**Author’s response to reviews**

**Title:** Ruptured hepatic metastases of cutaneous melanoma during treatment with vemurafenib: an autopsy case report

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Elaine Zhang
Executive Editor
BMC Clinical Pathology

Re: Manuscript 1168073522171651, “Ruptured hepatic metastases of cutaneous melanoma during treatment with vemurafenib: an autopsy case report”

Dear Drs. Elaine Zhang:

We would like to thank the editor and the reviewers for their helpful and thorough review of our manuscript. We have revised our manuscript in response to their comments, and we believe that our manuscript is significantly improved as a result. In the text, our revisions are marked in red font. Please find below a point-by-point response to the reviewers’ comments.

RESPONSE TO REVIEWER (Emyr Wyn Benbow):

We wish to express our appreciation to the reviewer for the insightful comments, which have helped us significantly improve the paper.

Major Compulsory Revisions

Comment 1: vemurafenib is hepatotoxic in some patients, and in some circumstances. There should be a comment on the background liver at autopsy.

Response: We appreciate the reviewer's comment on this point. We agree that this point requires clarification, and have added the following text (page 7, lines 87-89):

The background liver was completely normal, whereas exposed necrotic tissue and intratumoral hemorrhage were observed at the site of tumor rupture.

Comment 2: part of the description of the findings are included in the "conclusions", in particular the molecular biological findings. These are part of the case report, and should be moved there.
Response: We agree with the reviewer's comment. The molecular/biological findings that had previously been described in the “conclusion” section have now been moved to the “case presentation” section (page 7, line 91 through page 8, line 98):

Finally, for improved understanding of the mechanism of refractory metastasis, we conducted an immunohistochemical analysis of the signal transduction molecules, phosphorylated extracellular signal-regulated kinase (p-ERK), and phosphorylated Akt (p-Akt), as well as the melanocyte marker Melan-A and Ki-67 in tumor cells of the primary malignant melanoma obtained from the right lower leg and in hepatic and lymph node metastases obtained on autopsy (Figure 4). Our findings showed that hepatic and lymph node metastases were positive for p-ERK and negative for p-AKT, even though the primary tumor was negative for both.

Comment 3: the timeline of the vemurafenib treatment, in relationship to the complications, needs to be made clear

Response: In accordance with the reviewer's comment, we have added the following text (page 9, line 114 through page 10, line 117):

In addition, Anker et al. described synergistic toxicity from the combination of radiation and vemurafenib [17], but fortunately, our patient did not experience complications, such as liver and skin toxicity, during the course of radiation and vemurafenib treatment.

Comment 4: increased intra-tumoral pressure because of rapid growth is the only mechanism put forward for the rupture; other mechanisms, such as intra-tumoral bleeding, need to be considered.

Response: The reviewer's comment is correct. We have therefore added the following to the text regarding this issue (page 9, lines 106-108):

In our autopsy case, gross examination and a microscopic study of the liver suggested that the increased intratumoral pressure by rapid growth, acute intratumoral bleeding,
and the subsequent tumor necrosis resulted in rupture (Figure 3).

**Discretionary Revisions**

**Comment:** the authors may wish to read, and perhaps cite, Anker CJ, Ribas A, Grossmann AH, Chen X, Narra KK, Akerley W, Andtbacka RH, Noyes RD, Shrieve DC, Grossmann KF. Severe liver and skin toxicity after radiation and vemurafenib in metastatic melanoma. *J Clin Oncol.* 2013 Jun 10;31(17):e283-7. doi: 10.1200/JCO.2012.44.7755.

**Response:** We appreciate the reviewer's suggestion and agree with the relevance of this reference. We have added it to the Discussion (page 9, line 115) and to the References.

**Quality of written English:** Needs some language corrections before being published

**Response:** The paper has been edited and rewritten by an experienced scientific editor from Editage (www.editage.jp), who improved the grammar and stylistic expression of the manuscript.
RESPONSE TO REVIEWER (Ryan Sullivan):

We wish to express our appreciation to the Reviewer for his insightful comments, which have helped us significantly improve the paper.

Comment 1: Given the rapid growth of the patients hepatic metastasis in the setting of only 2 months of vemurafenib therapy, it is important to describe the type of BRAF analysis assay that was used and explain why the primary tumor was assayed instead of a metastatic lesion. There is expanding data that primary melanomas may be heterogeneous with respect to BRAF/NRAS mutational status, thus analysis of the primary tumors should be avoided as a general rule since the results may not reflect the BRAF/NRAS status of the metastatic clone. One exception to this would be a thick melanoma (especially a nodular melanoma), since the expanding clone(s) likely reflect those which will metastasize and thus would be expected to share the same BRAF mutational status. While the likeliest scenario in this case is that the patient had either primary refractory clones in the hepatic metastasis that ultimately grew and ruptured, it is important to better explain the BRAF testing methodology.

Response: We appreciate the reviewer's comment on this point. In accordance with the reviewer's comment, we have changed the description of the type of BRAF analysis used (page 6, lines 68-70):

from

“based on the finding of a positive BRAF V600E mutation in the resected primary site of the skin.”

to

“based on the finding of a positive BRAF V600E mutation in the resected primary site of the skin, which was analyzed by direct sequencing analysis using DNA from the paraffin-embedded primary cutaneous melanoma.”

In addition, we agree that we should have assayed for the type of BRAF mutation using several histologic samples from metastatic sites. Regrettably, because it was immediately after the rupture and because of the patient’s subsequent short life span, we were unable to perform the examination that had a risk of rupture. We assayed the
primary tumor again, but the histological finding was not nodular melanoma. However the patient tolerated the vemurafenib treatment remarkably well and the size of multiple hepatic and lung metastases decreased, we considered that the metastases have the same \textit{BRAF} mutational status.

\textbf{Comment 2:} \textit{It is critical to know whether the patient had widespread growth of metastatic disease in the setting of vemurafenib or disease control everywhere but the metastatic lesion in the liver. Please provide a more comprehensive description of what was happening to her disease otherwise. For example, where the brain mets or lung mets larger, was it just one liver met or multiple that were expanding, was the disease found at autopsy in the kidneys, adrenal gland, and lymph nodes growing on imaging (or even seen on imaging).}

\textbf{Response:} We agree that this point requires better explanation and have changed the following text describing the other metastases (page 6, lines 70-72):

from

“She tolerated the treatment remarkably well, and the size of the hepatic and lung metastases decreased.”

to

“She tolerated the treatment remarkably well, and the size of the multiple hepatic and lung metastases decreased, while the size of the brain metastases did not.”

In addition, we changed the following text (page 7, lines 85-87):

from

“Metastases were also discovered in the brain, lungs, kidneys, adrenal gland, and lymph nodes.”

to

“Metastases were also discovered in the brain and lungs as well as in the kidneys, adrenal gland, and lymph nodes, although these had not been detected on imaging
while she was alive."

Comment 3: When describing vemurafenib, it would be better to use primary data (Flaherty et al. NEJM 2010 - phase I trial, or Chapman et al. NEJM 2011 - phase III trial) instead of a review article. Along these lines, the mechanism of resistance data should also be primary data. There are two very comprehensive papers (Shi et al. Cancer Discovery 2014: 4:80-93; Van Allen et al. Cancer Discovery 2014: 4:94-109) that describe the various mechanisms of BRAF inhibitor therapy resistance on numerous samples. These should be used in place of the review article from Swiaka et al. Also, vemurafenib is a selective inhibitor of the mutant BRAF protein or gene product, not the mutant BRAF gene. This should be changed (first sentence, second paragraph of the conclusions section).

Response: We appreciate the Reviewer’s comment on this point and agree with the relevance of these references. They have been added to the text (page 9, line 111 and page 10, line 124) and to the References.

Moreover, we have changed the text, as follows (page 3, lines 29-31):

from

“In addition, vemurafenib, a selective inhibitor of the mutant BRAF gene, has been reported to be extremely effective in patients with metastatic melanoma who harbor a BRAF V600E mutation.”

to

“In addition, vemurafenib, a selective inhibitor of the mutant BRAF protein or gene product, has been reported to be extremely effective in patients with metastatic melanoma who harbor a BRAF V600E mutation.”

We have also changed the following (page 9, lines 109-111):

from

“The recently developed vemurafenib, a selective inhibitor of the mutated BRAF gene, has been reported to be extremely effective in patients with metastatic melanoma who
harbor a \textit{BRAF V600E} mutation.”

to

“The recently developed vemurafenib, a selective inhibitor of the mutant BRAF protein or gene product, has been reported to be extremely effective in patients with metastatic melanoma who harbor a \textit{BRAF V600E} mutation.”

\textbf{Comment 4}: \textit{The IHC is underwhelming and does not help determine what happened.} Again, the primary tumor from 2008 may not be the best source of comparison to an autopsy specimen from Dec 2012 / Jan 2013. Since the ERK staining from the primary tumor and the hepatic tumor is sparse, it is impossible to make any conclusions about the effects of vemurafenib. It is interesting that there appears to be a difference in the ERK staining from the hepatic and lymph node met, but there is no context about the lymph node. Was this from the right inguinal surgery in 2008? from the autopsy? This must be clarified. In truth, performing more expanded exome sequencing on this patient would be the best way to try and figure out what happened. The mechanism of resistance, even the pathway that is unregulated at time of resistance is not clear, since it appears that both the MAPK and PI3K/AKT pathway is not unregulated in the hepatic met. This last aspect is very important, because mechanisms of resistance that bypass MAPK or PI3K pathway (re)activation are not well described.

\textbf{Response}: We appreciate the reviewer’s important suggestion. Firstly, the lymph node that was studied in the immunohistochemical analysis (shown in Figure 4) was obtained on autopsy. Before staining, we thought that the primary cutaneous melanoma might be positive for p-ERK staining because the \textit{BRAF} mutation was positive; however, it was sparse. We did not have a good explanation for this, but the p-ERK protein level in the primary melanoma might have been less than that in the metastases. However, we were not able to elucidate this discrepancy because of insufficient samples. In addition, there was a difference in p-ERK staining in the hepatic and lymph node metastases, as the reviewer suggested. These differences might reflect the growth rate and malignancy, but we could not compare the size of lymph nodes because we had not detected the metastases on imaging, and they were only detected on autopsy.

Secondly, as the reviewer noted, performing more expanded exome sequencing on this
patient would be crucial and informative for a complete understanding of the disease, but such sequencing is impossible now because we have no samples for such an analysis.

Finally, the mechanism of resistance had not become completely clear after the immunohistochemical analysis. Because p-ERK staining of the hepatic metastases was positive, though sparse, and p-Akt staining was negative, activation of the MAPK pathway was one of the conceivable reasons for the secondary resistance. We should have examined \textit{NRAS} and \textit{MEK} mutations and the CRAF protein levels for definitive proof of reactivation.

We modified the following description in the text (page 10, lines 128-131):

from

“This findings indicated that reactivation of the MAPK pathway occurred without activation of the PI3K/AKT/mTOR pathway in the hepatic and lymph node metastases.”

to

“These findings suggested the strong possibility that reactivation of the MAPK pathway had occurred without activation of the PI3K/AKT/mTOR pathway in the hepatic and lymph node metastases.”

We again thank the reviewers for their helpful comments on our paper. We trust that the revised manuscript is suitable for publication. Please do not hesitate to contact me if you require anything further. We look forward to hearing from you soon.

With best regards,

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