Author's response to reviews

Title: Overexpression of miR-9 in mast cells is associated with invasive behavior and spontaneous metastasis

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

We would like to thank the reviewers for their comments regarding our submission to BMC Cancer “Overexpression of miR-9 in mast cells is associated with invasive behavior and spontaneous metastasis” by Fenger et al. The following is a point by point response to each of the concerns raised by the editor and reviewers. We have made edits to the manuscript to reflect these changes. Changes made in the text of the manuscript are indicated in underlined text.

RESPONSE TO EDITORIAL COMMENTS:

1. The generally positive nature of the reviews means that your paper can be accepted once you have addressed the major points raised. In addition, please include specific names and affiliations of all ethics committees that approved the protocols.

We have included names and affiliation of all ethics committees and IACUC protocol numbers in the Methods section (Cell lines, primary cell cultures, primary tumor samples).

RESPONSE TO REVIEWER 1

Overall this paper represents a significant amount of work and the results are interesting and potentially clinically relevant. I do believe that conclusions would be greatly strengthened by demonstration of altered protein expression (of select proteins in murine cell lines) since mRNA does not always translate to changes at the protein level. If unable to address, this should at least be discussed as a weakness. Inhibiting miR-9 in canine cell lines would provide further convincing evidence of its importance in MC migration/invasion, but understandably, this may represent a future area of study.

Specific comments and suggestions:

Major compulsory revisions:
1. Because of the many differences in miRNA expression, the authors should provide rationale for why they chose to focus on miR-9 in subsequent experiments.

Rationale for the focus of miR-9 based on evidence in human cancers for miR-9 contributing to metastatic behavior has been added in the Results section. In addition, Table 2 has been edited and now contains p-values/fold-change in expression for differentially expressed miRNAs; this shows miR-9 is one of the most significantly dysregulated miRNAs identified in our analysis.

2. You mention review of clinical data for one outlier in low-grade tumor group. This is interesting and I certainly understand why it was reviewed, however, this could represent bias unless all tumors were reviewed similarly. Potential bias should be mentioned.

Clinical data for all tumors was reviewed similarly and we have clarified this in the manuscript/Results. Given that clinical data for all tumors was treated similarly, we do not feel that this is a potential bias and thus is not mentioned.

3. Statistical analysis describes student’s t-test for two group means, but some analyses consist of more than two groups. One-way ANOVA would be appropriate for such comparisons.

One-way ANOVA has been performed for experiments involving multi-variable comparisons (Figures 3A, 3B, 6B, 6C) and the description of this statistical analysis has been added to the Methods (statistical analysis) section.

4. It is stated that 24, 48, and 72 hours were time points examined for apoptosis and proliferation of C57 and P815 but 72h is not shown for proliferation (Figure 3D).

24, 48, and 72 hour time points were evaluated for proliferation assays and this is shown in Figure 3C.
24 and 48 hour time points were evaluated for apoptosis assays and this is shown in Figure 3D.

5. There is no real discussion of why discrepancy between miR-9 expression in murine and canine cell lines when compared to mBMMC and cBMMC respectively.

The mouse P815 mastocytoma cell line is derived from a leukemia of mast cell origin (Gajewski TF, Markiewicz MA, Uyttenhove C. The P815 mastocytoma tumor model. Curr Protoc Immunol. 2001 20:Unit 20.4). In contrast, the canine BR and C2 mastocytoma cells are derived from cutaneous mast cell tumors (Lazarus SC, Devinney R, McCabe LJ, Finkbeiner WE, Elias DJ, Gold WM. Isolated canine mastocytoma cells: propagation and characterization of two cell lines. Am J Physiol. 1986 251(6 Pt 1):C935-944; Devinney R, Gold WM. Establishment of two dog mastocytoma cell lines in continuous culture. Am J Respir Cell Mol Biol. 1990 3(5):413-420). The differences in the biology of these diseases may account for the observed differences in miR-9 expression in canine and murine cell lines. Low miR-9 expression in P815 cells may reflect the fact that these cells represent a true leukemia, in contrast to the BR/C2 cell lines which are derived from cutaneous tumors that would metastasize via the lymphatic system. We have
Minor Essential Revisions:

6. It is stated that 151 of the 377 human miRNAs are conserved between human and dog. Can the authors further specify/discuss conservation between canine/human/murine miR-9?

This has been addressed in the Methods section (RNA isolation and quantitative PCR).

7. Can the authors provide more detail on methods for migration/invasion assay? Often cells on top of the chamber are removed with a swab but there is no mention of this.

A more detailed description of this methodology has been added to the Methods section and clarified in the Figure 4B legend.

Discretionary Revisions:

8. While I understand that the biological grade is potentially more powerful than histology (and represents a strength of this study), I would suggest that the authors consider including a summary of some of the routine clinical information (sex, breed, primary or metastatic site, outcome, follow-up time etc.) and histologic grade of tumors examined as supplemental information for reference by clinical veterinarians.

A table of the clinical/patient information for tumors examined in this study has been provided as a supplementary table.

9. I would suggest including fold change, and perhaps p-value of each miRNA that was differentially expressed in biologically high vs. low grade tumors

These changes have been incorporated into Table 1.

REFEREE 2

The study is well designed and the results are clearly illustrated.

Discretionary Revisions:

1. Methods - Quantitative reverse-transcription-PCR profiling of mature miRNA expression in MCT biopsies, first paragraph:

Normalizer gene for miRNA expression analysis. The authors selected snRNA U6 as a normalizer gene after miRNA array due to its stable expression in the sample set. In the
past years, small RNAs other than miRNAs, like U6, were reported to be unsuitable reference genes for miRNA expression data in different human and canine tumors (e.g. see Peltier and Latham, 2008. Normalization of miRNA expression levels in quantitative RT-PCR assays: Identification of suitable reference RNA targets in normal and cancerous human solid tissues. RNA 14, 844-852; Mortarino et al., 2010. Identification of suitable endogenous controls and differentially expressed miRNAs in canine fresh frozen and FFPE lymphoma samples. Leukemia Research 34: 1070-1078). A short statement discussing the result from the present study in comparison with abovementioned studies should be added to the manuscript.

The human Taqman MicroRNA Arrays contains 3 endogenous controls (snRNA U6, RNU44, RNU48). Since equivalent amounts of total RNA was added to each RT reaction, snRNA U6 was validated as the internal control by comparing the mean expression among tumor samples or cell lines (Lee EJ, Gusev Y, Jiang J, Nuovo GJ, Lerner MR, Frankel WL, Morgan DL, Postier RG, Brackett DJ, Schmittgen TD. Expression profiling identifies microRNA signature in pancreatic cancer. Int J Cancer. 2007 120(5):1046-1054; Schmittgen TD, Zakrjesk BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods. 2000 46(1-2):69-81). In contrast to previous reports evaluating the stability and suitability of small RNAs such as U6 as reference genes (Peltier and Latham, 2008. Normalization of miRNA expression levels in quantitative RT-PCR assays: Identification of suitable reference RNA targets in normal and cancerous human solid tissues. RNA 14, 844-852; Mortarino et al., 2010. Identification of suitable endogenous controls and differentially expressed miRNAs in canine fresh frozen and FFPE lymphoma samples. Leukemia Research 34: 1070-1078), the current study found no statistically significant difference in the mean U6 expression between tumor samples (p < 0.05) or cell lines (p < 0.05; p-values generated by ANOVA). Since there was no significant difference in the U6 expression between tumors or cell lines, snRNA U6 was selected as the internal control gene in the study.

2. Methods – RNA isolation and quantitative real-time PCR, second paragraph:

Normalizer gene for mRNA expression analysis. The authors should explain the rationale of selecting 18S gene as reference gene.

18S rRNA was validated as the internal control by comparing the mean expression among cell lines as described above (Lee EJ, Gusev Y, Jiang J, Nuovo GJ, Lerner MR, Frankel WL, Morgan DL, Postier RG, Brackett DJ, Schmittgen TD. Expression profiling identifies microRNA signature in pancreatic cancer. Int J Cancer. 2007 120(5):1046-1054; Schmittgen TD, Zakrjesk BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods. 2000 46(1-2):69-81). Since there was no significant difference in the 18S rRNA expression between cell lines (p < 0.05; p-values generated by ANOVA), 18S was selected as the reference gene in the study.

3. Results – miR9 is overexpressed in biologically high-grade canine MCTs:
Case H11, corresponding to a dog with biologically high-grade MCT, clustered with the biologically low-grade tumors after miRNA array (Figure 1) and expressed miR-9 as lowly as low-grade tumors except L12 (Figure 2A). This unexpected result should be discussed in the manuscript as done for case L12.

We have addressed these findings in the Results section and have discussed this in the manuscript text.

Minor essential revisions:

All of the requested minor changes/corrections/edits have been completed.

REFEREE 3

In this paper, authors investigated the role of microRNA dysregulation in mast cell tumours, and further explored the role of miR-9 in metastasis of mast cell tumours. The authors show that metastatic, high-grade canine mast cell tumours demonstrate a unique microRNA profile signature that is distinct from benign, canine mast cell tumours. MicroRNA miR-9 was shown to be upregulated in high-grade mast cell tumours, and authors further explored the role of miR-9 in metastasis and cell invasion, in vitro, in normal and malignant mast cells. The authors also investigated the molecular mechanisms by which miR-9 may drive invasion/metastasis of mast cell tumours. Transcriptional profiling of miR-9 transduced mast cells (mBMMCs) and P815 mast cell tumour cells was performed to identify potential miR-9 targets. The paper is well written, the research question is well defined and the experiments are well controlled. There are some concerns regarding the data, however, which will need to be discussed by the authors further:

Major Compulsory revisions:

1. The unsupervised hierarchical clustering of microRNA profiling on canine low-grade and high-grade tumours, shows that in general, high-grade and low-grade tumours cluster together and have a unique microRNA signature. Authors have identified and discussed the attributes of the one low-grade tumour sample (L12*) that clustered with, and showed comparable miR-9 expression to the high-grade tumours. However, high-grade tumour sample H11 appears to cluster with the low-grade tumours (Figure 1) and Figure 2A shows that at least 2 out of 12 high-grade tumours have comparable miR-9 expression to the low-grade tumours. The authors should address in the results and discussion of the paper.

We have addressed these findings in the Results section and have discussed this in the manuscript text.

2. Authors describe that potential targets regulated by miR-9 overexpression differ between normal (mBMMCs) and malignant (P815) cells and choose to further validate two of the seven genes that were common between the both normal and malignant cells. The authors have chosen CMA1 and IFITM3. The rationale for choosing CMA1 is clear in the
discussion, as it is a gene that has previously been implicated in the regulation of MMPs, and in cell metastasis. The authors, however, mention that the role of IFITM3 in mast cells is unclear, and do not provide their reasoning for the choice to validate this target, instead of the other 6 differentially regulated targets. It would enhance the paper if some of these other differentially expressed genes were also subject to validation (independent of the actual results).

These gene transcripts were selected for qRT-PCR validation based on any known role/implication in the metastatic phenotype (CMA1) or most significant fold-change in gene expression between groups (IFITM3). While the other gene transcripts (PDZK1IP1, MGL1, TMEM223, SLAMF1, CLEC4E) found to be dysregulated in P815/mBMMCs do not have demonstrated roles in contributing to invasion/migration of mast cells, we agree that it would enhance the paper if some of these other genes were also subject to validation. We have subsequently performed qRT-PCR for PDZK1IP1, as this gene was identified on our microarray analysis as being significantly up-regulated following miR-9 overexpression (similar to IFITM3). We have provided this data in Figure/legend 6C and addressed this in the manuscript text.

**Minor Essential revisions:**

1. **Methods/Paragraph 5:** Can the authors described how RNA was extracted from samples for transcriptional profiling of cells transduced with miR-9 lentivirus. I ask this because of some of the recent controversy regarding Trizol extractions for miR analysis.

   We have provided a detailed description in the Methods section describing the RNA purification method, RNA cleanup, and RNA integrity analysis (Agilent 2100 Bioanalysis) performed on samples for transcriptional profiling.

2. **Figure 2A:** Authors refer to a 3.2 fold change in miR-9 expression in high-grade tumours relative to the low-grade tumours, but do not state if this is a fold change in the mean expression levels or otherwise.

   This has been amended in the Figure legend.

3. **Figure Legends/Figure 3C:** In this figure, the measurements of cell proliferation are presented as a percentage of control. Can the authors more clearly define what the ‘non-treated control’ wells are. It is unclear if the non-treated controls referred to by the authors, are cells not transduced with lentivirus, or are EV/miR-9 transduced cells not treated with CyQuant.

   We have clarified in the Methods section and Figure 3C legend that nontransduced cells served as a negative ("non-treated") control for CyQuant cell proliferation assays.

4. **Figure 5:** The heatmap for transcriptional profiling of miR-9 and EV transduced
mBMMCs could be more clearly labeled. Does the heat map show only the differentially regulated targets or all targets profiled? Also, are Ct values for each sample or fold change relative to a control presented in the heat map.

The heat map shows only differentially regulated targets and this has been addressed and clarified in the Figure 5 legend. Data presented in the heat map represent relative expression of each gene after log2 transformation with respect to the average signal for each comparison (EV or miR9). We have provided this information and additional clarification in the legend.

5. Figure 6C: There is inconsistency in the way samples are labeled in the two bar graphs shown, one plot refers to the non-transduced P815 cells as ‘‘P815’’, while the other graph refers to the sample as ‘‘WT’’.

The figure has been corrected.

We hope that the changes we made sufficiently address the concerns of the reviewers.

Sincerely,

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