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LncRNA INHBA-AS1 promotes colorectal cancer cell proliferation by sponging miR-422a to increase AKT1 axis

H. LIN[1], Y.-G. HONG[2], J.-D. ZHOU[2], X.-H. GAO[2], P.-H. YUAN[3], C. XIN[2], Z.-P. HUANG[4], W. ZHANG[2], L.-Q. HAO[2], K.-Z. HOU[1]

[1]The First Department of General Surgery, Shidong Hospital, Yangpu District, Shanghai, Anhui Medical University, University of Shanghai for Science and Technology, Shanghai, P.R. China
[2]Department of Colorectal Surgery, Changhai Hospital, Second Military Medical University, Shanghai, P.R. China
[3]Department of the Operating Room, Shidong Hospital, Yangpu District, Shanghai, Anhui Medical University, University of shanghai for Science and Technology, Shanghai, P.R. China
[4]Department of Hepatobiliary Surgery, General Hospital of Southern Theatre Command, Guangzhou, P.R. China

Hui Lin, Yong-gang Hong, Ji-dian Zhou and Xian-hua Gao contributed equality

Abstract. – OBJECTIVE: In recent years, long non-coding RNAs (lncRNAs) have emerged for regulating the development, as well as progression in colorectal cancer (CRC), which assists in finding new targets for CRC treatment. A previous study indicated that INHBA-AS1 promotes oral squamous cell progression by sponging miR-143-3p. However, the exact function possessed by lncRNA INHBA-AS1 in CRC development remains unclear.

PATIENTS AND METHODS: The expression level of INHBA-AS1 in CRC tissues and cell lines was determined by qRT-PCR. The functional role of INHBA-AS1 in CRC was investigated by a series of in vitro assays. RNA immunoprecipitation (RIP), bioinformatics analysis was utilized to explore the potential mechanisms of INHBA-AS1.

RESULTS: The present study identified INHBA-AS1 as a kind of lncRNA with high expression in CRC tissues and cells. Functionally, NHBA-AS1 downregulation in CRC cells suppressed CRC cell proliferation as well as colony formability. Mechanistically, INHBA-AS1/miR-422a/AKT1 established the ceRNA network to regulate MMP-2, -7, -9 expressions that participated the modulation of CRC progression.

CONCLUSIONS: In summary, LncRNA INHBA-AS1 contributes to CRC progression through AKT1 pathway, and provides a new mechanism to regulate CRC development, as well as a potential target for treating CRC.

Key Words:
AKT1, CRC, INHBA-AS1, MiR-422a, Progression.

Introduction

Colorectal cancer (CRC) is a common type of cancer, with patient death typically resulting from tumor metastasis[1]. When CRC tumors are detected in their early stages, they can often be resected or effectively treated by other means. However, when the disease is not detected until it is already in an advanced state, few effective treatment options are available to affected patients[2,3]. Although many studies to date have explored the mechanisms governing CRC metastasis, this process remains complex and incompletely understood. Thence, new biomarker for the diagnosis and prognosis shall be identified for improving the survival rates of patients.

Long non-coding RNA (lncRNAs) is long RNA transcripts (> 200 nucleotides) that do not possess protein-coding capabilities. They can significantly regulate different cancer types with regard to development, treatment and prognosis[4,5]. By now, studies have identified that plenty lncRNAs act in CRC, regarding the diagnosis[6], cell the differentiation, metastasis, and growth of cells[7,8] the chemoresistance[9], as well as the prediction on therapy outcome[10] etc. LncRNA INHBA-AS1 was a novelty lncRNA that was associated with oral squamous cell progression by sponging miR-143-3p[11]. However, the accurate molecular mechanisms possessed by

Corresponding Authors: Li-qiang Hao, MD; email: 3502006005@smmu.edu.cn
Ke-zhu Hou, MD; email: houkezhu@126.com

INHBA-AS1 in CRC remain largely unclear. The study holds the purpose of investigating the novelty mechanism of INHBA-AS1 in CRC progression.

Patients and Methods

Patients and Sample Collection

32 CRC tissues together with corresponding healthy tissue in samples from Changhai Hospital were included in the study. The study has obtained the approval of the Ethics Committee of the Changhai Hospital, as well as the written informed consent of all the participants. Patient information related to their identification was confidential across the whole study.

qRT-PCR Analysis

We added the TRIzol (Thermo Fisher Scientific, Inc., Waltham, MA, USA) to cell/tissues for extracting RNA, and a nanodrop was applied to the quantification. The qRT-PCR analysis assisted in quantifying the gene expression of synthesized cDNA[12,13]. Primers were purchased from RiboBio (RiboBio, Inc., Guangzhou, Guangdong, China).

Cell Based Assays

The cell lines for CRC cell lines (LOVO, HT29, SW620, and SW480) and non-cancer cells (NCM460) were provided by the Nanjing KeyGen Biotech. Co. Ltd, in Nanjing (Nanjing, China). The maintenance of cell lines for NSCLC was done in the Dulbecco's Modified Eagle's Medium Medium (DMEM) with FBS (10%), and 100 μg/mL each of streptomycin plus 100 Ul/mL penicillin (all purchased from Thermo Fisher Scientific, Inc., Waltham, MA, USA). Lentivirus-mediated shRNA or short hairpin RNA, to target INHBA-AS1 (si-INHBA-AS1) and NC or shCon (negative control) vector came from Genechem in Shanghai (Shanghai, China). Small interfering RNA (siRNA) to target INHBA-AS1 (siINHBA-AS1) and negative control or siCon, the mimic of miR-422a, miR-NC or the negative control, inhibitors of miR-422a as well as miRNA (miR-NC inhibitor) were prepared and provided by Genechem in Shanghai (Shanghai, China). Lipofectamine^{3000} from Invitrogen was used for cell transfections (Carlsbad, CA, USA).

Cell Proliferation

Cells of CRC were added (2×10^3 cells/well) into plates of 96 wells. After indicated times, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (20 μL) (Beyotime, Shanghai, China) were added into wells and kept for 20 min with dimethyl sulfoxide (DMSO) (200 μL). The estimation of optical density (OD) values were done at 490 nm on an analyzer for Enzyme Immunoassays from Bio-Rad Laboratories (Hercules, CA, USA).

Colony Formation Assay

Each group of treated cells (1×10^3 per well) was seeded into 10 cm culture dish, and cultured for 2 weeks. Finally, the colonies were stained using 1% crystal violet, together with the counting of cell colony number.

RNA Immunoprecipitation (RIP) Assay

RNA-binding protein immunoprecipitation Magna RIP kit from Promega (Promega, Madison, WI, USA) was applied to the RIP (RNA immunoprecipitation) evaluation. AGO2 and IgG antibodies were obtained from Abcam (Abcam, Cambridge, MA, USA). The beads were washed using the wash buffer, and to remove proteins, the incubation of complexes was done with SDS (0.1%)/proteinase K. Using the precipitated RNA qRT-PCR assay was done to reveal the binding targets.

Luciferase Reporter Assay

Culturing of CRC cell was done in plates of 24 wells. After incubating for one full day, transfection of the reporter vector pmirGLO from Promega (Promega, Madison, WI, USA) harboring the WT (wild-type) or MUT (mutant) INHBA-AS1 was done into CRC cell combined with miR-422a. Post-transfection of two full days, estimation of Luciferase activity was done through Reporter System of Dual-Luciferase from Promega (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used for all the statistical tests, with data expressed as means ± SEM. Student's *t*-tests, Wilcoxon signed-rank tests, Pearson correlation analyses, Pearson chi-squared tests, Fisher's exact tests, and nonparametric Mann-Whitney U tests were conducted to test data depending on condition, and p-value < 0.05 was considered as the significance threshold.

Results

INHBA-AS1 Was Increased in CRC

From 32 cases, tissues of CRC and equivalent healthy tissues were sampled and INHBA-AS1 expression was examined through qRT-PCR. As per Figure 1A, remarkable overexpression of INHBA-AS1 was observed in tissues of CRC than in adjacent (normal) tissue. Further enhanced INHBA-AS1 expression was also observed in CRC cells (LOVO, HT29, SW620, and SW480) in contrast to the control (NCM460) cells (Figure 1B). Thus, there is an over-regulation of INHBA-AS1 expression in CRC.

Effects of INHBA-AS1 Knockdown on CRC Cells

For examining how INHBA-AS1 affected CRC progression, we used siINHBA-AS1 (siRNA targeting INHBA-AS1) and knocked-down INHBA-AS1 in LOVO and HT29 cells (Figure 2A). Then, verification of INHBA-AS1 was done through qRT-PCR. As per Figure 2B, the viability of LOVO and HT29 cells was impeded by INHBA-AS1 silencing. Colony formation assays also achieved similar trend (Figure 2C and D). Hence, INHBA-AS1 is an oncogene in CRC.

INHBA-AS1 Controls MMP-2, -7, and -9 Expression Through AKT1 in CRC

With the purpose of investigating CRC regulation *via* INHBA-AS1, it was found that INHBA-AS1 was positively co-expression with MMP-2, -7, and -9 from the GEPIA (Gene Expression Profiling Interactive Analysis) data, respectively (Figure 3A). Meanwhile, in CRC cells knockdown INHBA-AS1, a marked decrease in MMP-2, -7 and -9 mRNA level was observed (Figure 3B). Furthermore, INHBA-AS1 was positively co-expression with AKT1 level from the GEPIA data (Figure 3C) and in CRC cells knock down INHBA-AS1, AKT1 mRNA level remarkably decreased (Figure 3D). Combined with the above results we observed that AKT1-MMP2/7/9 axis played key role in CRC progression. We speculated that INHBA-AS1 enhanced CRC progression *via* AKT1-MMP2/7/9 axis. To explore whether INHBA-AS1 regulates MMP-2, -7 and -9 expression through AKT1, and si-INHBA-AS1 plus LV-NC or LV-AKT1 was introduced into CRC cells. First, RT-qPCR helped to verify the LV-AKT1 transfection efficiency, finding that, relying on LV-AKT1 transfection, LV-AKT1 expression significantly increased in CRC cells (Figure 3E). Furthermore, the downregulation of MMP -2, -7 and -9 mRNA (Figure 3F) caused by INHBA-AS1 knockdown was reversed in CRC cells by increasing AKT1. All these results demonstrated that INHBA-AS1 could mediate its role in CRC progression through MMP -2, -7, and -9 by AKT1.

INHBA-AS1 Undergoes a Direct Interaction with MiR-422a in CRC as a Molecular miRNA Sponge to Induce AKT1 Level

As proved by new studies, lncRNAs participated many molecular biological activities being competing endogenous RNA (ceRNA)

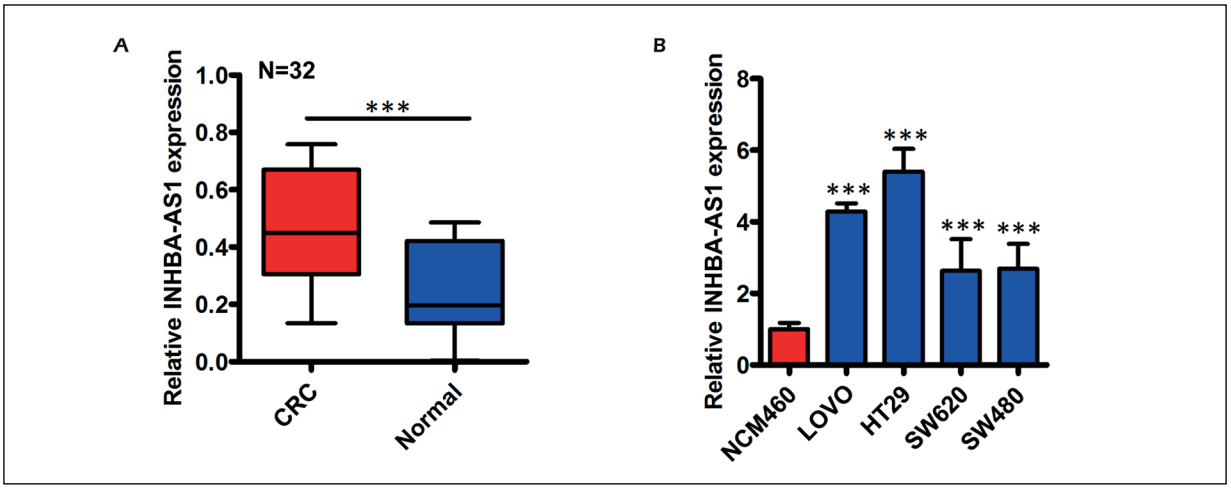

Figure 1. INHBA-AS1 is increased in CRC. **A,** INHBA-AS1 expression was identified in 32 paired samples of CRC tissues. **B,** INHBA-AS1 expression in the CRC cell lines and in the normal cell (NCM460). **p* <0.05; ***p* <0.01; ****p* <0.001

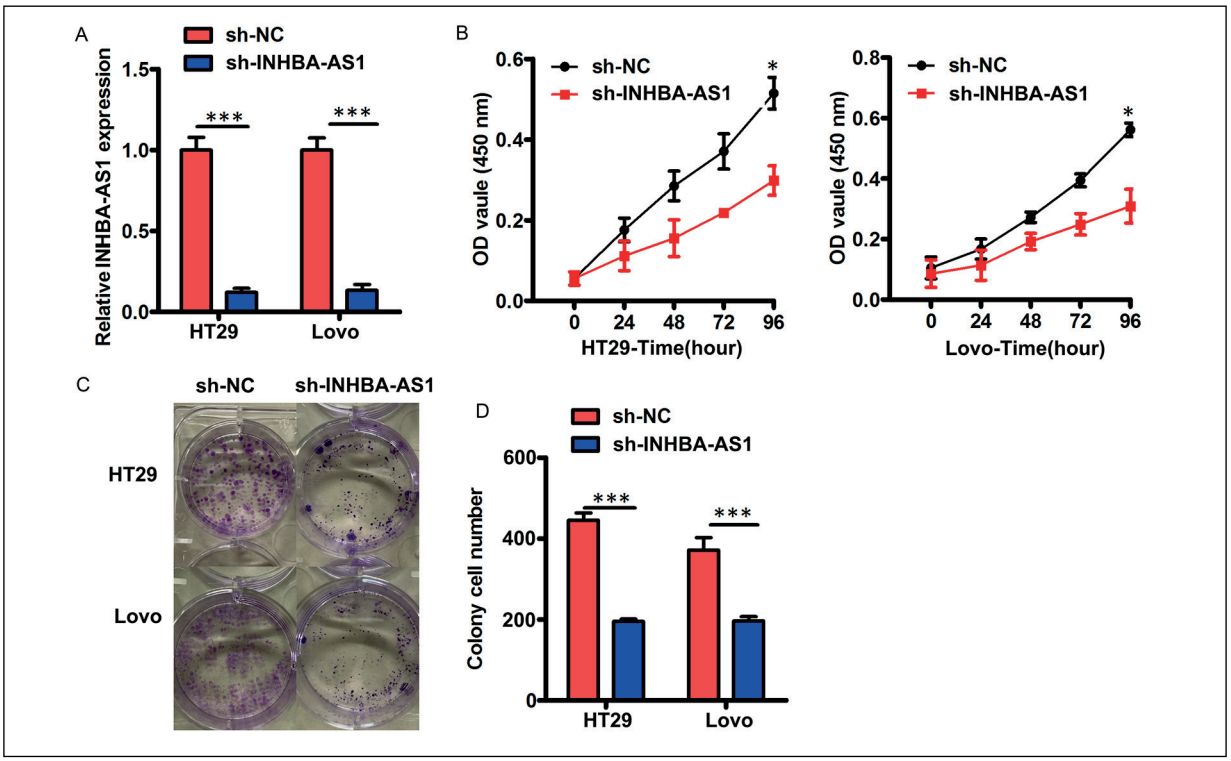

Figure 2. A decreases in INHBA-AS1 expression enhance CRC cell proliferation. **A,** RT-qPCR analysis on the expression of INHBA-AS1 in indicated CRC cells following si-INHBA-AS1 or si-NC transfection. **B,** Cell Counting Kit-8 assay assisted in detecting the indicated CRC cell proliferation that lack INHBA-AS1. **C, D,** Colony form assays helped to analyze indicated CRC cells after being transfected with si-INHBA-AS1 or si-NC. Colonies were observed and counted with an inverted light microscope (×200 magnification). **p* <0.05; ***p* <0.01; ****p* <0.001.

specific to miRNAs. Then, the publicly available algorithm starBase 3.0 assisted in predicting the direct interaction between miRNAs and INHBA-AS1. As reported, there were complementary binding sites between MiR-422a (Figure 4A) and INHBA-AS1, and miRNA could oppress CRC progression, more importantly, *via* targeting AKT1 in CRC, and thereby it was used for the further verification[14]. MiR-422a expression presented an evident increase in CRC cells transfected by agomiR-422a (Figure 4B). Next, co-transfection of pmirGLO reporter vector with either INHBA-AS1-WT (WT INHBA-AS1) or INHBA-AS1-MUT (MUT) combined with miR-422a was done in CRC cell. As per Figure 4C, the activity of Luciferase reduced remarkably due to miR-422a in cell transfected with INHBA-AS1-WT, although it did not suppress the Luciferase activity in INHBA-AS1-MUT transfected cells. Next, the miR-422a and INHBA-AS1 relationship was confirmed through the RIP assay. The miR-422a and INHBA-AS1 combination complex precipitated in AGO2 (Figure 4D). The introduction of si-INHBA-AS1 together with antagomiR-422a or antagomir-NC into CRC cells aimed at figuring out if INHBA-AS1 sponged miR-422a for regulating the AKT1 expression. First, RT-qPCR assisted in verifying the antagomiR-422a transfection efficiency. As found, antagomiR-422a transfection caused an evident decreased miR-422a expression in CRC cells (Figure 4E). Besides, AKT1 mRNA downregulation (Figure 4F) caused by INHBA-AS1 knockdown was reversed in CRC cells relying on the antagomiR-422a re-introduction. As suggested by all above findings, INHBA-AS1 sponged miR-422a for regulating the expression of AKT1 in CRC cells in a positive manner.

Discussion

As a most common tumor, CRC causes large amounts of deaths[15,16]. LncRNAs exert important roles in the development and progression

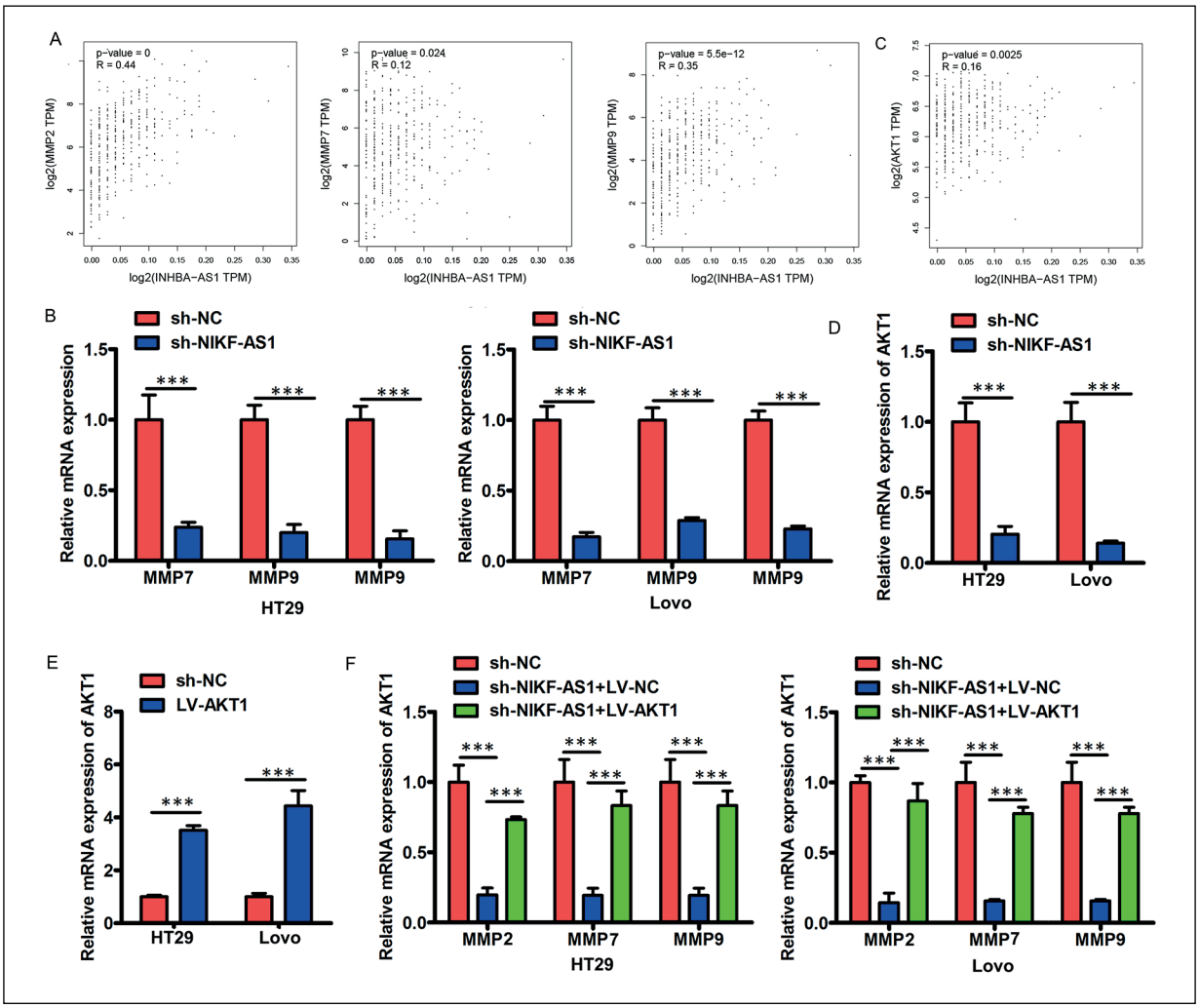

Figure 3. INHBA-AS1 controls MMP -2, -7 and -9 expressions through AKT1 in CRC. **A,** GEPIA tool indicted that the INHBA-AS1 expression was significantly and positively correlated with MMP -2, -7, and -9 expression in these CRC tissue samples, respective. **B,** MMP -2, -7 and -9 mRNA level expression analysis in indicated CRC cells with INHBA-AS1 knock down. **C,** GEPIA tool indicted that INHBA-AS1 was significantly and positively correlated with AKT1 in these CRC tissue samples. **D,** AKT1 mRNA level expression analysis in indicated CRC cells with INHBA-AS1 knock down. **E,** AKT1 mRNA was expressed in indicated CRC cells under the transfection of LV-NC or LV-AKT1 using RT-qPCR. **F,** Indicated CRC cells received transfection of LV-NC or LV-AKT1 when there was si-INHBA-AS1. After transfection, RT-qPCR assay was performed to detect AKT1 mRNA expressions. *p <0.05; **p <0.01; ***p <0.001.

in CRC[17-19], such as lncRNA GAS5/miR-222-3p/GAS5/PTEN axis play a key role in CRC progression[20]. LncH19 acted as key role in Epithelial-Mesenchymal Transition of CRC[21]. Previous scholars[22] reported that LncRNA INHBA-AS1 was a new lncRNA that was associated with oral squamous cell progression by sponging miR-143-3p. However, researchers fail to find the accurate molecular mechanisms with regard to INHBA-AS1 in CRC. Here, we validated a novelty mechanism of INHBA-AS1 which induced CRC progression *via* AKT1-MMP2/7/9 axis.

As demonstrated by previous studies, when the proteolytic activity exhibited by cancer cell expressed MMPs is enhanced, it will assist in medicating stroma degradation regarding neighboring cells, as well as enhance tumor cell spread, which constitutes an essential step in the process of tumor invasion[23]. MMP-7, also called matrilysin, is capable of cleaving a lot of protein components constituting the ECM, such as entactin, collagen, osteopontin, laminin, fibronectin, proteoglycans and elastin, as well as cleaving also pro-MMP-2 and pro-MMP-9, and other proteins[24]. MMP-7

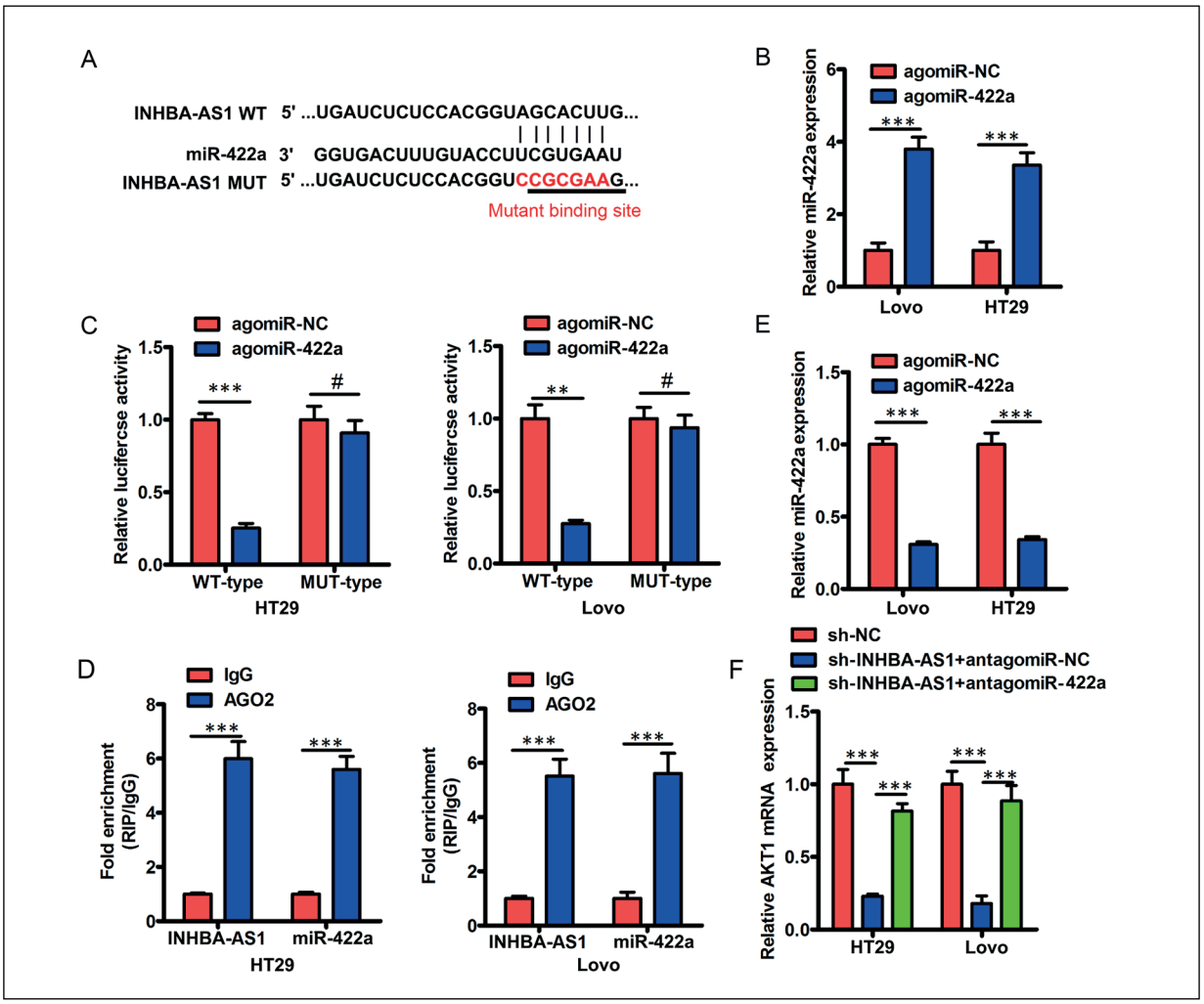

Figure 4. INHBA-AS1 presents a direct interaction with miR-422a in CRC as a molecular miRNA sponge to induce AKT1 level. **A,** Bioinformatics analysis found the bindings sites of WT and MUT miR-422a within INHBA-AS1. **B,** miR-422a expression analysis on indicated CRC cells under the transfection of agomiR-422a or agomir-NC. **C,** WT-INHBA-AS1 or MUT-INHBA-AS1 received co-transfection of agomiR-422a or agomir-NC into Indicated CRC cells, followed by the detection of luciferase activity 48 h later. **D,** INHBA-AS1 and miR-422a exhibited enrichment in immunoprecipitation contained Ago2 relative to the IgG control. **E,** RT-qPCR detected the miR-422a expression in indicated CRC cells under the transfection of antagomiR-422a or antagomir-NC. **F,** Indicated CRC cells underwent the transfection of antagomiR-422a or antagomir-NC when there was si-INHBA-AS1. Then, RT-qPCR assay was performed to detect AKT1 mRNA expressions. *p <0.05; **p <0.01; ***p <0.001; # p >0.05.

was reported to play key role in enhancing HCC cell migration and invasion ability.[25]. MMP-9 takes charge of ECM degradation, IL-1β activation, and chemokine cleavage[26]. It seems that MMP critically intervenes in regulating the angiogenesis of growth plate and recruiting endothelial stem cells, thereby mainly affecting the tumor angiogenesis[27]. High MMP-9 was associated with advantaged TNM stage, present of lymph node invasion, poor differentiation, and poor overall prognosis[28]. Combined with GEPIA and our researches it has been showed that INHBA-AS1 was closely positively associated with MMP2/7/9 and AKT1 level in CRC tissues and that the knockdown of INHBA-AS1 leading MMP-2/7/9 and AKT1 level was downregulated in CRC cell lines. Meanwhile, the downregulation of MMP -2, -7, and -9 mRNA caused by INHBA-AS1 knockdown was reversed in CRC cells by increasing AKT1. These findings suggested that INHBA-AS1 mediated its effects on CRC progression through MMP -7, and -9 by AKT1.

LncRNAs may act as ceRNA to sponge miRNAs in many types of cancers[29-33]. Such as, KCN-

Q1OT1 sponged miR-34 to enhance colon cancer chemoresistance by increasing ATG4B expression[34]. MALAT1/miR-20b-5p/Oct4 axis induces colorectal cancer cells stemness[35]. Meanwhile, DGCR5 inhibits HCC progression *via* sponging miR-346 and activating KLF1[36,37]. Ma et al[38] indicated that INHBA-AS1 may sponge miR-143-3p. Following, we wonder whether INHBA-AS1 exerts roles by sponging other miRNAs to regulate AKT1 level. First, it was predicted that there were complementary binding sites between miR-442a and INHBA-AS1. Luciferase reporter together with RIP assay helped to confirm their interaction and binding. Also, INHBA-AS1 downregulation caused an increase in the miR-422a expression, and the AKT1 expression decreased accordingly. In addition, downregulated miR-422a has been reported to be associated with enhancing CRC progression. Fourth, miR-422a has been reported to target AKT1 in CRC cell carcinoma. Based on all these findings, a ceRNA model was revealed which included INHBA-AS1, miR-422a, and AKT1 in CRC cells.

Conclusions

Collectively, INHBA-AS1 overexpression promotes CRC development by regulating MMP-2/7/9s axis *via* AKT1. Our research provides a new mechanism regulating CRC tumorigenesis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Availability of Data and Materials

All data used and analyzed throughout the study can be found in the published article.

Ethics Approval and Consent to Participate

The present study obtained the approval of the Ethics Committee of Changhai Hospital, as well as the written informed consent from all participants in advance.

Authors' Contribution

KZ H and LQ H conceived the study. H L, YG H and JD Z wrote the manuscript. XH G, PH Y, and C X designed and revised the manuscript. ZP H analyzed and interpreted the data. ZP H assisted in data analysis. The final version of the manuscript had been read and approved by all the authors.

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