Peer Review File

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Comment 1: page 4: “However, the other functional roles of miR-3614-3p in (add here) BC migration and invasion in BC (move this up before migration) are poorly understood. Thus, we speculated whether miR-3614 affects the invasion and migration of BC by regulating the expression of the downstream gene TRIM25.”

These two sentences are confusing—should work the follow of these sentences. Authors really mean that “in a previous study, they found the role of miR-3614-3p in proliferation of BC cells through downregulation of TRIM25, but other functional roles remain unknown. They speculate the same mechanisms (downregulating TRIM25, or other target genes) may also contribute to other functions if there are. They also mentioned studies on the miR-3614-5p in colon cancer, I think they should write like, although no other studies have reported the role of miR-3614-3p in BC, the -5p of this miR gene was found to be associated with colon cancer…..

And then in the following paragraph, they reported what they found. I think they should describe what they will do (after the above reasons) in the current study in the last paragraph of the Introduction section, not to report what they found. The introduction is to introduce your research question, why (rationale) and what you will do in the current study to address the question.

Reply 1: ***Thank you for your positive advice! We have revised this part according to your comments in this revised manuscript. (See page 4, line 64-82)***

In our published study, we found that miR-3614 expression was upregulated in BC tissues, and its expression was higher in the MCF-7 and MDA-MB-231 *BC* ***cell lines. We examined the levels of mature miR-3614-3p and miR-3614-5p by qRT-PCR, the expression of miR-3614-5p was almost undetectable, likely because miR-3614-3p maturation is prevalent in breast cancer. pri-miR-3614 and TRIM25 are share the same promoter, they displayed similar expression patterns. We also found IGF2BP3, an RNA banding protein, inhibit miRNA-3614 maturation, thereby protecting TRIM25 mRNA from miR-3614-mediated degradation. The overexpression of miR-3614 significantly inhibits BC cell growth through the downregulation of TRIM25. Thus, we think miR-3614 is a tumor suppressor. The***

data from Han confirmed our opinion, they also observed miR-3614 inhibits the proliferation of colon cancer cells, and found it impaired the P53 pathway and P38MAPK pathway. Huang reported another function of miR-3614, which regulates inflammatory response via targeting TRAF6-mediated MAPKs and NF-κB signaling in coronary artery disease (CAD). Other functional roles of miR-3614 in cancer remain unknown. Thus, here we investigated whether miR-3614 overexpression affects the motility of breast cancer cells, and whether this effect achieved by regulating the expression of TRIM25. This study will complement our understanding of the function of miR-3614 and discover its potential as a therapeutic target for breast cancer.

Comment 2a: "The cells used in this study consisted of three cell lines MDA-MB-231, MCF-7, and 293T(HEK) cells": why these three cell lines were selected—representing different BC Tumor subtypes, and but why use 293T(HEK) cells (Kidney cells) (control)?

Reply 2a: ***In our previous study, we investigated proliferation function of miR3614 in ER$^+$ MCF-7 and ER$^-$ MDA-MB-231 breast cancer cells. Thus, we choose MCF-7 and MDA-MB-231 for migration and invasion function analysis in this study.***

In dual-fluorescence reporter gene experimental analysis, according to previously published literature and the instruction of kit, usually choose HEK293T for transfection and dual-fluorescence assay, because this cell has a high transfection efficiency. Thus, we choose HEK293T cell for dual-fluorescence reporter gene experimental analysis.

Comment 2b: To explore the biological significance of miR-3614-3p in BC, MCF-7 and MDA-MB-231 cells were transfected with pre-miR-3614 plasmid to overexpress miR-3614-3p: This step could also overexpress the miR-3614-5p? so how you know not the effect from -5p, although you confirmed -3p overexpressed?

Reply 2b: ***Thank you for your advice! In our previous study, we examined the levels of mature miR-3614-3p and miR-3614-5p by qRT-PCR. The expression of miR-3614-5p was almost undetectable, likely because miR-3614-3p maturation is prevalent in breast cancer. And also previously, we mainly studied the proliferation of miR-3614-3p in breast cancer, so this study focuses on the invasion and***

metastasis of miR-3614-3p in breast cancer. Nevertheless, we agree with your opinion that after transfection of miR-3614 plasmid, the level of miR-3614-3p and -5p are increased, in order to describe the experimental results more accurately, we modified the result part 1 to include miR-3614-3p changed to miR-3614.

Comment 2c: In addition, the transwell migration assay showed that overexpression of miR-3614-3p dramatically inhibited MCF-7 and MDA-MB-231 cell migration compared to control cells: specific the control cells (the two cell lines without overexpressed -3p)?—can you confirm that)

Reply 2c: ***Thank you for your positive advice! In our previous study, we examined the levels of mature miR-3614-3p and miR-3614-5p by qRT-PCR. The expression of miR-3614-5p was almost undetectable, likely because miR-3614-3p maturation is prevalent in breast cancer. we agree with your opinion that after transfection of miR-3614 plasmid, the level of miR-3614-3p and -5p are increased, in order to describe the experimental results more accurately, we modified the result part 1 to include miR-3614-3p changed to miR-3614.***

Comment 3a: Page 10" "we searched for miR-3614-3p target genes using prediction programs and found that there are (I would add this in the sentence to be clear) miR-3614-3p binding sites in the AKT3 and HDAC1 3 UTR (Fig. 4A): mentioned using TargetScan to identify target genes in the discussion—but not in the result or method section for this analysis? Another question is that there must have a large number of predicted target genes, what criteria were used to limit to two genes, AKT3 and HDAC1?

Reply 3a: ***Thank you for your suggestion! We have revised this part according to your comments in this revised manuscript. (See page 10, line 200-202). We chose AKT3 and HDAC1 because we found that they have a stronger negative correlation with miR-3614 through database analysis. And also some studies have reported that AKT3 and HDAC1 are related to cell movement in tumors.***

Comment 3b: Again, in several experiments, the 293T(HEK) cells used, explain why?

Reply 3b: ***We chose 293T(HEK) cells for the dual-fluorescence reporter gene***

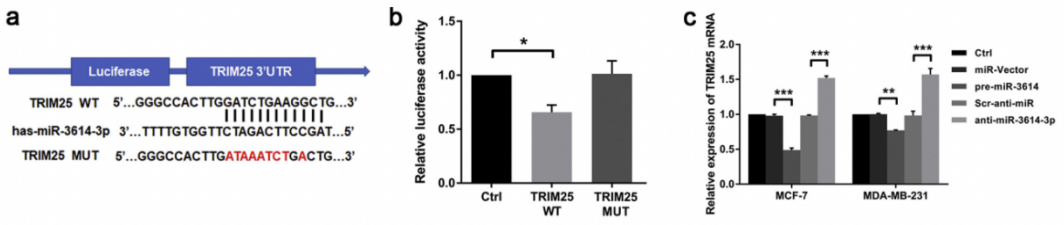
experimental analysis. The purpose is to verify the binding site of miR-3614-3p and the target gene, not to study its function. Therefore, we choose 293T(HEK) cells with high transfection efficiency.

Comment 4: In the discussion, authors listed studies showed associations of AKT3 and HDAC1 (by other miRs) with cancer, are there any studies showed miR-3614 with cancer (in addition to the -5p with colon cancer)?

Reply 4: Thank you for your advice! Through literature search, apart from what we mentioned in the introduction, we found no other studies on miR-3614 in cancer.

Comment 5: Regarding miR-3614-3p and its host gene TRIM25, Generally, miRs and its host gene—could co-expressed (same location—transcribed) in the same way —often have positive correlation in their gene expression. Authors mentioned miR-3614-3p targets TRIM25, I am wondering what the correlation in gene expression between miR-3614-3p and TRIM25 would be, may be use RNAseq and miRseq data in TCGA?

Reply 5: In our previous study, we used Targetscan and RegRNA prediction programs to identify putative miR-3614-3p binding sites in the TRIM25 transcript and found potential miR-3614-3p binding motif which is in the 3'-UTR region. To validate the theoretical relationship between miR-3614-3p and TRIM25, we constructed luciferase reporters by cloning the wildtype 3'-UTRs of TRIM25 (TRIM25-WT) or its mutant version (TRIM25-MUT) into the pmiRGLO dual-luciferase reporter vector. (Wang Z, Tong D, Han C, et al. Blockade of miR-3614 maturation by IGF2BP3 increases TRIM25 expression and promotes breast cancer cell proliferation. EBioMedicine 2019; 41: 357-369.)



Comment 6: Language may need some work:

a. Authors should read through the text, there are some typos, grammar issues or may write in a way as should be from English speakers.

b. the expression of mature miR-3614 and AKT3/HDAC1 mRNA

c. check: miR-3614-3p, not MiR-3614-3p

d. All gene name in the text should be as Italic.

<u>Reply 6:</u> Thank you for your advice! We have revised this part according to your comments in this revised manuscript.