Reviewer comments:

Reviewer #1:

The introductory section could be developed. Add the references: - Predescu D, Boeriu M, Constantin A, Socea B, Costea D, Constantinoiu S. Pregnancy and Colorectal Cancer, from Diagnosis to Therapeutical Management - Short Review. Chirurgia (Bucur). 2020 Sept-Oct;115(5):563-578. doi: 10.21614/chirurgia.115.5.563. - Gruia MI, Marinescu S, Predescu D, Jinescu G, Socea B, Gruia I. Oxidative stress level in onco-surgical treatment dynamics at patients with malignant colo-rectal tumors. Rev. Chim., 71 (5), 2020, 450-461. doi: 10.37358/RC.20.5.8157. - Păun I, Constantin VD, Socea B, Bobic S. The impact of environmental factors upon the incidence rate of colorectal cancer. Ciencia e Tecnica Vitivinicola, 2015, 30/2 (11): 99-133. The limitations of the study must be precised. The conclusions should be more concise and strictly reflect the results form the study.

Answers: Thank you for your kindly comments. As for the suggestion of adding references in the introduction, we have carefully read the original texts of the several literatures you provided, and believe that sufficient and relatively authoritative literatures have been quoted in our original manuscript to explain our views. The literatures you provided are not relevant to our research. So we do not think it is necessary to include it in our manuscript as a reference. As for the conclusion, we have adjusted it to make it more concise.

Reviewer #2:

A very good research study investigating a cancer resistance with the use of high-throughput sequencing. I do have several suggestions/concerns which are as follows:

1. Please add explanation of “ceRNA” in the main text (first appearance on page 1). I know it is explained in “Abbreviations” section, but I believe it should be (just like the other ones) also mentioned in the main text.

Answers: Thank you for your comments. We have made corrections to apply competitive endogenous RNA (ceRNA) when ceRNA first appears on the first page of the text.

2. Consider deleting the last commas in brackets for FOLFOX and FOLFIRI, I think it will be better when it comes to grammar

Answers: Thank you for your suggestion. We have revised it in the revised manuscript.

3. On page 3, there is a sentence: “CircRNAs regulate gene expression mainly at the transcriptional and posttranscriptional levels, and their main function is to act as miRNA sponges or to bind to other molecules is”. I suspect the “is” at the end could be deleted and the sentence will still make sense. Or maybe some part of the sentence was accidentally removed?

Answers: Thank you for your careful reading, and sorry for our carelessness. We have filled in the missing words.

4. The latest paragraph of “Introduction” section sounds like a combination of background, discussion and conclusions. Consider moving some of it to “Conclusion” section and/or limiting this part.

Answers: Thank you for your kindly comments. In the revised manuscript, we have made some modifications, which have partially deleted this paragraph.

5. On page 4, you can also correct “CO2” by adding bottom index for “2”. Also, delete the space between “37” and Celsius.

Answers: Thank you for your careful reading. As you suggested, we have made corrections

6. If I understand correctly, the study included four CRC cell lines while only two undergone RNA-seq. For validation, you used qRT-PCR in which step all four cell lines were again included. What is the reason for sequencing of only two cell lines and not four? I suspect the financial issues might be a case (HTS is not that cheap and you also done replicates) but just want to make sure.

Answers: Thank you. As you mentioned, the cost of sequencing was considered at the beginning of the experiment. Three samples from each group were sent for sequencing because of concerns about technical duplication. We sent two pairs of cell lines and a total of 12 samples for sequencing analysis, because we wanted to find potential research objects after analyzing the sequencing results of these two pairs of cell lines for subsequent verification.

7. On page 5 you wrote that human reference genome was UCSC hg19. Can I ask why not hg38? Were there any difficulties and/or disadvantages in using the newest reference version?

Answers: Thank you for your question. Thank you for your kind question. As for hg19 and hg38, our team has always referred to hg19 and ignored the latest updated hg38 due to habit. However, we know that hg38 added some

sequencing sites on the basis of hg19, but most of the sites can be found in both hg19 and hg38. Even so, we will refer to the latest hg38 in future research. Thank you for reminding us.

8. On the same page as above, you mentioned about Ensembl annotation file. In my opinion, GTF abbreviation should be capitalized and explained to the broader audience. The same for the explanation of "FPKM" abbreviation on the next page.

Answers: Thank you for your kindly comments. In the revised manuscript, we followed your suggestion and adopted the full acronyms in the main text and made notes in the Abbreviations.

9. In section "2.5 RNA extraction and qRT-PCR" you wrote that "the results were normalized with β-actin or U6 as a control". Actually, I see only β-actin in Supplementary Table 1. Is something missing by any chance?

Answers: Sorry for our carelessness. We only use β-actin as a control in this study, and we have made corrections.

10. In the first section of "Results", you mentioned about three biological replicates per group. I suspect you are concluding different cell stocks of the same cell line as "biological replicate". In my opinion, they are more like technical replicates. Please refer to Wales Gene Park's Tech Note about Biological vs technical replicates (https://www.walesgenepark.cardiff.ac.uk/wp-content/uploads/2020/10/WGPtech_replicates.pdf) and clarify the terminology. For example, if you would use RNA-seq on all your CRC cell lines and found some circRNAs that were differentially expressed (between respective groups within cell lines), I would say that you had four biological replicates.

Answers: Thank you for helping us point out the error. In this case, we used two pairs of cell lines, with three technical replicates in each group. We substituted biological replicate for technical replicates in the revised manuscript.

11. In the first section of "Results", there is a mention about "Additional file 2". First, I see no "Additional file 1" in the text and secondly, I have no access to any additional files (I do have access to supplementary table 1 and supplementary figures 1 and 2, though). Can you double-check that additional files are properly submitted in the system and/or referenced in the manuscript?

Answers: Thank you for helping us point out the error. We started with the supplement as a separate document and named it Additional file 1, but as followed the journal submission guidelines, we put the supplementary figures

at the end of the manuscript. We changed the original Additional file 2 into Additional file 1 and submitted it together with the revised manuscript.

12. Please double-check whether referring to subfigures fits the journal's guidelines, as this could be considered wrong in subsequent steps of manuscript processing. I think subfigures should be introduced one by one in sequence i.e. A -> B -> C -> etc. On page 7 you mentioned figure1A, then B + D, then C + E, then F. Alternatively, you can just change subfigures letters on the figure itself, and not change subfigures location.

Answers: Thank you for your kindly comments. We have made corrections in the revised manuscript.

13. The sentence "We also constructed a volcano plot to show the significant differentially expressed circRNAs (fold change > 2, and P < 0.05), and the scatter plot showed the variation in circRNA expression levels (Fig. 1C and E, and S1)" could be rephrased to avoid misleading where Fig1C and E are mentioned together with S1, while only S1 represents the scatter plot. You can change it to: "We also constructed a volcano plots (Fig. 1C and E) to depict the significant differentially expressed circRNAs (fold change > 2, and P < 0.05); the scatter plot showed the variation in circRNA expression levels (Fig. S1)".

Answers: Thank you for your comments. In order to avoid misunderstanding in language expression, we have made the modification according to your suggestion.

14. In the last sentence of section 3.1, I think you can delete "and identified potential circRNAs" at the end. Also, in the same sentence there is "crRNAs" which I believe should be "circRNAs".

Answers: Thank you for your comments. We have made corrections in the revised manuscript.

15. The first sentence of section 3.3 could be rewritten to "Focusing on the differentially expressed circRNAs from the sequencing results, we verified them using qRT-PCR in 4 pairs of CRC cell lines: HCT116, LoVo, HT29, and SW480."

Answers: Thank you for your suggestion. We think your modification could make the sentence more clear, we have followed your opinion in the revised manuscript. Thank you.

16. On page 9, there is a sentence "Combined analysis of the results from the two pairs of cell lines showed that 107 genes were upregulated in the 5-Fu resistance of the two pairs of cell lines". I think the part "5-Fu resistance of the two pairs of cell lines" could be changed to "5-Fu-resistant variants" to avoid repetition of "two pairs of cell lines".

Answers: Thank you for your comments, and we have followed your suggestion in the revised manuscript.

17. On page 11, the sentence “Moreover, the remaining circRNA, hsa_circ_0006877, also showed significantly high expression in the other three 5-Fu resistant cell lines in addition to the SW480 cell line” could be changed to “Moreover, the remaining circRNA, hsa_circ_0006877 showed significantly higher expression in 5-Fu-resistant variants of all cell lines except SW480”.

Answers: Thank you for your comments, and we have followed your suggestion in the revised manuscript.

18. Remove the space before full stop in the sentence “In our study, we performed high-throughput sequencing of two paired cell lines, and in addition to including circRNAs, we also measured mRNA expression levels .”

Answers: Thank you for your careful reading. As you suggested, we have made corrections.

19. In the last sentence of Discussion, you mentioned about “potential therapeutic targets”. Are there any therapies against e.g. FUT3 or TNS4?

Answers: Thank you for your question. Regarding these two potential targets, it is regrettable that there are currently no effective inhibitors against them in clinical practice. However, some preclinical studies have confirmed the role of the two genes in tumor genesis and development, laying a foundation for subsequent targeted therapies based on these two targets.

20. Suggestion for figure’s description: if you describe subfigures together e.g. “(C, D)”, you do not need to split them afterwards. If you do so, please do not forget to put separate marks e.g. (C) and (D) accordingly. For example, there is no “(D)” in “(B, D) Clustered heat map indicating differences in circRNA expression profiling between the HCT116 and HCT116 5-Fu resistant cell lines (B) and the LoVo and LoVo 5-Fu resistant cell lines.” which should be there based on what is in “(C, E) The volcano plot shows the expression profiling of circRNA between the HCT116 and HCT116 5-Fu resistant cell lines (C) and the LoVo and LoVo 5-Fu resistant cell lines (E)”.

Answers: Thank you for your comments, and we have followed your suggestion in the revised manuscript.

21. Figures 3 and 5 could have some short main title, not only subfigures descriptions.

Answers: Thank you for your comments. We have added the titles in Figure

3 and 5 in the revised manuscript.

22. For figure 4, is this possible to include which circ/mi/mRNA regulates the others in specific way (activation, inhibition etc.)?

Answers: For your question, we think for the mRNA part, there must be some regulation between the mRNAs. However, as for non-coding Rnas, most of the current studies show that their functional mechanism is mainly through the regulation of downstream target genes, among which circRNA's sponge effect on miRNA is one of the important ways. Therefore, we predicted and demonstrated the regulatory relationship of circRNA-mRNA-mRNA.

23. Lastly, why did you focus only on upregulated individuals? I spotted such approach when you selected five circRNAs at the very beginning (Figure 1), as well as when you found FUT3 and PLAG1 (Figure 5D)

Answers: Thank you for your question. Of course, for the selection of differentially expressed circRNA and mRNA, according to the sequencing results, there were candidate genes with high expression and candidate genes with low expression in 5-Fu-resistant cells. The expression of low expression candidate genes in drug-resistant cells is relatively low, which is not conducive to detection. Since one of the goals of our study is to find potential biomarkers, we believe that candidates with high expression would be easier to detect later.

Reviewer #3:

1. You have mentioned that this study was the first to study the potential role of circRNAs in 5-Fu resistance in CRC at the cellular level while there are some other studies

Answers: Thank you for the question. We think there may be some misunderstanding here. We mentioned in the manuscript that we for the first time used high-throughput sequencing technology on paired cells to study circRNAs in 5-Fu resistance in CRC.

2. You have mentioned detection of 17,939 cirRNA. What about some of the previously discovered CirRNA which are also involved in 5-FU resistance like (hsa_circ_0007031 and hsa_circ_0000504). Were they detected?

Answers: Thank you for the question. In this study, we adopted high-throughput sequencing technology on cell samples, and analyzed and

compared differentially expressed circRNAs in 5-Fu-resistant cells and their parents. Most circRNAs were detected, and the specific results can be seen in Additional file 1.

3. What about some important pathways and target genes that are commonly known to be associated with 5-FU resistance in colon cancer like STAT3/AKT3 signaling pathways, Bcl2, EGFR. Do they have a role with these circRNA?

Answers: Thank you for the question. In this study, we aimed to start with the differentially expressed circRNA, hoping to find the differentially expressed circRNA by comparing the 5-Fu resistant cells with the parental cells. The function and regulatory network of different circRNAs were predicted. We acknowledge these traditional drug resistance mechanisms like STAT3/AKT3 signaling pathways, Bcl2, EGFR, and do not deny their important roles, but it may not be included in the regulatory network predicted by circRNA for genetic differences only in this case.

4. What is the mechanism of FUT3 and PLAG in drug resistance?

Answers: Thank you for the question. About the FUT3, studies have shown that it is involved in the occurrence and development of a variety of cancers, including breast cancer, and can play a role by affecting glucose metabolism. However, further studies on its special 5-Fu resistance were needed.
Compared with FUT3, the mechanism of drug resistance of PLAG1 in cancer was relatively clear. PLAG1 silencing could promote cell chemosensitivity in ovarian cancer via the IGF2 signaling pathway. It has also been suggested that RNA methylation mediated inhibition of Mir-181a / 135a / 302c expression promotes the development of microsatellite unstable colorectal cancer and 5-Fu resistance by targeting PLAG1.

Reference.

1. *Albuquerque AP, Silva AL, Lima CA, Beltrão EI. FUT3 expression in human breast cancer cells under hypoxia and serum deprivation. Exp Oncol. 2019, 41(4): 318-322.*
2. *Drake RR, McDowell C, West C, et al. Defining the human kidney N-glycome in normal and cancer tissues using MALDI imaging mass spectrometry. J Mass Spectrom. 2020, 55(4): e4490.*
3. *Huang W, Li BR, Feng H. PLAG1 silencing promotes cell chemosensitivity in ovarian cancer via the IGF2 signaling pathway. Int J Mol Med. 2020, 45(3): 703-714.*
4. *Shi L, Li X, Wu Z, et al. DNA methylation-mediated repression of miR-181a/135a/302c expression promotes the microsatellite-unstable colorectal cancer development and 5-FU resistance via targeting PLAG1. J Genet Genomics. 2018, 45(4): 205-214.*

5. Wnt, mTOR, Cell adhesion are between the top 10 detected pathways. How did you test them?

Answers: Thank you for your question. As for the top 10 signaling pathways, we did not directly detect them through experiments in this study. We predicted the potential regulatory pathways of differentially expressed circRNA through sequencing results, and screened the top 10 of them according to the prediction results.

6. Revise the comments on figure 1

Answers: We have made corrections in the revised manuscript, thank you.

7. Reference No (16) is very old

Answers: Thank you for the comments. But in this part, we would like to introduce the discovery and research history of circRNA. This reference was published in Cell in 1980, which is one of the milestones of circRNA research. Therefore, we still insist on quoting this reference.