Author's response to reviews

Title: Genome wide single cell analysis of chemotherapy resistant metastatic cells in a case of gastroesophageal adenocarcinoma

Authors:

Geir Olav Hjortland (Geir.Olav.Hjortland@rr-research.no)
Leonardo A Meza-Zepeda (Leonardo.Meza-Zepeda@rr-research.no)
Klaus Beiske (klaus.beiske@oslo-universitetssykehus.no)
Anne H Ree (Anne.Hansen.Ree@rr-research.no)
Siri Tveito (Siri.Tveito@rr-research.no)
Hanne Hoifodt (Hanne.Kleppe.Hoifodt@rr-research.no)
Per J Bohler (Per.Johannes.Bohler@oslo-universitetssykehus.no)
Knut H Hole (Knut.Hakon.Hole@oslo-universitetssykehus.no)
Ola Myklebost (Ola.Myklebost@rr-research.no)
Oystein Fodstad (Oystein.Fodstad@rr-research.no)
Sigbjorn Smeland (Sigbjorn.Smeland@oslo-universitetssykehus.no)
Eivind Hovig (ehovig@ifi.uio.no)

Version: 3 Date: 30 September 2011

Author's response to reviews: see over
Dear BMC Cancer editorial team:

A revision of our article “Genome wide single cell analysis of chemotherapy resistant metastatic cells in a case of gastroesophageal adenocarcinoma” has now been submitted as requested. The confidential consent declaration and the revised manuscript with visible changes are uploaded as additional files. Please find our detailed responses to the reviewers’ comments below.

Yours sincerely

Geir Olav Hjortland,
Consultant, MD PhD
Corresponding author

Department of Oncology
Oslo University Hospital
Po Box 4950 Nydalen,
N-0424 Oslo
Norway
Phone: +4722934000 / +4792214010
Reviewer's report
Title: Genome wide single cell analysis of chemotherapy resistant metastatic cells in a case of gastroesophageal adenocarcinoma
Version: 2 Date: 30 August 2011
Reviewer: Tsz-Kwong Man
Reviewer's report:
- Major Compulsory Revisions

1. Thank you for the authors' responses to my original comments and clarification of the issues raised in the last review. For instance, in Fig. 5 the authors now show the FISH images of the ERBB2 and KRAS in the primary and metastatic lesions, which are crucial to the interpretation of the study. However, it is still difficult to correlate the images with their statement that ERBB2 was heterogeneously amplified in the primary tumor, but homogenously amplified in the metastatic lesion. For example, I can still see negative cells in the Fig. 5B, thought they were not highlighted. Can the authors please clarify this issue further? Also, even with the understanding that this is a case study, with such a small number of cells, can they really make the claim of enrichment without any statistics, such as % of amplified cells versus non-amplified cells?

Answer:
A correct interpretation of FISH results can only be performed in a fluorescence microscope. Images prepared for publication do not display all optical details for the final conclusion. The negative cells in Fig. 5B also include normal lymphoid and myeloid cells which are present at higher numbers than in the primary tumor (Fig. 5A) since we deal with metastases to the bone marrow. The final distinction between normal and tumor cell nuclei must be left to the expert observer at the microscope. Moreover, one image does not necessarily reflect the proportion of amplified and non-amplified cells being calculated on the basis of a much higher number of cells in the sample. In order to document the relative enrichment of ERBB2 amplified cells in the bone marrow after chemotherapy, we have calculated the percentage of positive cells in both samples. In the primary tumor, 24.4% (66 out of 270 nuclei) showed ERBB2 amplification, while KRAS was amplified in 27.6% (124 out 450 nuclei). In contrast, 76.7% (92 out of 120 nuclei) of the tumor cells in the bone marrow were ERBB2 amplified. The number of metastatic tumor cells available for KRAS analysis was too low (8 of 22) for percent calculations. These numbers are now included on page 7-8 of the manuscript.

2. The main unresolved issue is that the authors' claim of ERBB2 amplified tumor cells enrichment after chemotherapy is heavily based on the fact that ERBB2 amplification was not detected in the bone metastasis sample before chemotherapy. However, because a small number of cells was analyzed, would it be possible that it is an artifact or sample bias that lead to the negative result? Perhaps they should discuss this further in the text and describe any controls they used to eliminate this possibility.

Answer:
In this case, several samples from the primary and metastatic lesions were available in the primary diagnostic setting, but only two samples in the post-chemotherapeutic
setting; i.e. the bone marrow biopsy from a progressing bone metastasis in addition to the bone marrow aspirate which was performed for the array CGH analysis. All biopsies were successfully sampled with representative biopsies from their respective tumors, and this is also highlighted in the article. The reviewer is however concerned about sample bias in the post-therapeutic setting. His concern contains two issues, as we interpret it, listed below in italic:

1) The reviewer raises the question that the negative result of ERBB2 expression in the pre-therapeutic setting could be an artifact, due to the small number of cells analyzed. Or stated in other words, could the normal ERBB2 expression found in the pre-chemotherapeutic bone marrow biopsy be a false negative result? Could the ERBB2 overexpression found in the post-chemo bone marrow biopsy be a false positive result?

The reviewer refers to ”the small number of cells analyzed”. However, in the primary diagnostic setting, whole biopsies were analyzed using immunohistochemistry, in which several hundreds of tumor cells were stained. In addition to the immunohistochemistry analyses, fluorescence in situ hybridizations were performed on all biopsy samples, giving results corresponding to the immunohistochemistry results. In the pre-therapeutic bone marrow biopsy, 400 FISH signals were evaluated in 90 nuclei for ERBB2/Cen17 ratio, giving an average ratio of 1.3, i.e. no ERBB2 amplification. This corresponded to the 1+(clinically negative) immunohistochemistry result. In the post-therapeutic bone marrow biopsy, 600 FISH signals were evaluated in 51 nuclei, and average ERBB2 copy number per nucleus was 10.4, with 1.4 average cen17 copies, giving a ratio of 7.6. The corresponding immunohistochemistry result showed strong membrane staining (3+) of almost all tumor cells. The number of tumor cells analyzed in the pre-therapeutic bone marrow (91) is considerably larger than the number of cells analyzed after chemotherapy (51) and clearly exceeds the minimum of cells to be analyzed for ERBB2 amplification according to ASCO guidelines. Thus, we do not have indications that the negative pre-therapeutic result could be due to a bias introduced by a small number of investigated cells. Moreover our analyses were performed by experienced clinical pathologists, specifically trained for the respective methodologies and routinely screening the whole sample before a decision is made. These methods are well validated for clinical use, and results from similar ERBB2 analyses serves as important arguments for treatment decisions on a routine basis. Using different methodology, both immunohistochemistry and FISH, as well as performing all analyses on multiple biopsies from different metastases, we feel certain that the ERBB2 expression levels found in the sampled biopsies are real and not artifactual results.

2) Even though the extensive analyses performed showed that tumor cells in the bone marrow biopsy sampled after chemotherapy highly overexpress ERBB2 compared to the tumor cells from the bone marrow biopsy before chemotherapy, could this be a sample bias, as ERBB2 expression could be heterogeneous and with expression levels varying between tumor foci?

As shown in this case, both interlesional and intralesional heterogeneity of ERBB2 expression is found, as described in the article using the immunohistochemistry results, as well as the FISH results, as basis. If very few tumor cells are sampled,
theoretically one could hit specific tumor cell colonies that differ in ERBB2 expression from other tumor cell colonies that are not harvested. To overcome such a sampling bias, samples should be as representative as possible, harvesting a substantial amount of good quality tumor tissue. The biopsy samples in this case were samples of good quality with pieces of representative tumor tissue containing several hundreds of cells. In addition, radiology exams show regression of almost all metastatic lesions, except for bone metastasis, from which two new samples were harvested: 1) A new bone marrow biopsy, and 2) a bone marrow aspirate. The ERBB2 amplification found in the latter sample using array CGH on DNA from 14 single cells sampled, corresponded well to both the immunohistochemical result of strong c-erbB2 staining (3+) and highly ERBB2 amplified cells in the first sample. As this bone metastatic focus was also progressing during chemotherapy, as shown through the radiology exams, this metastasis was particularly interesting. Analyzing these two separate samples (aspirate + biopsy) from this particular metastasis, gave results serving as controls for each other, as highly ERBB2 amplified tumor cells were found in both samples. Thus, the ERBB2 amplification found in the fourteen single cells analysis of DNA copy number changes using array CGH, was confirmed by the more validated FISH analysis and supported by the immunohistochemistry results showing strong ERBB2 gene product expression in the corresponding biopsy. Even more, the tumor cells selected from the bone marrow aspirate sample were identified by the rosetting of ESA-coated magnetic beads and thus selected. The probability of the random sampling of 14 highly ERBB2 amplified tumor cells in an otherwise low/or no amplification background, would be low.

As suggested by the reviewer, the paper is now revised to include clarifying statements on these topics in the discussion section on pages 9-10.

- Minor Essential Revisions

1. If it is due to scarcity of the biopsy material that the authors could not verify KRAS overexpression in the amplified cases. I think it is necessary to mention in the text, so that the readers would interpret the results cautiously.

   **Answer:**
   This is now addressed in page 8, line 2-4 in the revised paper.

2. Some minor points: A period is needed after the last sentence in the consent section.

   **Answer:**
   This has now been corrected.

The semicolon should be replaced by a period after “resonance imaging” in the abbreviations section.

   **Answer:**
   This has now been corrected.
An additional error has also been corrected in the revised version: Reference no.11 was duplicated in the previous bibliography as no. 11 and 16. This has now been corrected.

- **Discretionary Revisions**
  None.

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**
I declare that I have no competing interests

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**Reviewer's report**

**Title:** Genome wide single cell analysis of chemotherapy resistant metastatic cells in a case of gastroesophageal adenocarcinoma

**Version:** 2 **Date:** 5 September 2011

**Reviewer:** gopeshwar narayan

**Reviewer's report:**
The authors have satisfactorily addressed the issues raised by both the reviewers. The manuscript is acceptable.

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**
I have no competing interests