Author's response to reviews

Title: Knocking down CDK4 mediates the elevation of let-7c suppressing cell growth in nasopharyngeal carcinoma

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Author's response to reviews: see over
Dear reviewer,

Thank you very much for your suggestions to our submission. We think your insightful comments are very helpful for improving the quality of our article before publication. Following your suggestions, we have revised the paper. We hope that our revision is what you were looking for.

Reviewer's report:
The present article by Liu Z. et al describes some aspects of the CDK4-let-7c interactions in nasopharyngeal carcinoma and analyzes CDK4 expression in this malignancy. Extensive revision is needed before this article is suitable for publication. Major and minor concerns are listed below:

Major Compulsory Revisions

**Question1.** The authors should analyze the expression of let-7c in all NPC tissues and evaluate its prognostic properties; this will strengthen the present study.

**Response:** Thanks reviewer for this good suggestion. Actually, we also want to evaluate the correlation of let-7c expression with prognostic properties of NPC patients. However, all NPC fresh samples used for the expression examination of let-7c are collected from one year ago. We only obtained the information of pathology diagnosis for these NPC patients. Thus, we have not the ability to evaluate the correlation of Let-7c with the prognostic properties. We wish that the reviewer can understand us.

**Question2.** Details are missing regarding sample collection, storage and preparation for analysis. What is more, since the tissue specimens are FFPE tissues, how was it possible to proceed to miRNA expression analysis without including a proteinase digestion step to remove methylol cross-links between RNA and proteins?

**Response:** Sorry for our mistake. 1) We checked our paper and found that we did not write details about the collection of fresh NPC and NP samples. We supplemented this information in Sample collection and cell culture of Materials and Methods: “All 56 fresh NPC and 15 NP samples were obtained from a professional otorhinolaryngology doctor by using nasal endoscope. Subsequently, all samples were immediately stored in liquid nitrogen after a part of each sample was isolated for pathology diagnosis. 2) We used fresh NPC and NP samples, but not FFPE tissues, for examining the expression of let-7c. So we did not remove methylol cross-links between RNA and proteins
We have used the same samples to examine the expression of miR-184 and miR-18b in NPC and NP tissues and papers for theses miRNAs were published or accepted in cell death disease


2. Yan Zhen, Zhen Liu, Huiling Yang, Xiaoli Yu, Qiangyun Wu, Shengni Hua, Xiaobin Long, Qingping Jiang, Ye Song, Chao Cheng, Hao Wang, Menyang Zhao, Qiaofen Fu, Xiaoming Lyu, Yiyu Chen Yue Fan, Yan Liu, Xin Li, Wei yi Fang. Tumor suppressor PDCD4 modulates miR-184-mediated direct suppression of C-MYC and BCL2 blocking cell growth and survival in nasopharyngeal carcinoma. Cell Death Dis(Accepted)

**Question 3.** P value for survival analysis is missing. Additionally, how was the cut-off value chosen for dichotomizing CDK4 expression values into high/low expression?

**Response:** Sorry for our carelessness. P value is equal to 0.045. We have added it to Figure 5. CDK4 is a nuclear and cytoplasmic factor. In some NPC tissues, we found that CDK4 is only expressed in cytoplasm. In other tissues, CDK4 is coexpressed in nuclear and cytoplasm. Therefore, we respectively evaluated the expression level of CDK4 in nuclear and cytoplasm. According to our previous study of CDK4 expression in lung cancer (Journal of Translational Medicine 2011, 9:38), the expression of CDK4 in nuclear and cytoplasm was respectively scored. In cytoplasm, the staining intensity was scored “0-3” where 0 represents negative expression, 1 is weak expression, 2 is positive expression, and 3 is strong expression. Furthermore, the percentage of positive staining areas of cells was defined as a scale of 0 to 3 where 0 represents <10%, 1 is 10-25%, 2 is 26-75%, and 3 is ≥76%. Therefore, the score of CDK4 cytoplasm expression was 0-6. Consistent with staining intensity assessment of CDK4 in cytoplasm, its staining intensity score in nuclear was also defined as ”0-3”. Further, the number of nuclear staining was also scored as follows: 0 represents <10%, 1 is 10-50%, 2 is 51-80%, and 3 is ≥80%. Therefore, the score of CDK4 nuclear expression was also 0-6. The sum of the cytoplasm and nuclear staining scores was used as the final staining score for CDK4 (0-12). For statistical analysis, a final staining score of 0-6 or 7-12 was respectively considered to be low or high expression.
In this research, we chose “6” as cut-off value basing on the following considerations. 1) In 133 paraffin-embedded undifferentiated NPC specimens, 75 patients showed the low expression of CDK4, and 58 cases indicated high CDK4 expression. According to the number distribution of patient in low and high expression, it is fit for statistical analysis. 2) CDK4 as a potential oncogene, the patients with CDK4 overexpression only has 58 cases, which was below the number of CDK4 downregulation. Therefore, we thought that the cut-off value should not be higher than that of “6”. 3) In preliminary analysis, we used “4”, “6” and “8” respectively as cut-off value, we found that no statistical significance was shown in “4”. However, in “8”, attributing to insufficient patient number for high CDK4 high expression, p value was indicated for 0.21. Therefore, we finally chose “6” as cut-off value for this study.

**Question 4.** The authors should proceed to univariate and multivariate Cox regression survival analyses.
We supplemented the univariate and multivariate Cox regression survival analyses (See table 3) The results indicated that although CDK expression was not significantly associated with overall survival of NPC patients according to univariate analyses, its overexpression is a independent prognostic factor for NPC patients regardless of its patients' disease status based on Multivariate analyses.

**Question 5.** More details regarding the calculations used to analyze the data of qPCR experiments should be provided.
**Response:** We supplemented the detail of analyzing the data of qPCR experiments. “In each panel of qPCR experiment, every sample has its \( \Delta C_t \) (Targeted gene Ct-Housekeeping gene Ct). We usually chose a maximal \( \Delta C_t \) value (the lowest expression) as a reference. All sample \( \Delta C_t \) values (objective samples and control samples) including this maximal \( \Delta C_t \) value subtract this maximal \( \Delta C_t \), which form \( -\Delta \Delta C_t \). Furthermore, all data was transformed by \( 2^{-\Delta \Delta C_t} \). It is obvious that the expression of level of the sample with the maximal \( \Delta C_t \) value is 1 and other samples has more the expression fold value compared to “1”. Further, we calculated the differential expression level by T test between NPC samples and control samples.

**Summary:**
1) Firstly, calculating \( \Delta C_t \) value of Each sample (Targeted gene Ct value-Housekeeping gene Ct value); 2) Secondly, calculating \( -\Delta \Delta C_t \) value of each sample (Each sample \( \Delta C_t \)
value - the maximal ΔCt value of all sample ΔCt values; 3) Thirdly, the expression level of each sample was transformed to fold-related value including that sample with maximal ΔCt value by 2 −ΔΔCt method. 4) Finally, the differential expression level was analyzed between objective group and control group by t test.

**Question 6.** The authors mention that “Knocking down E2F1 by specific siRNA-E2F1 elevated the expression of Let-7C.” How can this be explained? In an article by Bueno MJ et al (Mol Cell Biol. 2010 Jun;30(12):2983-95) it is shown that E2F1 induces the expression of let-7 microRNAs.

**Response:** Thanks for pointing out this problem. This question is very interesting. In recent investigation from our cancer institute, Prof. Xiao (Corresponding author) found that c-Myc may induce mesenchymal-epithelial transition (MET) in pig fibroblasts (Cell Cycle. 2013 Apr 1;12(7):1119-27). However, in tumor pathogenesis, c-Myc was taken as an oncogene inducing epithelial-mesenchymal transition (EMT) and promoting cell invasion and metastasis in tumors. C-Myc shows different functions in different cells. E2F1, an oncogene that positively directly regulated the expression of c-Myc, was reported to induce the expression of let-7 microRNAs in Mouse embryonic fibroblasts. Similar to Prof. Xiao’s study from our cancer institute, Bueno MJ et al used fibroblasts from mouse embryon to explore the E2F1-regulated genes. As you know, fibroblasts is a mesenchyma-original and non-tumor cell, whereas NPC cells were an epithelium-original tumor cells. Therefore, it is possible that E2F1 may exert different function and different molecular mechanism in different cells. Our results indicated that knocking down E2F1 by specific siRNA-E2F1 elevated the expression of let-7c in NPC, whereas Bueno’s report indicated that E2F1 induces the expression of let-7 microRNAs in MEF cells (Let-7c was not be reported to be induced by E2F1 in MEF cells). Furthermore, downregulated expression of let-7 microRNAs as tumor suppressors has been shown in many tumors, which promoted these tumor pathogenesis. Whereas E2F1 is a known oncogene that stimulated cell cycle transition from G1 to S in tumors. Let-7 microRNAs function is inverse with E2F1 action in tumor pathogenesis. Therefore, E2F1 negatively modulating the expression of let-7c was totally possible.

**Question 7.** It is mentioned that let-7c decreases the expression of CDK4 and decreased CDK4 expression leads to downregulation of E2F1 which in turn leads to let-7c
overexpression. Can this positive feedback loop be biologically relevant? The authors should discuss this finding, mentioning similar examples from the literature.

**Response:** According to this study, we thought that this positive feedback loop of CDK4-E2F1-let-7c was biologically relevant in NPC. In recent study, we also confirmed a positive feedback loop for CTGF-C-Jun/C-Myc-miR-18b-CTGF in NPC pathogenesis(1. Yu X, Zhen Y, Yang H, Wang H, Zhou Y, Wang E, Marincola FM, Mai C, Chen Y, Wei H, Song Y, Lyu X, Ye Y, Cai L, Wu Q, Zhao M, Hua S, Fu Q, Zhang Y, Yao K, Liu Z, Li X, Fang W. Loss of connective tissue growth factor as an unfavorable prognosis factor activates miR-18b by PI3K/AKT/C-Jun and C-Myc and promotes cell growth in nasopharyngeal carcinoma. Cell Death Dis. 2013 May 16;4:e634.). According to the reviewer’s suggestion, we supplemented these contents into article.

**Question** 8. English language should be comprehensively checked throughout the manuscript; some parts are really hard to follow.

**Response:** Sorry for low quality of English languages for this paper. We have invited a researcher from USA whose native language is English to revise this paper.

**Minor Essential Revisions**

**Question** 9. The term “Let-7C” should change to “let-7c” or “miR-let-7c”.

**Response:** Thanks for pointing out this question. We have revised Let-7C to let-7c”

**Question** 10. Abstract, Methods: Does the term “nasopharyngeal tissues (NPs)” correspond to normal/unaffected nasopharyngeal tissues? This should be also clarified throughout the manuscript.

**Response:** In our collected 15 nasopharyngeal tissues (NPs), most of them were chronic nasopharyngitis and only 2 nasopharyngeal tissues were normal nasopharyngeal tissues. Therefore, we called chronic nasopharyngitis and normal nasopharyngeal tissues as nasopharyngeal tissues. We added the explanation of nasopharyngeal tissues to the Sample collection and cell culture of Materials and Methods.

**Discretionary Revisions**

**Question** 11. Introduction: It is mentioned that: “Synergetic effects of viral infections, genetic alterations, and environmental factors are thought to the key factors driving the aberrant activity of a variety of genes and signal pathways, such as EZH2, HDGF,
EMT-associated genes, cell cycle, and epidermal growth factor receptor signaling pathway et al, which caused the pathogenesis of NPC.” This is information is too general. The authors should try to be more precise and provide the most salient examples.

**Response:** We chose several salient examples to explain the action of viral infections, genetic alterations, and environmental factors.

“Synergetic effects of viral infections, genetic alterations, and environmental factors are thought to be the key factors driving the aberrant activity of a variety of genes and signal pathways, which caused the pathogenesis of NPC. Epstein-Barr virus-encoded LMP1 promotes proliferation and transformation of human nasopharyngeal epithelial cells by inhibiting LKB1-AMPK pathway[1]. A single nucleotide polymorphism -32G/A in the promoter region of LOC344967 gene creates an activator protein (AP-1)-binding site in its transcriptional regulatory region, which significantly enhanced the binding of AP-1 to the promoter region of LOC344967 and activated its expression in vivo. [2]. Tobacco smoking as a risk factor for NPC has been supported by multiple studies[3,4] and cigarette smoke extract promoted EBV replication, induced the expression of the immediate-early transcriptional activators Zta and Rta, and increased transcriptional expression levels of BFRF3 and gp350 in the lytic phase[3].”

**Reviewer's report 2**

Major Essential Revisions:

**Question 1.** 1) The authors should show whether E2F1 binds to the promoter of let-7c.

**Response:** Firstly, we declared that the technology of Chromatin immunoprecipitation assay is a route work for our research group. In recent study, we found that oncogene transcription factors c-Myc and c-Jun can bind predicted promoter of miR-184 and miR-18b by Chromatin immunoprecipitation assay. Two papers” Loss of connective tissue growth factor as an unfavorable prognosis factor activates miR-18b by PI3K/AKT/C-Jun and C-Myc and promotes cell growth in nasopharyngeal carcinoma” and “Tumor suppressor PDCD4 modulates miR-184-mediated direct suppression of C-MYC and BCL2 blocking cell growth and survival in nasopharyngeal carcinoma” have been published or accepted by Cell Death & Disease(1. Yu X, Zhen Y, Yang H, Wang H, Zhou Y, Wang E, Marincola FM, Mai C, Chen Y, Wei H, Song Y, Lyu X, Ye Y, Cai L, Wu Q, Zhao M, Hua S, Fu Q, Zhang Y, Yao K, Liu Z, Li X, Fang W. Loss of connective tissue growth factor as an unfavorable prognosis factor activates miR-18b by
PI3K/AKT/C-Jun and C-Myc and promotes cell growth in nasopharyngeal carcinoma. Cell Death Dis. 2013 May 16;4:e634.2. Yan Zhen, Zhen Liu, Huiling Yang, Xiaoli Yu, Qiangyun Wu, Shengni Hua, Xiaobin Long, Qingping Jiang, Ye Song, Chao Cheng, Hao Wang, Menyang Zhao, Qiaofen Fu, Xiaoming Lyu, Yiyu Chen Yue Fan, Yan Liu, Xin Li, Weiyi Fang. Tumor suppressor PDCD4 modulates miR-184-mediated direct suppression of C-MYC and BCL2 blocking cell growth and survival in nasopharyngeal carcinoma. Cell Death Dis(Accepted))

Furthermore, we also used ZEB2 antibody to pulled down DNA fragments and successfully explored the binding of ZEB2 and miR-200b promoter. The result was show as follows:

In this research, we also want to prove that E2F1 binds to the promoter of let-7c. However, we used E2F1 antibody from Abcam, Cell Signaling Technology, and Merck Millipore Incorporation for ChIP test, However, E2F1 antibody can not pull down DNA fragments, which may attribute to the poor quality of E2F1 antibody for ChIP assay. We finally had to give this test up.

**Question 2)** Fig. 3C does not convey the conclusion that let-7c inhibitor or mimic exert any significant difference on cell cycle progression. A more convincing figure is needed.

**Response:** Thanks for pointing out this problem. The following picture is cell cycle examination figures(single time) for let-7c mimics and inhibitor in NPC cells.
Question 3) The authors state that let-7c affects invasion ability of NPC cells, but no data are presented to this effect.

Response: Sorry for our carelessness. In previous investigation, we observed that let-7c suppressed the migration and invasion of NPC cells by transwell and boyden assay, and this result was presented as following:
In initiation of writing this paper, we added the results of let-7c suppressing cell migration and invasion. However, in finally version of this paper, attributing to the molecular basis of let-7c suppressing cell migration and invasion was not observed, we removed the result of migration and invasion. We are sorry for stating that let-7c affects invasion ability of NPC cells in discussion section.

Minor Essential revisions:

**Question 4**) Fig 1D-labeling should be corrected.
**Response:** Sorry for these errors. We have revised these mistakes.

**Question 5**) Fig. 3A-both groups are labeled NPC. Which is NP?
**Response:** Sorry for this labeling mistakes. We have corrected this mistake.

**Question 6**) At a few places in the manuscript, the authors refer to CDK4 as CKD4.
**Response:** Thanks for pointing out this mistake. We have revised this error.

**Question 7**) Several grammatical and syntax errors need to be corrected. In its present form the manuscript is difficult to comprehend at places.
**Response:** Sorry for these for this paper. We have invited a researcher from USA whose native language is English to revise this paper.