MicroRNA-320 family is downregulated in colorectal adenoma and affects tumor proliferation by targeting CDK6

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Comment to the reviewers.

We wish to thank you for your helpful comments on our manuscript. Our replies are as follows.

Reviewed by 01588319

1. In the "Results" of the "Abstract" section, this study identified miR-320 family, including miR-320a, miR-320b, miR-320c, miR-320d, and miR-320e, were differentially expressed in adenoma and submucosal invasive carcinoma. However, the miR-320a did not shown in "Core tip", please explain it.

We noted only novel miRNAs in “Core tip” section, so miR-320a was not shown because it was previously reported. But it is very confusing, we corrected.

2. In Table 1, the expression level was present as the "mean" without standard deviation/error, does it make sense? Especially, this experiment was only

performed in 3 paired samples.

We updated results with standard deviation.

3. Several items should be justified and consistent in the beginning of this study. Such as LSTs and protruded tumors ; adjacent non-neoplastic mucosa,adenoma and submucosal invasive carcinoma. Please clarify which groups are the specific compared targets of this study.

As the reviewer pointed out, the aim of this study written in the background was unclear. The main aim of this study was to find the sequential changes of miRNA from normal mucosa through adenoma to cancer. But we have to consider the differences between the LST and protruded tumors according to the previous results. We revised the background section to clarify the main aim of this study,

4. In the "Gene expression analysis and miRNA target prediction" of the "Materials and Methods" section, why the authors only performed the transfection experiment with a mimic control or miR-320a mimics? How about other so-called significant miR-320 family?

We used miR-320a as a representative of miR-320 family to predict the target genes. CDK6 was one of the candidates, and the following analysis were performed using not only miR-320a but also 320b, 320c, 320d and 320e. We understand that this is one of the limitations in this study, but it could be available just to find out the candidates.

5. In the " Statistical analysis", the authors claimed that "The difference between two groups was analyzed by the Student's t-test.", is it appropriate for the comparison of the expression levels derived from paired samples, especially for 3 paired tissues only.

Thank you for pointing it out. The analysis of miRNA array was performed using the paired t-test for 3 paired samples. We compared miRNA expressions of 15 samples by qRT-PCR, but some of the 15 samples didn't contain adenoma, so we couldn't match samples. Therefore, we dealt the qRT-PCR data from normal, adenoma and cancer as independent groups, and compare those using Student's t-test. We revised it.

We agree the sample size (n=3) was very small, but the purpose of the miRNA microarray analysis was just to find out the possible candidates, not to see statistical significance.

6. On comparing adenomas and carcinomas, expression levels of the miR-320 family, except for 320d, in carcinomas were lower than that in adenomas; however, differences were not statistically significant. Based on this finding, the interpretation of "These results indicated that the expression of the miR-320 family progressively decreased from the early stages of the adenoma–carcinoma sequence." is not appropriate.

We revised the sentence by deleting the word, "progressively".

7. Regarding the " miR-320a targets CDK6", the authors stated that.....CDK6 expression was shown to be associated with prognosis in patients with CRC;,

please identify where are the results from Tables? or Figures?

Thank you for pointing it out, we added the reference [24].

8. In the "Discussion" section, the contents should be more concise , for example: "The most commonly used approach to find the target genes of miRNA is through..... ; however, recent reports have provided evidence that miRNAs may downregulate a greater number of transcripts than previously appreciated [22]." and " Finally, we narrowed down the candidate target genes of the miR-320 family using two bioinformatics algorithms and our results of the mRNA array; we selected seven genes as common to all of these analyses."

We revised the discussion more concise as the reviewer suggested.

Reviewed by 00068256

1. MicroRNA-320a is a well known tumour suppressive miRNA in colorectal cancer, many papers have reported its roles, especially anti-metastasis, in colorectal cancer, including several publications as followed (in addition to the papers that had been cited in the manuscript).

We added the references to these reports into the discussion section.

2. A luciferase reporter assay should be performed using wild-type and mutant-type of CDK6 3'UTR to confirm the direct regulation of miR-320s on CDK6 in a sequence-specific manner.

We agree with the reviewer, and we have already tried the luciferase reporter

assay as a pilot trial. Unfortunately, miR-320a precursor suppressed a luciferase reporter WITHOUT 3'UTR region of CDK6 gene. We could not rule out off-target effects in this assay. Further analysis will be needed as a next step.

3. A western blotting analysis also need to be performed to detect the CDK6 protein expression in miR-320s-ovexpressed and/or miR-320s-silenced CRC cells.

Protein levels of CDK6 were significantly suppressed in CRC cell line with miR-320 family mimics. These results were in the results and figure 4.

Reviewed by 03003235

1. The authors concluded that that miR-320 repress SW480 proliferation. As a target gene of miR-320, the expression of CDK6 mRNA and protein are both downregulated by miR-320. Could these data indicate that miR-320 inhibits SW480 proliferation by targeting CDK6? To make this point, the authors should observe whether deficiency of CDK6 similarly inhibit SW480 proliferation and perform a rescue experiment in which ectopic expression of CDK6 in miR-320 overexpressed cells restore the cells proliferation. Besides SW480, how about other colorectal cancer cell lines?

   We agree with the reviewer; these analyses will support our data.

2. In Figure 4, according to the results of miRNA binding-site prediction analyses, the authors indicated that CDK6 has a putative miR-320 family's binding site that is mapped to the 3'UTR. The authors should further

performed dual-Luciferase reporter assay to test the specific regulation through the predicted binding sites.

Please refer the reply to the reviewer 00068256.

3. The tissue sample size is not enough in this study.

We understand this is the limitation of this study. However, this analysis was performed using precious "matched" samples from one specimen, and we think it was enough to see sequential changes of mi-RNA.