Author's response to reviews

Title: Analysis of the Mitogen-activated protein kinase kinase 4 (MAP2K4) tumor suppressor gene in ovarian cancer

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Author's response to reviews: see over
To the editor:

We thank the reviewers for their constructive criticisms, which we have addressed as indicated below. We hope these changes are satisfactory and will enable acceptance of the manuscript for publication by BMC Cancer.

We also wish to advise that in preparing the resubmission we reanalyzed our HRM data and identified one additional mutation in MAP2K4 (c.282delAGAG). This mutation does not substantially change the text or overall conclusions to the paper, and we would like to include the new data in the resubmission. To this end, we have altered the manuscript as indicated below the response to reviewers and hope this is acceptable.

Yours sincerely,

Kylie Gorringe

Response to reviewers

Reviewer 1

1. Clarify whether genetic alteration studies were performed on 149 or 89 different clinical specimens

As indicated on line 99, the study comprised 161 individual tumours. As stated on line 192, 149 were used in the HRM mutation screen, of which 89 were also analysed by SNP array (line 212).

2. Stipulate the % of MAP2K4 loci loss

72/106 (68%) of samples analysed by SNP array showed LOH, and 40/72 samples with LOH (56%) showed copy number loss. No samples had CN loss without LOH. Thus overall copy number loss of MAP2K4 locus was 40/106 (38%). This has been clarified on line 232 by the addition of “(38% of samples overall)”

3. discuss why frequent alterations in copy number did not translate into altered gene expression

As stated on lines 236-237, we did in fact observe significant decreases in MAP2K4 expression correlating with copy number in three microarray datasets. The reviewer may be referring to the lack of association of MAP2K4 expression with clinical outcome in one data set analysed (lines 250-251).

4. highlight the specific nature of frequent hemizygous deletions in the abstract.

We have added “Hemizygous deletion of MAP2K4 was observed in 38% of samples.” to the abstract (line 41).

5. Show PCR data demonstrating MAP2K4 siRNA mediated knockdown
Please see additional figure, Supplementary Figure 3

Reviewer 2

1. On page 10: In the TCGA public copy number dataset an additional three homozygous deletions were found among 157 ovarian cancers. Did these deletions specifically target the MAP2K4 gene or were other genes/miRNAs also targeted? Can the authors speculate a bit on whether it is likely that MAP2K4 is (partly) responsible for the 17p loss?

The three deletions were fairly specific being 29kb, 36kb and 807kb. The largest of these deletions contained just three genes and one microRNA, while the smaller two overlapped only with MAP2K4. Consequently we believe that the deletions are targeting MAP2K4. To reflect this we have added “..., two of which specifically targeted only MAP2K4.” To line 219.

2. On page 11: Is it not contradictory that all five genetic changes were discovered in high grade tumors whereas reduced expression of MAP2K4 was associated with a better survival? How do the authors explain this apparent discrepancy? Is there information on the grade of those tumors or was this a selected sample set? Also, in literature there was a report on MAP2K4 expression being associated with a reduction in overt metastasis (Yamada et al.).

The analysis of survival was conducted on data sets that were predominantly (90%+) high grade serous samples. Thus, within this group with generally poor survival, reduced MAP2K4 may reflect a tumour phenotype with relatively better survival. To clarify this point we have added “Both data sets were comprised of primarily high grade serous carcinomas.” to line 246.

3. On page 12: The authors saw a reduction in cell numbers upon silencing MAP2K4 in one of the two ovarian cell lines. In contrast, the stable clones generated in the study of Yeasmin et al. did not show a reduction in cell proliferation. However, they did observe EMT-like changes. Did the authors observe any morphological EMT-like changes in their system?

We did not observe measurable EMT-like changes, however the HOSE cell line is already mesenchymal in phenotype.

Reviewer 3

1. In “Methods”, the authors stated that DNA for mutation screening underwent WGA. Then in Figure 1 legend, IC489T is a non-WGA tumor DNA. Did the authors use non-WGA DNA to verify the result after finding the mutation in WGA sample? Please clarify this.

The mutation was indeed verified in non-WGA DNA, as indicated on lines 121-123.

2. The protein termination caused by the 16 bp deletion (p.Asp263fs) is not
We have added the description to the text at line 197.

3. Quantitative PCR results and/or a Western blot image if available should be shown in a supplementary figure to support the authors' claim of significantly reduced expression of MAP2K4 in JAM and HOSE cell lines.

See above.

4. The authors may also include the following reference by Kan et al., Nature 466: 869-873 when discuss the effect of MAP2K4 knockdown from line 259 to 266.

We have included the reference and cited it in line 276: “Another recent study found that ectopic expression of MAP2K4 mutants increased anchorage-independent growth in NIH3T3 cells [32].”

Alterations to manuscript following data reanalysis

1. Additional author added (Georgina Ryland)

2. Change to Supplementary Table 1 to include the new mutation for sample IC128T.

3. Change to abstract (lines 36-38):
   “In addition to 4 previously detected homozygous deletions, we identified a homozygous 16 bp truncating deletion and a heterozygous 4 bp deletion, each in one ovarian tumor. No promoter methylation was detected. The frequency of MAP2K4 homozygous inactivation was 5.6% overall, and 9.8% in high-grade serous cases.”

4. Change to results:
   (lines 191-200)
   We identified two somatic alterations, a homozygous 16 bp deletion in exon 7 in a serous ovarian tumor, (Figure 1) and a heterozygous 4 bp deletion in exon 3 in an endometrioid tumor. The 16 bp deletion in IC489 causes a frameshift alteration that would lead to protein termination 7 amino acids downstream (p.Asp263fs). The mutation represented more than 70% of the bases in the sequence trace, thus we consider it to be homozygous, correlating with the SNP array data for this tumor showing chromosome 17 LOH across the MAP2K4 locus. The 4 bp deletion at the start of exon 3 would cause protein termination two amino acids downstream (p.Glu74fs) and is heterozygous in accordance with the lack of LOH at the locus in IC128 by SNP array.

   (Line 210)
   Combining the detection of mutation and deletion data, the overall frequency of homozygous inactivation of MAP2K4...