

Point-to point response to the reviewer's comments

Peer-Review Report:

Reviewer #1:

The manuscript is well written and the topics is interesting and timely, however, several criticisms should be addressed as below.

Author's response: We appreciate the reviewer's favorable comments.

Major:

The miRNAs of 3168, 223-3p, 455-5p, 30c-5p, 584-5p, 30c-5p, 106-3p, 628-3p, 941 do not play a role as biomarkers for HCC development diagnosis. The impact for readers of this submitted manuscript is not so high, however, several important and new evidences are reported, and the manuscript will be acceptable.

Author's response: Thank you for the reviewer's favorable comment and valuable question. Indeed, the miRNAs mentioned in this study were previously found to play a role as biomarkers for diagnosis of the development of HCC. However, several studies have shown that a some miRNAs, including miR-223-3p (PMID: 29467847; PMID: 32330203), miR-455-5p (PMID: 31732382), miR-584-5p (PMID: 31044566; PMID: 32522621), miR-584-5p (PMID: 31044566; PMID: 32522621), miR-628-3p (PMID: 31956442), and miR-941 (PMID: 27755585) were associated with the development of HCC, suggesting that these miRNAs merit further study as biomarkers for the diagnosis of HCC.

Minor:

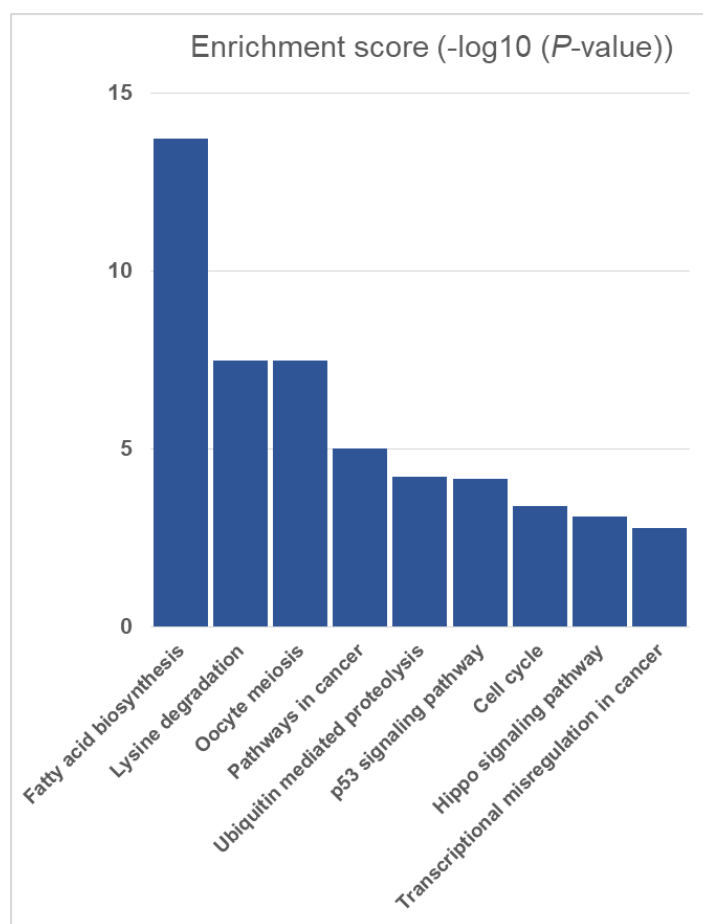
1. I think the functional disorders of each miRNAs may cause the carcinogenesis and development of HCCs. what functions?, what genes? Why thease miRNAs were selected in your analysis?

Author's response: We thank the reviewer for the critical comment and questions.

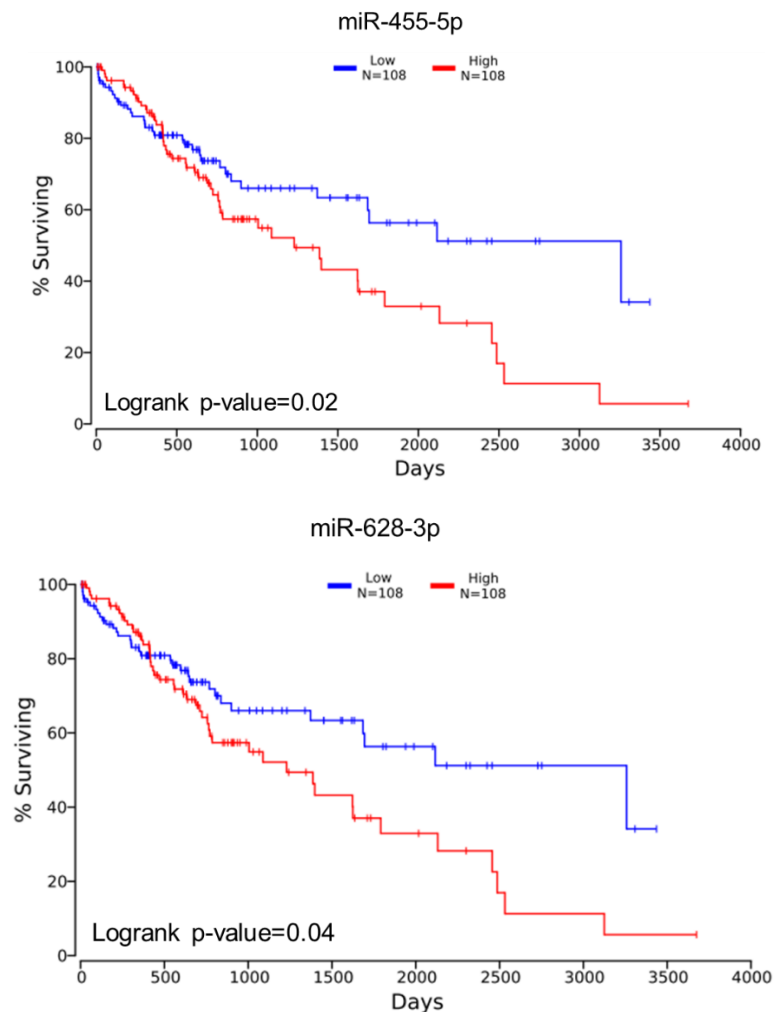
1) what functions?

Author's response: To evaluate the potential functions of miRNAs, the target genes of the eight

miRNAs mentioned in this study were summarized in the pathway analysis. The analyses revealed that the target genes were significantly enriched in many pathways related to tumorigenesis and progression processes, such as fatty acid biosynthesis, p53 signaling pathway, and the hippo signalling pathway (Response Figure 1). The high level of expression of hsa-miR-455-5p and the low level of expression of miR-628-3p in tumor tissues from the TCGA dataset showed better overall survival than the two miRNAs downregulated or upregulated in the tumor tissues (Response Figure 2). The functional enrichment analysis showed the eight biomarkers involved in the tumorigenesis and progress of HCC cancer.



Response Figure 1. Top ten KEGG pathways. To evaluate the potential function of eight miRNAs, the eight-miRNA target genes were summarized in the pathway analysis. Biological process of the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways enrichment of the experimentally validated targets of miRNAs was performed using mirPath v.3 which provided the Expression Analysis Systematic Explorer (EASE) score and false discovery rates using the Fisher's exact tests and unbiased empirical distributions (PMID: 25977294).



Response Figure 2. Survival curves of the samples with different expression levels of miR-628-3p and hsa-miR-455-5p using data from the TCGA database (top 30% versus bottom 30%). The Kaplan-Meier plot analysis of The Cancer Genome Atlas (TCGA) data was performed using OncoLnc (DOI:10.7287/peerj.preprints.1780v1).

2) what genes?

Author's response: To explore predict the target genes of eight miRNAs, miRPath v3.0 (<http://www.microrna.gr/miRPathv3>) an online software was used (PMID: 25977294). The results showed that some target genes of eight miRNAs are belong to tumor-related regulatory genes, such as *ARID2*, *KMT2C*, *IGF1R*, *PARP1*, *IRS2*, *SMAD2*, *APC*, *KRAS*, *AR*, *IDH1*, *HDAC1*, *ESR1*, *BRAF*, and so on (Response Table 1).

Response Table 1. Cancer-related target genes of eight miRNAs predicted using miRPath v3.0.

miRNAs	cancer-related target genes	miRNAs	cancer-related target genes
hsa-miR-3168	PTPRD	hsa-miR-584-5p	NBN
hsa-miR-3168	MGA	hsa-miR-584-5p	AFF4
hsa-miR-3168	SF3B1	hsa-miR-584-5p	INPP4B
hsa-miR-3168	EPHA7	hsa-miR-584-5p	KRAS
hsa-miR-3168	ARID2	hsa-miR-584-5p	PHF6
hsa-miR-3168	NFIB	hsa-miR-584-5p	MPLKIP
hsa-miR-3168	REV3L	hsa-miR-584-5p	ATRAX
hsa-miR-3168	BMPRI1A	hsa-miR-584-5p	NCOR1
hsa-miR-3168	MYCN	hsa-miR-584-5p	RHOH
hsa-miR-3168	KMT2C	hsa-miR-584-5p	H3F3A
hsa-miR-3168	RAD9B	hsa-miR-584-5p	USP8
hsa-miR-3168	FAM135B	hsa-miR-584-5p	REV3L
hsa-miR-223-3p	ACSL3	hsa-miR-584-5p	HOXA11
hsa-miR-223-3p	ELK4	hsa-miR-628-3p	KRAS
hsa-miR-223-3p	FBXW7	hsa-miR-628-3p	EPHA7
hsa-miR-223-3p	HMGA2	hsa-miR-628-3p	AR
hsa-miR-223-3p	NF2	hsa-miR-628-3p	EPHA3
hsa-miR-223-3p	SPRED1	hsa-miR-628-3p	AFF3
hsa-miR-223-3p	PAX5	hsa-miR-628-3p	TSHR
hsa-miR-223-3p	IGF1R	hsa-miR-628-3p	ATRAX
hsa-miR-223-3p	ZFHX3	hsa-miR-628-3p	BTK
hsa-miR-223-3p	CBFB	hsa-miR-628-3p	PPP4R1
hsa-miR-223-3p	XPA	hsa-miR-628-3p	GNAQ
hsa-miR-223-3p	PRDM1	hsa-miR-628-3p	PCDH9
hsa-miR-223-3p	FOXP1	hsa-miR-941	PRDM16
hsa-miR-223-3p	CBLB	hsa-miR-941	FAM135B
hsa-miR-223-3p	RASA1	hsa-miR-106a-3p	ESR1
hsa-miR-223-3p	UBE2A	hsa-miR-106a-3p	CAMTA1
hsa-miR-223-3p	EPHA3	hsa-miR-106a-3p	BRAF
hsa-miR-223-3p	GLI3	hsa-miR-106a-3p	NTRK2
hsa-miR-223-3p	ARID1A	hsa-miR-106a-3p	STAT3
hsa-miR-223-3p	ACVR2A	hsa-miR-106a-3p	PDGFRA
hsa-miR-223-3p	ERC1	hsa-miR-106a-3p	EXT1
hsa-miR-223-3p	PARP1	hsa-miR-106a-3p	SMAD2
hsa-miR-223-3p	IL6ST	hsa-miR-106a-3p	TET1
hsa-miR-223-3p	FAT1	hsa-miR-106a-3p	ELK4
hsa-miR-223-3p	XPO1	hsa-miR-106a-3p	TRIM37
hsa-miR-223-3p	KMT2C	hsa-miR-106a-3p	RUNX1
hsa-miR-223-3p	RPS6KB1	hsa-miR-106a-3p	HMGA2
hsa-miR-455-5p	SUZ12	hsa-miR-106a-3p	THBS2
hsa-miR-455-5p	RPS6KB1	hsa-miR-106a-3p	HOOK3
hsa-miR-30c-5p	CAMTA1	hsa-miR-106a-3p	MCL1

hsa-miR-30c-5p	IRS2	hsa-miR-106a-3p	CCND2
hsa-miR-30c-5p	PICALM	hsa-miR-106a-3p	SPRED1
hsa-miR-30c-5p	RAD23B	hsa-miR-106a-3p	FRS2
hsa-miR-30c-5p	SMAD2	hsa-miR-106a-3p	CD274
hsa-miR-30c-5p	SH2B3	hsa-miR-106a-3p	PTPRD
hsa-miR-30c-5p	TET1	hsa-miR-106a-3p	SMAD3
hsa-miR-30c-5p	APC	hsa-miR-106a-3p	FRK
hsa-miR-30c-5p	TRIM37	hsa-miR-106a-3p	EBF1
hsa-miR-30c-5p	RUNX1	hsa-miR-106a-3p	SF3B1
hsa-miR-30c-5p	HOOK3	hsa-miR-106a-3p	BCL2
hsa-miR-30c-5p	PPP4R4	hsa-miR-106a-3p	PPP6C
hsa-miR-30c-5p	GNA13	hsa-miR-106a-3p	FGF4
hsa-miR-30c-5p	REV1	hsa-miR-106a-3p	NOTCH2
hsa-miR-30c-5p	FRK	hsa-miR-106a-3p	MAP3K1
hsa-miR-30c-5p	NUP93	hsa-miR-106a-3p	EPAS1
hsa-miR-30c-5p	BCL10	hsa-miR-106a-3p	EPHA7
hsa-miR-30c-5p	AFF4	hsa-miR-106a-3p	FAT4
hsa-miR-30c-5p	STAG2	hsa-miR-106a-3p	NCOA3
hsa-miR-30c-5p	IGF1R	hsa-miR-106a-3p	PHF6
hsa-miR-30c-5p	EED	hsa-miR-106a-3p	PPP4R2
hsa-miR-30c-5p	KRAS	hsa-miR-106a-3p	DPYD
hsa-miR-30c-5p	TDG	hsa-miR-106a-3p	NPM1
hsa-miR-30c-5p	ACVR1	hsa-miR-106a-3p	FOXP1
hsa-miR-30c-5p	CBFB	hsa-miR-106a-3p	ZNF217
hsa-miR-30c-5p	PRDM1	hsa-miR-106a-3p	RASA1
hsa-miR-30c-5p	PTPN13	hsa-miR-106a-3p	FOXL2
hsa-miR-30c-5p	CBLB	hsa-miR-106a-3p	RIF1
hsa-miR-30c-5p	RASA1	hsa-miR-106a-3p	USP6
hsa-miR-30c-5p	AR	hsa-miR-106a-3p	CCND1
hsa-miR-30c-5p	NCOR2	hsa-miR-106a-3p	SMAD4
hsa-miR-30c-5p	PIK3CD	hsa-miR-106a-3p	SRGAP3
hsa-miR-30c-5p	SPEN	hsa-miR-106a-3p	PPP2R2A
hsa-miR-30c-5p	SRGAP3	hsa-miR-106a-3p	PBRM1
hsa-miR-30c-5p	SOX9	hsa-miR-106a-3p	FLT1
hsa-miR-30c-5p	SOCS1	hsa-miR-106a-3p	SOS1
hsa-miR-30c-5p	PBRM1	hsa-miR-106a-3p	ACVR2A
hsa-miR-30c-5p	NRG3	hsa-miR-106a-3p	DDX5
hsa-miR-30c-5p	LIFR	hsa-miR-106a-3p	SHOC2
hsa-miR-30c-5p	ARID1A	hsa-miR-106a-3p	SHPRH
hsa-miR-30c-5p	BAZ1A	hsa-miR-106a-3p	MDM4
hsa-miR-30c-5p	SOS1	hsa-miR-106a-3p	ERC1
hsa-miR-30c-5p	IDH1	hsa-miR-106a-3p	PRKAR1A
hsa-miR-30c-5p	DDX5	hsa-miR-106a-3p	CASP8
hsa-miR-30c-5p	SHOC2	hsa-miR-106a-3p	NFIB

hsa-miR-30c-5p	DNMT3A	hsa-miR-106a-3p	REV3L
hsa-miR-30c-5p	RANBP2	hsa-miR-106a-3p	CDK12
hsa-miR-30c-5p	BCL6	hsa-miR-106a-3p	POLQ
hsa-miR-30c-5p	UBE2V2	hsa-miR-106a-3p	IL6ST
hsa-miR-30c-5p	GNAQ	hsa-miR-106a-3p	FUBP1
hsa-miR-30c-5p	NFIB	hsa-miR-106a-3p	KMT2A
hsa-miR-30c-5p	REV3L	hsa-miR-106a-3p	MYCN
hsa-miR-30c-5p	CDK12	hsa-miR-106a-3p	PDPK1
hsa-miR-30c-5p	KMT2A	hsa-miR-106a-3p	BCL11B
hsa-miR-30c-5p	ELL	hsa-miR-106a-3p	PDCD1LG2
hsa-miR-30c-5p	HNRNPA2B1	hsa-miR-106a-3p	TMEM127
hsa-miR-30c-5p	PER2	hsa-miR-106a-3p	PTPRT
hsa-miR-30c-5p	BCL11B	hsa-miR-106a-3p	UBE2B
hsa-miR-30c-5p	BCOR	hsa-miR-106a-3p	MAPK1
hsa-miR-30c-5p	HOXA11	hsa-miR-106a-3p	RAD21
hsa-miR-30c-5p	XPO1	hsa-miR-106a-3p	TET2
hsa-miR-30c-5p	AMER1	hsa-miR-106a-3p	KMT2C
hsa-miR-30c-5p	GRIN2A	hsa-miR-106a-3p	LRP1B
hsa-miR-30c-5p	KMT2C	hsa-miR-106a-3p	TGFBR2
hsa-miR-30c-5p	TBL1XR1	hsa-miR-106a-3p	FAM135B
hsa-miR-30c-5p	ABL1	hsa-miR-106a-3p	ARHGAP5
hsa-miR-30c-5p	PAX3	hsa-miR-106a-3p	JAK1
hsa-miR-584-5p	HLTF	hsa-miR-106a-3p	ERBB4
hsa-miR-584-5p	HDAC1	hsa-miR-106a-3p	EREG

3) Why these miRNAs were selected in your analysis?

Author's response: Since blood exosomal miRNAs might derive from various sources, normalization of all miRNAs with the same size factor (or the same housekeeping miRNA) might not reveal the real difference of each miRNA between HCC and non-HCC patients. Similar to the normalization concept of qPCR, the quotient between a pair of miRNAs, where one is considered the target gene and the other one is considered the reference gene, was calculated. This exhaustive method was used to find the best reference gene to normalize each target gene, and their quotients were considered the candidate biomarkers. To avoid potential batch effects, the patients were allocated to two cohorts according to their experimental date. The accuracy of each pair of miRNAs were measured by area under the curve (AUC) of receiver operating characteristic (ROC). The pairs of miRNAs were ranked according to the average AUC between two cohorts, where the average AUC should be larger than 0.7 (Fig. 4a). The top two pairs of miRNAs were miR-628-3p/miR-941 and miR-584-5/miR-106b-3p, and their average AUCs were 0.7423 and 0.7421, respectively.

2. I think alcohol excessive intake may cause increased amount of miR-3168 and miR-223-3p, and no relationship between these miRNAs and HCC development/carcinogenesis was shown. Please clarify it. (Figure 2.CD).

Author's response: Thanks a lot for the reviewer's comments and questions. Indeed, daily alcohol consumption (excessive alcohol intake) may cause increased amount of miR-3168 and miR-223-3p in HBV positive non-HCC patients as shown in Figure 2C and 2D. However, daily alcohol consumption may cause the different trends in miR-3168 and miR-223-3p in HBV positive HCC patients. These results were not intended to illustrate the relationship between these miRNAs and HCC development/carcinogenesis, but to indicate that daily alcohol consumption could alter the blood exosomal miRNA profiles of HBV positive non-HCC and HCC patients, increasing the difficulty of distinguishing between non-HCC and HCC patients.

3. Figure 1. Please correct the explanation of the red and green deposits. Change "Cancer" into "HCC".

Author's response: We thank the reviewer for the important remind in time. We have corrected the explanation of the red and green deposits in Figure 1.

4. Table 1. Drinking history: please add the clear definition of No daily drinking and Daily drinking.

Author's response: We thank the reviewer for the important remind. We defined daily alcohol consumption under 12 g of alcohol as "No daily drinking." We defined daily drinking greater than or equal to 12 g of alcohol as "Daily drinking." We have added the description to the materials and methods section of the revised manuscript.