Author's response to reviews

Title: WDR66 is a novel Marker for Risk Stratification and involved in Epithelial-Mesenchymal Transition of Esophageal Squamous Cell Carcinoma

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Version: 3 Date: 2 January 2013

Author's response to reviews: see over
Dear editorial board of BMC Cancer,

Thank you for your email from Dec 6, 2012 with regard to our manuscript for publication in BMC Cancer. Please find enclosed a carefully revised manuscript entitled “WDR66 is a novel Marker for Risk Stratification and involved in Epithelial-Mesenchymal Transition of Esophageal Squamous Cell Carcinoma” by Qing Wang, Chenming Ma and Wolfgang Kemmner from the Research Group Surgical Oncology, Experimental and Clinical Research Center, a joint cooperation between the Charité Medical Faculty and the Max-Delbrück Center for Molecular Medicine, Berlin, Germany.

We want to thank the reviewers for their suggestions regarding our manuscript. Below, please find a point-by-point response to the comments of the reviewers. We still believe that our findings are of interest for the readers of BMC Cancer, because to our best knowledge, this is the first report investigating the role of WDR66 in esophageal cancer development and progression. This article can improve our understanding of the biology of ESCC, and thereby identify goals for further investigation of selective targeting of WDR66 as a novel strategy for ESCC treatment.

I certify that this manuscript, or any part of it, has not been published and will not be submitted elsewhere for publication while being considered by BMC Cancer.

Thank you for your time and consideration. I look forward to hearing from you.

Sincerely,

Chenming Ma, MD
Point-by-point response to the comments of the reviewers

**First reviewer's report**
**Title:** WDR66 is a novel Marker for Risk Stratification and involved in Epithelial-Mesenchymal Transition of Esophageal Squamous Cell Carcinoma  
**Version:** 2  
**Date:** 13 November 2012  
**Reviewer:** Takehiko Yokobori  
**Reviewer's report:**  
Minor Essential Revisions  
**Level of interest:** An article of outstanding merit and interest in its field  
**Quality of written English:** Needs some language corrections before being published  
**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Comments to the editor**
The authors described the clinical significance of WDR66 in esophageal squamous cell carcinoma (ESCC). WDR66 was identified by microarray analysis of ESCC and normal squamous cell, and the expression was associated with poor prognosis in clinical ESCC patients and proliferation and invasion in ESCC cell lines. Moreover, WDR66 was related with EMT related genes. This report is very intriguing and will meet the criteria of this journal, and should be published soon. We raised several points to improve the contents.

**Comments to the author**
The authors described the clinical significance of WDR66 in esophageal squamous cell carcinoma (ESCC). WDR66 was identified by microarray analysis of ESCC and normal squamous cell, and the expression was associated with poor prognosis in clinical ESCC patients and proliferation and invasion in ESCC cell lines. Moreover, WDR66 was related with EMT related genes. This report is intriguing and will meet the criteria of this journal. There are still several points that should to be addressed prior to publication of the manuscript in this journal.

**Queries**
**Abstract section:**
1. Please describe the sample number for microarray and clinical samples.

2. Off course, EMT is known to be associated with cancer metastasis, invasion, and cancer stem cell. However, authors may overstate the correlation of WDR66 and ESCC carcionogenesis in conclusion. Please change “esophageal squamous cell carcinogenesis as a positive ....” to “esophageal squamous cell carcinoma growth and invasion as a positive ......”. Moreover, please delete “Thus, our functional studies point toward an important role of WDR66 for squamous carcinoma cell growth and invasion.” to eliminate redundancy.

3. Please describe the relationship of clinicopathological status and WDR66 expression.
   
   **Author’s response: The abstract section was carefully modified according to the suggestions of**
Introduction section:
1. The purpose of this study is not described clearly. What did authors want to clarify using the microarray analysis? Please show your purpose and motive of this study.

2. P6 line12-14
The relation of WDR66 and EMT should be described in result section. Moreover, please describe the WDR66 related report (only 2 reports in Pubmed) and WD repeated proteins’ function in several cancers in detail for readers.

3. Please spell out EMT.
Author's response: The introduction section was carefully modified according to the suggestions of the reviewer.

Material and method section:
1. Please summarize the clinical sample characteristic (including TNM classification) in Table. Especially, sample information for microarray and prognostic evaluation was confusing.
Author’s response: The clinical sample characteristics are summarized in the supplement.

2. Is the following sentence correct? NE? “Desired tumor cell or BE areas were selected…..”
Author’s response: The material and method section was carefully modified according to the suggestions of the reviewer.

Result section:
1. P13 line 5:
Please revise all Kyse520 to KYSE520. KYSE520 is not a squamous cell line.
Please change to squamous cell carcinoma cell line KYSE520.
Author’s response: We agree and revise all Kyse520 to KYSE520, and change to squamous cell carcinoma cell line KYSE520.

2. Was KYSE520 obtained from ATCC? KYSE series were established by Dr. Shimada and now supplied by JCRB in Japan. In this study, KYSE520 was used in in vitro analysis. Therefore I think that the background of the cell line is very important.
Author’s response: We confirm that KYSE520 was obtained from American Type Culture Collection (ATCC, Manassas, VA)

3. P13 line 9:
The significance of WDR66 as CTA should be described in discussion section, not in result section.
Author’s response: We agree and show only the result here, and discuss the result in the appropriate discussion section.
4. Please describe the relationship of clinicopathological status and WDR66 expression level in an independent set of ESCC examples as supplementary data

Author’s response: The clinical sample characteristics are summarized in the supplement.

5. P14:
Authors should describe the methods of microarray analysis in WDR66 suppressed cells in Materials and Methods section.

Author’s response: We describe the methods of microarray analysis in WDR66 suppressed cells in material and method section according to the suggestions of the reviewer.

6. If authors describe the correlation of EMT and WDR66, the expression data of E-cad and established other EMT markers by microarray was important. Authors should show even negative data as supplementary data.

Author’s response: We show all results in the result section and in discussion section with regard to EMT and WDR66.

7. P15 line9
Suggestion about WDR66 function was described in dissection section.

Discussion section:
1. Please describe the background information of WD-repeat proteins’ function in introduction section.

Author’s response: The discussion section was modified according to the suggestions of the reviewer.

Figures;
1. The sample number of high or low expression group was described in Figure2 legends. Moreover, optimal cut off point was described in the legends.

Author’s response: Figure 2 legend section was modified according to the suggestions of the reviewer.

2. NTC and Allstar was very confusing. Please change NTC to KYSE520 or parent cell, and Allstar to negative control or control siRNA.

Author’s response: The figure legend section was modified according to the suggestions of the reviewer.

3. WDR66 suppression data (transfection efficiency) by RT-PCR was shown in Figure3.

4. WB data of beta-actin in Figure3 was poor. Please revise it.

Author’s response: Figure3 was modified according to the suggestions of the reviewer.

5. Wound healing assay data in 24h was not informative to understand the WDR66 function. Please delete the data or add other figure at the point of 12h or 16h before scratch closure.
Author’s response: Figure 4 was modified according the suggestions of the reviewer.

6. Please describe the evaluation time (24h?) in Figure 4A legend
Author’s response: The evaluation time for cell migration is 16h. Figure 4A legend was modified according to the suggestions of the reviewer.

Second reviewer’s report
Title: WDR66 is a novel Marker for Risk Stratification and involved in Epithelial-Mesenchymal Transition of Esophageal Squamous Cell Carcinoma
Version: 2 Date: 30 November 2012
Reviewer: Rupert Langer
Reviewer’s report:
The article of Wang et al presents the identification of WDR66 as a possible specific and prognostic marker for esophageal squamous cell carcinoma. The study delivers interesting results and is well performed, the study outline well-structured and logic.
Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: Yes, and I have assessed the statistics in my report.

Major Compulsory Revisions:

My major concern is that the tissue which was used for analysis is not characterized very well. The authors state that they obtained all tissue (fresh frozen) from the tumorbank of the CCC Charite. However, several times the authors mention the usage of FFPE tissue.

1) Are these FFPE probes from the tumorbank as well?
Author’s response: All FFPE specimens are from the tumorbank of the CCC Charite.

2) In contrast, the normal esophageal tissue was also fresh frozen? Can the authors assure that the investigated tissue (microdissected and non-microdissected) is really cancer (any histo-pathological expertise?).
Author’s response: The normal esophageal tissue was also fresh frozen. Each single specimen included in this study was histopathologically approved independently by a pathologist with long term histo-pathological expertise (Prof. Michael Vieth (University Bayreuth) in the Acknowledgement.

3) Were the clinical follow up data associated with the probes also provided by the tumorbank?
Author’s response: Yes, all clinical and histopathological data were provided from the tumorbank.
4) Is there any policy in the CCC for using these data (e.g. co-authorships, acknowledgements etc.)

Author’s response: Senior author Wolfgang Kemmner belongs to the Tumorbank.

5) The authors could provide a table with the clinicopathologic characterization of the ESCC collective which they used for survival analysis. Are they really all G1?

Author’s response: The clinical sample characteristics for survival analysis are summarized in the supplement.

6) The second major issue is the statistics regarding the prognostic role of WDR66 expression. The authors should provide data about any association to established prognostic factors like T category, N category, grading.

Author’s response: The clinical sample characteristics for survival analysis are summarized in the supplement.

7) Moreover, had the factors included in the cox analysis prognostic impact on univariate analysis? It is somehow surprising that lymph node status had such a bad performance.

Author’s response: The stratified Cox-regression model was used to determine prognostic factors in multivariate analysis, not in univariate analysis.

8) Moreover, the authors include TNM stage, pT category and pN category in the same multivariate analysis. This approach is not quite correct, as TNM stage is composed of these two factors. How are the results, if the analysis is performed with TNM stage alone or alternatively pT and pN category.

Author’s response: TNM Classification of Malignant Tumours (TNM) is a cancer staging system composing three factors, the size and extension of the primary tumor, its lymphatic involvement, and the presence of metastases. We use this TNM staging system to give an indication of prognosis and to assist in the evaluation of the results of treatment, independent of pT and pN category.

Further comments:

9) Abstract: The abstract should be modified:
- Background: the aim is very vague (this is what biomarker research is in general..). The authors should be more specific.
- Methods: please specify: whole human gene expression profiling was performed comparing normal with neoplastic tissue/ESCC. The second sentence could be shortened (e.g....whole human gene expression was performed after laser microdissection comparing...; “promising candidate”: what for;
- Conclusion: please explain shortly why WDR66 might be a novel drug target.

Author’s response: The abstract section was modified according to the suggestions of the reviewer.

Introduction:

10) Although the article is well written in general, some lines and sentences would request a
more careful editing. The paper would also become easier to read, if the authors would describe their stepwise approach in the introduction.

11) 3rd paragraph: did the first microarray analysis really identify WDR66 as a biomarker for risk stratification or deliver information about its role for EMT? As far as I understood this step was used to identify the gene as being expressed in ESCC in contrast to normal tissue and other cancer types. Please clarify.

Author’s response: The introduction section was modified according to the suggestions of the reviewer.

12) Materials and Methods (see above)
- What are “International Federation of Gastrointestinal criteria”?
- Next paragraph: “desired tumor cell or “BE” areas, better “NE”?”

Author’s response: The materials and Methods section was modified according to the suggestions of the reviewer.

Results:
13) Could the authors provide information about the other differentially expressed genes?

Author’s response: We provided the other differentially expressed genes in the supplement.

14) Validation cohort: the authors have analyzed a series of different gastrointestinal cancers. Were they from all tumor stages/grading?

Author’s response: All the surgical specimens of patients with known gastrointestinal cancers are of histological grading G1, UICC stage II and III.

15) The authors describe the RNA In situ hybridization being applied on FFPE tissue. Did they fix the frozen tissue for that? This has not been described in the material and methods section.

Author’s response: No, we did not fix the frozen tissue. The FFPE specimens were obtained from tumor bank, were 5 um dewaxed FFPE section They were used after treatment with proteinase K (2µg/ml in PBS) at 37°C for 15 min, and hybridized with the Digoxigenin-labeled WDR66 probe at 65~C overnight in a humid chamber. After 3 washes to remove the nonspecific binding or unbound probes, digoxigenin-labeled probe was detected using alkaline phosphatase method.

Minor essential Revisions
16) Figure 1 has poor quality.

Discretionary Revisions/additional comment:
17) A last comment regarding authorship: reading the author’s contributions it is not clear why being involved in drafting/revising the manuscript (CM) should qualify as first authorship. Maybe the authors forgot to mention some more impact?

Author’s response: The authors’ contributions section was modified according to the suggestions of the reviewer.