Author's response to reviews

Title: Somatic mutation analysis of MYH11 in breast and prostate cancer

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Author's response to reviews: see over
To the Editor,

Enclosed please find the revised version of the manuscript “Somatic mutation analysis of MYH11 in breast and prostate cancer” (MS:1482949605196577) submitted for possible publication in BMC Cancer. The manuscript has been revised along the lines suggested by the referees and every point raised has been addressed. We thank the reviewers for their valuable contributions and think these changes have improved the manuscript. You can find below a list of referees’ comments and how each one of them is addressed. Also the ethics board approvals and informed consent documentation have been added, as requested by the journal. We appreciate the favorable reviewers’ comments and thank you for the possibility to resubmit our work.

Referee 1

The abstract and discussion should clearly state the fraction of MYH11 coding sequence that was analyzed, in order to facilitate interpretation of the study.

Altogether 8 exons out of 42 coding MYH11 exons were analyzed covering approximately 18% of the coding region. This information has been added to abstract and discussion (page 8):

“A total of 155 breast cancer and 71 prostate cancer samples were analyzed for those regions in MYH11 (altogether 8 exons out of 42 coding exons) that harboured mutations in colorectal cancer in our previous study.”

“Here we have extended the analysis of the role of myosins in tumor development and analyzed sizable sets of breast and prostate cancers for somatic mutations in the key regions of the MYH11 gene (altogether 8 out of 42 coding exons, covering approximately 18% of the coding region).”

Please indicate MYH11 mutation frequencies in AML and non-MSI colorectal cancers.
Inversion at the MYH11 locus resulting in fusion gene CBFB/MYH11 occurs in approximately 8% of AMLs, most commonly in the M4Eo subtype (Liu et al 1993). We have analyzed the entire coding region of MYH11 in 30 microsatellite stable CRCs. One tumor harbored two MYH11 mutations (Alhopuro et al 2008).

“**Inversion at the MYH11 locus inv(16)(p13q22) is one of the most frequent chromosomal translocations found in acute myeloid leukemia (AML) and accounts for approximately 8% of all AML cases, especially those of the M4Eo subtype [6].**”

“**MYH11 mutations were also found in one microsatellite stable CRC (altogether 30 MSS CRCs analyzed) and in the germline of one patient with Peutz-Jeghers syndrome.”**

**The reason for increased exon 9 failure in PrC should be clarified, is there a potential for translocations involving this exon?**

The failure of exon 9 is most likely due to a technical artifact, as all samples on certain part of the plate amplified poorly in PCR. As more DNA was not available, the fragment was not re-amplified. (Results, page 6)

“**In prostate cancers, exon 9 was successfully analyzed only in 33% of samples due to technical problems,”**

**On a more general note, the selection of exons is based only on two mutations plus a MSI hotspot location, which I find a questionable approach. Although the gene is large, it would make more sense to analyze the entire coding region in a more limited set of samples. The manuscript is otherwise concisely written and of appropriate length.**

The authors agree that studying a smaller set of samples for the entire coding region of MYH11 would be interesting; however, there was not enough DNA available from breast cancers for this type of approach. If screening of the entire coding region is required for publication of the article, the authors are willing to set new collaborations to get more appropriate material for this purpose. Because that would mean a substantial delay, we suggest not doing that, and explain our rationale for exon selection here. We concentrated on the 8 MYH11 exons, which were mutated in MSI CRC. Based on current structural models of myosin regulation, most mutations in the MYH11 head or in the rod domain would not be expected to abolish regulation. Therefore we hypothesized that mutations only in certain regions of MYH11 contribute to tumor formation. Although an approach studying the whole coding region would have been interesting, analyzing regions mutated in CRC in sizable sample sets of breast and prostate cancers would point to a role of similar mutations in the development of non-CRC tumors. The strategy was to analyze the regions with the greatest potential for discovery in a large sample set. The approach suggested by the reviewer has been acknowledged (Discussion, page 8-9).

“The strategy was to analyze the regions with the greatest potential for discovery of mutations in a large set of non-CRC tumors.”
“It would be of interest to study smaller sets of breast and prostate tumors for the entire coding region of MYH11.”

Referee 2

The paper is clearly written and the manuscript is easy to read. On the positive side is that the authors studied a reasonably large sample size (155 breast and 71 prostate). The authors investigate only 8 of the 42 exons of the gene (with one of these exons being only marginally covered in breast cancer samples). If the important finding is the lack of mutations of this gene in these samples, it seems to me that further work needs to be done to ensure that this is not due to technical problems.

1) Most importantly, one reason why somatic mutation may be missed is if the samples are contaminated by normal tissue. This will lead to the presence of less than heterozygous mutations that can be missed by sequencing. Therefore it is important to get an idea about the level of contamination in these samples. One way to do that, is to look for loss of heterozygosity (LOH) in regions in the genome known to frequently undergoes LOH in these cancer types. If the sample is not contaminated at all, regions of LOH should be detected (heterozygous in the normal tissue but homozygous in the tumor). The presence of contamination will keep both alleles present but causes one of the alleles to be lower than the other one. Quantitative analysis of the reduction can help in the estimate of the degree of contamination.

The concern of normal tissue contamination raised by the referee is important in interpretation of the results. Prior to DNA extraction, tissue sections were HE-stained and evaluated by a pathologist to confirm sufficient quantity of tumor cells. All prostate cancer samples contained >60% of malignant tissue. The breast cancer samples contained at minimum 30% of tumor cells, and on average the tumor cell content was 50-70%. In our experience, based on our 170 cancer genetics publications, a tumor cell content >30-50% should enable detection of somatic mutations by sequencing. Also, possible normal tissue contamination was considered when scoring the sequence traces. Only sequence traces with no background signals were deemed successful to enable detection of even small mutation peaks (see also below). These data now appears (Materials and Methods, page 4-5)

“Tumor samples were evaluated by a pathologist prior to DNA extraction and contained at minimum 30% of tumor cells. On average the tumor cell content was 50-70%, which should enable detection of somatic mutations by sequencing.”

“Prior to DNA extraction the samples were evaluated by a pathologist to confirm sufficient content of malignant tissue. All tumor samples contained >60% of malignant tissue.”

On the minor side:
1) The authors that most of the exons are covered in 80-100% of samples. What are the criteria used to determine something is covered or not? Is it some phred
score-that needs to be clarified. Can the authors comment on the potential false negative rate?

The exon was determined to be covered when every basepair in the exon as well as the nucleotides flanking the exon-intron boundary could be visually analyzed. The reasons for a cover rate <100% were i) background signal in all or part of the sequence trace (which could have hindered detection of small mutation peaks e.g. in a subset of cells) or ii) failure to amplify in PCR most likely due to quality of DNA. This information has been added to the manuscript (Results, page 6):

“As somatic mutations may be missed due to normal tissue contamination, only sequence traces with no background signal were scored successful to enable detection of even small mutation peaks.”

2) The authors refer to c.5798delC as a somatic mutation. Since its absence in the normal sample could not be verified, it should be described as a potential or candidate somatic mutation.

c.5798delC found in the prostate cancer sample is now described as candidate somatic mutation (Results, page 7, Discussion, page 9 as well as Table 1, page 12).

“The sample (LuCaP73) harboring the candidate somatic MYH11 frameshift mutation was a xenograft specimen originating from a hormone-refractory prostate cancer.”

“In prostate cancers, an ATPase activating candidate somatic MYH11 mutation (c.5798delC) was identified in one xenograft sample only.”