Author's response to reviews

Title: Mutational profiling reveals PIK3CA mutations in gallbladder carcinoma

Authors:

Aram Hezel (aram_hezel@urmc.rochester.edu)
Vikram Deshpande (vdeshpande@partners.org)
Afamefuna Nduaguba (afmajo@gmail.com)
Stephanie Zimmerman (scmzimmerman@stanford.edu)
Sarah Kehoe (SarahM_Kehoe@DFCI.HARVARd.edu)
Laura MacConaill (Laura_Macconailil@dfci.harvard.edu)
Gregory Lauwers (glauwers@partners.org)
Cristina Ferrone (CFERRONE@PARTNERS.ORG)
Nabeel Bardeesy (Bardeesy.Nabeel@MGH.HARVARD.EDU)
Andrew Zhu (AZHU@PARTNERS.ORG)

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Author's response to reviews: see over
Dear Dr Federica Di Nicolantonio,

Thank you for the very thorough and instructive review of our manuscript. We are pleased to resubmit this manuscript with each comment addressed as outlined below.

Sincerely,

Aram Hezel

**General Comments:**

Abstract - Please amend your abstract to comply with the following guidelines:

Background: This should place the study into the context of the current knowledge in its field and list the purpose of the work; in other words, the authors should summarise why they carried out their research.
Methods: This section should summarize how the study was performed and mention the different techniques employed.
Results: This section should describe the main findings of the study.
Conclusions: A brief summary of the content of the manuscript and the potential implications of its results.

*Reply:* We will amend the abstract as outlined above.

*Ethics - Experimental research that is reported in the manuscript must have been performed with the approval of an appropriate ethics committee. Research carried out on humans must be in compliance with the Helsinki Declaration (http://www.wma.net/e/policy/b3.htm), and any experimental research on animals must follow internationally recognized guidelines. A statement to this effect must appear in the Methods section of the manuscript, including the name of the body which gave approval, with a reference number where appropriate.

*Reply:* We will include such a statement as outlined above.

**Comments from Associate Editor:**

*The manuscript is written in a clear and concise way. The authors appropriately review in their introduction previous reports investigating the frequency of common oncogenic variants in gallbladder carcinomas. The aim of the study is clearly stated, and the methods are accurately reported. The results are
sufficiently well described, although they would benefit from the addition of a table comparing the frequency of known cancer variants observed in this study with those from previously published work and/or Cosmic databases”.

**Reply:** We will include a table (Table 4) describing established mutations in which our work is placed.

**Reviewer 2:**

My greatest concern regarding this study relates to the low number of mutations that were ultimately identified after hME analysis. It would be good if the authors could provide a little more explanation on the hME methodology. In the second paragraph of the Results the authors state the hME method is “more sensitive and specific” than the OncoMap technology. I do believe that it is more specific than the OncoMap technology and that that of course is the reason that they are using to confirm the OncoMap mutations. But I suspect the hME is significantly less sensitive than the OncoMap technology (i.e. higher false negative rate). I suspect that some of the OncoMap mutations that were not confirmed by hME method were real mutations but that the hME method was not analytically sensitive enough to confirm them. Do the authors have a good feel for the analytical sensitivity of the hME assay? I do think it is good that the authors are favoring specificity over sensitivity but they may be excluding real mutations. It seems that this is especially possible given the surprisingly low number of mutations that were ultimately identified by the study.

**Reply:** Thank you, we will refrain from comments on specificity and sensitivity on each individual aspect of the mutational screen since these are not published. Instead we would refer to established parameters that described the entire process of mutation screening including both the Oncomap and hME components. As described in the PLoS One paper using this same technique, we found the OncoMap sensitivity and specificity to be 93.8% and 100%, respectively, in DNA from fresh/frozen tissue; and 89.3% and 99.4% in FFPE-derived DNA. In general, these numbers for the overall sensitivity and specificity of the platform, allow a more meaningful comparison to other orthogonal technologies such as Sanger or Illumina sequencing.

Overall, there isn’t a high false negative rate with hME, rather we accept a high false positive rate with the initial iPLEX step, followed by validation using an orthogonal chemistry. This combined allows us to get an overall sensitivity of 89.3% and specificity 99.4% in FFPE-derived DNA (MacConaill et al., 2009). Compared to orthogonal technologies (Sanger, Illumina and pyrosequencing), OncoMap can detect an allele at an overall frequency of 5-10%, which is more sensitive than Sanger, and almost as sensitive as pyrosequencing (~4%). Going forward, alleles that are present at a lower frequency in samples (eg samples
with a lower tumor% or polyclonal tumors) will be detected by more sensitive methodologies such as deep sequencing with Illumina.

Regarding the analytic sensitivity of the hME assay- the overall sensitivity of the assay platform as compared to CLIA-certified pyrosequencing is published (Badalian-Very et al., Blood 2010, Supplemental data). Based on this, the lower limit of detection of an allele using OncoMap is 5-10%. A similar result was obtained in the PLoS ONE paper comparing to a ground-truth dataset of Illumina sequencing. Compare this to Sanger that has a lower limit of ~20%.

The sensitivity of OncoMap is influenced by inherent technological parameters, individual mutation assay performance characteristics, and the quality and purity of tumor tissue. The 89-94% assay sensitivity observed in this study is sufficient for many translational and clinical applications; however, there are of course circumstances where even higher assay sensitivities will be desirable. Enrichment of tumor cells using core needle dissection or laser-capture microdissection prior to mutation profiling may offer one avenue to enhance sensitivity, particularly in tumors where the stromal or inflammatory content is high. At the same time, the sensitivity of OncoMap vastly exceeds that of Sanger sequencing, which remains the gold standard for many genetic diagnostic approaches.

Note: the words “sensitive and” should be removed from the sentence on page 8 that says “Candidate mutations across 12 genes (ABL1, APC, BRAF, EGFR, FGFR3, FLT3, KIT, KRAS, NRAS, PDGFRα, PIK3CA, MYC,) were then evaluated using a more sensitive and specific homologous mass extend (hME) approach on non WGA DNA using independent primers and probes…”

Reply: We will remove sensitive here as requested.

In the fourth paragraph of the Discussion, the authors state “Our careful histological evaluation suggests that adenocarcinoma involving both the mid portion of the bile duct and intra-pancreatic bile duct lack KRAS mutations.” This is inconsistent with previously published findings which show that extrahepatic cholangiocarcinomas do harbor KRAS mutations. Further discussion is needed regarding this discrepant finding.

Reply: We will enhance discussion in this area. It also remains entirely possible that this was due to relatively low numbers of cases of extra-hepatic tumors and by chance alone and thus will remove emphasis here. This is further addressed in comments below is response to reviewer 3.

The authors should use the correct nomenclature for genes. For example, KRAS should be KRAS. Also I believe that the officially accepted nomenclature for HER2 and NEU is ERBB2.
**Reply:** We agree with this comment and will change the text accordingly.

The first sentence of page 11 (Discussion section) states that “Taken together this data points towards deregulation of PI3K signaling as a key event in the molecular pathogenesis of BTC.” I would say it is a key event in the molecular pathogenesis of some BTC.

**Reply:** We agree with this comment and will change the text accordingly.

**Reviewer 3:**

The authors stated that there are important differences among tumors having different anatomic origin in the biliary tree, but they have chosen to group intra-hepatic and peri-hilar carcinoma. Since the characteristics and the prognosis of these two entities are different (J Surg Oncol. 2010 101:111-5), they should explain this choice.

**Reply:** Thank you, yes we agree and will appropriately divide them in the results, table and discussion.

-Not only resected tumors have been included in the analysis. In fact in “Methods” it is described that samples were identified from “resected or biopsied” archived tissues. If this is true, I cannot understand how (Table 3) all of the 33 gallbladder carcinoma have a complete TNM staging. Seven patients have a stage IV disease, and they have been classified as T1-4 and N0-2. In these cases a Tx or Nx is rather expected.

**Reply:** While there were some biopsied hepatic cholangiocarcinomas all the gallbladder cases were in fact complete resections and thus full staging information is available and provided.

-Distal extra-hepatic (n=6) and intrapancreatic biliary carcinoma (n=9) (in Table 1 referred as to middle CBD and intrapancreatic cholangiocarcinoma) have been distinguished by peri-hilar carcinoma (n=5). One out of five peri-hilar tumor has KRAS mutation, but no KRAS mutation have been found in 13 extra-hepatic carcinoma. Then, the author conclude that it is possible that KRAS mutation previously described in extra-hepatic biliary tumors may be misdiagnosed peri-biliary pancreatic carcinoma. I believe that the statement is not supported by the evidence. A statistical analysis should be included to define whether KRAS mutation may be confined to intra-hepatic/peri-hilar carcinoma (4/27).

**Reply:** We did not detect any KRAS mutation among the 13 distal extra-hepatic and intrapancreatic biliary carcinoma. Analysis of the absence of KRAS
mutations in extra-hepatic and intrapancreatic biliary carcinoma in comparison to
the 4 mutations identified intrahepatic and perihilar cholangiocarcinomas shows
this relationship to be insignificant, p=0.28 (Fishers exact test). It is possible that
the absence of KRAS mutations in our screen was a chance event. As such we
will omit our comment about possible misdiagnosis of pancreatic cancer from this
manuscript though other more comprehensive ongoing work suggests this as
well. Additionally we will enhance discussion on this point.

-Since this work is descriptive and does not contain novelty which can
immediately affect the menagement of patients with biliary cancer, preclinical
models should be set up to argument the statement “this data points towards
deregulation of PI3K signaling as a key event in the molecular pathogenesis of
BTC (“Discussion”).

Reply: We agree and will amend this comment as outlined above in our
response to Reviewer #2.

Minor Essential Revisions:

The abbreviation CBD is not explained in the text. Does it mean common bile
duct?

Reply: Yes this means common bile duct and will be explained.

The abbreviation IBD is not explained in the text. Does it mean internal review
board?

Reply: Yes, this means internal review board and will be explained.

In Table 3 the four PIK3CA mutations are all E545K whereas in Table 2, two of
them are E542K

Reply: Thank you Table 3 is incorrect and will be amended.

Discretionary Revisions

A systematic review of previously identified genetic alteration of key regulatory
genes involved in biliary carcinoma cell proliferation and invasiveness might
improve the global impact of this work.

Reply: We will provide a table of established mutation as suggested above and
will refer the reader to recent reviews on this topic. Given the limitations on
space and the breadth of this topic we do not feel we would be able review this
adequately in this paper.