Author's response to reviews

Title: Deep Sequencing the microRNA profile in rhabdomyosarcoma reveals down-regulation of miR-378 family members.

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Author's response to reviews: see over
Dear Editor

Please find the revised version of the manuscript n. 2134482199130065, entitled "Deep Sequencing the microRNA profile in rhabdomyosarcoma reveals down-regulation of miR-378 family members", which we would like to resubmit to the BMC Cancer. We thank you for this opportunity.

We changed part of the main text considering the comments and the experiments requested by the reviewers. We added supplementary figures 1 and 2, and supplementary table 1 for the experiments requested by the reviewers. We re-numerated supplementary table 1 as supplementary table 2. We also performed experiments on apoptosis in the RD cell line, an in vitro model of embryonal rhabdomyosarcoma, as requested. We provided the name of the ethics committees (Policlinico Umberto I’s Ethics Committee and Alder Hey Children’s NHS Foundation Trust Ethics Committee) which approved our study in the methods section of the manuscript. We eliminated “age” and “gender” from Table 1 in order to anonymize this table. We added some references and re-numerated all the bibliography.

We thank all the reviewers and editorial board for their valuable comments and suggestions, and we are looking forward to your response.

Sincerely yours,

Francesca Megiorni Carlo Dominici
Response to Reviewer Beat Schafer:

Major concern

The authors refer to several crucial experiments as data not shown. Q-PCR data to confirm differential expression as imposed by NGS needs to be presented.

**R:** We added the quantitative Real Time PCR data as Supplementary Figure 1.

Similarly, expression level of the transfected miR-387a needs to be included in the Figure and compared to expression levels in normal muscle.

**R:** We added the expression level of miR-378a-3p in miR-378a-3p-transfected cells as Supplementary Figure 2.

Some key experiments such as induction of apoptosis need to be repeated in a second RMS cell line.

**R:** We performed some experiments also in RD cells, an in vitro model of human embryonal RMS. Indeed, we assessed apoptosis by FACS analysis also in RD cells transfected with miR-378a-3p (miR-Ctr as mocked control) or treated with 5-aza-dC (DMSO as mocked control). The percentages of apoptosis in the RD cells have been detailed in the main text. We also checked the cell cycle distribution in RD cells treated with 5-aza-dC and we added the results in the main text.

Minor points

The authors conclude in the abstract that miR-387 may represent a novel possible therapeutic target. This needs to be rephrased since restoring its expression would be of therapeutic benefit.

**R:** We corrected the sentence.

On line 152, references should be added.

**R:** We added references.

In Figure 3, total AKT level should be included on the blot. Please indicated time point when samples were analysed.

**R:** We included total AKT levels in Figure 3. The time points of the experiments were indicated in the “Methods” section. We reported the time point of the western blot experiments also in Figure 3 legend.

The study did not identify miR-206 which is one of the prominent myomirs known (e.g. Macquarrie et al. 2012, Missiaglia et al. 2010, Taulli et al. 2009). The authors should comment on this in their discussion.

**R:** We report here the values derived from the differential analysis of the microRNA datasets for miR-206:

<table>
<thead>
<tr>
<th>Comparison</th>
<th>logFC</th>
<th>logCPM</th>
<th>P-value</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERMS vs NMS</td>
<td>-1.44</td>
<td>14.65</td>
<td>0.27847</td>
<td>0.54603</td>
</tr>
<tr>
<td>ARMS vs NMS</td>
<td>-1.92</td>
<td>14.65</td>
<td>0.16616</td>
<td>0.49965</td>
</tr>
<tr>
<td>ARMS vs ERMS</td>
<td>-0.48</td>
<td>14.65</td>
<td>0.67892</td>
<td>0.94528</td>
</tr>
<tr>
<td>NMS vs RMS</td>
<td>-1.46</td>
<td>14.55</td>
<td>0.21667</td>
<td>0.66728</td>
</tr>
</tbody>
</table>

MiR-206 was always present with a detectable negative fold change in all comparisons, but its expression never reached statistical significance for uncorrected p-values. Hence, miR-206 has not been included in the results. It is well worth of note that also in a recent article describing microarray experiments in the same biological model (Li et al., 2012), hsa-mir-206 appears to be regulated but without being associated with a significant statistical index in the final analysis results. According to the Reviewer request, we added a sentence in the “Discussion” section, we added a sentence in the “Discussion” section.

References:
- Li L, Sarver AL, Alamgir S, Subramanian S. Downregulation of microRNAs miR-1, -206 and -29 stabilizes PAX3 and CCND2 expression in rhabdomyosarcoma. Lab Invest. 2012 Apr;92(4):571-83
Response to Reviewer Alicja Jozkowicz:

Major concerns

- Line 461: Increased expression of miR-378 results in reduced activity of Akt. However, it is a correlation, not a prove that biological effects of miR-378 upregulation are mediated by inhibition of Akt. Please, perform experiment e.g. with constitutively active form of Akt or rewrite this fragment to avoid the impression of mechanistic connection.
R: We eliminated the final sentence in order to avoid the impression of a mechanistic connection between miR-378 up-regulation and Akt inhibition.

- Line 513: Comparison of effect of miR-378 overexpression with those of 5-aza-dC treatment is not the evidence of mediating the 5-aza-dC activity through miR-378 upregulation. To prove such a conclusion, please, perform experiments with miR-378 antagonomirs. In other case, please, rewrite this subchapter.
R: We reformulated the initial and final sentences of the paragraph underlying that the 5-aza-dC effects are not limited to the up-regulation of miR-378a-3p, but to the re-expression of several epigenetically silenced genes, which also warrant further studies.

Minor concerns and suggestions

- Line 145-146: It would be good to add one sentence about studies on transgenic animals, which indicate that translocations resulting in Pax3/FoxO1 or Pax7/FoxO1 proteins are not sufficient to induce RMS.
R: We added one sentence about studies on transgenic animals with the corresponding references.

- Line 153-155: Information that binding of miRNA to non-perfect complementary sequence results in translational repression and/or mRNA degradation is not precise, as the complete base-pairing leads to mRNA degradation. Please, correct.
R: We corrected the sentence.

- Line 190 and following subchapter (or table 1): Please, add information which samples have been used for deep sequencing – especially what was the translocation status of aRMS samples and the clinical stage.
R: We specified the number of tumour samples that were used in deep sequencing analysis in the “Methods” section and in the Table 1 legend. The translocation status of ARMS samples and the clinical stage are already described in Table 1 (“Fusion status” and “Group” columns, respectively). We renamed “Group” with “Clinical stage”.

- Please, add in the Discussion section a short rationale why RNA from a pool of NMS, not from individual samples, has been used as a control.
R: Since the mainly infiltrative growth pattern of RMS, the quantity of available tumour samples is very often a limiting factor, especially for controls and experiments with NGS. Hence, we used a set of pre-pooled NSM samples from already available normal donors. Apart this factor, we know that in general microRNA expression variability between single individuals is high. By pooling different samples, we averaged out the variance, at the expense of some loss in biological variability. If the differential expression or representation of single microRNA molecules is true and relevant, though, it will still stand out clearly in a comparison of individual samples versus a pooled control, minimizing the inter-individual 'transcriptional noise'.

- Line 441-442: What was the increase in miR-378 expression after transfection? Please, add information on approximate range (2-fold? 10-fold?).
R: Expression of transfected miR-378a-3p mimics was measured by quantitative Real Time PCR. We added Supplementary Figure 2.

- Line 471: Information that myoD are normally detected in post-mitotic myoblasts can be misleading, as myoD is also present in activated, proliferating myoblasts, and myoD KO satellite cells have a lower proliferation potential. Please, make this sentence it more precise.
R: We specified that MyoD is detected in committed proliferating myoblasts and MyHC in post-mitotic muscle cells.
- Experiments performed on RH30 cell line – please, add information on number of independent experiments performed (in figure legend or the methods section).

R: We specified how many times any experiment was performed in the “Methods” section.

- Fig. 4A: Increase in MyoD on the blot is not convincing. Please, add more samples or show the results of densitometry.

R: As already stated in the main text, we only observed a slight up-regulation of MyoD1 levels in RH30 cells transfected with miR-378a-3p in comparison to miR-Ctr control. We used Quantity One software to analyse MyoD1 western blot by densitometry and we obtained a 1.6-fold increase. We added this value in the legend of Figure 4A.

- Fig. 6. Treatment with 5-aza-dC results in up to 40% apoptosis rate (Fig. 6A). However, no apoptotic cells are visible on cell cycle histograms (Fig. 6D) or immunohistochemical staining (Fig. 6E). Please, comment.

R: Figure 6A describes the cell cycle distribution of RH30 cells treated or not with 5-aza-dC. Cells were fixed in cold ethanol, immediately stained with PI and analysed by FACS using a linear (not logarithmic) scale by acquiring gating exclusively viable cells and leaving out dead cells. Thus, we obtained typical histograms of DNA content frequency representing cells untreated and treated with the drug that affects the cell cycle distribution. Moreover, often apoptotic cells have fractional DNA content and during the staining procedure some cells lose DNA by shedding apoptotic bodies, so that only a fraction of DNA remains within the apoptotic cells. In these conditions, the difference in the DNA fluorescence of normal and apoptotic cells is minimal or undetectable. Indeed, because we didn’t have a software to deconvolute DNA histograms in order to identify and quantify the sub-G1 apoptotic cell population, for detecting apoptosis we used the more accurate method of Annexin V/7-AAD double staining with the appropriate kit and FACS settings.

As concerning Figure 6E, we eliminated dead or dying cells floating in the medium before fixing the adherent cells so that the percentage of apoptosis may be underestimated. Moreover, the figure depicts only a field of the chamber slide in which are present MyHC-positive cells but apoptotic cells are not evident.

- Line 152: “(reference)” is not specified.

R: We specified references.
Response to Reviewer Steven Cheng:

1. The authors claimed in the Introduction that members of the entire miR-378 cluster were “strongly under-represented in ARMS and ERMS samples. However, the data are not clearly presented to substantiate this point.

R: We added Supplementary Table 1: (A)miR-378 family members differentially expressed in ARMs vs. NMS, with NMS baseline; (B)miR-378 family members differentially expressed in ERMS vs. NMS, with NMS baseline. The members of the miR-378 family have FC values always negative and significant in both comparisons, indicating an under-expression in the investigated ARMS and ERMS tumour samples.

Also, are these miRNAs produced in a polycistronic transcription unit or scattered in different chromosomal loci? If the latter is the case, are those separate genes regulated differently?

R: The current release of Mirbase (Release 21, June 2014) reports 11 mature miRNAs belonging to the has-mir-378 family as reported in Table 4: hsa-miR-378a to hsa-miR-378j; has-miR-378a has two active mature forms, hsa-miR-378a-5p and hsa-miR-378a-3p. All these molecules are mapped in different parts of the genome (except of course hsa-miR-378a-5p and its cognate -3p). In order to check for the existence of commonly shared promoter motifs (following evidences from Mas Montey et al., 2010; Baer et al., 2012), we retrieved a 56 kb of upstream sequence from the Transcription Start Site of hsa-miR-378a to hsa-miR-378f included. We then randomized the same genomic sequences to produce a set of background random controls, with the same nucleotide composition than the test set. We searched for significant Transcription Factor Binding sites (using EMBOSS 6.5.7 tfscan program and associated publicly available human Transfac SITE dataset) in both set of genomic sequences. Sites which were common to more than one of the promoter sequences and none of the random controls were identified and labelled as putative ‘mir-378 promoter modules’. TFBS derived from these analyses and associated only with miR-378 putative promoters were c-Jun, NF and WT1.

References:

2. The authors also claimed that miR-378 targets IGF1 and presented one Western blot to support that (Fig. 2B). It is not clear if this regulation is direct or indirect? Is there miR-378 recognition sequences in IGF1 3’UTR, coding region? An experiment using luciferase reporter construct with IGF1 3’UTR is necessary to address this issue.

R: Knezevic et al., 2012 and Ganesan et al., 2013 demonstrated the direct regulation of IGF1R by miR-378a-3p. Indeed, the authors showed the localization of predicted binding sites for human miR-378a-3p in the 3’-untranslated (3’-UTR) region of IGF1R mRNA and performed luciferase assays using reporter constructs carrying the IGF1R 3’-UTR in the presence of miR-378a-3p or mimic control (miR-Ctr). We already cited Ganesan et al., 2013 in the “Results” section to corroborate the down-regulation of IGF1R protein levels in RH30 transfected with miR-378a-3p. The reduction of IGF1R expression was also obtained in RD cells transfected with miR-378a-3p (we added a sentence in the “Results”). Furthermore, a recent work by Li et al., [2014] showed that miR-378a-3p over-expression can inhibit IGF1R expression in colorectal cancer cells. Therefore, we think that an additional in vitro luciferase experiment would not add further substantial evidence to our data. Anyway, we added a panel (C) in Figure 2 showing the position of miR-378a-3p binding sites and the species conservation in the IGF1R 3’-UTR obtained by TargetScan interrogation. DIANA-microT version 3.0 (http://diana.cslab.ece.ntua.gr/microT/) and miRanda (http://www.microrna.org/microrna/home.do) algorithms also identified miR-378a-3p target sites in the IGF1R 3’-UTR sequence.

References:

3. RMS samples consisting of both ARMS and ERMS types. However, the experimental testing was done only in the embryonal type RH30 cells. It is necessary to repeat the testing with additional RMS cell lines, particularly the ARMS type.
R: We performed experiments in RH30 human cell line, a model of alveolar RMS. We repeated some tests in RD human cell line, a model of embryonal RMS.

4. The authors showed that over-expression of miR-378 induced a number of myogenic markers, implying that it has a function of promoting myogenic differentiation of RH30 cells. However, the experiment supporting that claim was only the transient transfection. The proper way to make that point is to induce myogenic differentiation in vitro without or with ectopically expressed miR-378 in RH30 and other RMS cells.
R: Spontaneous differentiation to a muscle phenotype was determined in a percentage of transfected cells by MyHC expression, whose positivity is considered a good marker of myogenesis in different papers using transient transfection assays.

5. The 5’-Aza experiments were confusing and seemed to be irrelevant, since miR-378 promoter (which one?) was not methylated. This observation suggests that the observed 5’-Aza effect might have been indirect, despite it induced miR-378 expression.
R: We discussed this observation and hypothesised that 5’-aza-dC might have an indirect effect on miR-378a expression by acting on a transcriptional factor able to bind the miR-378a promoter region. This will be addressed in future investigations. Anyway, we more accurately specified the sentence in the “Discussion”.

6. The authors neglected to cite several important recent articles on miRNA in rhabdomyosarcoma. Please cite Huang et al, MiR-214 and N-ras regulatory loop suppresses rhabdomyosarcoma cell growth and xenograft tumorigenesis. Oncotarget 5:2161-75, 2014; and Diao et al miR-203, a tumor suppressor frequently down-regulated by promoter hypermethylation in rhabdomyosarcoma. JBC 289:529-39, 2014.
R: We added the paper by Huang et al. 2014 in “Background” and “References” sections. We had already cited the paper by Diao et al., 2014 as reference n. 25.