Author’s response to reviews

Title: High Nuclear/Cytoplasmic Ratio of Cdk1 Expression Predicts Poor Prognosis in Colorectal Cancer Patients

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Version: 4 Date: 11 November 2014

Author’s response to reviews: see over
Response to the questions of the editor and reviewers

Editor's Comments:

Q1. The reviewers have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, I invite you to respond to the reviewers' comments and revise your manuscript. As stated by both reviewers additional/detailed information regarding to the used immunohistochemical protocols and evaluation of the stainings should be provided. Furthermore, an additional figure with adequate controls (positive-, negative-, system- and isotype-controls) should be added to the manuscript.

Answer:

Thank you for review our manuscript. We revised our manuscript according to the comments of reviewers. Thank you.

Q2. We recommend that you copyedit the paper to improve the style of written English. If this is not possible, you may need to use a professional language editing service. For authors who wish to have the language in their manuscript edited by a native-English speaker with scientific expertise, BioMed Central recommends Edanz (www.edanzediting.com/bmc1). BioMed Central has negotiated a 10% discount to the fee charged to BioMed Central authors by Edanz. Use of an editing service is neither a requirement nor a guarantee of acceptance for publication. For more information, see our FAQ on language editing services at http://www.biomedcentral.com/authors/authorfaq/editing.

Answer:
This manuscript was edited by a native-English speaker with scientific expertise before submission. The receipt is shown below. If further edit was needed, please informed us. Thank you.
Reviewer #1:

Minor comments:

Q1. Methods (IHC staining) are appropriate; however, a negative control for the specificity of the antibody is missing. Moreover, as with most “new” or “novel” prognostic markers proposed, an independent validation cohort of patients would have been adequate and important (although not compulsory, of course). However, there are some (minor) points missing: how were the cut-points defined? Empirically or by log-rank statistics for maximum discrimination power? This is not entirely clear from the text.

Minor essential revisions -as stated in the written report (e.g., provide negative control for antibody staining) However, the prognostic value of CDK1 N/C staining should be tested on an independent test set of samples, this would add greatly to the impact of the manuscript.

Answer:

We agree with the comment of the reviewer. We added positive and negative control of Cdk1 IHC staining in Supplementary Figure 1. Normal colon tissue was used as the positive control and showed weak Cdk1 immunostain. Also seen was some Cdk1 positive lymphocytes infiltration in the colon tissues. PBS was used instead of primary antibodies as a negative control. The same normal colon tissue core showed no Cdk1 immunoreactivity including colon glands and lymphocytes.

We used the median Cdk1 expression score was 200 for cytoplasm staining and 180 for nucleus staining and we used the median value as cut-off point for further analysis.
The quartile and median values of Cdk1 expression in the cytoplasm and nucleus (cut-off points were 180, 200, and 285 for the cytoplasm; and 150, 180, and 255 for the nucleus) were used for further evaluation.

We also agree with the comment that an independent test set of samples would add greatly to the impact of the manuscript. However, we have difficulty to collect enough new cases with survival data in this revision.

We revised our manuscript as following (line 9, page 24):

Supplementary Figure 1. Positive and negative control of Cdk1 IHC staining. (A) Normal colon tissue was used as the positive control and showed weak Cdk1 immunostain. Also seen was some Cdk1 positive lymphocytes infiltration in the colon tissues. (B) PBS was used instead of primary antibodies as a negative control. The same normal colon tissue core showed no Cdk1 immunoreactivity including colon glands and lymphocytes. (Magnification: 200X)

(line 14, page 11):

The median Cdk1 expression score was 200 for cytoplasm staining and 180 for nucleus staining and we used the median value as cut-off point for further analysis.
We evaluated the possible correlation of Cdk1 expression and clinical outcome by grouping patients into four subgroups according to the quartile and median values of Cdk1 expression in the cytoplasm and nucleus (cut-off points were 180, 200, and 285 for the cytoplasm; and 150, 180, and 255 for the nucleus).

Q2. The authors state in the “Results” section: "Overall, 1 Cdk1 expression in the cytoplasm and nucleus was not related to clinical parameters in our study population.” However, from table 1, it is evident that CDK1 cytoplasm staining is significantly associated with T-stage, which is indeed a relevant clinical parameter. Therefore, the conclusion that there is no relation of “separate” stainings for cytoplasm and nucleus to prognosis is not justified.

Answer:

We agree with the comment of the reviewer and revised the statement (line 1, page 12):

Overall, Cdk1 expression in the cytoplasm and nucleus was not related to clinical parameters except cytoplasm Cdk1 expression in T stage in our study population.

Q3. The last phrase in the discussion (“this (…) requires further study and (…) should be validated”) is very general and could be used for basically any scientific publication, I propose to withdraw the whole sentence. Moreover, the authors could have validated their new prognostic score “independently” themselves quite easily – by using an independent set of patient samples, or by dividing their patient cohort in a training/test set. This would have added to the importance to their findings.
Thank you for your nice comment. We withdraw this sentence. There are some difficulties to do cohort study or find another independent set of patient samples in this revision. Indeed, our research is a IRB approval, retrospective study that we could not change.

We revised our manuscript as following (line 4, page 18):

However, this possibility requires further study and the findings presented here should be validated by other investigative groups.

Q4. p. 11: patient collective it is stated that “50 patients had stage III tumors, and 28 patients had stage IV tumors”. However, the next phrase states “Thirty patients had metastasis at diagnosis” – this is a discrepancy to the 28 stage IV patients mentioned earlier. Why are there 2 patients more with distant metastasis? Moreover, also the stage III patients do present “metastasis” (lymph node, that is) at the time of diagnosis, therefore the term “distant metastasis” would be more appropriate and precise

Answer:

We rechecked all database, and corrected the mistakes of table 1. Also, we replaced “metastasis” with “distant metastasis.”

We revised our manuscript as following (line 9, page 11):

Twenty-seven patients had distant metastasis at diagnosis.

Q5. Table 3+5: univariate /Multivariate analysis
TNM-stage was analyzed in a “grouped” fashion: the authors compared stage I against the rest. In my opinion, it would make more sense clinically to compare stage I+II against III+IV? (locally restricted vs. metastatic lesions, moreover stage I is relatively rare)

Answer:

We compared stage I+II against III+IV, but it did not have significant difference (HR=1.311, 95% CI=0.938-1.832, p=0.113). This could be explained by related more cases in stage II and III, which had close prognosis. However, Cdk1 N/C ratio still showed significant difference in the multivariate model (HR=1.733, 95% CI=1.040-2.890, p=0.035). Thus, using stage I+II against III+IV or stage I against II+III+IV would not change our result.

Q6. Fig. 2: please indicate in the figure/legend the number of cases in the respective groups of the KM-analysis

Answer:

We added the number of cases according to N/C ratio subgroups in the legend as following (line 5, page 24):

Figure 2. Kaplan-Meier actuarial analysis of overall survival according to (A) N/C ratio subgroups and (B) N/C ratio cut by 1.5 of Cdk1 immunostaining expression in colorectal cancer patients. The case number was 18, 101, 26, and 19 according to N/C ratio subgroups, respectively.

Q7. Fig. 2: negative control missing, specificity of the Ab? Please indicate size bar
We added positive and negative control of Cdk1 IHC staining in Supplementary Figure 1. We revised legend as following (line 2, page 24):

Figure 1. Representative immunostaining of Cdk1 in colorectal cancer in tissue arrays according to the N/C ratio. N/C ratio of Cdk1 were (A) 0.00-0.49; (B) 0.50-0.99; (C) 1.00-1.49; (D) ≥1.5. *(Magnification: 200X)*

We added the scale bar and magnification in figure and legend as following (Figure 1):
We revised our manuscript as following (line 9, page 24):

Supplementary Figure 1. Positive and negative control of Cdk1 IHC staining. (A) Normal colon tissue was used as the positive control and showed weak Cdk1 immunostain. Also seen was some Cdk1 positive lymphocytes infiltration in the colon tissues. (B) PBS was used instead of primary antibodies as a negative control. The same normal colon tissue core showed no Cdk1 immunoreactivity including colon glands and lymphocytes. (Magnification: 200X)
Q8. Discussion: the authors clearly show that the amount of nuclear vs. cytosolic pools of CDK1 is important for tumor development and prognosis, rather than just the overall expression. In accordance, earlier studies showed that the enzymatic activity of CDK1 is important for tumorigenesis, rather than just overall expression levels. Since the authors propose a putative and elegant mechanism (14-3-3sigma protein) to explain for the intracellular localisation differences of CDK1, why did the not analyze the 14-3-3sigma expression on parallel sections, to find out whether tumor cases with loss-of-expression of 14-3-3s have indeed higher nuclear levels of CDK1? This could also make the clinical score more robust.

Answer:

Thank you for your nice comment. It will take much time to get new IRB approval for 14-3-3sigma protein staining. We plan to propose new project for further evaluation of possible mechanism. However, in this vision, we cannot do the experiment without IRB approval.
Reviewer #2:

Major comments:

Q1. Since this study was based on immunoreactive staining score, how to control the time of HRP-DAB IHC staining to decrease the influence of technical artifact? The authors should show the detail of the IHC protocol, such as the microscope, the magnification of lens, et al.

Answer:

We agree with the concern of the reviewer. The IHC staining was performed at the Department of Surgical Pathology, Changhua Christian Hospital which is qualified by College of American Pathologists and Joint Commission International. All the experiment was performed by qualified technician. In order to decrease the influence of technical artifact between samples, we used tissue microarray rather than whole mount section. Otherwise, we added the scale bar and magnification in figures and legends to clarify the magnification of tissue samples. We revised IHC protocol of the Materials and Methods as following (line 16, page 8):

*Immunohistochemistry Staining and Evaluation of Cdk1 Immunoreactivity*

Immunohistochemistry (IHC) staining was performed at the Department of Surgical Pathology, Changhua Christian Hospital, as previously described [16, 17]. IHC analyses were performed on tissue microarray sections (4 µm) of formalin-fixed, paraffin-embedded, pre-chemotherapy primary colorectal tumors. The sections were placed on coated slides, washed with xylene to remove the paraffin, and rehydrated through serial dilutions of alcohol, followed by washings with a solution of phosphate
buffered saline, PBS (pH = 7.2). Endogenous peroxidase activity was blocked with 3% H2O2. Antigen retrieval was performed by boiling in citrate buffer (10 mM) for 20 min. After incubation with the anti-human Cdk1 antibody (Cdc2 p34 antibody, 1:180 dilution; sc-166135, Santa Cruz Biotechnology) for 20 min at room temperature and thorough washing (three times with PBS). The immunoreaction was visualized using polymer-based MACH4 DAB Detection Kit (Biocare Medical) according to the manufacturer’s instructions to obtain optimal immunoreactivity and least background artifact. The slides were incubated with a horseradish peroxidase (HRP)/Fab polymer conjugate for another 30 min. The sites of peroxidase activity were visualized using 3,3’-diamino-benzidine tetrahydrochloride as the substrate for 300 seconds and hematoxylin as the counterstain. Pathologically verified normal colon specimens were used as positive control (Supplementary Figure 1). PBS was used instead of primary antibodies as a negative control (Supplementary Figure 1). Immunoreactivity scores were analyzed by pathologists using scores defined as previously described [17, 18]. In brief, immunoreactivity scores were defined as the cell staining intensity (0 = nil; 1 = weak; 2 = moderate; and 3 = strong) multiplied by the percentage of stained cells (0–100%), leading to scores from 0 to 300. Cdk1 immunoexpression was assessed semiquantitatively by 2 pathologists (YML and CJC), who independently scored coded sections based on the staining score without knowledge of clinical and follow-up information. A final agreement was obtained for each score by using a multiheaded microscope (Olympus BX51 10 headed microscopes).

We revised legend as following (line 2, page 24):

Figure 1. Representative immunostaining of Cdk1 in colorectal cancer in tissue arrays according to the N/C ratio. N/C ratio of Cdk1 were (A) 0.00-0.49; (B) 0.50-0.99; (C)
Q2. How to control the variance with each pathologist when analyzing immunoreactivity scores? Why the authors did not use the software such as Image-Pro Analyzer software or Image J to scan the staining area to directly calculate the ratio of CDK1 in nuclei and cytoplasm?

Answer:

We agree with the comment of the reviewer. However, we do not have this expansive equipment. In addition, Cdk1 could exist in some non-tumor cells such as lymphocytes and this phenomenon could somehow obscure the Cdk1 expression in colon cancer especially when intratumoral lymphocyte presents by automatic image analyzer. In this study, Cdk1 immunoexpression was assessed semiquantitatively by 2 pathologists (Dr. YML and Dr. CJC), who independently scored coded sections based on the staining score without knowledge of clinical and follow-up information. A final agreement was obtained for each score by using a multiheaded microscope.

We added this statement to the Materials and Methods as following (line 3, page 10):

Cdk1 immunoexpression was assessed semiquantitatively by 2 pathologists (YML and CJC), who independently scored coded sections based on the staining score without knowledge of clinical and follow-up information. A final agreement was obtained for each score by using a multiheaded microscope (Olympus BX51 10 headed microscopes).

Minor comments:

Q1. In the figure 2, the author should show the nuclei and cytoplasm expression of
CDK1, including the bar, exposure time et al.

Answer:

We added the scale bar in figure and mentioned the exposure time in the Materials and Methods (line 14, page 9):

The sites of peroxidase activity were visualized using 3,3’-diamino-benzidine tetrahydrochloride as the substrate for 300 seconds and hematoxylin as the counterstain.

We added the scale bar in figure as following (Figure 1):

(A)  
(B)  
(C)  
(D)