RESPONSE TO REVIEWERS

REVIEWER #1

This manuscript highlights the interaction between miRNA and colon cancer, because the Colorectal cancer (CRC) account ~ 10% of all new cancer cases in 2022 and is the third-most prevalent cancer in the world, I think it is very important to try identifying a novel parameter that might cause the disease. Furthermore, identifying the miRNA that causes the disease can be considered as a promising diagnostic biomarker or a novel therapeutic. Thus, in my opinion this kind of paper is important. Considering this specific manuscript, I think the topic is important. Yet, before it is published, some revisions are required to improve the manuscript: Although the authors validate the results with TCGA COAD, I'm not convinced about the control samples, which were used in the experiments. This is because they were taken from different tissues of the patient "Adjacent normal colonic mucosa of each tumour was used as the corresponding control", and as it is known that miRNA expression is tissue specific. Thus, it might not be a good control. The authors may consider taking samples from healthy individuals. In addition, the tissue samples, which were taken from the patients, were taken from different areas in the colon, and from tumors of different Grades. What is the evidence that the gene expression level is the same in the different tissues and in the different tumors Grades?

Response: We understand and appreciate the concern raised by the reviewer. However, we would like explain our logic and reasoning behind the usage of tumour adjacent normal as control for our experiments.

Firstly, we would like to make it clear that the control samples were not taken from "different tissues of the patient" but from the same tissue **as that of the respective tumour (colon/rectum) from a region atleast 5cm away from the visible tumour margin**. Therefore, the control tissue is expected to have **similar** tissue-specific profile as in tumour tissue apart from the alterations involved in tumorigenesis. As miRNA expression is tissue specific, we collected the 'tumour adjacent normal colonic mucosa' to ensure that the control samples reflected the same tissue backdrop as that of the tumour. The tumour adjacent normal colonic mucosa represents tissue from

the **same individual** and the **same organ** and also reflects the **microenvironmental milieu shared by the tumour in the absence of tumour development**.

The custom for this type of gene expression studies [**1**] has been to use tumour adjacent normal colonic tissue beyond the tumour margin. In fact, even The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC) have followed the same method.

Studies involving circulating RNA and Extracellular Vesicles use normal tissues from healthy individuals as in these cases blood is the biological tissue being collected. Our study deals with Colorectal Cancer where tumours of the colonic/rectal mucosa are being studied. No Ethics Committee would give permission to extract normal colonic tissue from healthy individuals. Alternatively getting colonic tissues from patients undergoing colonoscopy or being treated for other diseases would be unethical and hamper patient benefit due to associated risks like bleeding. Secondly comparing tumour tissue from one individual with normal tissue from another can always lead to inter-individual differences being counted as False Positives. Following these arguments, we deemed it best that comparing miRNA expression between tumour and adjacent normal of 'SAME' colonic tissue of the 'SAME' individual is the best possible scenario. Hence we used tumour adjacent normal colonic mucosa as a control for our study as the difference in miRNA expression between a tumour and its respective adjacent normal could directly be linked to the development of tumorigenesis.

We agree with the reviewer that gene expression levels are not the same in different areas of the colon and rectum and/ or in tumours of different grades. The tumour samples used in our study mostly belonged to Grade 2 (G2). In our discovery cohort 4/5 tumours were of G2 Grade. Our validation cohorts had 10/16 G2 tumours in Early-Onset (<50 years old) patients and 9/11 G2 tumours in Aged onset (>55 years old) patients. Hence even though the tumours are of different grades, most of the tumours in both the discovery and validation cohorts belonged to the grade G2.

We admit that there could be differences in gene expression between tumours from different segments of the colon. The aim of our study was to identify miRNAs showing different levels of expression in Early-Onset (<50 years old) and Aged onset (>55 years old) tumours in colorectal tissue irrespective of tumour grade and/or exact tumour location. Initially we wanted to see if at all there are any differences in miRNA expression without incorporating any other variables (like site of tumour). Hence, we took care to eliminate other canonical factors like MSI and *APC*

inactivation (nuclear β catenin, *APC* mutations) from our discovery and validation cohort to ensure that we were looking at non-canonical drivers of pathogenesis. By choosing a wide tissue and tumour stage cohort we aimed to identify miRNAs deregulated in **all Early Onset CRC (EOCRC) cases** as compared to their adjacent normal. In the clinic, colorectal cases show tumour location from different areas of the colon/rectum and with varying tumour grades. Hence identifying deregulated miRNAs common to all locations and grades would make our study more clinically viable.

If the young and old tissues show significant tissue specific changes in miRNA expression which are being reflected as False Positives in the comparison between the young and old tumours, then this difference would also be reflected in the comparison between the normal tissues (as the normals come from the same tissues as that of the respective tumours). To compensate for the tissue-specific difference in miRNA expression in different areas of the colon/rectum, we have compared **miRNA levels between young and old normal tissues** and **excluded** miRNAs showing significant differences from further analyses (Fig 6). Hence, miRNAs that show **age-specific** and/or **tissue specific** expression would be eliminated in this analysis. Only those miRNAs were chosen that show **non-significant differences between normal tissues** and **significant difference between tumour tissues** of young and old patients. Thus, the analysis outcome of our study identifies miRNAs that show only **EOCRC-specific deregulation in tumour**. We admit that it would have been better to establish cohorts belonging to a specific area of the colorectum (either colon or rectum) and a specific tumour grade, but since our cohort size was limited, we performed the pilot study incorporating tumours of different grades and from different regions of the colorectum. For a full-fledged follow-up study we plan to investigate site-specific differences by establishing separate cohorts corresponding to the colon and the rectum.

References

1. Liu C, Wu W, Chang W, Wu R, Sun X, Wu H, et al. miR-31-5p-DMD axis as a novel biomarker for predicting the development and prognosis of sporadic early-onset colorectal cancer. *Oncol Lett.* **(2022),** *23*, 157.