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Upregulation of lncRNA HITT promotes cell apoptosis by suppressing the maturation of miR-602 in gastric cancer

Yun Chen[1]*, Canhui Ouyang[1]*, Lingyun Liao[1], Yun Zhou[1], Fan Meng[1], Yao Liu[1] and Jing Ye[2]

[1]Department of Gastroenterology, The first Affiliated Hospital of Gannan Medical College, Ganzhou City and [2]Office of Academic Affairs of Jiangxi University of Traditional Chinese Medicine, Nanchang City, Jiangxi Province, PR China

*Yun Chen and Canhui Ouyang contributed equally to this work

Summary. It has been reported that HITT can inhibit colon cancer. However, the role of HITT in gastric cancer (GC) is unknown. Our preliminary sequencing data revealed the altered expression of HITT in GC and its close correlation with miR-602, suggesting the involvement of HITT and its potential interaction with miR-602 in GC. This study explored the role of HITT and its crosstalk with miR-602 in GC. In this study, the expression of HITT, premature and mature miR-602 in paired GC and normal tissues (62 patients) was detected by RT-qPCR. RNA pull-down assay was performed to evaluate the direct interaction between HITT and mature miR-602. The subcellular location of HITT was assessed by nuclear fractionation assay. The role of HITT in regulating miR-602 maturation was explored by overexpression assay. Cell apoptosis was analyzed by flow cytometry. Our data illustrated that HITT was highly upregulated and mature miR-602 was downregulated in GC. No alteration in premature miR-602 in GC was observed. HITT was located in both nucleus and cytoplasm, and it can directly interact with miR-602. In addition, overexpression of HITT in GC cells increased the expression levels of mature miR-602 but not premature miR-602. Overexpression of HITT further increased GC cell apoptosis and suppressed the role of miR-602 in inhibiting GC cell apoptosis. In conclusion, HITT may promote GC cell apoptosis by suppressing the maturation of miR-602.

Key words: HITT, Gastric cancer, miR-602, Apoptosis

Corresponding Author: Jing Ye, Office of Academic Affairs of Jiangxi University of Traditional Chinese Medicine, No. 1688 Meiling Avenue, Nanchang City, Jiangxi Province, 330004, PR China. e-mail: jingyejiangxi@163.com

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Introduction

As a major burden of global health, gastric cancer (GC) affects about 1 million people worldwide each year (Thrift and El-Serag, 2020). At present, patients with early stage GC in many cases can be cured by surgical resection (Yang and Hu, 2017). However, in most countries except for Japan, most GC patients are diagnosed with inoperable conditions, or develop recurrent tumors after resection with curative intent (Van Cutsem et al., 2016; Japanese gastric cancer treatment guidelines, 2014 (ver. 4), 2017). Once tumor metastasis to distant ends has occurred, surgical resection will be inappropriate (Smyth and Moehler, 2019). Although chemotherapy or radio therapy can be applied to treat GC, full recovery is rare and prognosis is generally poor (Ilson, 2019). Therefore, the field of GC treatment needs more effective therapeutic approaches.

With the advantages of less adverse effects and higher accuracy, molecular targeted therapies are emerging approaches for the treatment of cancers including GC (Pellino et al., 2019; Patel and Cecchini, 2020). For instance, regulating the expression of HER2 and miR-374a-5p, which play critical roles in GC, has shown potential in the treatment of advanced GC (Ji et al., 2019; Meric-Bernstam et al., 2019). However, more therapeutic targets are still needed to achieve better treatment outcomes (Figueiredo et al., 2013). Micro RNAs (miRNAs) and long non-coding RNAs (lncRNAs) are ncRNAs that have no coding capacity but regulate protein synthesis to participate in most, if not all types of cancers, including GC (Hao et al., 2017; Peng et al., 2017). Therefore, lncRNAs and miRNAs are promising targets for GC treatment. A recent study showed that HITT could inhibit colon cancer (Wang et al., 2020). Our preliminary microarray analysis showed the altered expression of HITT in GC. In addition, our preliminary data also showed that HITT was closely and specifically correlated with miR-602, but not other miRNAs. MiR-602 is an important player in cancer biology (Zhou et al., 2020). We hypothesized that HITT may interact with

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miR-602 to participate in GC. We then explored the interaction between HITT and miR-602 in GC.

Materials and methods

Tissue specimens

HITT is a novel lncRNA and its expression data are lacking in public datasets. To investigate the expression pattern of HITT in GC, 62 paired GC and non-tumor tissue specimens were obtained from the first Affiliated Hospital of Gannan Medical College (Ethics Committee of this hospital approved this study). The specimens were stored at −80 °C prior to subsequent assays. The tumor types and stages were analyzed, diagnosed and confirmed by 3 experienced pathologists. The 62 cases included 30 cases of gastric adenocarcinoma and 32 cases of gastric carcinoma. Based on AJCC staging system, the 62 cases included 28 cases at stage I or II, and 34 cases at stage III or IV. All these patients signed the informed consent. Based on medical records, none of these patients received therapy prior to the collection of specimens.

GC cells

To match the patients included in this study, this study included two GC cell lines SNU-1 (carcinoma) and AGS (adenocarcinoma), which were purchased from ATCC (USA). These cells were cultivated in 1640 medium with 10% FBS. Regular mycoplasma test and STR profiling were performed (Mykoalert detection kit).

Cell transfections

HITT and miR-602 were overexpressed in SNU-1 and AGS cells through transfections mediated by Neon Transfection System (Thermo Fisher Scientific), in which miR-602 mimic or pcDNA3.1- HITT expression vector (Invitrogen) was used. In all cases, two controls, including the negative control (NC, empty vector- or NC miRNA-transfection) and control (untransfected cells) were included. The overexpression of HITT and miR-602 were confirmed every 24h until the end of all assays that included in the overexpression experiments.

RNA samples and RT-qPCRs

Following RNA extraction using RNAzol reagent (Sigma-Aldrich), DNase I (Sangon) was used to remove genomic DNA. The ratio of OD260/280 reached to about 2.0 in all samples, indicative of pure RNA samples. Urea-PAGE gels (4.5%) were used to separate RNA samples, followed by ethidium bromide staining and visualization under UV lights.

To detect the expression of HITT and premature miR-218, RNA samples were used to prepare cDNA samples. With cDNA samples as template and 18S

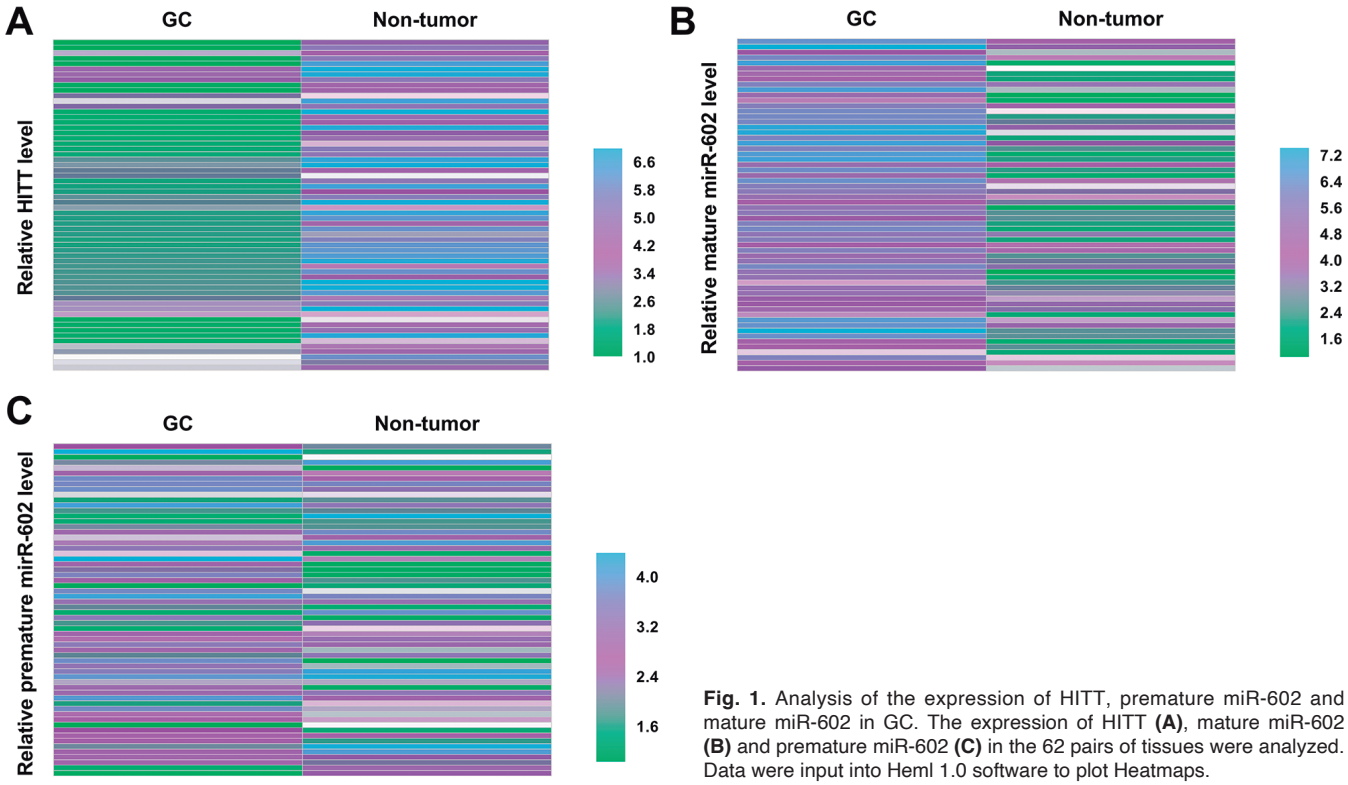

Fig. 1. Analysis of the expression of HITT, premature miR-602 and mature miR-602 in GC. The expression of HITT **(A)**, mature miR-602 **(B)** and premature miR-602 **(C)** in the 62 pairs of tissues were analyzed. Data were input into Heml 1.0 software to plot Heatmaps.

rRNA as the internal control, qPCRs were performed to measure the expression levels of HITT across samples. The expression of mature miR-218 was measured using All-in-One™ miRNA qRT-PCR Detection Kit (Genecopoeia). The $2^{-\Delta\Delta CT}$ method was used for the normalization of Ct values.

Nuclear fractionation assay

SNU-1 and AGS cells were used to prepare nuclear and cytosol samples with BioVision Nuclear/Cytosol Fractionation Kit (# K266). Both samples were subjected to the preparation of RNA samples. The same amount of RNA samples was used to prepare cDNA samples, which were used as the template to perform semi-quantitative PCR amplifications. Different PCR cycles were used to avoid over-amplification. In this experiment, amplification of GAPDH was performed and used as a positive control.

RNA pull-down assay

Biotinylated premature miR-602 (Bio-premature miR-602) and NC (Bio-NC) were transfected into SNU-1 and AGS cells. At 48h post-transfection, cell lysis was prepared and was subjected to separation using Streptaviden magnetic beads (Invitrogen). After that, the expression of HITT was analyzed by RT-qPCR.

Cell apoptosis assay

SNU-1 and AGS cells were harvested at the time point of 48h post-transfection. These cells were cultivated in non-serum medium at 37°C for 48h. After that, cells were washed with ice-cold PBS twice and then stained with FITC Annexin-V and PI (Abcam) in the dark for 15 min. Apoptotic cells were analyzed by flow cytometry.

Statistical analysis

Heatmaps plotted using Heml 1.0 software were used to present the differential gene expression in paired tissue samples. Data collected from different cell transfection groups (more than 3) were compared by ANOVA Tukey's test. $p<0.05$ was considered as statistically significant.

Results

The expression of HITT and mature miR-602, but not premature miR-602 were altered in GC

The expression of HITT, premature and mature miR-602 in the 62 pairs of tissues were analyzed. Data were inputted into Heml 1.0 software to plot Heatmaps. The results illustrated that HITT was highly upregulatred in GC tissues (Fig. 1A). In contrast, mature miR-602 was downregulated in GC tissues (Fig. 1B). However, the expression of premature miR-602 was not significantly different between paired tissues (Fig. 1C, $p>0.05$).

HITT was located in both nucleus and cytoplasm

Nuclear fractionation assay was performed to determine the subcellular location of HITT. Our data illustrated that, unlike GAPDH, which is a cytoplasm marker, HITT was localized in both nucleus and cytoplasm (Fig. 2). Therefore, HITT may transport from nucleus to cytoplasm.

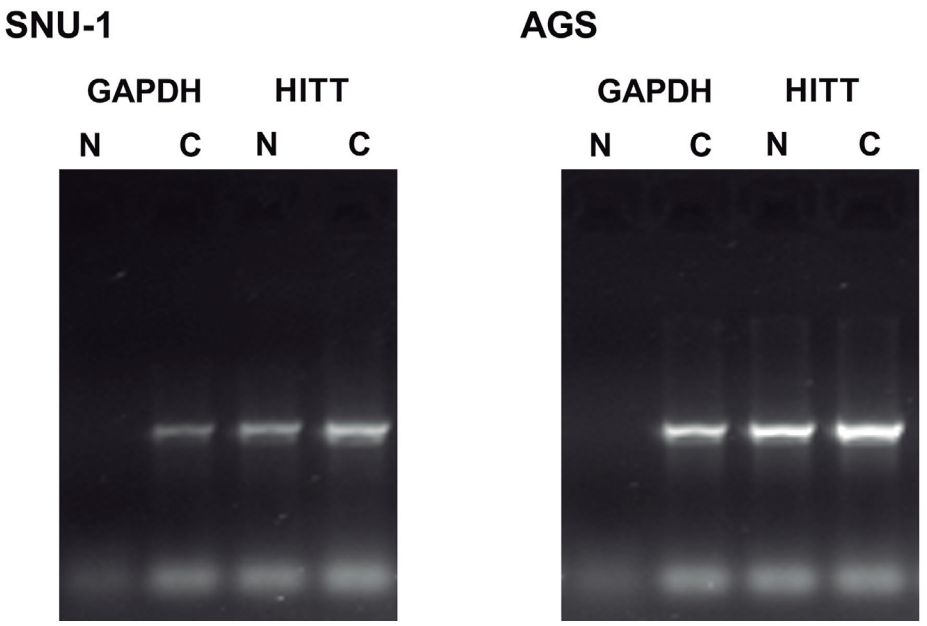

Fig. 2. HITT was located in both nucleus and cytoplasm. Nuclear fractionation assay was performed to determine the subcellular location of HITT. PCR products were subjected to 1% agarose gel electrophoresis, followed by EB staining. N, nucleus; C, cytoplasm.

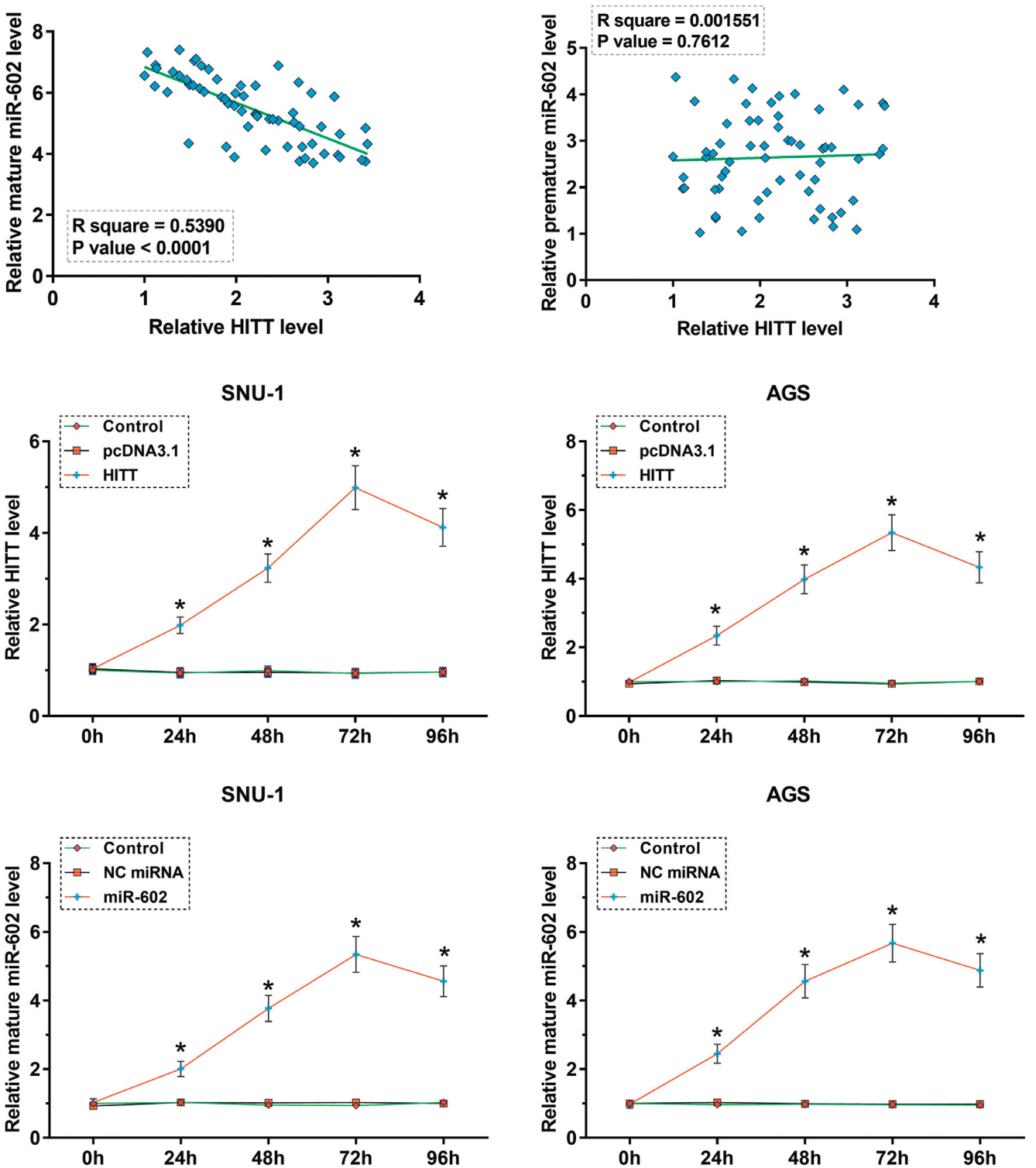

Fig. 3. The correlation between HITT and miR-602, and the upregulation of HITT and miR-602 in GC cells. Pearson's correlation coefficient analysis was performed to analyze the correlations between HITT and mature miR-602 **(A)** or premature miR-602 **(B)** across GC tissues. HITT and miR-602 were overexpressed in SNU-1 and AGS cells and the overexpression was confirmed every 24h until 96h **(C)**. Control, control cells without transfections; pcDNA3.1, cells transfected with empty pcDNA3.1 vector; HITT, cells transfected with HITT expression vector; NC miRNA, cells transfected NC miRNA; miR-602, cells transfected with miR-602 mimic. *, $p<0.05$.

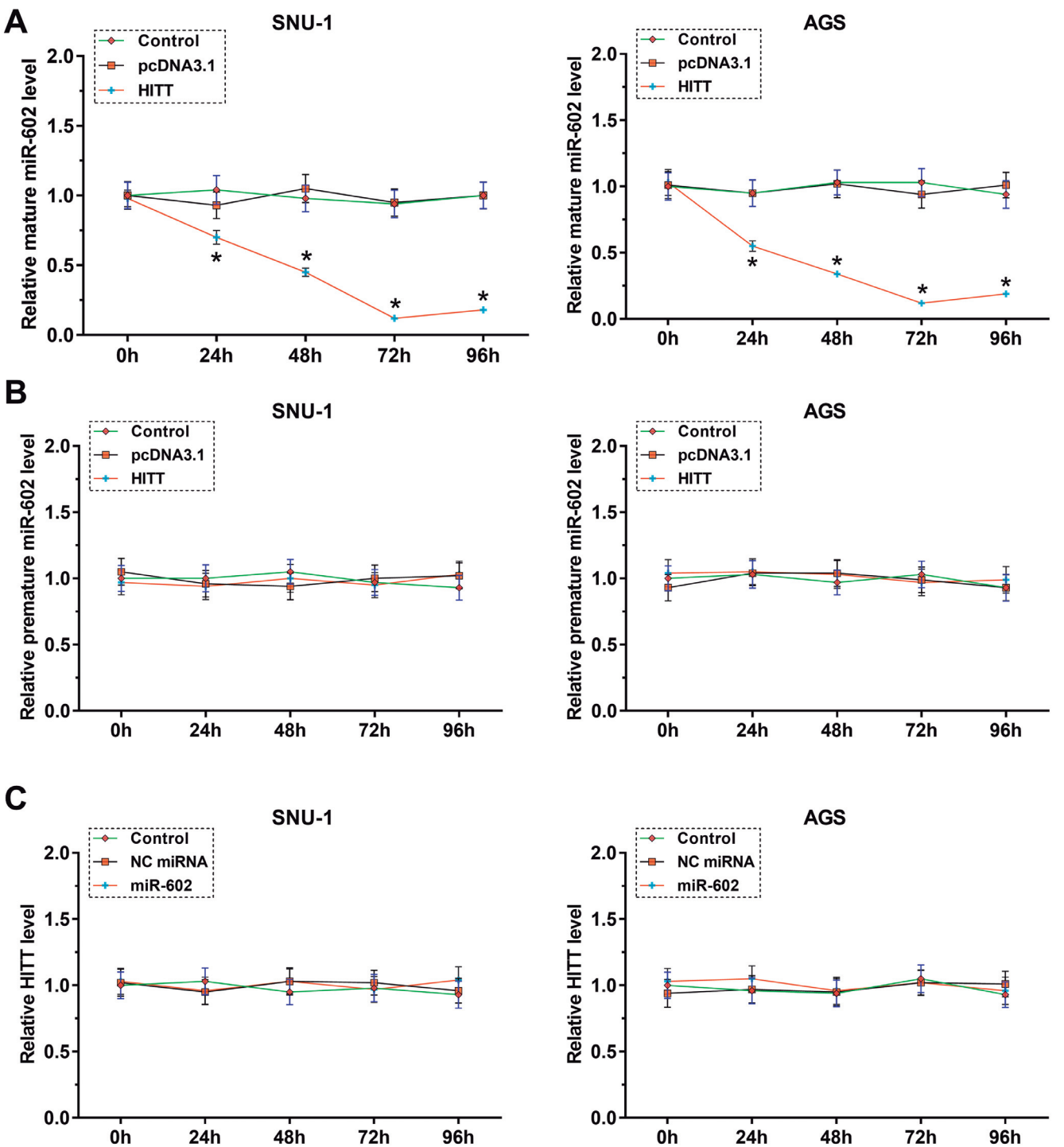

Fig. 4. The role of HITT in regulating the maturation of miR-602 in GC cells. The alteration in the expression of mature miR-602 **(A)** and premature miR-602 **(B)** after overexpression of HITT were analyzed by RT-qPCR. In addition, the alteration in the expression of HITT after overexpression of mature miR-602 were also analyzed by RT-qPCR **(C)**. Control, control cells without transfections; pcDNA3.1, cells transfected with empty pcDNA3.1 vector; HITT, cells transfected with HITT expression vector; NC miRNA, cells transfected NC miRNA; miR-602, cells transfected with miR-602 mimic. *, $p<0.05$.

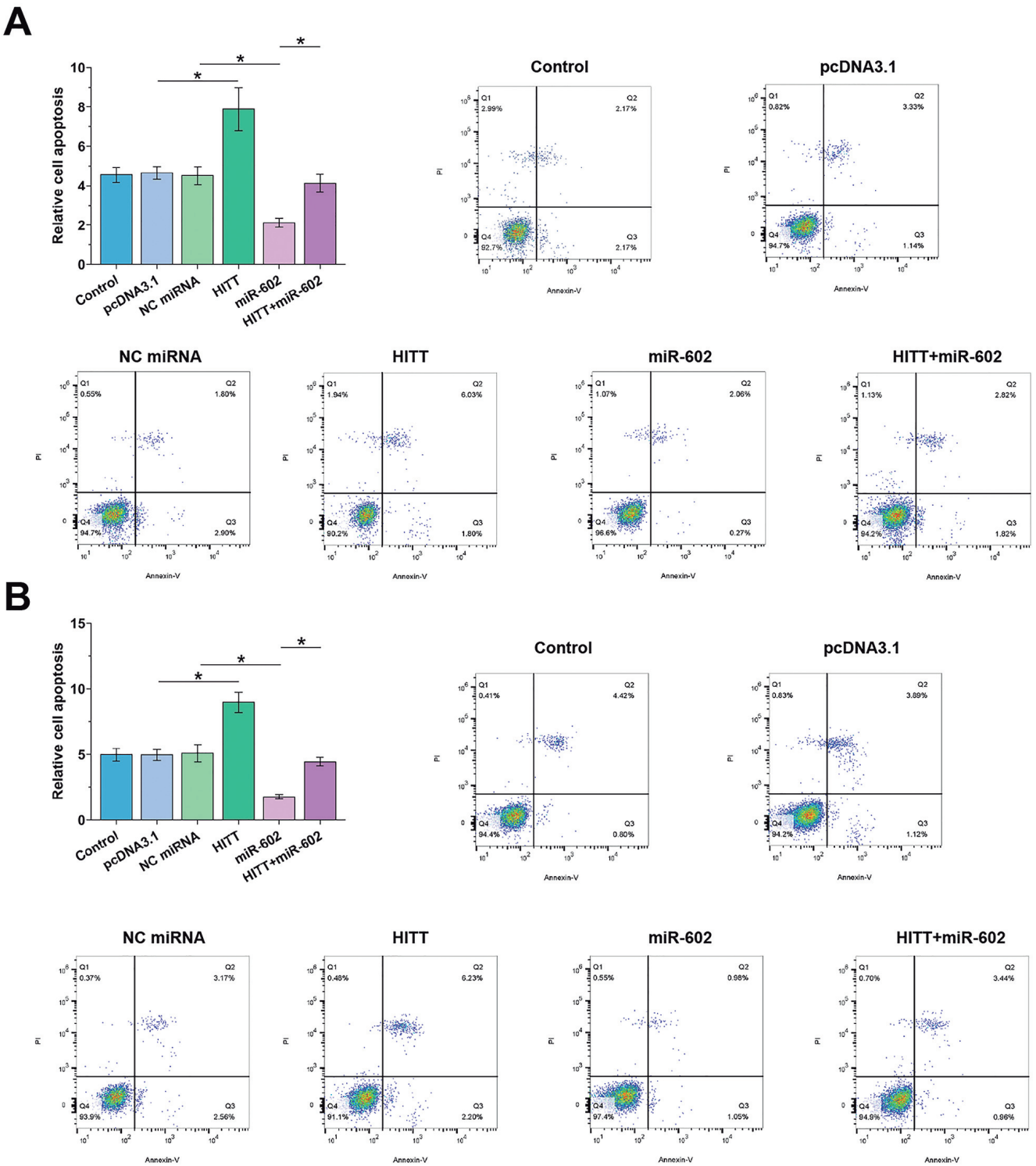

Fig. 5. Overexpression of HITT increased GC cell apoptosis through miR-602. The roles of HITT and miR-602 in regulating the apoptosis of SNU-1 **(A)** and AGS **(B)** cells were analyzed by cell apoptosis assay. Control, control cells without transfections; pcDNA3.1, cells transfected with empty pcDNA3.1 vector; HITT, cells transfected with HITT expression vector; NC miRNA, cells transfected NC miRNA; miR-602, cells transfected with miR-602 mimic; HITT+miR-602, cells co-transfected with HITT expression vector and miR-602. *, $p<0.05$.

HITT was inversely correlated with mature miR-602 across GC samples and suppressed the maturation of miR-602 in GC cells

Pearson's correlation coefficient analysis showed that, HITT was inversely and closely correlated with the expression of mature miR-602 across GC tissues (Fig. 3A). In contrast, HITT and mature miR-602 were not closely correlated across GC tissues (Fig. 3B). HITT and miR-602 were overexpressed in SNU-1 and AGS cells, and the overexpression was confirmed every 24 h until 96 h (Fig. 3C, $p<0.05$). The results illustrated that overexpression of HITT significantly decreased the expression levels of mature miR-602 in both cell lines (Fig. 4A, $p<0.05$). However, no significant alteration in the expression of mature miR-602 was observed (Fig. 4B, $p<0.05$). Moreover, no significant alteration in the expression of HITT was observed after overexpression of miR-602 (Fig. 4C).

Overexpression of HITT increased GC cell apoptosis through miR-602

The role of HITT and miR-602 in regulating the apoptosis of SNU-1 and AGS cells was evaluated by cell apoptosis assay. Our data illustrated that overexpression of HITT increased GC cell apoptosis, while overexpression of miR-602 inhibited cell apoptosis. Moreover, overexpression of HITT suppressed the role of miR-602 in inhibiting SNU-1 (Fig. 5A, $p<0.05$) and AGS (Fig. 5B, $p<0.05$) cell apoptosis.

HITT directly interacted with premature miR-602

RNA pull-down assay was performed to analyze the direct interaction between HITT and miR-602. Compared to bio-NC pull-down group, the bio-premature miR-602 pull-down group exhibited significantly higher expression levels of HITT, suggesting the direct interaction between HITT and premature miR-602 (Fig. 6, $p<0.05$).

Discussion

The expression of HITT in GC and its interaction with miR-602 was explored in this study. We found that HITT was highly upregulated in GC. In addition, HITT may suppress the maturation of miR-602 to induce the apoptosis of GC.

HITT is a recently characterized lncRNA with tumor suppressive roles in colon cancer (Wang et al., 2020). It was observed that HITT was downregulated in colon cancer and the downregulation of HITT is required for the expression of HIF-1α induced by hypoxia, thereby regulating angiogenesis and tumor growth (Wang et al., 2020). Based on our knowledge, the role of HITT in other cancers is unknown. In this study we showed that HITT was upregulated in GC, and the upregulation of HITT increased the apoptosis of GC cells. Therefore, HITT is likely a tumor suppressor in GC and overexpression of HITT may serve as a potential target for the treatment of GC.

The oncogenic role of miR-502 has been investigated in several types of cancer (Yang et al., 2010). For instance, miR-602 is upregulated in liver cancer and suppresses the expression of tumor suppressor RASSF1A to promote cancer development (Yang et al., 2010). In addition, miR-602 is upregulated in esophageal squamous cell carcinoma and forms a negative feedback loop with FOXK2 to regulate cancer progression (Liu et al., 2019). In this study we observed the upregulation of miR-602 in GC and its inhibitory

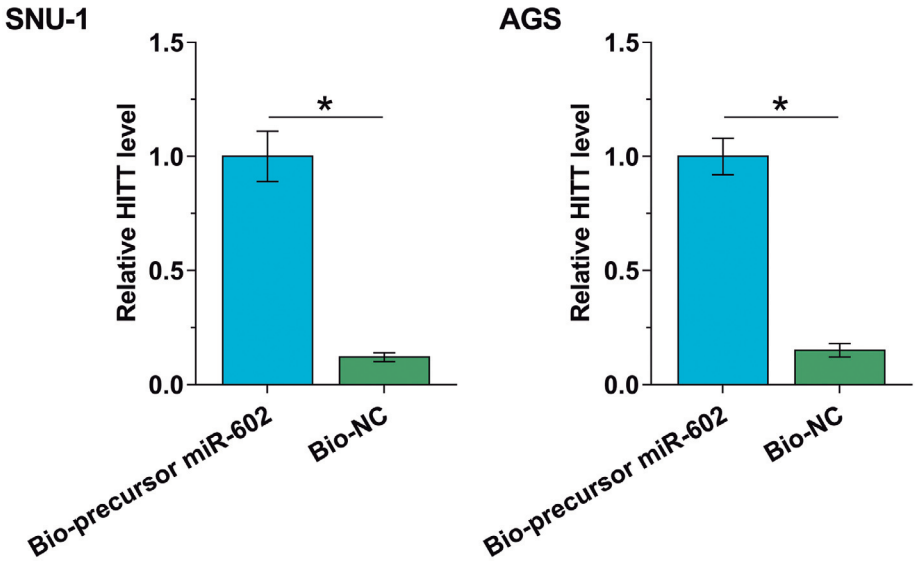

Fig. 6. HITT directly interacted with premature miR-602. RNA pull-down assay was performed to analyze the direct interaction between HITT and miR-602. The RNA pull-down assay was performed following the methods described above. *, $p<0.05$.

effects on GC cell apoptosis, suggesting the oncogenic role of miR-602 in GC.

Interestingly, no significant alteration in the expression of premature miR-602 was observed in GC patients, suggesting that the maturation, but not transcription of miR-602 was altered in GC. We showed that HITT could suppress the maturation of miR-602 in GC cells. HITT was found to be located in both nucleus and cytoplasm. Furthermore, it can directly interact with premature miR-602. It has been well established that the movement of premature miRNAs from nucleus to cytoplasm is required for the maturation of miRNAs (Murchison and Hannon, 2004). We therefore speculated that HITT could suppress the movement of premature miR-602 to reduce the production of mature miR-602.

This study proposed that the maturation of a miRNA can be regulated by a lncRNA, which is the novelty of this study. However, we did not perform *in vivo* animal model experiments to confirm the conclusion. In addition, the number of patients is small. Further validations are still needed.

Conclusion

In conclusion, HITT is downregulated in GC and it may suppress the maturation of miR-602 to induce the apoptosis of GC.

Acknowledgements. Not Applicable.

Ethical Approval and Consent to participate. Informed consent was obtained from all individual participants included in the study. All procedures were approved by the First Affiliated Hospital of Gannan Medical College Ethics Committee. Procedures operated in this research were completed in keeping with the standards set out in the Announcement of Helsinki and laboratory guidelines of research in China.

Consent to publish. Not applicable.

Availability of supporting data. The data that support the findings of this study are available on request from the corresponding author.

Competing interests. All other authors have no conflicts of interest.

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Authors' contributions. Yun Chen, Jing Ye: study concepts, literature research, clinical studies, data analysis, experimental studies, manuscript writing and review; Canhui Ouyang: study design, literature research, experimental studies and manuscript editing; Lingyun Liao: definition of intellectual content, clinical studies, data acquisition and statistical analysis; Yun Zhou: data acquisition, manuscript preparation and data analysis; Fan Meng, Yao Liu: data acquisition and statistical analysis. All authors have read and approve the submission of the manuscript.

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