Author's response to reviews

Title: Rapid detection of SMARCB1 sequence variation using high resolution melting.

Authors:

Vinod Dagar (vinod.dagar@mcri.edu.au)
Chung-Wo Chow (cw.chow@rch.org.au)
David M Ashley (david.ashley@rch.org.au)
Elizabeth M Algar (elizabeth.algar@rch.org.au)

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Author's response to reviews: see over
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Dear Editor

RE; MS 1074529053273607.

We wish to submit a revised version of our research article entitled “Rapid detection of SMARCB1 sequence variation using high resolution melting” for consideration by BMC Cancer.

Specific responses to the associate editor and reviewers are detailed below. All changes in the manuscript have been underlined.

Associate Editor
Additional experimental detail has been added to the manuscript as described in the response to reviewer 2. References to zygosity in the tumor series have been removed due to insufficient information required to fully describe the copy number of the mutation in each tumor (see additional comments under reviewers 1 and 3).

We believe that HRM will be widely adopted as a mutation screening tool. While it may not give information as to the mutational mechanism it is an extremely useful and cost effective technique for screening for sequence variation. Nevertheless the specific sentences referring to wide adoption of HRM in SMARCB1 screening have been removed from the manuscript.

Reviewer 1
(1) We have removed reference to the mechanism of SMARCB1 inactivation in rhabdoid tumor because we feel there is insufficient evidence in the absence of copy number analysis to describe a mutation as homozygous or hemizygous.

(2) All sample analyses were performed in duplicate. The HRM software can be set to “group” samples and in doing so generates average melting data from duplicates. The legends to figures 1 and 2 have been amended to describe this.

(3) Twenty-five amplicons from the digital HRM shown were sequenced. All genotypes were obtained and are shown in Figure 3.

(4) Reference 4 has now been amended.
(5) The cut-off we set for scoring a sample as variant is necessarily stringent (90%). False positives often fall just below this threshold value. True variants are usually well below this value. We feel therefore that inclusion of data for tumor 3161 in Figure 1 showing a significant deviation from the melting curves of normal controls is representative and not likely to be impacted significantly by artefact associated with any impurities.

Reviewer 2

Major revisions

Reviewer 2 has major criticisms of the manuscript due to a lack of methodological detail.

(1) We do not think that it is necessary to provide detail for very standard methods such as DNA extraction and sequencing when these protocols are widely available with commercial kits including DNA extraction from paraffin embedded tissues. A reference to manufacturer’s instructions has been made, see page 5 under methods.

Issues raised in (2), (3) and (4) have been addressed in the methods and results sections (underlined on pages 6, 7 and 11). The reaction mixes, annealing temperatures etc that were used for HRM have been stated in the second paragraph of the methods section. A third paragraph now describes additional detail specifically relevant to the digital HRM.

With respect to point (5) all of the sequences identified following digital PCR reflected the sequence present in the original tumor sample. We did not discover any unexpected sequence variations by performing the dilution. We think that it is extremely unlikely that mutations could be introduced by mis-incorporation in this particular situation. Our amplicons are on average only 200 bp in length and the chance of mis-incorporation would be extremely low given the probability of an error rate of 1 in 9000. The same probability (ie 1 in 9000) applies for each single amplicon amplified and is not additive, thus we believe that the reasoning behind this criticism is perhaps erroneous. We were also able to detect the sequence variation in numerous replicate samples as shown in Figure 3, arguing most strongly against PCR artefact.

(6). Compositions of Sensimix and EvaGreen dye are proprietor information therefore we cannot precisely explain why Sensimix with EvaGreen was superior to Syto 9 and LC Green. The available information suggests that EvaGreen dye has high stability, high fluorescence and much lower propensity for PCR inhibition than other dyes.
Minor revisions

(1) Spaces between units and numbers have been inserted.

(2) The sentence has been deleted.

Reviewer 3.

(1) Method Validation
The method validation has been expanded to include 14 tumors analysed. These tumors were both sequenced and analysed by HRM. The false negative rate remained at zero. See Table 2 and amendment page 8.

(2) Table 2 (now Table 3 in revised manuscript).
The table has been amended and reference to zygosity has been removed for clarity. Without performing an extensive copy number determination analysis in this tumor group we cannot address the issue of zygosity reliably (see comment to reviewer 1). We feel that this is not a central theme of the paper and the absence of this information does not detract from its significance.

(3) Sensitivity
We agree with the reviewer that the sensitivity for mutation detection will also be influenced by the proportion of normal to tumor cells in each tumor specimen at the outset and have amended the text to include this (Results page 10).

Other minor revisions.
(1) and (2). We agree with the reviewer that use of the term mosaic could be misleading within the context in which it is used in the paper. Use of this term in reference to mutations has been removed.
(3) We have checked the mutation nomenclature against HGVS recommendations and now believe that it is correct.
(4) Primer sequences, annealing temperatures and amplicon sizes have been place in a table (Table 1).
(5) The asterisk has been removed from Table 3 (previously Table 2).
(6) In Figure 1 the control and tumor sequences are labelled and the mutation position has been asterisked.
(7) Figure 3 has now been replaced.
We thank you for re-considering this new version of our manuscript.

Yours sincerely

Elizabeth Algar PhD mHGSA