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RAPID COMMUNICATION

Circular RNA circEIF3C promotes intrahepatic cholangiocarcinoma progression and immune evasion via the miR-34a-5p/B7–H4 axis

Circular RNA (circRNA) is a novel type of noncoding RNA that originates from eukaryotic precursor messenger RNA (pre-mRNA).[1] These circRNAs regulate tumor processes through diverse mechanisms, including serving as a miRNA sponge to modulate expression of miRNA-target genes.[2] Eukaryotic translation initiation factor 3 subunit C (eIF3c) is a core subunit of the IF3 complex, involving in the initiation of the translation process of tumor cells.[3] Currently, little is known about the underlying molecular mechanisms of circEIF3C in the pathogenesis of intrahepatic cholangiocarcinoma (ICC). A better understanding of the biological functions of circEIF3C in response to tumor cellular growth and how this affects the tumor signaling pathways would provide important clinical value related to the pathogenesis of ICC.

In the current study, we detected expression of 23 circRNAs of the eIF3c gene (predicated by starBase v3.0) in three pairs of ICC compared to adjacent liver tissue. We found that hsa_circ_0005602 (circEIF3C) was significantly upregulated in ICC, and was further identified by sequencing (Fig. S1A; Fig. 1A). Next, we measured circEIF3C expression in 50 ICC and corresponding matched liver samples, and found that circEIF3C was significantly overexpressed in ICC samples (Fig. 1B). The intensity of circEIF3C staining further confirmed that circEIF3C was upregulated in ICC related to matched liver tissues (Fig. S1B, C). Moreover, our results demonstrated that circEIF3C expression was closely related to tumor stage, with higher expression in stage III-IV than in stage I-II (Table S1). Next, we divided patients into circEIF3C^{high} and circEIF3C^{low} groups. Kaplan–Meier analysis suggested that ICC patients in the circEIF3C^{high} group had a high recurrence rate and poor prognosis (Fig. S1D, 1C). Thus, these data demonstrate that circEIF3C expression in ICC is higher than that in paratumorous tissues, and increased circEIF3C expression correlate with a worse prognosis in ICC patients.

Subsequently, we determined circEIF3C expression in ICC cells. As shown in Figure S2A, circEIF3C expression in RBE and HUCCT1 cells was significantly lower than that in HCCC9810 and QBC939 cells. Next, we overexpressed circEIF3C in RBE cells by transfecting LV-circEIF3C plasmids, and qRT-PCR showed that the circEIF3C was effectively overexpressed (Fig. S2B). Additionally, we effectively inhibited circEIF3 expression in QBC939 cells (Fig. S2C, D). Transwell assays demonstrated that elevated circEIF3C expression increased RBE cell invasion (Fig. S2E, F). CCK-8 assays showed that cell viability was boosted following altered circEIF3C expression in QBC939 and RBE cells (Fig. S2G). Importantly, *in vivo* tumor assays demonstrated that the tumor volume in RBE-circEIF3C and QBC-939-NC cells was much larger than that of the RBE-Control and QBC-939-shcircEIF3C cells (Fig. S2H, I). Together, these data suggest that circEIF3C promotes the progression of ICC.

We used circRNA-RIP to purify circEIF3C-interacting miRNAs, which were specifically against circEIF3C, and used qRT-PCR to analyze the 52 candidate miRNAs (predicated by starBase v3.0). Interestingly, we uncovered a specific enrichment of circEIF3C and miR-34a-5p compared to the negative control. However, other miRNAs showed little to no enrichment (Fig. 1D), indicating that miR-34a-5p functions as a circEIF3C-interacting miRNA in QBC939 cells. Moreover, circEIF3C and miR-34a-5p were pulled down using AGO2 antibodies (Fig. S3A). To further validate the role of miR-34a-5p as a circEIF3C RNA sponge, a dual-luciferase reporter assay was performed in HEK293 T cells. Wild type (wt) circEIF3C and mutant circEIF3C were cloned into a luciferase expression vector. The results demonstrated that circEIF3C

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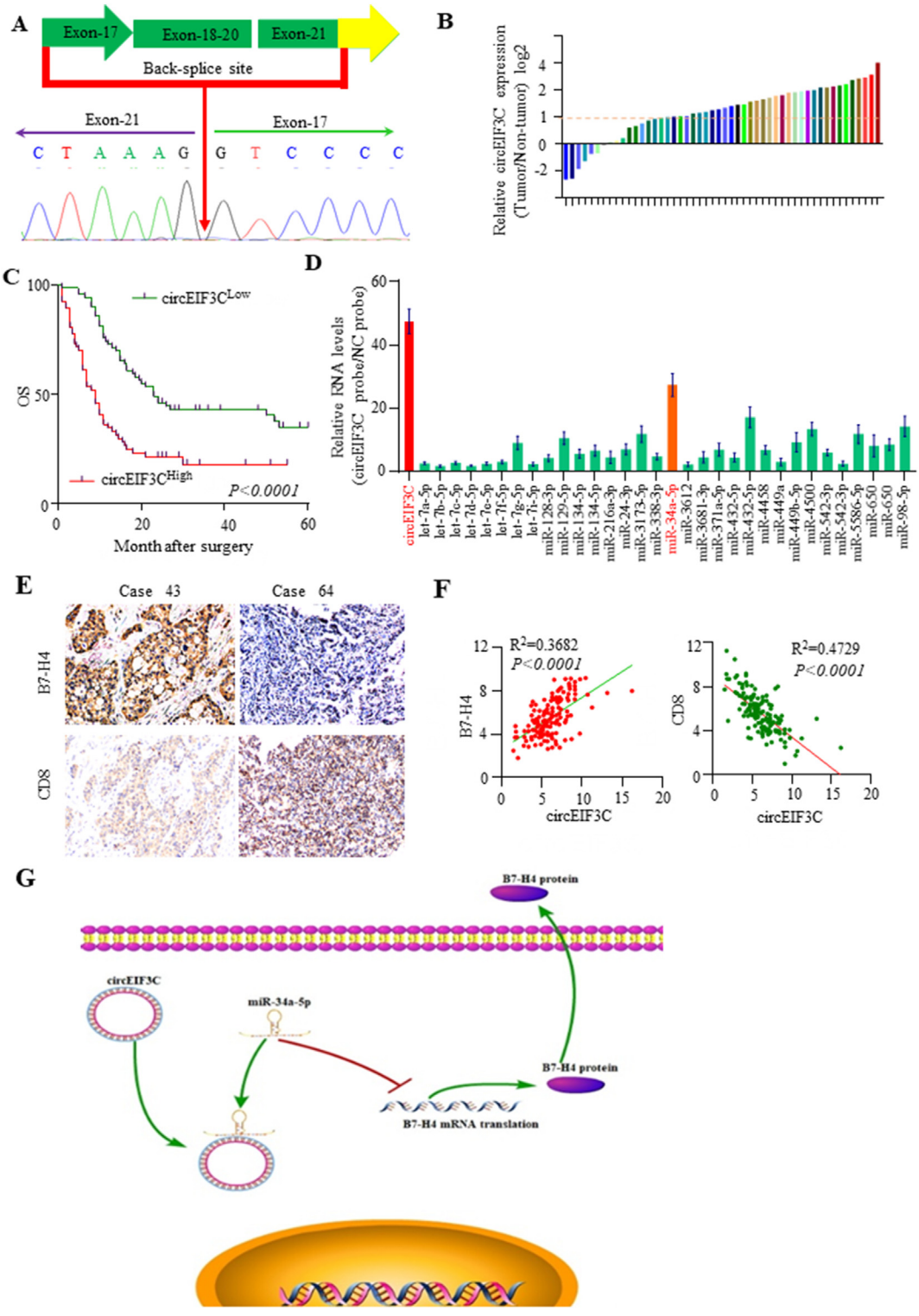

Figure 1 Circular RNA circEIF3C promotes intrahepatic cholangiocarcinoma progression and immune evasion via the miR-34a-5p/B7—H4 axis. **(A)** Schematic illustration to display the circularization of EIF3Cexons 17—21 to form circEIF3C. **(B)** A diagram depicting how circEIF3C expression was determined in ICC samples from 50 patients. **(C)** Kaplan—Meier method was used to analyze the overall survival (OS) rate in 140 patients with ICC. **(D)** Results of circRIP exhibited a specific enrichment between circEIF3C and miR-34a-5p. **(E)** Representative images of B7—H4 and CD8 staining in serial TMA (Bar = 100 μm). **(F)** A diagram showing a positive correlation between circEIF3C and B7—H4 expression, and a negative correlation between CD8 and circEIF3C expression. **(G)** Work model: High circEIF3C expression promotes the progression of ICC by sponging miR-34a-5p, thereby increasing the translation of B7—H4.

(wt) significantly decreased the luciferase activity of miR-34a-5p, but not mutant circEIF3C (Fig. S3B). Here, the luciferase activity was decreased more than 35%, which indicated a direct interaction between circEIF3C and miR-34a-5p. Furthermore, miR-34a-5p showed obvious fluctuations following overexpression or knockdown of circEIF3C in RBE or QBC939 cells (Fig. S3D, E). These data confirm that circEIF3C may act as a sponge for miR-34a-5p.

Initially, B7—H4 is present on the surface of antigen-presenting cells including tumor cells and T cells, which contributes to inhibition of T cell function and tumor immune escape.[4] As a result, B7—H4 dysregulation is closely correlated with tumor progression.[5] We demonstrated that B7—H4 might be a target of miR-34a-5p and identified a binding site for miR-34a-5p in the 3′-UTR of B7—H4 through bioinformatics analysis (Fig. S4A). To verify the relationship among circEIF3C, miR-34a-5p, and B7—H4, we constructed miR-34a-5p and B7—H4 pLG3 luciferase reporter plasmids with mutated and wild type potential binding sites. As expected, the dual-luciferase reporter assay further revealed obviously reduced levels of luciferase activity with wt-B7-H4 compared to mu-B7-H4 (Fig. S4B). Moreover, we found that B7—H4 was elevated in RBE-shmiR-34a-5p (Fig. S4C, D) but was markedly reduced in GBC939-shcircEIF3C (Fig. S4E, F). We also showed that circEIF3C-upregulated cells secreted more B7—H4 compared to circEIF3C-downregulated cells (Fig. S4G). Finally, to test specific effects of B7—H4 on pro-apoptosis of $CD8^+$ T cells, we performed an *in vitro* functional assay. Supernatants that were collected from the circEIF3C-overexpressing cells induced high apoptosis rate of $CD8^+$ T cells compared to control cells (Fig. S4H—K), suggesting a positive correlation between circEIF3C and B7—H4 expression, but a negative correlation between circEIF3C expression and $CD8^+$ T cell infiltration in ICC tissues (Fig. 1E, F). Together, these data further suggest that circEIF3C induces ICC progression and immunosuppression via the miR-34a-5p/B7—H4 axis in ICC cells.

We aimed to address whether the oncogenic function of circEIF3C could be reversed by interfering with B7—H4 expression. Thus, RBE-circEIF3C cells were transfected with a B7—H4 shRNA plasmid, followed by quantification of shRNA knockdown efficacy via Western blotting and qRT-PCR. As expected, B7—H4 expression was reduced in plasmid-transfected cells compared to the control group (Fig. S5A, B). Importantly, B7—H4 knockdown greatly suppressed cell migration and proliferation induced by circEIF3C overexpression *in vitro* (Fig. S5C, D). Together, these data demonstrate that circEIF3C promotes ICC development via the miR-34a-5p/B7—H4 axis (Fig. 1G).

In this study, we found that overexpression of circEIF3C was associated with the low overall survival and high recurrence in ICC patients. Intriguingly, circRNA-RIP, pull down and luciferase reporter assays with wt/mutant circEIF3C or knockdown of circEIF3C showed the specific interaction between circEIF3C and miR-34a-5p. In addition, our data showed that 3′-UTR of B7—H4 directly binds and recognizes miR-34a-5p, serving as an RNA binding protein, which induced apoptosis of $CD8^+$ T cells. Collectively, these data indicate that high levels of circEIF3C act reliably to promote ICC progression via sponging miR-34a-5p to upregulate B7—H4.

Author contributions

Conceived and designed the experiments: XM Zhong. Revised the manuscript: CX Ji. Analyzed the data: DB Ren. Performed the experiments: AW Ke. Wrote the paper: ZW Yang.

Conflict of interests

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.05.005.

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