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RAPID COMMUNICATION

A novel microRNA miR-MTCO3P38 inhibits malignant progression via STAT3/PTTG1/MYC in hepatocellular carcinoma

To the Editor,

Hepatocellular carcinoma (HCC) is a malignant tumor with the fourth highest incidence and the third highest mortality among all tumor sites in China.[1] Finding new diagnostic markers and therapeutic molecules may be of great value in the treatment of HCC and the improvement of prognosis. MicroRNA (miRNA) is a type of short, approximately 18～22 nucleotides (nt) long, single-stranded noncoding RNA. Increasing evidence indicates microRNAs are involved in various cellular and biological processes of HCC.[2] However, a complete picture of the miRNA targetome, which defines the number of oncogenes or tumor suppressors targeted by a particular miRNA, has yet to be generated. To investigate novel differentially expressed miRNAs in HCC, we performed small RNA deep sequencing analysis in 15 liver tissue samples and validated a novel miRNA (tentatively named miR-MTCO3P38), which derived from 5′-end of the pseudogene *MTCO3P38*. MiR-MTCO3P38 was downregulated in HCC tissues and significantly related to the overall survival (OS) of HCC patients. Further study showed miR-MTCO3P38 inhibits malignant progression via STAT3/PTTG1/MYC in hepatocellular carcinoma.

To identify differentially expressed known and novel miRNAs in HCC tissues and paired adjacent non-tumor tissues, 15 human liver tissues (3 normal liver tissues, 6 HCC tissues and 6 para-tumor tissues) were collected for small RNA deep sequencing. A total of 526 unique reads were annotated as novel miRNAs. Among them, 25 unique reads were counted with reads number $>$ 100 (Table S1). An unannotated new miRNA (5′ - uaggagggcugagagggc - 3′) was consistently downregulated in HCC tissues compared with para-tumor tissues and normal liver tissues (Fig. 1A, B and Table S2). The host gene of this novel miRNA was validated, then pseudogene *MTCO3P38* was identified (Fig. S1A). Therefore, we tentatively named this miRNA miR-MTCO3P38. The pre-miRNA secondary structure of miR-MTCO3P38 was calculated and a stable stem-loop structure was predicted (Fig. S1A). Meanwhile, the base bias was evaluated and showed in Figure S1B and C. Northern blot assay proved that miR-MTCO3P38 was derived from host gene *MTCO3P38* (Fig. 1C).

MiR-MTCO3P38 was significantly downregulated in HCC tissues and liver cancer cell lines compared to para-tumor tissues or cell line L02 (Fig. 1D, S2A). Patients of higher miR-MTCO3P38 expression in HCC tissues experienced longer OS than those of lower miR-MTCO3P38 expression (Fig. 1E). Furthermore, multivariate analysis suggested that miR-MTCO3P38 expression was a predictive factor of OS time (Fig. S2B).

To explore its role in the development of HCC, miRNA mimic and inhibitor were separately used in liver cancer cell lines. Colony formation and CCK8 assays showed that the proliferative capacity of tumor cells was decreased by miR-MTCO3P38, but increased by inhibitor (Fig. 1F, S3A, B). Scratch wound-healing experiments and Transwell assay showed the migration ability and invasion ability were severely impaired in cells transfected with miR-MTCO3P38 mimics, while cells treated with inhibitor showed the opposite phenomenon (Fig. S3C–H). A lung metastasis model by tail vein injection and subcutaneous tumor xenografts were used to determine the impact of miR-MTCO3P38 separately in HCC *in vitro*. In the groups of overexpressing miR-MTCO3P38, less lung metastases in the metastasis models, less tumor volume and tumor weight in subcutaneous tumor xenografts were observed (Fig. 1G, H, S3I, J).

To explore the molecular mechanism, transcriptome sequencing of cells treated with miR-MTCO3P38 mimic and NC mimic were performed (Table S3). Gene set enrichment analysis (GSEA) revealed Myc target pathway (Fig. 1I), which has been reported relating to the angiogenesis, tumorigenesis and metastasis of liver cancer,[3]

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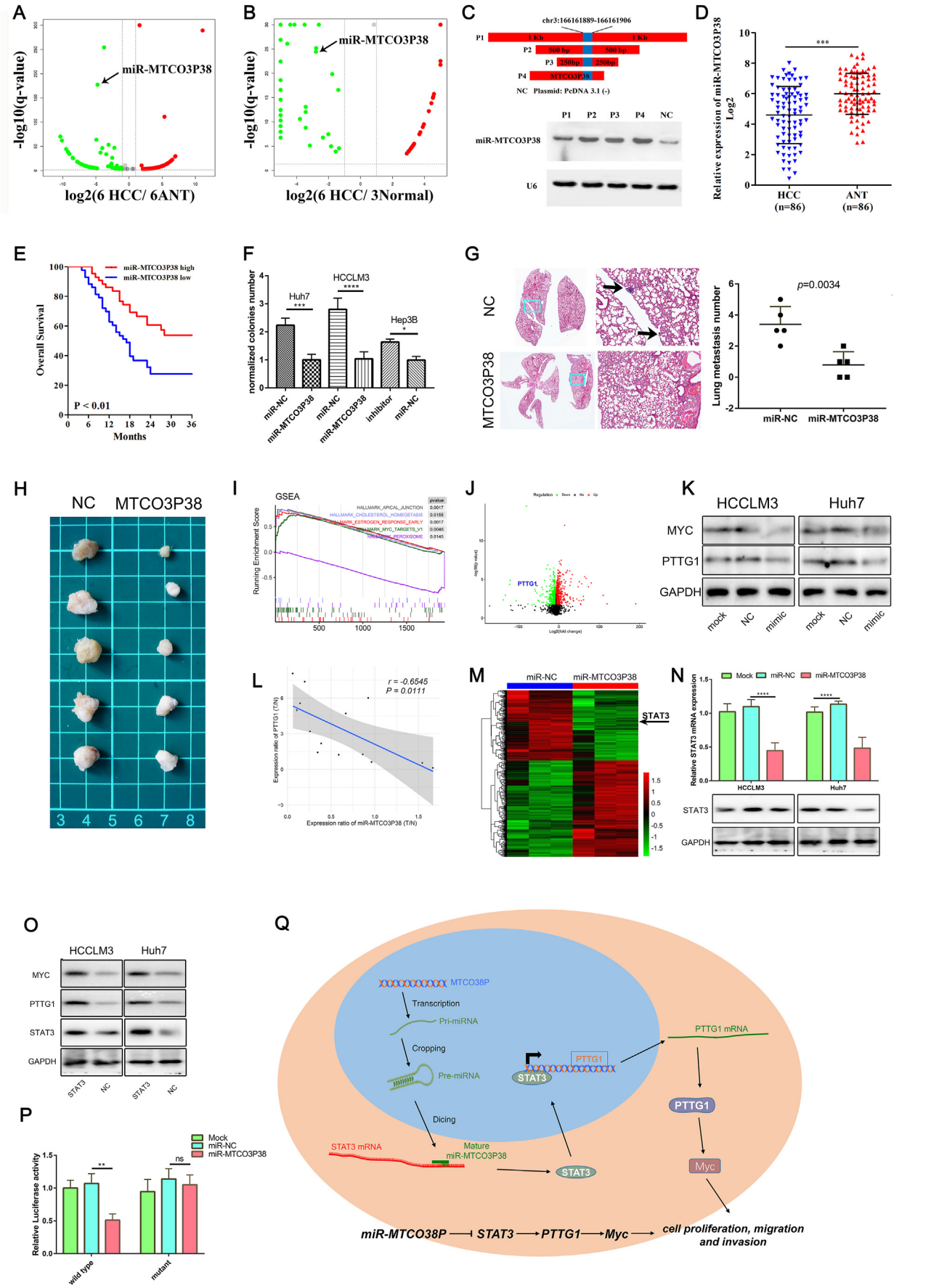
A
-log10(q-value)
miR-MTCO3P38
log2(6 HCC/ 6ANT)
B
-log10(q-value)
miR-MTCO3P38
log2(6 HCC/ 3Normal)
C
chr3:166161889-166161906
P1
P2
P3
P4
NC
Plasmid: PcDNA 3.1 (-)
miR-MTCO3P38
U6
D
Relative expression of miR-MTCO3P38 Log2
***
HCC (n=86)
ANT (n=86)
E
miR-MTCO3P38 high
miR-MTCO3P38 low
Overall Survival
P < 0.01
Months
F
normalized colonies number
Huh7
HCCLM3
Hep3B
miR-NC
miR-MTCO3P38
inhibitor
G
NC
MTCO3P38
p=0.0034
Lung metastasis number
miR-NC
miR-MTCO3P38
H
NC
MTCO3P38
I
GSEA
Running Enrichment Score
J
PTTG1
K
HCCLM3
Huh7
MYC
PTTG1
GAPDH
mock
NC
mimic
L
r = -0.6545
P = 0.0111
Expression ratio of PTTG1 (T/N)
Expression ratio of miR-MTCO3P38 (T/N)
M
miR-NC
miR-MTCO3P38
STAT3
N
Mock
miR-NC
miR-MTCO3P38
Relative STAT3 mRNA expression
HCCLM3
Huh7
STAT3
GAPDH
O
HCCLM3
Huh7
MYC
PTTG1
STAT3
GAPDH
STAT3
NC
P
Mock
miR-NC
miR-MTCO3P38
Relative Luciferase activity
**
ns
wild type
mutant
Q
MTCO38P
Transcription
Pri-miRNA
Cropping
Pre-miRNA
Dicing
STAT3 mRNA
Mature miR-MTCO3P38
STAT3
PTTG1
PTTG1 mRNA
PTTG1
Myc
miR-MTCO38P ⊣ STAT3 → PTTG1 → Myc → cell proliferation, migration and invasion

was involved. We speculated miR-MTCO3P38 affects the biology of HCC through the Myc target pathway. Western blotting confirmed Myc was down-regulated by miR-MTCO3P38 (Fig. 1K). However, the mechanism is still unclear. We analyzed downregulated genes with significant differences from the transcriptome sequencing results and Myc associated regulators in the peer-reviewed literatures. PTTG1, which has been reported to regulate the Myc pathway,[4] was identified (Fig. 1J). We found PTTG1 decreased significantly in RNA and protein level when overexpressing miR-MTCO3P38 (Fig. 1K, S4A). In tissues from patients, expression of miR-MTCO3P38 and PTTG1 were negatively correlated (Fig. 1L, S4B). Interestingly, PTTG1 was highly expressed in HCC tissues compared to non-tumor tissues and patients of HCC with high expression of PTTG1 exhibited significantly shorter DFS and OS compared to patients with low expression of PTTG1 (Fig. S4B–E).

However, luciferase reporter assays showed miR-MTCO3P38 did not act on the 3′-UTR of PTTG1 mRNA (Fig. S4F). We suspect that miR-MTCO3P38 regulating PTTG1 via other mechanism except targeting the 3′UTR region of PTTG1 mRNA. Actinomycin D was used to block new synthesis of mRNAs, and the decay of already-synthesized PTTG1 mRNA has no difference between cells treated with miR-NC and miR-inhibitor (Fig. S4G). However, in cells transfected with miR-inhibitor, PTTG1 decreased significantly after actinomycin D treatment compared to DMSO (Fig. S4G). Therefore, we suspect that miR-MTCO3P38 affects the expression of PTTG1 by a transcription-dependent manner.

Then, we conducted interactive studies on multiple data sets (genes predicted as the target of miR-MTCO3P38, genes downregulated in the transcriptome, and known transcription factors (TFs). Details in Table S4). Intersection of the data sets focused on STAT3 (Fig. S5A), a TF widely mentioned in the literature referring to cancer-related events.[5] Meanwhile, TFs prediction using KnockTF, a database performing hypergeometric test between submitted genes and all DEGs regulated by each TF, predicted STAT3 the upstream regulator of PTTG1 (Table S6). Subsequently, STAT3 was downregulated by miR-MTCO3P38 in cells consistent with the result of transcriptome (Fig. 1M, N). Meanwhile, the protein level of PTTG1 and Myc were up-regulated in cells transfected with STAT3 (Fig. 1O). Interestingly, expression of miR-MTCO3P38 and STAT3 were negatively correlated while expression of STAT3 and PTTG1 were positively correlated in tissues from patients (Fig. S5B, C). Thus, we speculate that miR-MTCO3P38 mediates the down-regulating of PTTG1 by targeting STAT3. Dual luciferase reporter assays were performed, the luciferase activity of STAT3-wt construct declined after the upregulation of miR-MTCO3P38, whereas no significant change of luciferase activity was shown in the STAT3-mut construct (Fig. 1P, S5D). The upstream (chr5: 160,420,855–160,421,854) of the *PTTG1* gene were analyzed using JASPAR and the loci of top five sore on the sense strand were chosen for subsequent experiments (Table S6). Truncated and full-length (FL) of the promoter region were constructed (Fig. S5E), and the results showed the relative transcriptional activity of P2 promotor region has a significant enhance compared to NC group (Fig. S5F). This was further confirmed by ChIP-PCR analysis (Fig. S5G, H). Then the predicted two loci in P2 promotor region were mutated separately. The results showed that the luciferase activity of mutated truncation P2-M1 was strongly repressed (Fig. S5I). Here, we conclude that the binding site of STAT3 on the promotor region of PTTG1 locates from chr5: 160,421,678–160,421,688.

To further determine the regulatory relationship, we subsequently conducted the rescue experiments, the results confirmed that STAT3 mediated the inhibition of miR-MTCO3P38 to PTTG1 (Fig. S6).

In the present study, we identified a novel miRNA (miR-MTCO3P38) and found that it was downregulated in HCC tissues and was significantly related to HCC patient overall survival. Overexpression of miR-MTCO3P38 inhibits

Figure 1 A novel microRNA miR-MTCO3P38 inhibits malignant progression via STAT3/PTTG1/MYC in hepatocellular carcinoma. **(A, B)** Differential expression of miR-MTCO3P38 in the deep sequencing of 3 normal liver tissues and in 6 HCC tissues and paired adjacent non-tumor tissues. **(C)** Northern blot analysis of total RNA from HCCLM9 cells that were transiently transfected with pcDNA3.1(−) or with a plasmid encoding a precursor of miR-MTCO3P38. **(D)** The relative expression of miR-MTCO3P38 in 86 HCC tissues and paired adjacent non-tumor tissues. ***$P < 0.001$. **(E)** Analysis of the effect of miR-MTCO3P38 on survival with Kaplan–Meier analysis. $P < 0.01$. **(F)** The number of formatted colonies was reduced by miR-MTCO3P38, but increased by inhibitor in colony formation assays. **(G)** Overexpression of miR-MTCO3P38 promoted lung metastasis less efficiently as compared to vector. $n = 5$/group. **(H)** Growth suppression by miR-MTCO3P38 on subcutaneous tumor xenografts, 4 weeks after injection, is shown. $n = 5$/group. **(I)** Gene set enrichment analysis (GSEA) using transcriptome sequencing of groups miR-MTCO3P38 mimic and miR-NC mimic revealed that Myc target pathway was involved. **(J)** The volcano plot of DEGs showed PTTG1 was found a significant difference in expression. **(K)** c-Myc, PTTG1 decreased significantly in protein level in the miR-MTCO3P38 mimic group. **(L)** The correlation between miR-MTCO3P38 and PTTG1 were calculated. MiR-MTCO3P38 was evaluated using RT-qPCR assay (cancer/peritumor ratio) and PTTG1 (cancer/peritumor ratio) was evaluated in Figure S4B. **(M)** Heatmap conducted on transcriptome showed the expression pattern of STAT3 has obvious differences between the miR-NC group and miR-MTCO3P38 group. **(N)** The mRNA and protein level of STAT3 were downregulated in cells transfected with miR-MTCO3P38. Means ± SE ($n = 3$ independent experiments). **(O)** The protein level of PTTG1 and Myc were up-regulated accordingly in cells transfected with plasmid STAT3. **(P)** The luciferase activity of STAT3-wt construct declined after the upregulation of miR-MTCO3P38, whereas no significant change of luciferase activity was shown in the STAT3-mut construct. WT: wild-type; MUT: mutant sequences; miR: miR-MTCO3P38 mimics; NC: negative control for mimics. Data are shown as the mean ± s.d. from three independent experiments. **$P < 0.01$. **(Q)** The mechanism graph of the regulatory network and function of miR-MTCO3P38 in inhibiting the proliferation, migration and invasion of hepatocellular carcinoma cells via STAT3/PTTG1/MYC. MiR-MTCO3P38 regulates Myc target pathway by suppressing PTTG1 indirectly. STAT3 acts as a broker in promoting the transcription of PTTG1 and being downregulated by miR-MTCO3P38 via binding its 3′-untranslated region.

malignant progression of liver cancer cells. Mechanistically, miR-MTCO3P38 regulates Myc target pathway by suppressing PTTG1 indirectly. STAT3 acts as a broker in promoting the transcription of PTTG1, and it is downregulated by miR-MTCO3P38 via binding its 3′-untranslated region (Fig. 1Q). Our findings open new avenues for future investigations on this novel miRNA, which may be a potential therapeutic target for the treatment of HCC.

Ethics approval and consent to participate

Ethical approval was obtained from Ethics Committee of Zhongnan Hospital of Wuhan University, and written informed consent was obtained from each patient.

Consent for publication

All authors have agreed to publish this manuscript.

Availability of data and materials

The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

Q. L. and P. W. were the principal investigators for the study. D. G., Y. G. and D. M. conceived the study and carried out the major part of the project. D. G. also performed figure editing according to the journal's request. P. L. and B. C. participated in this project and collected and analyzed the data. D. G. and P. W. wrote the manuscript. Y. G. collected clinical and experimental data and made significant improvement to the preliminary data. Q. L. and Z. L. provided the funding and contributed to critical review of the manuscript. The authors read and approved the final manuscript.

Conflict of interests

All the authors declared no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2021.11.006.

Abbreviations

AMSCs	adipose tissue-derived mesenchymal stem cells
HCC	hepatocellular carcinoma
RT-qPCR	quantitative real-time polymerase chain reaction
MiRNA	microRNA; nt: nucleotides
PVTT	portal vein tumor thrombus
3′-UTR	3′-untranslated region

References

1. Chen W, Zheng R, Baade PD, et al. Cancer statistics in China, 2015. *CA A Cancer J Clin*. 2016;66(2):115—132.
2. Klingenberg M, Matsuda A, Diederichs S, Patel T. Non-coding RNA in hepatocellular carcinoma: mechanisms, biomarkers and therapeutic targets. *J Hepatol*. 2017;67(3):603—618.
3. Zhou X, Wen Y, Tian Y, et al. Heat shock protein 90α-dependent B-cell-2-associated transcription factor 1 promotes hepatocellular carcinoma proliferation by regulating MYC proto-oncogene c-MYC mRNA stability. *Hepatology*. 2019;69(4):1564—1581.
4. Lin X, Yang Y, Guo Y, et al. PTTG1 is involved in TNF-α-related hepatocellular carcinoma via the induction of c-myc. *Cancer Med*. 2019;8(12):5702—5715.
5. Yang Y, Zheng B, Han Q, Zhang C, Tian Z, Zhang J. Targeting blockage of STAT3 inhibits hepatitis B virus-related hepatocellular carcinoma. *Cancer Biol Ther*. 2016;17(4):449—456.

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