Peer Review File

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Reviewer A

The article is devoted to a current research topic. Namely, the search for epigenetic approaches to expand the understanding of the molecular pathogenesis of thyroid cancer.

Reply 1: Thank you for your comment. In our present study, we posit that a newly identified signal transduction pathway, OIP5-AS1/miR-455-3p/MFAP2, may contribute to the malignant progression of thyroid cancer. Utilizing bioinformatics, we confirmed the gene expression of key components within this signaling pathway and assessed the protein expression of MFAP2 in relevant tissues. Subsequently, we conducted a detailed analysis of the cell biological implications of the identified targets. Ultimately, our findings suggest that the long non-coding RNA OIP5-AS1 exerts a pivotal role in the progression of thyroid carcinoma by the inhibition of miR-455-3p to activate MFAP2.

Changes in the text: none

Reviewer B

The paper titled "OIP5-AS1/miR-455-3p/microfibril-associated protein 2 axis exacerbates the progression of thyroid carcinoma" is interesting. The results suggest that lncRNA OIP5-AS1 plays a crucial role in the advancement of thyroid carcinoma by inhibiting miR-455-3p to activate MFAP2. However, there are several minor issues that if addressed would significantly improve the manuscript.

1)The description of the methods "Histologic examination", "Cell proliferation, migration, invasion, and apoptosis" in this study is too simplistic, please describe in detail.

Reply 1: Thank you for your comment. The methodologies of histologic examination, cell proliferation, migration, invasion, and apoptosis were rearranged to meet your requirements.

Changes in the text: overnight at 4°C . The tissue sections underwent treatment with a biotinylated anti-rabbit secondary antibody (Thermo Fisher Scientific, MA), subsequent incubation with the streptavidin-horseradish peroxidase complex (Thermo Fisher Scientific), immersion in 3,3′-diaminobenzidine, counterstaining with 10% Mayer's hematoxylin, dehydration, and mounting. The sections were evaluated based on the proportion of positively stained tumor cells, with scores ranging from 0 (no

positive tumor cells) to 3 (>50% positive tumor cells). Staining intensity was graded on a scale from 0 (no staining) to 3 (strong staining). The staining index (SI) was determined by multiplying the staining intensity score by the proportion of positive tumor cells. We assessed expression of the MFAP2 in IHC-stained tumor sections based on the SI scores as 0, 1, 2, 3, 4, 6 and 9. Cells (5 x 10^3/mL) were seeded into the wells of 96-well plates and incubated for 24 hours prior to transfection. Following transfection for 48 h, 10 μL of CCK8 reagent was added to each well and incubated at 37°C for 1 hour. Cell viability in each well was assessed by measuring absorbance at 450 nm with a SpectraMax M5 ELISA plate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The Transwell migration (without Matrigel) assay and Matrigel invasion assay (Corning Incorporated, Corning, NY, USA) were performed for migration and invasion, respectively. Cells that had migrated and invaded the bottom surface of the membrane were immobilized in 1% paraformaldehyde, stained with hematoxylin, photographed, and quantified by enumerating the number of cells in five randomly selected fields at a magnification of 200×.

TUNEL kit (Roche, Basel, Switzerland) were used to evaluate cell apoptosis. In brief, the TUNEL assay results were observed using fluorescence microscopy (Olympus BX53, Japan), with TUNEL-positive nuclei appearing red in the fluorescence images. The apoptotic rate was determined by calculating the proportion of TUNEL-positive cells per field using Image-Pro Plus software.

2)There are many detection methods for cell proliferation, migration, invasion, and apoptosis. Why this study only uses one method? If multiple methods are used, the results may be more reliable. It is suggested to add test results of other methods.

Reply 2: Thank the reviewers for these precious comments and suggestions. We concur on the significance of employing diverse methodologies to corroborate a shared conclusion. Owing to the constraints of experimental settings, each selected experiment represents the most authoritative approach and guarantees the precision of results. The outcomes of this study align with established experimental norms and findings in existing literature, thereby enhancing the reliability of our conclusions.

Changes in the text: none

3)All figures have no scale bars. Please add relevant information.

Reply 3: Thank the reviewers for these precious comments and suggestions. Scale bars have been included in all figures.

4)The sample size of this study is too small. It is recommended to increase the sample size to increase the credibility of the study.

Reply 4: Thank the reviewers for these precious comments and suggestions. As previously mentioned, the sample size utilized in this study was limited primarily to the detection of MFAP2 expression and did not encompass an analysis of survival rates or clinical baseline data. Consequently, we contend that the sample size employed in this study is appropriate for its intended purpose.

Changes in the text: none

5)Does the ceRNA regulatory network in this study affect the radioresistance of thyroid carcinoma? What impact might it have? It is recommended to add relevant contents.

Reply 5: Thank the reviewers for these precious comments and suggestions. As is well understood, the management of tumors is multifaceted and encompassing, involving systematic chemotherapy, localized radiotherapy, targeted therapy aimed at specific proteins, and immunotherapy. Given the diverse nature of biological mechanisms, a single mechanism may impact chemotherapy, tumor stem cell, radiotherapy, and immunotherapy. Currently, our understanding is limited to elucidating the role of the OIP5-AS1/miR-455-3p/MFAP2 signaling axis in the advancement of thyroid gland pathogenesis, offering promising avenues for targeted therapeutic interventions such as small molecule compounds, antibody-drug conjugates, and small nucleic acid drugs. However, the influence of this signaling axis on radiotherapy remains unexplored. Furthermore, a cell model corresponding to radiotherapy resistance or sensitivity was not established, thus hindering our ability to elucidate its impact on radiotherapy. We value your constructive feedback and commit to exploring the significance of the OIP5-AS1/miR-455-3p/MFAP2 signaling axis in relation to radiotherapy resistance in forthcoming research endeavors.

Changes in the text: none

6)The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as “New insights into COL26A1 in thyroid carcinoma: prognostic prediction, functional characterization, immunological drug target and ceRNA network, PMID:38197076”. It is recommended to quote the article.

Reply 6: Thank you for your constructive comments. This study is deemed significant, as evidenced by the inclusion of secondary literature in the introduction section and the subsequent revision of pertinent content.

Changes in the text: This reference was added in the text as reference 14.

7)What are the problems and challenges that need to be overcome in the clinical application of lncRNA? It is recommended to add relevant content.

Reply 7: Thank you for your constructive comments. Currently, the primary constraint impeding the clinical utilization of lncRNA is the delivery technology. It is

well established that small nucleic acid drugs are extensively employed in clinical practice, with a predominant focus on intrahepatic or hepatic metabolism diseases utilizing GalNAc targeting technology (1,2). Targeting tissues outside of the liver has historically posed challenges in clinical treatment. However, advancements in scientific research, particularly in the development of lipid nanoparticle (LNP) technology, have offered promising solutions to enhance the delivery of nucleic acid drugs to these tissues despite their large molecular weight (3). Consequently, the primary barriers hindering the clinical application of lncRNA, small interfering RNA, and microRNA are predominantly attributed to limitations in delivery technology. Addressing these challenges holds the potential to significantly advance the utilization of nucleic acid drugs in clinical settings.

References

1. Ranasinghe P, Addison ML, Webb DJ. Small interfering RNA: Discovery, pharmacology and clinical development-An introductory review. Br J Pharmacol 2023;180:2697-720.

2. Won Lee J, Kyu Shim M, Kim H, et al. RNAi therapies: Expanding applications for extrahepatic diseases and overcoming delivery challenges. Adv Drug Deliv Rev 2023;201:115073.

3. Eygeris Y, Gupta M, Kim J, et al. Chemistry of Lipid Nanoparticles for RNA Delivery. Acc Chem Res 2022;55:2-12.

Changes in the text: reference 36-38

Reviewer C

1. **Please spell out the abbreviation at its first use both in the text:**

176 TUNEL kit (Roche, Basel, Switzerland) were used to evaluate cell a

Reply: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)

2. **Figure 1**

Please explain (*, **, ***, ns) in the figure legend.

ns * ** ***

Reply: * $P < 0.05$, ** $P < 0.05$, or *** $P < 0.001$ represents tumor group as compared to

normal group. ns, no significant

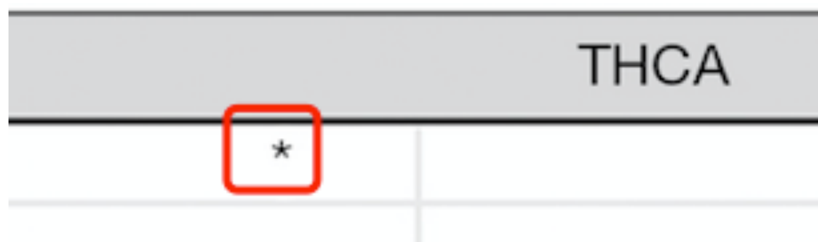
3. Figure 3

Please indicate the staining method of figure 3C in the figure legend.

Reply: transwell migration assay without matrigel by crystal violet staining

4. Figure 4

Please explain asterisk (*) in the figure legend.



Reply: Upon thorough examination of the data, the decision was made to eliminate the symbol (*) from the figure 4 to prevent any potential confusion among readers.

5. Figure 5

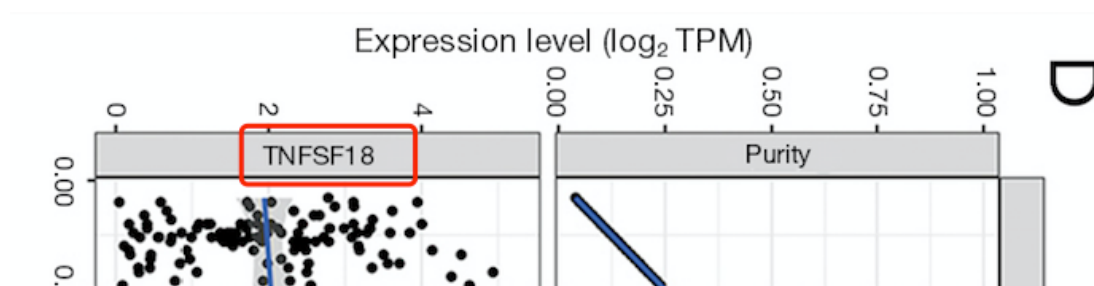
It is "TNFRSF18" in the paper, but it is "TNFSF18" in figure 5D. Please revise.

524 (B), CD40 (C), TNFRSF18 (D), TNFSF18 (E), a

251 also known as CD40) (r=0.429, P<0.001; **Figure 5C**), TNFRSF18 (r=0.444, P<0.001; **Figure 5D**),

252 TNFSF18 (r=0.300, P<0.001; **Figure 5E**), and butyrophilin 2A1 (BTN2A1) (r=0.260, P<0.001;

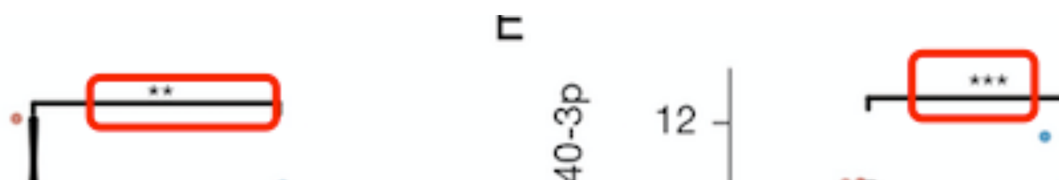
253 **Figure 5F**).



Reply: "TNFSF18" was replaced by "TNFRSF18" in figure 5D.

6. Figure 6

Please explain asterisks (*, ***) in the figure legend.



Reply: ** $P < 0.05$, or *** $P < 0.001$ represents tumor group as compared to normal

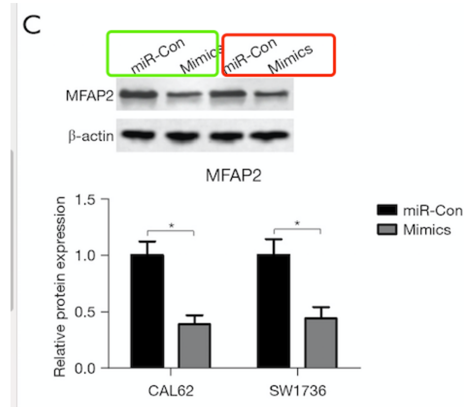
group.

7. Figure 7

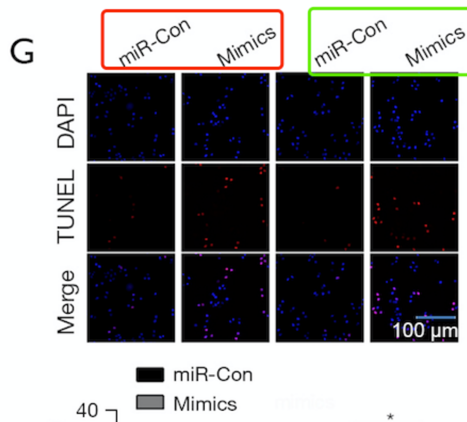
a. Please indicate the staining method of figure 7F in the figure legend.

Reply: transwell migration assay without matrigel by crystal violet staining

b. Please specify which part is for CAL62 or SW1736 cells, respectively. Please check figure 7C and 7G.



Reply: The outcomes of experiments conducted with CAL62 or SW1736 cells are depicted in Figure 7C.



Reply: The outcomes of experiments conducted with CAL62 or SW1736 cells are depicted in Figure 7G.

c. We cannot open this URL. Please check and revise.

531 Figure 7 miR-455-3p directly targets MFAP2 and inhibit

532 On-line bioinformatics software Targetscan (

533 (http://carolina.imis.athena-innovation.gr/diana) were

Reply: http://www.microrna.gr/microT-CDS

8. Figure 8

1) There seems to be no "RT-qPCR" in Figure 8, while it was explained in the legend. Please check and revise.
2) ALL abbreviations used in each figure or figure description should be defined in a

footnote below the corresponding figure. Please check all figures and provide correspondingly. Eg. RT-PCR in Figure 8

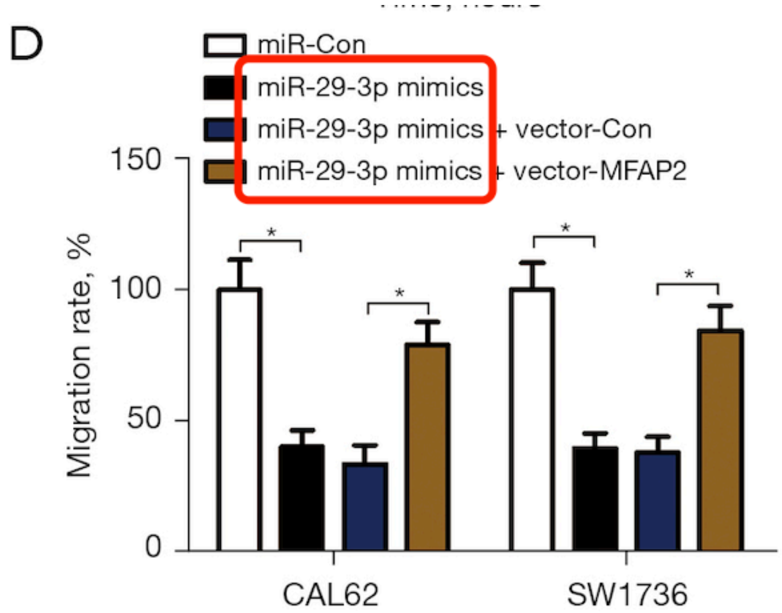
Reply: We validated the utilization of RT-PCR in Figures 8B-8F, and meticulously verified all abbreviations in Figure legends to ensure their accuracy.

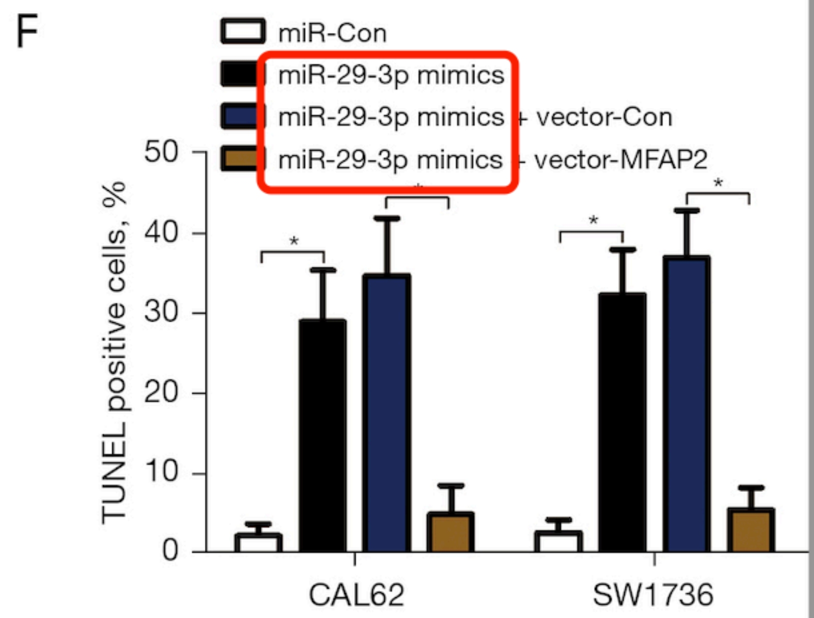
9. Figure 9

miR-455-3p in legend while miR-29-3p in Figure.

Please double check and confirm whether the legend for Figure 9 is correct.

596 Figure 9 The overexpression of MFAP2 reversed the antineoplastic activities of the sh-OIP5-AS1 and
597 miR-455-3p mimics in vitro. Cell proliferation (A), migration (C), and apoptosis (E) were assessed in
598 the CAL62 and SW1736 cells after co-transfection with the sh-OIP5-AS1 and MFAP2 overexpressed
599 plasmids using CCK-8, transwell, and TUNEL assays, respectively. Cell proliferation (B), migration
600 (D), and apoptosis (F) were detected in the CAL62 and SW1736 cells after co-transfection with the
601 miR-455-3p mimics and MFAP2 overexpressed plasmids using CCK-8, transwell, and TUNEL assays,





Reply: The legend for Figure 9 is correct, and we revised the Figure 9.