Author’s response to reviews

Title: CHCHD2 is a potential prognostic factor for NSCLC and is associated with HIF-1a expression

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Dear Maire Clayton,

Thank you and other reviewers for your valuable suggestions and questions about my manuscript. I will reply them point-by-point.

Kangsheng Tu (Reviewer1):

Q1: Representative IHC pictures for different staining grades should be provided.

A1: In Fig.2, I have added the representative IHC pictures of CHCHD2 and HIF-1a at different staining levels in adenocarcinoma, squamous cell carcinoma and normal tissues and have modified the Figure title and legend (figure title and legend section, line 502-512, page 19).

Q2: The expression difference of CHCHD2 between NSCLC and normal tissues, as well as its prognostic value should be further confirmed in TCGA database.

A2: The difference expression of CHCHD2 between NSCLC and normal tissues and its prognostic value were further confirmed by Oncomine database, and I have supplemented and modified corresponding content in the manuscript (methods section, line 153-165, page 7; results section, line 184-189, page 8; discussion section, line 290-293, page 12; figure title and legend.
section, line 491-501, page 19; references section, line 461-472, page 18;). I wonder if it can meet the requirements for the journal?

To use TCGA database requires a basic of R language, and master it need to some time. In order not to affect the progress of the manuscript, the TCGA database was replaced by Oncomine database to analyses the expression of CHCHD2 in cancer and normal tissues and its prognostic value. The value of CHCHD2 expression is not related to the survival time in the oncomine database, may be due to factors such as heterogeneity of samples, sample size, and incomplete consistency of mRNA and protein expressions (In some samples, mRNA expression is high, while protein expression is low), this result is contrary to that in our study. Therefore, I have not add the outcome of CHCHD2 prognostic from OncoMe database analysis to the manuscript. I wonder if you agree with me.

Q3: HIF-1a and HIF-2a are two main effectors of hypoxia. Thus, it is necessary to analyze the correlation between CHCHD2 and HIF-2a.

A3: Above all, HIF-1 is the first hypoxia-inducible factor to be discovered, and HIF-1a is its functional subunit. Secondly, although HIF-2a and HIF-1a are structurally similar, HIF-1a is widely expressed in various types of cells and organs, while HIF-2a is mainly expressed in vascularized organs and tissues, such as endothelial cells, Macrophages and so on. Thirdly, HIF-1a is mainly responsible for the transcription of glycolytic and apoptotic proteins, and CHCHD2 is directly related to glycolytic enzymes in the co-expression network, which suggests that the expression of CHCHD2 should be related to the expression of HIF-1a. Fourthly, HIF-1a overexpression can induce high expression of CHCHD4. Can HIF-1a overexpression induce high expression of CHCHD2? This needs to be proved. Therefore, our study concentrates on correlation between HIF-1a and CHCHD2.

Q4: Does the combination of CHCHD2 and HIF-1a predict the poorest survival of patients?

A4: As we all know, HIF-1a is an important nuclear transcription factor. The purpose of this study is to show that CHCHD2 expression related to HIF-1a, it may be a target gene of HIF-1a. CHCHD2 may be an adverse prognostic factor of NSCLC.

Jochen Wilhelm, Ph.D. (Reviewer 2):

Q1: It remains unclear what the used criterion of disease severity is to judge the prognostic value of the target gene expressions. Initially, the 5-year survival rate is mentioned, but the study was too short (3 years median follow-up time) to estimate this criterion. Hazard ratios in survival model were neither addressed nor discussed.

A1: In this study, we followed up the patients for 5 years. Based on the survival time of each patient, the median value is 36 months. In the results of “High levels of CHCHD2, HIF-1α predict poor prognosis of NSCLC patients”, I supplement the description and discussion of hazard ratio in survival model (results section , line 254-261, page 11; discussion section , line 309-329, page 13-14).
Q2: In general, it is unclear to me where mRNA expression and where protein expression was analysed. The figures and tables are all labelled with CHCHD2 and HIF-1a, and these all-capital symbols indicate that the protein expression is meant (but I don't think that this is intended).

A2: In order to make it clear, I re-labeled the mRNA and protein expressions of CHCHD2, HIF-1a in title and legend of Fig.1 (figure title and legend section, line 491-501, page 19) and tables (tiles of table 1, 2, 4).

Q3: The quantitative RT-PCR is not described in enough detail to understand the results. It is not clear if the assays were validated (amplification specificity, efficiency, dynamic range?). In the methods it is written in line 122 that mRNA levels are expressed as CT values, but these are nowhere presented. Then it is stated that the amount of target gene is measured using the $2^{\Delta\Delta CT}$ method. There is no reference to a publication explaining this method (it's likely to be the paper by Livak and Schmittgen, what is statistically flawed; but that's a different topic). It is not described how expressions are compared statistically.

A3: I can provide the pictures of amplification plots and dissociation curve of quantitative RT-PCR and try to illustrate the amplification specificity and efficiency, but I don't think it is related to the topical subject of the research content, so I think these pictures should not be present in the manuscript. I wonder if you agree with me.

In the procedures of PCR, three parallel reactions are set for each sample. The mRNA expression value were calculated by the MxPro QPCR Software 4.10 (Mx3005P, Stratagene, Agilent Technologies) according to $2^{\Delta\Delta CT}$ method (added in methods section, line 122-124, page 6).

Q4: Statistical analysis: Tables 1+2 show contingency tables of categorized expression values. It is not described how this categorization was done. The analysis via the signed rank test is extremely crude. As it is well-known that the conditional distribution of ddCT values is approximately normal, linear models could have been used to include the effects of covariables (see Table 2). It would also be really helpful to provide some indication about the direction of the association (e.g. is the expression higher in smokers or in non-smokers, in larger or in smaller tumors? etc.).

Using a significance level of 0.05 in Tables 2 and 3 is too liberal, given a sample size of 209 and multiple testing. You should consider Bonferroni-Holm adjustment to judge statistical significance.

Where were U-tests and H-tests used?

A4: The Wilcoxon (W) test was used to evaluate the comparison of CHCHD2 and HIF-1a protein expression between NSCLC and corresponding normal tissue. Associations between immunohistochemical expression and clinical variables were evaluated by Mann-Whitney U (among tow groups), Kruskal-Wallis H test (among multiple groups) and Spearman’s rank correlation analysis as appropriate (modified in methods section, line 168-172, page 7-8).

Q5: Western blot: Which lysis buffer was used? Where have the antibodies been obtained from?
A5: I have added the description of the RIPALysis Buffer in the part of western blot method (methods section, line 131-133, page 6). The description of the antibodies in the part of immunohistochemistry method (methods section, line 111-113, page 5).

Q6: It seems that the variables Age, Tumor size and expression for the survival analysis have been discretized (dichotomized). This is not adequate, unless reasonable and externally validated choices of for the dichotomization are given. If the relationships of these variables with the log hazard ratios are clearly non-linear, one could consider a spline model of appropriate flexibility.

A6: The median value of a grouping variable is generally used as the cutoff value. The median age of the patients was 62, according to lung cancer TNM staging regulations the tissue size ≤ 5cm was T2a. In this study, the degree of expression of CHCHD2 and HIF in tissues were divided into four levels (-, +, ++, +++), Can be divided into low (-, +) and high (++ , +++ ) expression.

Maire Clayton (editor):

Q1: We notice significant text overlap between your manuscript and previously published materials. Please reformulate the large sections of overlapping text present in your discussion section. Please ensure that, where relevant, these sources are also referenced as appropriate.

A1: I have re-edited the discussion section (discussion section, line 280-288, 290-295, 304-329, page 12-14) and added appropriate references.(discussion section, line 333, page 14; references section, line 485-488, page 18;)

Q2: On the title page of your manuscript please indicate only one corresponding author. Please ensure that the sole corresponding author on the title page is consistent with the corresponding author declared in the submission data.

A2: Dear editor, I would like to request you approve me that the informations of two corresponding authors be presented in the title page of my manuscript, as it will better reflect the author's contribution to this article. If you cannot approve, Please inform me, and I will consider changing the authors order.

Q3: Figure 3 and 4 are referenced in the manuscript, but are not included. Please add these figures or revise accordingly.

A3: I have added Figure 3 and 4.

Q4: Please remove the revise reports from the supplementary material.

A4: I have removed the revise reports from the supplementary material.
Other modifications:

1. Modified all “adjacent non-cancerous” or “ANC” in the manuscript to “normal”.


3. Modified “Envision ×400” to “All images are magnified at ×400”

4. Modified “Among factors in Table2, the tumor size, TNM stage, differentiation and lymph node metastasis were significantly associated with patient’s survival.” to “Among factors in Table2, the tumor size, TNM stage, differentiation and lymph node metastasis were significantly associated with the expression of CHCHD2, here we analyzed the relationship between these factors and patient’s survival.” (results section, line 213-215, page 9)

If there are any other requirements, please inform me, and I will do my best to cooperate to ensure the progress of the manuscript.

Best wishes,

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