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RAPID COMMUNICATION

LncRNA MIR31HG controls the proliferation and metastasis of gastric cancer by c-CBL-mediated degradation of β-catenin

LncMIR31HG acts as a host gene for miR-31, also known as LncHIFCAR (long non-coding HIF-1 co-activating RNA), whose deregulation has been reported to promote the development of various human cancers, including lung cancer, colorectal cancer, *etc*.[1,2] However, the biological functions and molecular mechanisms of MIR31HG in gastric cancer are unclear. The lncRNA MIR31HG was identified as a possible therapeutic target for gastric cancer throughout our work. In summary, we found that MIR31HG plays an oncogene role in gastric cancer; the deletion of MIR31HG significantly reduced gastric cancer cell proliferation, metastasis, and sphere-forming ability; and the overexpression of MIR31HG promoted these capacities. FH535, a small molecule targeting β-catenin, was utilized to confirm that MIR31HG, which directs the malignant progression of gastric cancer, is β-catenin dependent. We revealed that MIR31HG positively regulated β-catenin stability by reducing β-catenin ubiquitination and increasing β-catenin steady-state expression. Furthermore, this is the first time that MIR31HG has been shown to interact with the E3 ligase c-CBL (dendritic cell (DC)-specific defect of casitas B-lineage lymphoma), and that c-CBL is reported to be involved in regulating β-catenin ubiquitination in cancer cells. MIR31HG interacted with c-CBL and caused its instability to protect β-catenin stability. Thus, our study provided insights into the applicability of using the MIR31HG-c-CBL-β-catenin axis as a potential therapeutic target in gastric cancer.

To evaluate the possible role of MIR31HG in GC cells, we found that increased expression of MIR31HG was significantly associated with poor prognosis in gastric cancer patients (Fig. S1A). Furthermore, we found that MIR31HG was generally highly expressed in gastric cancer cell lines, and the expression of MIR31HG was the highest in MKN45 and SGC7901 cells, so we selected these two cell lines in subsequent experiments (Fig. S1B). Subsequently, we designed two independent short hairpin RNAs (shRNAs) to knock down the expression of MIR31HG, with shGFP as a control. Quantitative real-time PCR results revealed that shMIR31HG#1 and shMIR31HG#2 effectively reduced endogenous MIR31HG levels in MKN45 and SGC7901 cells (Fig. S1C). At the same time, the morphology of gastric cancer was altered, and the number of cells was dramatically reduced as compared to the control group (Fig. S2A). Therefore, we speculated that MIR31HG knockdown could inhibit the proliferation of MKN45 and SGC7901 cells. MTT assay, BrdU assay, cell cycle assay, wound-healing assay, Transwell assay, and Western blot assay were performed in MIR31HG-knockdown MKN45 and SGC7901 cells (Fig. S1C–G; Fig. S3A–C), and their results revealed that MIR31HG is required for cell development, proliferation, migration, and invasion in gastric cancer cells. In addition, we overexpressed MIR31HG in MKN45 and SGC7901 cells to further confirm the above results (Fig. S1H–I; Fig. S2B, C, 3D).

To detect the effect of MIR31HG on clone formation *in vitro* and *in vivo*, we performed soft agar and xenograft tumor growth assays. Loss of MIR31HG resulted in a considerable drop in colony size and number (Fig. S4A). Moreover, gastric cancer cells with MIR31HG knockdown were injected into nude mice to form xenograft tumors. It was obvious that the volume and weight of the transplanted tumor were smaller than the control group (Fig. S4B, C), which confirmed the effect of MIR31HG on the self-renewal capability of gastric cancer cells. Furthermore, the data in Figure S4D demonstrated that the knockdown of MIR31HG dramatically reduced the number of Ki67-positive cancer cells, showing that the knockdown of MIR31HG impeded cell growth in xenograft tumors. These findings indicated that loss of MIR31HG dramatically reduced the self-renew ability of gastric cancer cells, showing that MIR31HG is crucial for gastric cancer cell cloning and carcinogenesis.

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The Wnt/β-catenin signaling system is a complicated pathway that participates in embryonic development, tissue homeostasis, and a variety of clinical illnesses.[3] As a multifunctional protein, β-catenin plays a critical role in forming malignancies. Pathological activation of the Wnt/β-catenin signaling pathway is observed in roughly 30% of stomach malignancies, and it may be a pivotal step leading to tumorigenicity. The abnormal β-catenin expression can increase the transcription of oncogenes such as c-MYC and CyclinD1, resulting in cancer development. Therefore, strict supervision of β-catenin expression is necessary. Previous studies have shown that MIR31HG expression is strongly associated with Wnt/β-catenin pathway activation in tumors. However, the author did not further clarify the relationship between MIR31HG and Wnt/β-catenin.[4] Since β-catenin is the key component of the Wnt/β-catenin signaling pathway, it is essential for controlling cell proliferation, embryonic patterning, and tumorigenesis. We investigated the relationship between MIR31HG and β-catenin in depth. After knocking down MIR31HG, we noticed that the protein level of β-catenin was significantly reduced, and then we detected the expression level of c-MYC and cyclinD1, which are downstream target genes of Wnt/β-catenin signaling pathways. These results showed that the expression of c-MYC and cyclinD1 were down-regulated when MIR31HG was knocked down (Fig. S5A), and their protein levels were up-regulated after MIR31HG overexpression (Fig. S5B). To further examine whether MIR31HG regulates the progression and development of gastric cancer through β-catenin, a gastric cancer cell model with MIR31HG overexpression was established. Then we used FH535, a small chemical inhibitor of the Wnt/β-catenin pathway, to treat the cell model above. Western blot assay showed that FH535 inhibited the activating effect of MIR31HG on β-catenin-mediated downstream signaling (Fig. S5C). The results of MTT and Transwell assays revealed that compared with the control cells, the proliferation and invasion of MIR31HG-overexpressing SGC7901 and MKN45 cells were inhibited by treatment with the β-catenin inhibitor FH535 (Fig. S5D—E). The above results indicate that MIR31HG regulates the proliferation and migration of gastric cancer cells through a β-catenin-mediated signaling pathway.

To investigate the relationship between MIR31HG and β-catenin, we first conducted a RIP (RNA immunoprecipitation) experiment. The results showed that there was no binding between MIR31HG and the protein of β-catenin (Fig. 1A). Subsequently, we down-regulated MIR31HG and detected the mRNA levels of β-catenin, and found no significant change in mRNA levels (Fig. 1B), and there was no significant association between MIR31HG and β-catenin mRNA levels in gastric cancer according to bioinformatics analysis (Fig. S6A). However, the expression of the β-catenin protein was significantly down-regulated in MIR31HG-knockdown MKN45 and SGC7901 cells (Fig. 1C). Because of this discovery, we speculated that MIR1HG regulates β-catenin through posttranscriptional regulation. To further confirm whether MIR31HG regulates the expression of β-catenin at the post-transcriptional level. We next used MG132 (proteasome inhibitor) to treat MKN45 and SGC7901 cells based on the knockdown of MIR31HG. The results showed that the down-regulation of β-catenin caused by the knockdown of MIR31HG was eliminated upon MG132 treatment (Fig. S7A). Then, we performed an *in vitro* ubiquitination assay, which expressed exogenous HA-UB in MKN45 and SGC7901 cells, and found that the ubiquitination of β-catenin was up-regulated after the loss of MIR31HG (Fig. 1D). To further confirm that MIR31HG promotes the stability of β-catenin, we performed a CHX (cycloheximide) assay and found that overexpression of MIR31HG could prolong the half-life of β-catenin after the addition of CHX (Fig. 1E). The above results indicated that MIR31HG stabilized the expression of β-catenin by reducing its ubiquitination level. Protein ubiquitination is accomplished through a series of enzyme cascades. To determine whether E3 ubiquitin ligases were involved in the regulation of β-catenin ubiquitination by MIR31HG, we assessed the expression of the E3 ligase of β-catenin after the loss of MIR31HG. After the interference with MIR31HG, the expression of c-CBL was significantly up-regulated (Fig. S8A). However, the c-CBL RNA level was not significantly up-regulated (Fig. 1G). Bioinformatics analysis also showed that MIR31HG was not statistically associated with c-CBL at the mRNA level in gastric cancer tissues (Fig. S6B). In order to further prove that MIR31HG stabilizes the expression of β-catenin by c-CBL, we performed RIP and RNA pulldown assays and found that the protein of c-CBL interacted with MIR31HG (Fig. 1H, I). In addition, an *in vitro* ubiquitination assay was performed, these results suggested that MIR31HG affected c-CBL expression by interacting with c-CBL and regulating c-CBL ubiquitination (Fig. 1J, K). As a highly conserved E3 ubiquitin ligase, c-CBL-mediated ubiquitination not only modulates the function of signaling proteins by changing cellular localization but also recruits target molecules to degrade them. Previous studies have also shown that c-CBL acts as a tumor suppressor gene in many tumors to degrade epidermal growth factor receptor (EGFR) and β-catenin to slow down the malignant process of tumors.[5] We, therefore, aimed to verify whether c-CBL was a downstream effector of MIR31HG. After the loss of c-CBL expression in MIR31HG-knockdown MKN45 and SGC7901 cells, the expression of β-catenin was recovered (Fig. S8B). The MTT and Transwell experiments proved that silencing the expression of c-CBL could restore the inhibitory effect of MIR31HG down-regulation on the proliferation and migration of gastric cancer cells (Fig. S8C—E). Therefore, we validated that c-CBL, as a downstream effector of MIR31HG, is responsible for the proliferation, metastasis, and tumorigenesis of GC cells.

LncRNAs will now be excellent targets for cancer therapy since they are highly tissue-specific drivers of cancer characteristics. The development of RNA-targeting medicines opens more options for modulating lncRNAs for cancer prevention. In this study, we found that MIR31HG ultimately regulates the wnt/β-catenin signaling pathway by binding to c-CBL protein and reducing its stability. MIR31HG was discovered as a promising prognostic marker and therapeutic target in gastric cancer in this work, supported by reliable experimental data. MIR31HG enhanced the proliferation, invasion, and tumorigenesis of gastric cancer cells, according to a variety of *in vivo* and *in vitro* investigations. Furthermore, this report is the first to provide mechanistic insight into the direct interplay between MIR31HG and β-catenin. When MIR31HG was highly expressed in gastric

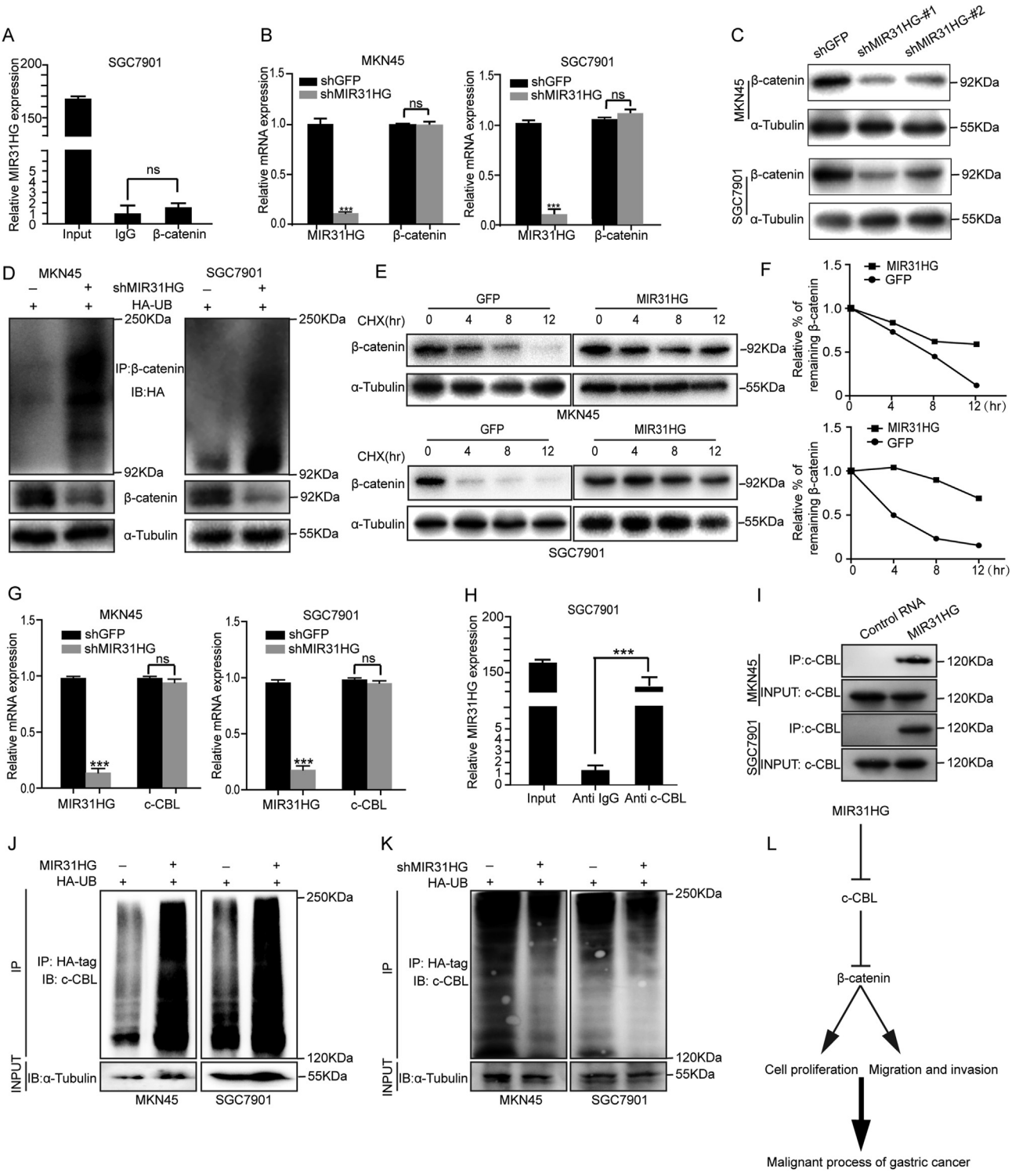

Fig. 1 MIR31HG reduced the ubiquitination of β-catenin via c-CBL to protect β-catenin stability. **(A)** The binding of MIR31HG with β-catenin protein was detected by RIP assay. **(B, C)** The mRNA and protein levels of β-catenin were detected after MIR31HG knockdown. **(D)** Ubiquitination of β-catenin *in vitro* was detected by co-IP. **(E, F)** The β-catenin turnover rate in MIR31HG-over-expressing GC cells was tested upon treatment with cycloheximide (chx). **(G)** The mRNA level of c-CBL was detected after MIR31HG knockdown. **(H)** The binding of c-CBL with MIR31HG was verified by RIP assay. **(I)** The binding of c-CBL with MIR31HG was verified by RNA pulldown assay. **(J, K)** Ubiquitination of c-CBL *in vitro* was detected by co-IP. **(L)** The schematic diagram of the mechanism of MIR31HG in gastric cancer. All data were expressed as mean ± standard deviation. Student's t-test was performed to analyze significance; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

cancer cells, it interacted with c-CBL protein and reduced c-CBL protein expression, thereby protecting the stability of β-catenin to direct the proliferation, migration, and invasion of gastric cancer cells via the wnt/β-catenin-mediated regulatory network (Fig. 1L). Our results indicated that the MIR31HG-c-CBL-β-catenin axis could be a potential therapeutic target in gastric cancer.

Author contributions

H.C., M.L., X.K., W.P. and J.Z. designed this study. W.P., J.Z. and H.C. performed all the experimental work. W.P., J.Z., S.W., F.W., K.W., R.G., X.D., J.Z., B.L., X.K., and H.C., M.L. and X.K. analyzed the experimental data. W.P., J.Z. and H.C. wrote drafts of the main text and collated diagrams. All authors have read, revised, and approved the final manuscript.

Conflict of interests

The authors declare no competing interests.

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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.2022.12.022.

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Wen Peng[a,d,1], Jiayi Zhang[a,c,1], Shenghao Wang[d], Feng Wang[a,d], Kun Wang[a,d], Rui Geng[d], Xiangfei Ding[d], Jingping Zhang[d], Biao Li[d], Xiaoxue Ke[a,d,***], Muhan Lü[b,**], Hongjuan Cui[a,d,e,*]

[a] *State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing 400716, China*
[b] *Department of Gastroenterology, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan 646000, China*
[c] *Chongqing Institute for Food and Drug Control, Chongqing 401121, China*
[d] *Cancer Center, Medical Research Institute, Southwest University, Chongqing 400716, China*
[e] *Jinfeng Laboratory, Chongqing 401329, China*

*Corresponding author. State Key Laboratory of Silkworm Genome Biology, Southwest University, No.2 Tiansheng street, Beibei District, Chongqing 400716, China.

**Corresponding author. Department of Gastroenterology, The Affiliated Hospital of Southwest Medical University, 25 Taiping Street, Jiangyang District, Luzhou City, Sichuan 646000, China.

***Corresponding author. State Key Laboratory of Silkworm Genome Biology, Cancer Center, Medical Research Institute, Southwest University, No.2 Tiansheng Street, Beibei District, Chongqing 400716, China.
E-mail addresses: kexiaoxue@126.com (X. Ke), 52721759@qq.com (M. Lü), hcui@swu.edu.cn, hongjuan.cui@gmail.com (H. Cui)

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