ANSWERING REVIEWERS

June 08, 2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 17517-review.doc).

Title: miR-30b suppresses tumor migration and invasion by targeting EIF5A2 in gastric cancer

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Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 17517

The manuscript has been improved according to the suggestions of reviewers:
1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer

Reviewer 1

(1) There have been several reports about the effect of miR-30b in gastric cancer cells. The authors found some genes that regulated by miR-30b were different from EIF5A2. Author should explain this problem.

Response: Thanks for your kind review. A multiple-to-multipe relationship exits between miRNAs and their targets. In gastric cancer, there is only one study described the targeted gene of miR-30b, which found that plasminogen activator inhibitor-1(PAI-1) was the potential target of miR-30b. Now in our research, EIF5A2 was identified as the target of miR-30b. So, more targets of miR-30b should be validated to investigate its function in gastric cancer.

(2) The research only detected the E-cadherin and Vimentin expression, which have effects on invasion and migration. Further molecular mechanism research should be applied (such as expression and function of MMPs and TIMPs).

Response: Thanks for your kind review. We found that epithelial markers E-cadherin and β-catenin expression dramatically increased in AGS and MGC803 cells infected with miR-30b, whereas silencing miR-30b suppressed E-cadherin and β-catenin expression.

In the following studies, we also detected the MMP-9 and TIMP-1 protein expression using Western blot. The results showed that no change was observed of MMP-9 and TIMP-1 level after transfection of miR-30b mimics or inhibitor. These results indicated that miR-30b inhibit gastric cancer cell invasion and migration not through the MMP-9 or TIMP-1.

(3) Author claimed that miR-30b could inhibit EIF5A2, and expression of E-cadherin and Vimentin changed simultaneously. However, author should expain which molecule(miR-30b or EIF5A2) regulated E-cadherin and Vimentin directly. And the mechanism also should be explored.

Response: Luciferase reportor asssys showed that EIF5A2 was the target gene, and overexpression miR-30b could low EIF5A2 protein expression. However, there was not binding site between miR-30b and E-cadherin and Vimentin through bioinformatic analysis. In our previous study, we also found that knockdown of EIF5A2 using siRNA could upregulate E-cadherin levels and downregulate vimentin expression. So, it was EIF5A2 that regulated E-cadherin and Vimentin directly.

(4) The first 2 paragraphs in 'Discussion' section were apart from significance of this research, so author should reorganize or delete them.

Response: These changes were made in the text as requested.

(5) There are some English writing defects in present paper.

Response: Further English language and style changes were made as requested.

Reviewer 2

Tian and colleagues have described a putative role of miR-30b in regulating EIFA2 expression and function. The article is well organized and well written other than some minor English language errors. The methods used are well studied and appropriate for the experimental design. The major concern of this reviewer is the lack of convincing functional studies to prove the author's conclusion. With appropriate revision this manuscript could be acceptable and relevant to the journal readership. I would like the authors to address the following issues;

Response: Thank you for your kind review.

(1) The authors should discuss and present data for relative levels of miR-30b overexpression. High overexpression of multiple miRNA can induce apoptosis, but the levels used in some experiments have not been observed endogenously.

Response: In the function test, we used miR-30b mimics of 50nM to verify the effect on proliferation, apoptosis, invasion and migration. Many miRNA could induce cell apoptosis, we used miR-30b mimics designed to mimic endogenous mature miRNA molecules when transfected into cells. We also detected miR-30b expression by transfection of miR-30b mimics in gastric cancer cells, the results of Real-time PCR showed that miR-30b was significantly overexpression(data not shown) compared with the control. So overexpression miR-30b induced cancer cell apoptosis after transfection.

(2) There is not enough evidence to support the statement in the discussion that miR-30b expression could serve as a biomarker in gastric cancer. If the authors presented to patient outcomes for high and low miR-30b expressing samples, then conclusions about biomarkers would be more feasible

Response: We detected the miR-30b expression in another 12 pairs gastric cancer tissues. The relationship between clinicopathological factors and the expression of miR-30b showed that only lymph node metastasis was associated with low miR-30 expression. Because the limited number of samples, and a shorter follow up time, we cannot do survival analysis. As you mentioned, we are not able to draw the conclusion that miR-30b could serve as a biomarker in gastric cancer. Some changes were made in the text.

(3) The effectiveness of the miR-30b knowckdown is not well depicted.

Response: The effectiveness of the miR-30b knockdown is detected using western blot in protein level. As show in figure 4, after transfection with miR-30b mimics, EIF5A2 protein expression was significantly reduced in AGS and MGC803 cells, indicating the knockdown effectiveness of miR-30b is high.

(4) The western blots in figure 4E do not clearly demonstrate the increase in E-cadherin that is discussed by the authors. I think it would be helpful to perform an EIF5A2 knockdown with siRNA or alternate method and then blot for Vimentin and E-cadherin after effective EIF5A2 knockdown. This would also help with the mechanism suggested by the authors. A quantitative analysis of the protein level in the western blots would also add further, and the rightmost lane in figure 4E using the MGC803 cell line seem to have a larger actin band.

Response: Thanks for your review. In our previous research, we found that E-cadherin levels were upregulated and vimentin was downregulated after the knockdown of EIF5A2 in MKN28 and HGC27 cells (Discussion and Reference 30 and 31). So EIF5A2 was the key modulator in regulating cell metastasis. Also, we detected the changes of these markers by transfecting miR-30b inhibitor into AGS

and MGC 803. Instead, we found that EIF5A2 was upregulated after transfection with miR-30b inhibitor compared with the control.

(5) In figure 1A the labeling perhaps should be changed. It appears that only the "combination" is significant to $p<0.05$, but I assume this is the average as several of the other samples 2, 5,6,7 must have significant differences in miR-30b

Response: Thanks for your constructive comments. To investigate the miR-30b expression in gastric cancer, we dected another 12 pairs primary cases. The results of Real-time PCR of a total of 23 cases revealed that miR-30b was down-regulated in gastric cancer tissues than that in adjacent non-tumor tissues (P=0.0016, Figure 1A). The miR-30b expression was significantly lower in gastric cancer cases with lymph node metastasis (P=0.021). No association was found between miR-30b expression and age, gender and Lauren type (Table 1).

(6) The authors should show the effect of other miR-30b targets, this would add to the paper.

Response: Thanks for your review. A multiple-to-multipe relationship exits between miRNAs and their targets. In gastric cancer, there is only one study described the targeted gene of miR-30b, which found that plasminogen activator inhibitor-1(PAI-1) was the potential target of miR-30b. MiR-30b regulated the apoptosis of gastric cancer cells by targeting PAI-1. Now in our research, EIF5A2 was identified as the target of miR-30b, which could enhance E-cadherin expression and eventually inhibit the EMT process in gastric cancer cells. So, more targets of miR-30b should be validated to investigate its function in gastric cancer.

Reviewer 3

(1) The author present that miR-30b can suppress proliferation, migration and invasion in gastric cancer. However, there are only 11 tissue samples enrolled for the expression assay in this study, which suggested the expression of miR-30b was differential between normal and cancer tissues. It will be more convincible that the authors enrolled more samples and perform the analysis on the relationship between expression of miR-30b with the metastasis in tissues.

Response: Thanks for your review. We detected the miR-30b expression in another 12 pairs gastric cancer tissues. MiR-30b expression was significantly lower in gastric cancer tissues (P=0.0016 ; Figure 1A). After that, we associated miR-30b expression with the conresponding clinicopathlogical data from the gastric caner patients. We observed that loss expression of miR-30b was correlated to tumor lymph nodes metastasis (P=0.021). However, there was no association between miR-30b expression and patient's age, gender and lauren type (Table 1).

(2) The expression of E-cadherin and Vimentin suggested the expression of miR-30b may responsible for the EMT of gastric cell line. But only these two proteins were not sufficient to cover EMT. It will be better to perform more experiments on MMPs, FN and Cytoskeleten to make the results more convincible.

Response: We detected the other molecular markers of EMT using Western blot in protein level. We examined the expression of the epithelial markers E-cadherin and β-catenin, as well as the mesenchymal marker vimentin. As show in Figure 4, we found that E-cadherin and β-catenin expression dramatically increased in AGS and MGC803 cells infected with miR-30b mimics, whereas silencing miR-30b suppressed E-cadherin and β-catenin expression, and induced vimentin expression in cance cells.

In additon, transfection with miR-30b mimics or inhibitor had no effect on the expression of MMP-9 and TIMP-1. These results indicated that miR-30b supress cancer cell metastasis was was via downregulation of EIF5A2.

(3) In Figure 2, the gate of the FCM seems not accurate, which separate one population into two. Please reset the gate and support more information to identify the conclusion of apoptosis such as expression of protein BCL-2 and BAX.

Response: Thanks for your review. We set a gate for viable cells on the FSC vs SSC dot plot. Early apoptosis cells with intact membranes showed Annexin V postive and Propidium Iodide (PI) negative. Viable cells often showed Annexin V and PI negative. End stage apoptosis and death cells showed both membrane staining by Annexin V and strong nuclear staining from the PI. Apoptosis is a normal continuous process, cells could be in the different stages of apoptosis (early, middle and late stage). So it may show two population in the gate of the FCM.

Because of the limited modification time, we cannot accomplish the work of detection of BCL-2 and BAX in protein level.

(4) Transfect the inhibitors of miR-30b into the cell lines can promote the cell migration and invasion in transwell assay. But in the EMT assay, the authors only showed the overexpression of miR-30b (transfected mimics). If the authors can present the results of Western blot of EIF5A2, E-cadherin and Vimentin in inhibitors transfected cell lines, which will be helpful to prove the mechanism of miR-30b/EIF5A2 pathway in EMT of gastric cell line.

Response: According to review opinions, we detected the EMT related marker after transfection with miR-30b inhibitor in gastric cancer cells. The results showed that downregulated expression miR-30b could increase the protein levels of EIF5A2 and vimentin, attenuate the expression of E-cadherin and β-catenin protein levels. Theses results suggest that miR-30b enhances E-cadherin expression by targeting EIF5A2 and eventually inhibit the EMT process in gastric cancer cells.

Table 1 The relationship between clinicopathological parameters and miR-30b expression

Clinicopathologic parameters	Number of cases	$2^{-\Delta\Delta CT}$(mean)	P value
Age (years)			0.621
≥60	8	0.2078±0.0285	
<60	15	0.2165±0.0143	
Gender			0.427
Male	16	0.1951±0.0198	
Female	7	0.2083±0.0239	
Lauren type			0.371
Intestinal type	11	0.2148±0.0316	
Diffuse type	12	0.1932±0.0257	
Lymph node metastasis			0.021
No	9	0.2693±0.0381	
Yes	14	0.1651±0.0259	

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,

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