. Gene burden analysis would be valuable.

Thank you for your kind suggestion, we agree that analyzing and comparing the phenotype of individuals with compound variants to those with a single variant would indeed provide valuable insights.

However, in our study, we only found 17 DCM patients carrying 16 different rare deleterious *FBN1* variants out of 1041 DCM patients, and only 1 control individual carrying 1 *FBN1* variant out of 414 controls. Therefore, we did not observe any individuals carrying more than one different deleterious *FBN1* variant. **(Table S4)**

As for the other 44 DCM-associated genes included in our study, we identified 172 pathogenic or likely pathogenic variants in 181 DCM patients according to the ACMG guidelines. Only two of these individuals also carried a rare deleterious *FBN1* variant.

Given the limited number of individuals with compound variants (n = 2), we believe that a comparison between phenotypes of individuals with compound variants and those with a single variant may not yield statistically convincing results. Therefore, we did not perform this comparison in our manuscript.

However, we did compare the clinical characteristics and outcomes among three groups: individuals carrying a rare deleterious *FBN1* variant, individuals carrying pathogenic or likely pathogenic variants in other DCM-associated genes, and individuals who do not carry any of these variants. While DCM patients carrying a rare deleterious *FBN1* variant showed higher incidence of certain arrhythmic events (such as atrial fibrillation), we did not find any significant differences in clinical outcome events among these three groups. One possible reason for this may be the limited sample size. **(Table S7, Figure S2, Figure S3)**

We have added these comparisons and discussions to the revised manuscript and we believe that these additional analyses further enrich our study. We appreciate your understanding and consideration.

7. Phenotypic data (Table 1) is inadequate and too general. For example, the category of "arrhythmia" is not very informative. All phenotypic data should be expanded and biomarkers of heart failure should be included.

We appreciate your suggestion for expanding and refining the phenotypic data. In the revised manuscript, we have provided more detailed and explicit phenotype data in **Table 1 and Table S7**, as well as in the results section of the manuscript.

As for the category of "arrhythmia," we have replaced the ambiguous term with "Any arrhythmia," which we have defined in the results section as "the collective incidence of atrial fibrillation, non-sustained ventricular tachycardia, and left bundle branch block."

Regarding the biomarkers of heart failure, we have added the most commonly used biomarker, NT-proBNP, to the updated Table 1.

Moreover, we have provided definitions for the terms "Aortic root dilatation," "Proximal ascending aorta dilatation," and "Any aorta dilatation" in the manuscript. These terms are also used in **Table 1 and Table S7**. The terms "Aortic root dilatation" and "Proximal ascending aorta dilatation" refer to conditions in which the diameters of the aortic root and proximal ascending aorta, respectively, exceed the upper reference limits adjusted for age and body surface area. The term "Any aorta dilatation" signifies the presence of either or both of these conditions.

We hope these changes will enhance the clarity and comprehensiveness of our phenotypic data, thereby addressing your concerns.

8. Echocardiographic data should be provided and assessed for the effects of the *FBN1* variants (carries vs. non-carriers DCM cases).

Thank you for the suggestion. In the revised manuscript, we have incorporated a comparison of echocardiographic data between DCM cases who carry *FBN1* variants and those who do not. This data comparison includes parameters such as left ventricular ejection fraction (LVEF), left ventricular end-diastolic diameter (LVEDD), and left atrial diameter (LAD), amongst others.

We found that the echocardiographic features between these two groups did not differ significantly, suggesting that the *FBN1* variants might not have a substantial impact on these cardiac structural and functional parameters. These details have been added to the results section in the revised manuscript.

9. Given that *FBN1* is a known gene for Marfan and thoracic aortic aneurysm, phenotypic data with regards to Marfan syndrome/aneurysm should be discussed in the paper. Did any of these patients had a known associated phenotype with the *FBN1* gene?

Thank you for bringing up the connection between *FBN1* and Marfan Syndrome (MFS)/thoracic aortic aneurysm. In response to your comment, we have indeed conducted a thorough review of the medical histories of the DCM patients carrying rare deleterious *FBN1* variants. We specifically looked for any signs of MFS-related phenotypes, such as lens dislocation, chest deformity, and skeletal system abnormalities.

Simultaneously, we have supplemented our baseline data with aortic root diameter and ascending aorta diameter measurements **(Table 1)**. Upon comparison, we found no significant difference in baseline aortic phenotype between patients carrying *FBN1* rare deleterious variants and those without. Also, none of the carriers were diagnosed with MFS by the end of our follow-up period. Their primary cardiovascular phenotype remained cardiac dilatation rather than severe aortic dilatation.

These findings suggest that while the *FBN1* variants we identified in our DCM cohort are indeed associated with DCM, they do not seem to lead to the typical phenotypes associated with MFS. This information is now integrated into the **results** and **discussion** sections of our manuscript.

10. Those with a family history of DCM should be analyzed separately (additional analysis) and the relatedness of the family members should be considered in the data analysis.

We appreciate the your suggestion for the further stratification and analysis of our cohort. However, we would like to clarify that our study comprised of 1041 unrelated and sporadic cases of DCM, without any familial aggregation. Despite our extensive efforts to uncover any familial history, by the end of our follow-up, we unfortunately did not identify any distinct family lines.

This may be due to factors such as the high genetic heterogeneity and incomplete penetrance associated with DCM, which could make shared variants difficult to discover in a relatively small cohort. We acknowledge that this is a limitation of our study and have included it in the 'Study Limitations' section of our revised manuscript.

We understand the importance of considering family history and the relatedness of family members in genetic analyses, as they can provide valuable insights into heritability and genetic architecture of diseases like DCM. While our current study design and dataset do not allow for such an analysis, we are committed to continuing our search for familial cases in the future, which will allow us to conduct such an analysis and potentially provide more detailed insights into the genetic basis of DCM.

We hope this clarifies our approach and the limitations of our study, and we appreciate the reviewer's understanding and consideration.

11. Data should not be over-interpreted to state that was an association between the *FBN1* gene variants and the clinical outcomes. The p value is 0.4, which is non-significant.

We appreciate your comment. In the revised manuscript, we have made it a point to accurately represent our data and avoid overinterpretation, particularly in relation to any clinical baseline or prognostic comparisons that did not reach statistical significance. We understand the importance of maintaining a balanced and cautious interpretation when discussing our findings. Your feedback has been instrumental in ensuring that we appropriately represent our results. Thank you for bringing this matter to our attention.

In conclusion, we would like to express our deepest gratitude for your time, expertise, and constructive comments that have greatly contributed to the improvement of our manuscript. Your thorough review has allowed us to refine our study and presentation in a more meaningful and accurate way. We sincerely appreciate your invaluable contribution to our work.

Reviewer 2 Report

Concern is the characterization of the variants:
1. -are they considered polymorphic variations or mendelian gene variants?

2. -rs763759308, rs148888513, rs143863014: in clinvar are benign or likely beign, therefore unclear how considered in the analysis.

3. -for the frequency, authors should also refer to MAF in other public databases such as gnomAD, specifically for the Han population.

4. -Unclear how the variants were classified as 'pathogenic': there is no reference to the ACMG criteria.

5. Line 237: The conclusions are based on nonsignificant differences therefore are incorrect. It is unclear how *FBN1* mutations can be considered.

Author Response

Concern is the characterization of the variants:
1.-are they considered polymorphic variations or mendelian gene variants?

Thank you for your insightful question. The variants we identified in our study are considered rare Mendelian gene variants rather than polymorphic variations. In alignment with the ACMG Guidelines for Sequence Variation Interpretation (PMID: 25741868), polymorphic variants are defined as having a frequency greater than 0.01. In contrast, the frequency of the identified candidate *FBN1* rare variants in our study is less than 0.001 in the general population, as per data from public databases (specific frequencies for the East Asian populations are detailed in **Table S4**). Therefore, we categorize these rare variants as

Mendelian rather than polymorphic. This clarification has now been incorporated into our revised manuscript for better understanding.

2. -rs763759308, rs148888513, rs143863014: in clinvar are benign or likely beign, therefore unclear how considered in the analysis.

Thank you for your comments regarding the classification of variants. Based on your feedback, we have re-examined the pathogenicity of all rare variants reported in ClinVar. rs763759308, rs148888513, rs143863014, and rs371375126 have been reported as having "Conflicting interpretations of pathogenicity," while other *FBN1* variants were listed as "Uncertain significance"or "Not Reported in ClinVar".

We have now further classified the pathogenicity of all rare *FBN1* variants (with MAF-EAS < 0.001) using the ACMG guidelines, as per your suggestion. The classification results can be found in **Table S2** of our revised manuscript. As you pointed out, rs148888513 was classified as "Likely benign" and was consequently excluded from our analysis. (PMID: 25741868).

Furthermore, rs763759308, rs143863014, and other *FBN1* rare missense variants were categorized as "Variants of Uncertain Significance" (VUS) according to the ACMG guidelines. We noted that the 2015 ACMG guidelines caution: "Care must be taken when applying these rules to candidate genes in the context of exome or genome studies, because this guidance is not intended to fulfill the needs of the research community in its effort to identify new genes in disease."

Therefore, for *FBN1* missense variants initially classified as "Uncertain Significance" in our study, we believe it is reasonable to include those rare variants predicted to be deleterious by multiple missense prediction softwares in our gene-based association analysis. This approach allows us to assess whether these predicted deleterious rare variants are significantly enriched in the case group, thereby analyzing the potential association between *FBN1* variants and DCM. This clarification has been added to our revised manuscript for better understanding.

3. -for the frequency, authors should also refer to MAF in other public databases such as gnomAD, specifically for the Han population.

We agree that the use of other public databases such as gnomAD for frequency comparison is important. We have included the minor allele frequencies (MAF) for East Asian population from gnomAD in the revised manuscript (**Table S4**).

4. -Unclear how the variants were classified as 'pathogenic': there is no reference to the ACMG criteria.

We apologize for not clearly describing the criteria used to classify variants as "pathogenic" in the initial manuscript. We agree with the reviewer's point and have made the necessary corrections. In the revised manuscript, we have thoroughly described the process and

criteria for the classification of "pathogenic" variants in the **METHODS** section under "Bioinformatics Analysis and Variants Deleteriousness Evaluation".

Particularly, for the candidate gene *FBN1*, a large number of missense variants were initially classified as Variants of Uncertain Significance (VUS) according to ACMG guidelines. To further evaluate these variants, we used multiple missense prediction software tools to assess the potential pathogenicity of these VUS. Only those variants predicted as deleterious were retained and together with the variants classified as "pathogenic" or "likely pathogenic" by ACMG guidelines were included in the subsequent gene-based association test.

The flowchart in **Figure S1** provides a brief overview of the screening process for *FBN1* rare deleterious variants. The detailed ACMG classification results can be found in **Table S2**, the deleterious missense prediction results are in **Table S3**, and the final list of rare deleterious variants in *FBN1* is provided in **Table S4**.

For other known DCM-associated genes, we adhered to the ACMG guidelines, retaining only rare variants classified as "pathogenic" or "likely pathogenic" as the final set of rare deleterious variants in other DCM-associated genes.

5. Line 237: The conclusions are based on nonsignificant differences therefore are incorrect. It is unclear how *FBN1* mutations can be considered.

Thank you for your insightful comments. We fully acknowledge and understand your concern about the interpretation of non-significant differences in our initial manuscript. We concur that it was incorrect to overinterpret the clinical outcomes that did not reach statistical significance.

In our revised manuscript, we have rectified this issue by focusing our conclusion primarily on the gene-based association test, which showed a higher incidence of rare deleterious variants in the *FBN1* gene in DCM cases compared to controls. We have carefully revised the sections involving clinical outcomes to ensure that non-significant results are not over-interpreted.

Furthermore, we now emphasize that our findings suggest a potential association, rather than a definitive causal relationship, between *FBN1* variants and DCM. We believe that larger studies are necessary to further validate our findings and to clarify the precise role of *FBN1* variants in DCM.

We appreciate your time and effort in reviewing our manuscript and providing these valuable insights, which have helped us to improve the quality of our work.

Round 2

Reviewer 1 Report

1. The manuscript should be carefully edited. There are too many errors. For example, line 65. The first sentence in the conclusion is repeated twice.

The title repeats *FBN1* twice and should be corrected as: "Possible association of *FBN1* Variants with Dilated Cardiomyopathy in the Chinse hand Population."

2. The problem with this manuscript is that the findings contradict authors own very recent publication in the Journal of Cardiovascular Aging. In the earlier report (*J Cardiovasc Aging* 2023;3:12. 10.20517/jca.2022.44) the authors concluded that when corrected for multiple testing *FBN1* variants were not associated with DCM. Here it is the opposite. One conclusion must be wrong and it cannot be both way. The reviewer worries about selection bias (selecting cases and controls as desired) to achieve the expected outcome as described below.

3. The study design does not benefit from a balance sample size. The sample size of the control group is small compared to the sample size of the DCM patients. This is perplexing as the authors already published a large sample size of 514 control population in a recent JCA paper (*J Cardiovasc Aging* 2023;3:12. 10.20517/jca.2022.44). what is (are) the reason(s) for reducing the sample size of the control group from 514 in the previous study to 414 in the present study?

4. The reviewer is also perplexed with a different sample size of DCM in the present study (N = 1,059) from that published recently by the authors (N = 1,041). It is unclear why 18 cases were moved from the present study. Overall, the approach gives the impression of selection bias, i.e., the authors have selected the sample size data to fit into their hypothesis.

5. The reviewer recommends including the baseline characteristics of the control group in Table 1, if echocardiographic data are available.

6. Line 82-84. Run on sentence. Needs to be corrected.

7. Abstract: line 45, delete "mere".

8. The following statement is not relevant to the present study (and is likely a carry over from the previous one by the authors), as here only *FBN1* variants are analyzed: Our attention was primarily directed towards the rare variants of 44 DCM-related genes as evaluated by ClinGen, specifically, 19 of these genes were determined by ClinGen to have high evidence, and the remaining 25 genes exhibited low or variable evidence[7].

9. Redundant statements. "Sixteen of these missense variants were predicted as deleterious, and the detailed predictive scores of these 16 rare missense variants as calculated by the software tools are provided in Table S3. Hence, through ACMG and software prediction, we identified 17 rare deleterious variants in DCM patients and controls, consisting of 16 missense and one frameshift insertion variant." Should be revised.

Author Response

1. The manuscript should be carefully edited. There are too many errors. For example, line 65. The first sentence in the conclusion is repeated twice. The title repeats *FBN1* twice and should be corrected as: “Possible association of *FBN1* Variants with Dilated Cardiomyopathy in the Chinse Han Population.”

Thank you for pointing out the need for careful editing in the manuscript. We have thoroughly revised the manuscript to rectify the errors.

In **line 65** with your observation regarding the redundancy in the title and the first sentence of the conclusion, we have made necessary amendments to remove the repetitions.

Taking into consideration your concern about our findings, we have adjusted the title to a more cautious tone: "*Possible Association of FBN1 Variants with Dilated Cardiomyopathy in the Chinese Han Population*".

We appreciate your constructive suggestions, which indeed have helped improve the clarity and precision of our manuscript.

2. The problem with this manuscript is that the findings contradict authors own very recent publication in the Journal of Cardiovascular Aging. In the earlier report *(J Cardiovasc Aging* 2023;3:12. 10.20517/jca.2022.44) the authors concluded that when corrected for multiple testing *FBN1* variants were not associated with DCM. Here it is the opposite. One conclusion must be wrong and it cannot be both way. The reviewer worries about selection bias (selecting cases and controls as desired) to achieve the expected outcome as described below.

We appreciate your attention to detail and your concerns regarding potential discrepancies between our current study's results and Dr. Sun Yang's recent publication in the Journal of Cardiovascular Aging. We would like to emphasize that these two studies were conceived with different research focuses and methodologies, which can account for the apparent differences in results.

Dr. Sun Yang's study was primarily intended to evaluate the association of all potential DCM-related genes with DCM phenotype in the entire population. A notable detail in Dr. Sun's methodology is that they were evaluating multiple genes simultaneously for their association with DCM. This approach indeed necessitates multiple testing corrections to control the false discovery rate due to the high number of concurrent hypotheses being tested, such as the Benjamini-Hochberg procedure used in that study.

It is worth noting that while the Benjamini-Hochberg procedure is very effective in controlling false discovery rate, it can be over-conservative in some instances. This aspect may lead to an increased probability of Type II errors (false negatives), where truly associated genes, particularly those with smaller effect sizes, may be incorrectly excluded

from the significant findings (**PMID: 33300887**). This statistical nature might have contributed to the non-significant finding of *FBN1* in Dr. Sun's study.

In contrast, our present study was designed with a more targeted approach. We focused specifically on *FBN1*, assessing its association with DCM without the need for multiple testing corrections because we are testing a single hypothesis. The absence of multiple testing corrections in our study, however, may increase the possibility of false-positive findings, which is a limitation we acknowledge and have included in the 'Study Limitations' section of our manuscript. We may have been able to detect associations that the broader approach employed in Dr. Sun's study might have been more cautious in asserting.

We believe our findings regarding the potential role of *FBN1* variants in our cohort, particularly in differentiating MFS-related phenotypes from DCM, are meaningful and contribute to the ongoing research in this field. Our targeted approach in the current study should not be viewed as a contradiction or invalidation of the findings in Dr. Sun's work. Instead, these two studies complement each other, each providing valuable insights from different perspectives - one offering a broad view of DCM-related genes across a population, and the other focusing on the specific role of *FBN1* variants in DCM.

In summary, the methodologies and purposes of these two studies are not identical, thus their conclusions are not directly comparable. However, both studies, when considered together, contribute to a richer understanding of the complex genetic landscape of DCM.

We appreciate your constructive suggestions, which indeed have helped improve the clarity and precision of our manuscript. We believe that the detailed explanation provided here resolves the seeming contradiction and hope it meets your concerns.

3. The study design does not benefit from a balance sample size. The sample size of the control group is small compared to the sample size of the DCM patients. This is perplexing as the authors already published a large sample size of 514 control population in a recent JCA paper (*J Cardiovasc Aging* 2023;3:12. 10.20517/jca.2022.44). what is (are) the reason(s) for reducing the sample size of the control group from 514 in the previous study to 414 in the present study?

Thank you for your observation regarding the sample size discrepancy between our study and the recent publication in the *Journal of Cardiovascular Aging* (*JCA*).

When the data for our study was collated in early 2021, we had a cohort of 414 healthy controls and 1041 DCM patients. This is corroborated by two other articles from our research group published around the same period (**PMID: 33996946 and PMID: 34333030**). As the research progressed, additional DCM cases and controls were incorporated, resulting in the larger sample sizes in Dr. Sun's *JCA* paper.

This variation is not a consequence of deliberate selection bias, but a reflection of different points in time in the progression of our ongoing research. We acknowledge that we failed to fully address this aspect in our previous response and apologize for any confusion that

this may have caused. To address your concern, in this revision, we have revised the cohort size to match that of Dr. Sun's study and reanalyzed the data using our established methodology.

4. The reviewer is also perplexed with a different sample size of DCM in the present study (N = 1,059) from that published recently by the authors (N = 1,041). It is unclear why 18 cases were moved from the present study. Overall, the approach gives the impression of selection bias, i.e., the authors have selected the sample size data to fit into their hypothesis.

We appreciate your attention to the variation in the DCM sample sizes between our current study and the recent *JCA* paper.

As explained in the response to Comment 3, this discrepancy is due to the different time points of data collection and the continuous progression of our study. We assure you that there was no intent to introduce selection bias. In our revised manuscript, we will adjust our DCM patient cohort size to align with the one reported in the *JCA* paper and conduct our analyses accordingly. Again, we are grateful for your constructive comments, which help us improve the transparency and reliability of our work.

5. The reviewer recommends including the baseline characteristics of the control group in Table 1, if echocardiographic data are available.

We appreciate the reviewer's point about the importance of providing a comprehensive overview of both the DCM patients and control groups for improved transparency and comparability. Therefore, in the revised manuscript, we will supplement the echocardiographic data of the control group in **Table 1**, as suggested by the reviewer.

6. Line 82-84. Run on sentence. Needs to be corrected.

Thank you for your careful reading and valuable comments. We appreciate your suggestion to clarify the sentence in line 82-84.

In response to your comment, we have revised the sentence to improve its readability:

"Over 50 genes associated with DCM have been identified. However, the combined genetic contribution of these genes explains about 35% of idiopathic dilated cardiomyopathy cases. This suggests the existence of as-yet-undiscovered genes or mechanisms associated with DCM".

7. Abstract: line 45, delete “mere”.

Thank you for your suggestion to delete the word "mere" from the sentence in question. Upon reflection, we agree that this term may inadvertently convey an unintended tone. We have therefore revised the sentence to say:

"Although over 50 genes have been associated with DCM, these collectively explain 35% of idiopathic DCM cases."

We appreciate your keen eye for detail and believe that this revision improves the clarity of the text.

8. The following statement is not relevant to the present study (and is likely a carry over from the previous one by the authors), as here only *FBN1* variants are analyzed: Our attention was primarily directed towards the rare variants of 44 DCM-related genes as evaluated by ClinGen, specifically, 19 of these genes were determined by ClinGen to have high evidence, and the remaining 25 genes exhibited low or variable evidence[7].

Thank you for your insights and for raising a valid concern. The reference to the 44 DCM-related genes in our study is not merely a carryover from Dr. Sun's previous study, but rather an integral part of our methodology. While our focus is on *FBN1*, understanding its role within the broader context of DCM-related genes is crucial.

In this study, we have consciously addressed the potential confounding impact of variants in other DCM-associated genes on our analysis of *FBN1*. These 44 DCM-associated genes, evaluated by ClinGen, represent a significant pool of genes that could potentially confound the association between *FBN1* variants and DCM if they co-occur in our patient samples. To mitigate this, we explicitly excluded both patient and control samples carrying pathogenic or likely pathogenic variants in these genes from the final SKAT-O analysis. This step was imperative to ensure that the associations we found for *FBN1* were not influenced by these other known DCM-associated genes.

This unique approach distinguishes our study from Dr. Sun's previous work. While his study considered a large panel of genes simultaneously, we specifically focused on *FBN1* and conscientiously controlled for the effect of other known DCM-associated genes. This strategic focus has allowed us to uncover and highlight the potential importance of *FBN1* in DCM, a finding that was not apparent in broader gene panel studies.

We apologize if this was not clearly articulated in the manuscript. We will revise the text to better communicate the unique contributions of our study and the rationale behind considering the 44 DCM-associated genes. We greatly appreciate your careful review and constructive comments.

9. Redundant statements. “Sixteen of these missense variants were predicted as deleterious, and the detailed predictive scores of these 16 rare missense variants as calculated by the software tools are provided in Table S3. Hence, through ACMG and software prediction, we identified 17 rare deleterious variants in DCM patients and controls, consisting of 16 missense and one frameshift insertion variant.” Should be revised.

Thank you for your suggestion. We understand that the sections you pointed out might appear redundant. For the revised manuscript, we will be conducting a new analysis with an updated sample size to maintain consistency with Dr. Sun's study as suggested by the

editor, we will revise the mentioned sections based on the updated results, striving for a more concise and clear presentation. We appreciate your patience and understanding in this matter.

Finally, we would like to express our sincere appreciation for the reviewer's meticulous guidance during the two rounds of revision. We deeply respect the efforts you have made to enhance the scientific rigor and integrity of our study. We believe that your constructive suggestions will significantly improve the quality and credibility of our work. Thank you again for your time and effort.

Reviewer 2 Report

1. it has to be acknowledged that the authors replied to all the points (or at least they did their best) and toned down their conclusions. However, the paper still has important structural limitations. In particular, the control population is too small to derive conclusive results on the difference in frequency of mutations. Moreover, it is at least unusual that among the 17 mutations concluded as pathogenic or likely pathogenic, 16 are missense and there is only one frameshift (so potentially questionable for pathogenicity in absence of strong markers such as co-segregation). In relation to this last point, the authors affirmed that there was not family history in the patients (all 1041 unrelated!), still unusual for a genetic disease with, theoretically, autosomal dominant transmission.

Minor points:

1. - authors reported outcomes, but there is no adjusted analysis supporting the conclusion of lacking association with outcome.

2. - therapy is singular (90% on MRA, < 50% on BB).

3. - high prevalence of renal failure in *FBN1*$^{+}$ is worth to be mentioned.

4. - the overall text is too long and dispersive, and requires language editing as well.

Author Response

1. it has to be acknowledged that the authors replied to all the points (or at least they did their best) and toned down their conclusions. However, the paper still has important structural limitations. In particular, the control population is too small to derive conclusive results on the difference in frequency of mutations. Moreover, it is at least unusual that among the 17 mutations concluded as pathogenic or likely pathogenic, 16 are missense and there is only one frameshift (so potentially questionable for pathogenicity in absence of strong markers such as co-segregation). In relation to this last point, the authors affirmed that there was not family history in the patients (all 1041 unrelated!), still unusual for a genetic disease with, theoretically, autosomal dominant transmission.

Thank you for your constructive comments and acknowledging our efforts in addressing your previous points. We fully accept that our study has inherent structural limitations and have done our best to address these within the context of the present study.

Regarding your concern about the size of our control population, we understand that this could limit the conclusiveness of our results on the difference in frequency of mutations. We will strive to expand our control population in future studies to address this limitation.

As for the unusual predominance of missense mutations (16 out of 17 mutations) and only one frameshift, we concur that this distribution might seem atypical. However, it aligns with previous literature, which suggests that missense variants make up about two-thirds of all variants [**PMID: 27437668**]. We have employed a combination of multiple software tools to predict the effects of missense variants that the ACMG has classified as Variants of Uncertain Significance (VUS). Although this method reduces the rate of false predictions from individual software, we agree there remains the possibility of false positives. We have acknowledged this limitation in our study.

In terms of the absence of family history in our patients, our use of the term 'unrelated' referred to the lack of kinship among the patients, which we now realize might have caused confusion. We have therefore replaced 'unrelated' with 'sporadic' for clarity. We have managed to obtain DNA samples from a small number of patient family members during enrollment and follow-up, provided their understanding and consent. However, the points of interest identified in the patients did not receive validation from their relatives. Hence, based on our current knowledge, we cannot confirm the patients' family history. This is a significant limitation of our research and might be attributable to the incomplete penetrance and individual-specificity of DCM gene mutations. As you have rightly pointed out, the size of our sample indeed limits our potential for new discoveries.

We appreciate your valuable feedback, and we will continue to improve our research methodology and clarity of reporting in our future works.

Minor points:

1. - authors reported outcomes, but there is no adjusted analysis supporting the conclusion of lacking association with outcome.

Thank you for your comment, in response to your suggestion, we have added an adjusted analysis in our revised manuscript. In this analysis, we performed a Cox proportional hazards model to adjust for age at enrollment, gender, and the carriage of pathogenic or likely pathogenic variants in the other 44 known DCM-associated genes that we considered in our study.

Upon performing this adjustment, we did not observe a significant difference in the risk of primary endpoints (Hazard Ratio - HR: 1.38 [0.71, 2.68], $P = 0.25$). However, we did find a significantly higher risk of secondary endpoints in the FBN1^{+} group (HR: 1.78 [1.03, 3.10], $P = 0.019$).

We believe this revised analysis, considering your valuable suggestion, strengthens our study by adding further nuance to our understanding of the potential effects of *FBN1* variants in DCM patients. We are appreciative of your contribution to improving the scientific rigor of our research.

2. - therapy is singular (90% on MRA, < 50% on BB).

We acknowledge your observation about the singularity in the therapeutic approach, with 90% of patients on Mineralocorticoid Receptor Antagonists (MRA) and fewer than 50% on beta-blockers (BB). You are correct in your interpretation.

Our patient cohort had notably low LVEF scores, indicating significant cardiac dysfunction. Furthermore, the nature of our single-center cohort study may have introduced selection bias in treatment strategies, reflecting institutional preferences rather than broader clinical practice.

Additionally, some of the patients who enrolled early (around 2010) might not have been managed strictly according to the contemporary heart failure management guidelines. These factors likely account for the distribution of therapeutic approaches observed in our study.

We sincerely appreciate your attention to this matter and will make sure to highlight these points in our manuscript to provide context for our findings and to guide future research.

3. - high prevalence of renal failure in *FBN1*$^+$ is worth to be mentioned.

Thank you for your comment. Indeed, we have addressed the high prevalence of renal failure in the *FBN1*$^+$ group in the revised manuscript. Given that diseases related to *FBN1* are often systemic, affecting multiple organs to varying degrees, we've included our initial speculation in the manuscript: "Moreover, renal insufficiency was more prevalent in *FBN1*$^+$ patients (33.3% vs. 8.5%, $P < 0.001$), suggesting a potential link between *FBN1* variants and renal impairment. However, this hypothesis requires further robust evidence for validation."

We believe that this observation is important and provides an avenue for future research. We appreciate your suggestion to highlight this finding in our study.

4. - the overall text is too long and dispersive, and requires language editing as well.

Thank you for your valuable feedback.

We appreciate your comment on the length and dispersion of the manuscript. We have tried our best to streamline the text and make the language more concise in the revision. Furthermore, we have sought assistance from a professional language editing service to ensure the clarity and readability of the text.

This being the second review of our manuscript, we deeply appreciate your insightful comments and suggestions that have significantly contributed to the improvement of our paper. Your thorough and rigorous review process has undoubtedly enhanced the quality of our work. Thank you once again for your time and expertise.

Editor's Comments:

Thank you very much for addressing the concerns and submitting a revised manuscript. We are delighted to inform you that your manuscript is potentially acceptable for publication in the *JCA* pending minor revisions as follows:

1. Abstract Title: please delete the word "possible". Your data show that *FBN1* variants are associated with dilated cardiomyopathy.

The suggested title is: Rare Variants in the *FBN1* gene are associated with Sporadic Dilated Cardiomyopathy in a Chinese Han Population or simply delete the word "possible". Or "Enrichment of the Rare Deleterious Variants in the *FBN1* Gene in Patients with Sporadic Dilated Cardiomyopathy".

2. In the title: It is better to state "a Chinese Han Population" rather than "the Chinese Han population".

3. Lines 34-36. Change to: The objective of this study was to investigate the association between *FBN1* variants and DCM in the Chinese Han population.

4. Line 37: Given the changes above, please revise this sentence as: "We performed whole exome sequencing (WES) to identify ……

5. Line 47. Please revise as follows: The findings suggest an association between rare deleterious variants in the *FBN1* gene and DCM in a Chinese Han population.

Results:
6. Line 197: Suggest changing "Mutational profile of *FBN1*" to Profile of the rare variants in the *FBN1* gene.

Discussion:
7. Line 305. Please change "comprehension" to understanding.

8. Line 360 in the limitation section: Please add. *FBN1* is at best expressed at low levels in the cardiac myocytes but at high levels in cardiac fibroblasts. Therefore, the deleterious effect of the rare variants in the *FBN1* gene on cardiac function could be mediated through their effects on cardiac fibroblasts and the myocardial architecture.

9. Line 363: “We identified rare, deleterious *FBN1* variants in a significant DCM cohort”. This sentence should be revised as it is incomplete. Suggest: We found enrichment of rare, deleterious variants in the *FBN1* gene in patients with sporadic DCM.

Author Response

Thank you very much for addressing the concerns and submitting a revised manuscript. We are delighted to inform you that your manuscript is potentially acceptable for publication in the JCA pending minor revisions as follows:

1.Abstract Title: please delete the word “possible”. Your data show that *FBN1* variants are associated with dilated cardiomyopathy.

The suggested title is: Rare Variants in the *FBN1* gene are associated with Sporadic Dilated Cardiomyopathy in a Chinese Han Population or simply delete the word “possible”. Or “Enrichment of the Rare Deleterious Variants in the *FBN1* Gene in Patients with Sporadic Dilated Cardiomyopathy”.

We appreciate your suggestion regarding the title of the abstract. In line with your guidance, we have amended the title to: "Rare Variants in the *FBN1* gene are associated with Sporadic Dilated Cardiomyopathy in a Chinese Han Population". We believe that this revised title more accurately represents the findings of our study. Thank you for your constructive suggestion.

2.In the title: It is better to state “a Chinese Han Population” rather than “the Chinese Han population”.

We thank you for your careful attention to detail in the manuscript's title. We agree with your recommendation and have revised it accordingly to say "a Chinese Han Population". We appreciate your guidance on this matter.

3. Lines 34-36. Change to: The objective of this study was to investigate the association between *FBN1* variants and DCM in the Chinese Han population.

We appreciate your suggestion to enhance the clarity of our study's objective. As per your advice, we have modified lines 34-36 to: "The objective of this study was to investigate the association between *FBN1* variants and DCM in a Chinese Han population." We agree that this revised wording presents the study's objective in a more succinct and understandable manner.

4. Line 37: Given the changes above, please revise this sentence as: “We performed whole exome sequencing (WES) to identify ……..

Thank you for your suggestion. In light of your comments and the aforementioned revisions, we have revised line 37 to read: "We performed whole-exome sequencing (WES) to identify rare *FBN1* variants among 1059 DCM cases and 514 controls."

5. Line 47. Please revise as follows: The findings suggest an association between rare deleterious variants in the *FBN1* gene and DCM in a Chinese Han population.

Thank you for your guidance. We agree with your suggestion and have accordingly revised line 47 to read: "The findings suggest an association between rare deleterious variants in the *FBN1* gene and DCM in a Chinese Han population."

Results:
6. Line 197: Suggest changing "Mutational profile of *FBN1*" to Profile of the rare variants in the *FBN1* gene.

We appreciate your suggestion on the section title. We have revised the title in line 197 to 'Profile of the Rare Variants in the *FBN1* Gene', maintaining the convention of capitalizing the principal words in section headings.

Discussion:
7.Line 305. Please change "comprehension" to understanding.

We thank you for this linguistic improvement. The term "comprehension" in line 305 has been replaced with "understanding".

8. Line 360 in the limitation section: Please add. *FBN1* is at best expressed at low levels in the cardiac myocytes but at high levels in cardiac fibroblasts. Therefore, the deleterious effect of the rare variants in the *FBN1* gene on cardiac function could be mediated through their effects on cardiac fibroblasts and the myocardial architecture.

We appreciate the suggestion to include the following statement at the end of the limitation section: "*FBN1* is at best expressed at low levels in the cardiac myocytes but at high levels in cardiac fibroblasts. Therefore, the deleterious effect of the rare variants in the *FBN1* gene on cardiac function could be mediated through their effects on cardiac fibroblasts and the myocardial architecture." We acknowledge the importance of highlighting this aspect and have revised the manuscript accordingly.

9. Line 363: "We identified rare, deleterious *FBN1* variants in a significant DCM cohort". This sentence should be revised as it is incomplete. Suggest: We found enrichment of rare, deleterious variants in the *FBN1* gene in patients with sporadic DCM.

We appreciate the suggestion to revise the sentence in line 363. Instead of "We identified rare, deleterious *FBN1* variants in a significant DCM cohort," we have modified it as follows: "We found enrichment of rare, deleterious variants in the *FBN1* gene in patients with sporadic DCM." We acknowledge the clarity and completeness of the revised sentence and have incorporated this change in the manuscript. Thank you for your valuable input.

In addition to addressing your valuable comments, we would like to bring to your attention two minor corrections that we identified during our thorough review of the manuscript and figures:

1. In the legend section of **Figure S1 (Lines 539-540)**, "Kaplan-Meier curve illustrates survival free from primary endpoints, which include cardiac mortality or heart transplantation. (B) The curve represents survival free from secondary endpoints, comprising all-cause mortality or heart failure recurrence." We sought to maintain consistency with the corresponding parts of the legend of Figure 1 and the contents of the Y-axis labels of Figure S1. Therefore, we have revised the two "**free from**"to "**free of**".

2. To maintain consistency with the Legends corresponding to **Figure S1** and the format used in the Results section, we modified the legend format in Figure S1 by adding a "/" between *FBN1* and DCMGenes. Thus, "*FBN1*-DCMGenes+" and "*FBN1*-DCMGenes-" have been revised to "*FBN1*- / DCMGenes+" and "*FBN1*- / DCMGenes-", respectively.

We appreciate your understanding and apologize for any confusion caused by these discrepancies.

Acknowledgments:
We would like to express our sincere gratitude to the editors and reviewers for their valuable insights and assistance during the revision process. Their patience, dedication, and meticulous attention to scientific rigor have been truly invaluable. This collaborative process has not only allowed us to enhance the quality and scientific integrity of our research but has also provided us with valuable learning experiences. We are deeply appreciative of the time and effort they have invested in our manuscript and for their trust in our work.

Thank you once again for your invaluable contributions and support.