Author's response to reviews

Title: Enhancer of zeste homolog 2 (EZH2) expression is an independent prognostic factor in renal cell carcinoma

Authors:

Nina Wagener (n.wagener@dkfz.de)
Stephan Macher-Goeppinger (Stephan.Goeppinger@med.uni-heidelberg.de)
Maria Pritsch (pritsch@imbi.uni-heidelberg.de)
Johannes Hüsing (Johannes.Huesing@med.uni-heidelberg.de)
Karin Hoppe-Seyler (k.hoppe-seyler@dkfz.de)
Peter Schirmacher (Peter.Schirmacher@med.uni-heidelberg.de)
Jesco Pfitzenmaier (Jesco.Pfitzenmaier@med.uni-heidelberg.de)
Axel Haferkamp (Axel.Haferkamp@kgu.de)
Felix Hoppe-Seyler (hoppe-seyler@dkfz.de)
Markus Hohenfellner (Markus.Hohenfellner@med.uni-heidelberg.de)

Version: 2 Date: 25 August 2010

Author's response to reviews:

MS 2844523813743872

Dear Dr. Motoyoshi Tanaka,

thank you for your positive response. The reviewer’s comments were most helpful for revision of the paper and individual suggestions were considered in the revised manuscript as detailed below. All changes introduced into the revised version of the manuscript are indicated in red color (file: manuscript_rev).

Sincerely yours

F. Hoppe-Seyler

Rebuttal

Reviewer Tomoaki Fujioka:

1. The MSKCC (Memorial Sloan-Kettering Cancer Center) risk categorization is widely accepted as prognostic factor for metastatic RCC. The association between MSKCC risk categorization and EZH2 expression need to be demonstrated.

Response by the authors:

As requested, the association of nuclear EZH2 expression with the MSKCC (Memorial Sloan-Kettering Cancer Center) risk categorization or Motzer criteria
(Motzer et al., J Clin Oncol 2004, 22(3):454-463) has been evaluated by Fisher’s exact test. The following risk parameters were studied: hemoglobin, corrected calcium, and Karnofsky performance score. Altogether, 51 metastasized RCC patients were categorized to one of the following risk groups: low, intermediate and high risk. For 36 metastasized RCC patients, the variable corrected calcium could not be evaluated due to missing albumin measurements preoperatively. The data revealed that nuclear EZH2 expression and the Motzer criteria are independent parameters. Respective additions have been made in Table 2 and in the text (Material and Methods, p. 6, Results, p. 12).

2. Whereas there is a sentence that non-tumorous kidney tissue was mainly negative for EZH2 expression (page 10, lane 8), no concrete scores were described. Add the data into Table 2.

Response by the authors:

Five non-tumorous kidney tissue samples showed 1-5% nuclear EZH2 expression, only proximal and distal tubular epithelial cells stained positive for EZH2. All other 515 non-tumorous kidney tissue samples showed no nuclear EZH2 expression. Furthermore, in RCC and non-tumorous kidney tissue, infiltrating lymphocytes, if present in the sample, stained positive for EZH2. Due to the very limited staining of EZH2 in non-tumorous kidney tissue, the data were not included in Table 2.

Reviewer Kiyohide Fujimoto:

1. The authors describe that only 10 patients had high (>50%) nuclear EZH2 staining. The number of patients in this group seems to be small to adequately perform multivariate prognostic analysis. In fact, there were no statistically significance factors in analyzing this group. What was the reason for setting cutoff percent of EZH2 expression at 50% and 25%?

Response by the authors:

For immunohistochemical assessment of EZH2 expression, the frequency of nuclear staining was evaluated using a semiquantitative score: 0 = no expression; 1 = positivity in 1 to 5% = low expression; 2 = positivity in >5 to 25% = intermediate expression; 3 = positivity in >25 to 50% = high expression; and 4 = positivity in more than 50% = very high expression. To determine this semiquantitative score, 200 tissue specimens were independently scored by two researcher initially to analyse the distribution of nuclear EZH2 staining.

As pointed out in the methods section of the paper we did not pool any of these categories for the statistical evaluation because we did not want to disturb the confirmatory nature of the analysis by a data-dependent decision. We explored pooling adjacent categories and examined the results obtained by nested cross-validation. These results suggested different threshold for different cancer stages (data not shown). Yet the overall gain for precision was not enough to warrant this more complex approach.
2. Is there a significant difference between expression levels of EZH2 in the periphery and in the center of tumor? Previous reports described that EZH2 was related to invasion and progression of cancer cells. These results supported that EZH2 would be expressed predominately in cancer cells located in expanding peripheral zone. The authors need to describe the text about this issue.

Response by the authors:
As our study has been conducted according to a large cohort of patients to perform multivariate analyses, we used a tissue micro array (TMA) for immunohistochemical evaluation of EZH2 protein expression. For this, morphologically representative regions were chosen from each of the RCC and normal kidney tissue samples. Two cylindrical core tissue specimens per tumor block (diameter, 0.6 mm) were punched from these regions and arrayed into a recipient paraffin block. The specimens showed continuous staining of EZH2, without any predominately staining in any zone. To establish immunohistochemical staining, we initially analysed some whole tissue slides for EZH2 expression. In the same manner, there were no differences in staining in the central and peripheral zone of tumors. The respective additions have been made in the text (Results, p. 11).

3. Karnofsky PS, delay between diagnosis and treatment, serum LDH, corrected calcium and haemoglobin are widely known to be powerful prognostic factors in metastatic RCC. These variables should be included into the analyses for evaluating the true prognostic value of EZH2 expression in metastatic RCC (Table 4). The authors have to describe about this issue.

Response by the authors:
As requested, and in addition to the data newly added in Table 2, we have performed additional univariate and multivariate analyses including Karnofsky PS, corrected calcium, and hemoglobin (known as the Memorial Sloan-Kettering Cancer Center (MSKCC) risk categorization or Motzer criteria (Motzer et al., J Clin Oncol 2004, 22(3):454-463)). In an exploratory manner, we included EZH2 and the Motzer criteria as the only two factors in a multivariate cox proportional hazards model, which reviewed EZH2 as a borderline significant factor, despite the small number of patients in the analyses. The respective additions have been made in the text (Material and Methods, p. 6, Results, p. 13). The findings further corroborate the conclusion that EZH2 expression is an independent unfavourable prognostic marker for CSS in patients with metastatic RCC - as has been concluded in the first version of the manuscript. Assessment of the EZH2 status could therefore be integrated in established prognostic models in order to improve clinical management of RCC patients.

4. There is no explanation for Figure 1A in the text. This should be mentioned in the text.

Response by the authors:
As requested by the reviewer, we added an explanation of Figure 1A in the text.
5. In the section of Conclusion, the authors described that inhibition of EZH2 expression might be a novel therapeutic target. However, EZH2 was expressed in infiltrating lymphocytes, proximal and distal tubule epithelial cells, proliferating parabasal cells in the normal cervical epithelium, proliferating cells of normal mammary gland and mammary stem cells. Inhibition of EZH2 seems to potentially lead important human organs to various and undesirable toxicity if the authors have and use small molecules or neutralization antibody for targeting EZH2.

Response by the authors:
In our study, RCCs exhibited significantly higher EZH2 expression levels than histologically normal kidney. Increased EZH2 expression in tumorous versus corresponding normal tissue has been also reported for other cancers as well, including malignant melanoma, prostate carcinoma, breast cancer and hepatocellular carcinoma (Kleer et al., Proc Natl Acad Sci U S A 2003, 100(20):11606-11611, Varambally et al., Nature 2002, 419(6907):624-629, Bachmann et al., J Clin Oncol 2006, 24(2):268-273, Sudo et al., Br J Cancer 2005, 92(9):1754-1758). However, it should be noted that infiltrating lymphocytes and, sporadically, proximal and distal tubule epithelial cells stained positive for EZH2 in normal renal tissue. This indicates that detectable EZH2 expression is not stringently restricted to tumor cells. In line, EZH2 expression could be detected in the proliferating parabasal cell layer in normal cervical epithelium (Holland et al., Cancer Res 2008, 68(23):9964-9972) and in proliferating cells of normal mammary gland tissue and mammary stem cells (Pietersen et al., Breast Cancer Res 2008, 10(6):R109).

The low levels of EZH2 expression in specific cells of normal kidney suggest that its targeted inhibition of EZH2 will not be a strictly tumour-specific therapeutic strategy. This should be taken into account for the development of EZH2 inhibitors, to avoid unwanted side effects, such as nephrotoxicity. Yet, we also would like to stress the point that our findings do not disqualify EZH2 as a therapeutic target. As a matter of fact, intense effects are currently undertaken to target EZH2 in epigenetic therapies (Tuma, J Natl Cancer Inst 2009, 101(19):1300-1301). As only about 1% of patients express low nuclear EZH2 expression in certain cells, this may allow at least a preferential attack on tumour cells, depending on the therapeutic index of EZH2 inhibitors.

Minor Criticisms:
1. Page 8 Line 16, the abbreviated word “CCS” should be “CSS”.
Response by the authors:
“CCS” has been changed to “CSS”.

2. Table 2: A total number of patients in the sex row, the columns of >25-50% and >50% are incorrect.
Response by the authors:
The total number of patients in the row “sex” has been changed to 64 and 36 in the >25-50% column and to 60 and 40 in the >50% column.

3. Table 2: How about EZH2 expression of papillary and chromophobe RCC? These data are important to discuss tumor aggressiveness in the consecutive histological types.

Response by the authors:
The histopathological subtypes were divided into subgroups of clear cell RCC vs. non clear cell RCC for univariate and multivariate analyses. As requested, the association of nuclear EZH2 expression with all histopathological subtypes was evaluated by Fisher’s exact test using Monte Carlo Simulation. This revealed that EZH2 and the histopathological subtypes are not independent parameters. The data have been added in Table 2, respective additions have been made in the text (Material and Methods, p. 9 and 10 and Results, p. 12).

4. Figure 1: The magnification should be mentioned.

Response by the authors:
As indicated in the text, sections were analysed by light microscopy (Olympus Vanox-T, Hamburg, Germany), using a magnification of up to x 400. Scale bars are included in Figure 1, indicating a 5 µm distance in overview (left panel) and higher resolution (right panel) of RCC samples showing EZH2 protein expression.