Author's response to reviews

Title: KRAS, BRAF Genotyping Reveals Genetic Heterogeneity of Ovarian Borderline Tumors and Associated Implants

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Author's response to reviews:

Dear Editors-in-Chief, Dear Editorial Board Members,

Thank you for your e-mail of 20th August 2013 concerning the reviews of our manuscript entitled:
“KRAS, BRAF Genotyping and p53, p16 Expression Reveals Genetic Heterogeneity of Ovarian Borderline Tumors and Associated Implants”.

We are very grateful for the careful consideration given to our manuscript and the very helpful comments we received.

Please find below our point-by-point response to the reviewers’ comments within which we have addressed the comments of each reviewer. We hope that you will now consider our submission suitable for publication in BMC Cancer.

None of the authors has a conflict of interest to declare. In case of any questions please do not hesitate to contact me anytime.

Yours sincerely,
Doris Mayr, MD

Point-by-Point Response to the Reviewers’ Comments

We gratefully thank the reviewers for their interesting comments. Please find below our response to the reviewers’ comments.

Reviewer 1 (H. Brustmann):
The authors attempted to clarify the clonal origin of implants of borderline tumors (BOTs) by assessing BRAF/KRAS hot spot mutations and p53 as well as p16
INK4a immunostaining. They conclude on heterogeneity of their BOTs and implants. This study is interesting and contributes to this controversial topic. Some aspects need to be discussed.

Apparently the authors consider serous tumors. This does not come clear from the manuscript, and may be added (serous borderline tumor, s-BOT). Similarly, BOTs and IOC (invasive ovarian carcinomas) are discussed as the background of this study. However, it is not sufficient to just describe IOC with its invasive growth and strong cellular atypia (lines 39/40). As for serous carcinomas it may be interesting to apply the binary grading of Malpica, which distinguishes low and high grade tumors. This morphological grading system mirrors molecular events in low and high grade serous tumors by nuclear morphology and mitotic activity.

Of note, low grade serous carcinomas in this system are invasive, too, and may be life-threatening. Thus, the clinical course of low grade carcinomas in the Malpica system may take a longer time than in high grade carcinomas, but they develop high stages, too.

Authors' response: The term “BOT” has been replaced by “s-BOT” throughout the manuscript as instructed. A passage on ovarian cancer classification has been added to the first paragraph of the introduction.

In line with these comments the discussion on p53 or TP53 could be improved (lines 66 and 67, others). Low grade serous carcinomas even of high stage are similar to s-BOTs in this aspect. The loss of TP53 characterizes high grade serous carcinomas following the Malpica system, even in low stage cases. Of note, p53 immunohistochemistry reflects p53 mutations inaccurately. Mutations of the p53 gene can occur upstream of the protein segment targeted by immunohistochemistry. Such truncated mutated p53 protein will go undetected by this method. Consequently, this study focuses primarily on the evaluation of p53 protein overexpression. This should be included to the considerations of this matter. However, the scoring system (IRS) applied herein appropriately reflects this situation in s-BOTs.

Authors' response: The explanation on the role of p53 has been revised according to the reviewer’s comment. Further we see that immunohistochemistry may not fully replace p53 mutation analysis. However, assessing p53 by immunohistochemistry is a currently well-established method in various cancers as stated in the text. A comment dealing with the validity of p53 immunohistochemistry has been added to the text as instructed.

Lines 81 and 84: why is a patient included to the cohort without any primary ovarian lesion? How was this “implant” compared to an ovarian lesion?

Authors' response: One Patient was identified with seven implants though no ovarian lesion was found. Due to the missing ovarian s-BOT it was not possible to perform s-BOT - implant matching or s-BOT analysis. Data of this patient were included in the section reporting on implant genotypes / immunophenotypes (see “KRAS-/BRAF Genotypes in s-BOTs and Implants” of the results section). We reported on this case since the immunophenotype regarding p53 and p16 was different among six of seven implant samples. However, we agree with the
reviewer that including this case may not be necessary for the key message of the study and thus we have now excluded this patient from the cohort.

Indeed, there are different results concerning the clonality of implants and associated s-BOTs. I am reminded to the term “homoiology” introduced by comparative morphologists and evolution biologists to describe analogous developments based on homologous structures. This may be true on the genetic level, too. The matter of stem cells may play a future role in this research area. Such stem cells with serous potential may develop different mutations in different sites. Thus, I agree to the statement in the discussion at hand (lines 232 and 233). However, comparable to this study most of the previous studies examined small numbers of cases only on molecular basis. The problem of small cohorts may also be indicated in the conclusion.

Authors’ response: We thank the reviewer for this interesting explanation. A comment on the problem of small cohorts has been added to the text as instructed.

Reviewer 2 (Rachel Grisham):

This is an interesting article describing mutation profiling of primary serous borderline ovarian tumors and their associated noninvasive implants.

Minor Essential Revisions

1. The authors report here an unusually high number of concurrent KRAS and BRAF mutations identified within a single tumor tissue or implant (up to 26.7% of patients). As the authors acknowledge, these mutations are generally found to be mutually exclusive, and multiple prior publications examining serous borderline disease have confirmed these mutations to be mutually exclusive. I recommend that the authors confirm their results using Sanger sequencing.

Authors’ response: Unexpectedly, herein coexisting BRAF and KRAS mutations were observed. This finding is unlikely to be due to sequencing inconsistencies, as the methods employed to determine BRAF and KRAS mutation status had been intensively validated. KRAS mutation analysis was taken out at a German reference laboratory for KRAS mutation testing at our Department of Pathology and has already been published multiple times (Moosmann et al., 2011; Kriegl et al., 2011; Bellon et al., 2011; Modest et al., 2011; Modest et al., 2012b; Stintzing et al., 2012; Stintzing et al., 2013; Modest et al., 2012a; Heinemann et al., 2013; Boeck et al., 2013b; Kriegl et al., 2012; Boeck et al., 2013a; Modest et al., 2013; Neumann et al., 2013). This procedure was used to detect mutations in the KRAS proto-oncogene with a specificity of 0.98 and sensitivity of 0.99 (Modest et al., 2011, Neumann et al., 2009).

Though coexistence of mutations occurring in BRAF or KRAS has been assumed to be mutually elusive, such phenomena were recently observed in colorectal adenoma and ovarian malignancies. We further assume that coexisting KRAS, BRAF mutations in the same s-BOT may be indicative for a secondary genetic event or may reflect a possible polyclonal origin of s-BOTs and implants.

Implant formation is a relatively seldom event in s-BOT genesis. However since
just s-BOT patients diagnosed with concomitant implants were included in the current study, it is hard to compare our data to studies mostly reporting on BOTs in general (regardless of the diagnosis of implants).

Regarding the reporting on KRAS / BRAF mutated tumors in the manuscript text we have to clarify the following: As stated in the text four patients (26.7 %) presented with both KRAS and BRAF mutated s-BOTs in the same patient. However only three of these patients presented with combined KRAS / BRAF mutation in the same s-BOT, while the fourth patient showed KRAS mutation in the left ovarian s-BOT and BRAF mutation in the right ovarian s-BOT. This has been specified in the text as well.

KRAS, BRAF genotyping was done from the same DNA sample. Unfortunately, DNA material has been used up and thus cannot be analyzed by Sanger sequencing any more.

2. The same sentence is repeated in lines 82 and 84 of the manuscript.

Authors’ response: This sentence has been removed as instructed.

3. Table 1, it should be made clear how many of the implants displayed each mutation profile (i.e. in patient 16 there were 19 implants examined and 3 mutation profiles reported, how many implants displayed each profile?)

Authors’ response: The required data have been added to table 1 as instructed.

Reviewer 3 (Sigurd Lax):

The authors present an interesting and important study on the issue of peritoneal implants of ovarian serous borderline tumors. The application of modern molecular techniques allows them to analyze small lesions even with contamination of a significant amount of normal tissue. They find that implants and borderline tumors are genetically heterogenous with respect to mutations in K-Ras and B-RAF. They further found an association between mutated K-Ras and B-Raf, respectively and overexpression of p16.

Major compulsory revisions:

A major point is the classification of the implants. Invasive implants are associated with a significantly worse prognosis compared to non-invasive implants. Invasive and non-invasive implants are furthermore considered biologically distinct, the former invasive low grade serous carcinomas, the latter reactive changes. The authors mentioned that only non-invasive implants were included. However, the manuscript is missing criteria for the distinction between invasive and non-invasive implants. Some of the photomicrographs give the impression that invasive implants might have been included. Therefore, the implants need to be reviewed and further detailed. The criteria used for D need to be listed.

Authors’ response: We thank the reviewer for this important comment. Differentiation between non-invasive and invasive implants was performed according to criteria of WHO by two experienced gynecological pathologists at
the Department of Pathology of the Ludwig-Maximilians-University of Munich. Images shown in the manuscript (including Figure 1 D) were also selected according to the WHO criteria.

According to the WHO the diagnosis of non-invasive implants was performed when they were typically localized on the surface, in submesothelial spaces or with extension into interlobular fibrous septa without infiltration of the underlying tissue.

In contrast, diagnosis of invasive implants was made when the lesions disorderly infiltrated the normal tissue with irregular borders and showed nuclei resembling cells of low-grade serous adenocarcinoma.

A comment regarding the criteria used for histopathological differentiation of implants has been added to the manuscript text as instructed.

Minor essential revisions
The authors might comment on the reasons of the genetic heterogeneity of the analyzed implants and the associated serous BOT. Could this also be caused by technical factors or due to the small amount of analyzed tissue. Analysis on the single or few cell level would be of interest.

Authors’ response: The methods employed to determine BRAF and KRAS mutation status had been intensively validated even for small amounts of isolated DNA stemming from small lesions. KRAS mutation analysis was taken out at a German reference laboratory for KRAS mutation testing at our Department of Pathology and has already been published multiple times (Moosmann et al., 2011; Kriegl et al., 2011; Bellon et al., 2011; Modest et al., 2011; Modest et al., 2012b; Stintzing et al., 2012; Stintzing et al., 2013; Modest et al., 2012a; Heinemann et al., 2013; Boeck et al., 2013b; Kriegl et al., 2012; Boeck et al., 2013a; Modest et al., 2013; Neumann et al., 2013). This procedure was used to detect mutations in the KRAS proto-oncogene with a specificity of 0.98 and sensitivity of 0.99 (Modest et al., 2011, Neumann et al., 2009).

The study is lacking follow up data. Even if the follow up is less than 10 years, it should be included.

Authors’ response: Follow up data has been added to the “Patients” paragraph of the Patients and Methods section.

Please detail the implant with strong p53 or show a photo.

Authors’ response: Patient #3 (Table 1) presented with an implant strongly expressing both p53 and p16. Additionally this implant was found to carry KRAS p.G12D and BRAF p.V600E at the same time. A photomicrograph of this implant has been included as a supplementary data file (Additional file 1) and the specifications as made within this paragraph have been added to the manuscript text.

page 3: invasive implants affect prognosis stronger than non-invasive implants, this should be mentioned.
Authors’ response: A further comment on the impact of non-invasive vs. invasive implants on patients’ prognosis has been added to the text as instructed.