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ORIGINAL RESEARCH

Long Non-Coding RNA HAND2-AS1 Inhibits Growth and Migration of Gastric Cancer Cells Through Regulating the miR-590-3p/KCNT2 Axis

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Introduction: Long non-coding RNAs (lncRNAs) are regarded as crucial regulators for cancer initiation and progression. Heart and Neural Crest Derivatives Expressed 2 antisense RNA 1 (HAND2-AS1) was recently proposed to function as tumor suppressor in several human cancers. However, its role in gastric cancer (GC) remains unclear.

Methods: HAND2-AS1 expression in GC tissues and normal tissues was analyzed at GEPIA (a web server for gene expression profiling analysis). Moreover, RT-qPCR method was utilized to explore HAND2-AS1 expression in GC cells and normal cell. In vitro experiments were carried out using cell counting kit-8 assay, colony formation assay, and flow cytometry assay, respectively. Bioinformatic analysis and luciferase activity reporter assay were performed to identify the downstream targets of HAND2-AS1.

Results: We found HAND2-AS1 has decreased expression in both GC tissues and cells. Overexpression of HAND2-AS1 was able to inhibit GC cell proliferation, colony formation, but promote apoptosis. On the contrary, knockdown of HAND2-AS1 could cause the opposite effects on GC cells. Furthermore, HAND2-AS1 was shown to function as a competitive RNA that binds with microRNA-590-3p (miR-590-3p) to affect the expression of potassium sodium-activated channel subfamily T member 2 (KCNT2).

Discussion: Our results indicated the tumor suppressive role of HAND2-AS1 in GC. Also, the newly identified HAND2-AS1/miR-590-3p/KCNT2 axis will help us to understand the role of HAND2-AS1 in cancer.

Keywords: HAND2-AS1, miR-590-3p, KCNT2, gastric cancer

Introduction

Among all the cancer types in the digestive system, gastric cancer (GC) is one of the leading causes of newly occurring cancer types each year.[1] As predicted, the numbers for newly diagnosed and cancer deaths each year are 1,033,071 and 782,685, respectively.[1] The 5-year overall survival of GC patients receiving treatment at early stages can be about 95% owing to the improvements in surgery and targeted therapy methods.[2] However, for patients diagnosed at late stages, the best treatment window is closed.

Long noncoding RNAs (lncRNAs) are a family of RNAs with lengths of 200 nucleotides to 100 kilobases.[3] LncRNAs have typically been regarded as junk genes as they lack the ability to code proteins.[3] In 2011, Salmena et al[4] proposed a competitive RNA (ceRNA) theory that helped us to understand the functions of non-coding RNAs including lncRNA.

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In recent years, investigations of the biological roles of lncRNAs in disease progression, especially in cancers, have been the hotspots.[5] Heart and Neural Crest Derivatives Expressed 2 antisense RNA 1 (HAND2-AS1), located at chromosome 4q33-34, has been demonstrated to be a crucial regulator for cancer progression in the past 2 years.[6–9] In endometrial carcinoma, HAND2-AS1 was found to have decreased expression in tumor tissues.[6] Moreover, they found HAND2-AS1 overexpression could suppress cancer cell migration and invasion via inactivating neuromedin U 6. In colorectal cancer, down-regulation of HAND2-AS1 was found to have a negative correlation with tumor stages.[7] Also, they showed that the proliferation and invasion abilities of colorectal cancer cells can be suppressed by HAND2-AS1 overexpression.[7] A recent work performed on non-small cell lung cancer showed HAND2-AS1 can suppress cancer cell malignant behaviors with transforming growth factor β as a mediator.[8] Importantly, a similar expression trend of HAND2-AS1 was found in esophagus squamous cell carcinoma.[9] However, until now, the expression and functions of HAND2-AS1 in GC remains to be elucidated.

In this study, we explored the expression of HAND2-AS1 in both GC tissues and cell lines. Also, the effects of HAND2-AS1 on GC cell behaviors were explored using in vitro experiments. Importantly the possible involvement of microRNA-590-3p (miR-590-3p) and potassium sodium-activated channel subfamily member 2 (KCNT2) in the HAND2-AS1-mediated GC cell events was investigated.

Materials and Methods

Cell Lines and Cell Culture

Normal gastric mucosal epithelial cell (GES-1) and GC cells (SGC-7901 and SGC-8901) were bought from the Cell Collection Center of Chinese Academy of Sciences (Shanghai, People's Republic of China). RPMI 1640 with 10% fetal bovine serum (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplement was used to incubate these cells at a 37°C humidified incubator supplemented with 5% of CO_2.

Cell Transfection

Small interfering RNA targeting HAND2-AS1 (si-HAND2-AS1), negative control (si-NC), miR-590-3p mimic, and the corresponding negative control (mi-NC) were provided by GenePharm (Shanghai, People's Republic of China). The pcDNA3.1 with open reading frame of HAND2-AS1 or KCNT2 inserted was bought from Generay (Shanghai, People's Republic of China). These siRNAs, miRNAs, or pcDNAs were transfected into GC cells using Lipofectamine 2000 (Invitrogen) after incubating these cells to about 60% of confluence.

Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

RNA from cells were prepared using Trizol reagent (Invitrogen) according to the provided protocols. Complementary DNA was synthesized using PrimerScript reagent kit (Invitrogen). RT-qPCR was performed at ABIT 7500 system (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA) to detect the relative expression level of HAND2-AS1, miR-590-3p and KCNT2 using SYBR Green Mix (Takara, Dalian, Liaoning, People's Republic of China). The primers used were as follows: HAND2-AS1: 5′-GGC GTTTACGTAGACCAGAACC-3′ (forward) and 5′-CTTCCAAAAGCCTTCTGCCTTAG-3′ (reverse); KCNT 5′-TGCCTCCCAGGTACAGATTCCG TGA 3′ (forward) and 5′-TTGTTTCAAATAGACTTATC AATGCC GAGA-3′ (reverse); β-actin: 5′-GACCTCTA TC AACACAGT-3′ (forward) and 5′-AGTACTTGCG TCAGGAGGA-3′ (reverse); miR-590-3p: 5′-GCGCTA ATTTTATGTATAA-3′ (forward) and 5′-GTGCAGGGTC GAGGT-3′ (reverse); U6 snRNA: 5′-AGAGCCTGTG GTGTCCG-3′ (forward) and 5′-CATCTTCAAAGCAC TTCCCT-3′ (reverse). β-actin was used as internal control for HAND2-AS1 and KCNT2, while U6 snRNA was used as endogenous control for miR-590-3p.

Cell Counting Kit-8 (CCK-8) Assay

Cells were incubated into 96-well plates with the density of 5,000 cells/well. After 0, 24, 48, and 72 hours of incubation, CCK-8 reagent obtained from Beyotime (Haimen, Jiangsu, People's Republic of China) was added to each well and further incubated for 2 hours. Microplate reader was used to measure optical density at 450 nm.

Colony Formation Assay

Cells were seeded into 6-well plates with the density of 1,000 cells/well and incubated at the above-mentioned condition for 2 weeks to form colonies. Subsequently, colonies formed were fixed, stained, and counted under a microscope.

Cell Apoptosis Assay

The cultured cells were treated using trypsin, and then stained with Annexin V Fluorescein Isothiocyanate (FITC)/propidium iodide (PI) at room temperature for 15 minutes obtained from Beyotime. Cell apoptosis rate was analyzed at flow cytometry (BD Biosciences, San Jose, MA, USA). The cells with FITC staining were regarded as apoptosis cells.

Targets Prediction Using Bioinformatic Analysis Tools

The miRNA target for HAND2-AS1 was analyzed at LncBase Predicted v.2 (http://carolina.imis.athena-innovation.gr/diana_tools/) and miR-590-3p was found to be a putative target. The targets for miR-590-3p were analyzed at TargetScan V7.2 (http://www.targetscan.org/vert_72/) and KCNT2 was identified as a highly potential target for miR-590-3p.

Luciferase Activity Reporter Assay

According to the bioinformatic analysis results, luciferase activity vectors were constructed using pmirGLO vector (Promega, Madison, WI, USA). The wild-type and mutant sequences of HAND2-AS1 and KCNT2 were cloned into pmirGLO and named as wt/mt HAND2-AS1 or KCNT2, respectively. Cells were co-transfected with luciferase activity vectors and synthetic miRNAs using Lipofectamine 2000. A dual-luciferase activity system was used to measure relative luciferase activity.

RNA Immunoprecipitation (RIP)

RIP assay was utilized to investigate the connection of HAND2-AS1 and miR-590-3p using Magna RI RNA-Binding Protein Immunoprecipitation Kit (Millipore, Sigma Aldrich, Merck Millerica, MA, USA). RIP buffer was used to incubate cultured cells and treated with anti-Ago2 antibody and control IgG. Samples were then treated with Proteinase K and subjected to RT-qPCR analysis to analyze HAND2-AS1 and miR-590-3p levels.

RNA Pull-Down Assay

Cells were transfected with biotin-labeled miR-590-3p-wt and miR-590-3p-mt (bio-miR-590-3p-wt/mt). Then, cells were treated with lysis buffer and incubated with streptavidin magnetic beads (Sigma, St Louis, MO, USA) and rinsed with pre-cold lysis buffer and salt buffer. Trizol was used to extract the bound RNA and then detect the expression of HAND2-AS1 using RT-qPCR.

Gene Expression Level Exploration in GC Tissues at GEPIA or StarBase

Expression of HAND2-AS1 in GC tissues and normal tissues was analyzed at GEPIA (http://gepia2.cancer-pku.cn/#index).[10] Expression of miR-590-3p, and KCNT2 in GC tissues and normal tissues was analyzed at StarBase (http://starbase.sysu.edu.cn/).[11]

Statistical Analysis

SPSS 21.0 software (IBM Corporation, Armonk, NY, USA) was used for data analysis. Data were presented as mean ± SD. Differences in groups were analyzed with Student's *t*-test and one-way ANOVA with Tukey post-hoc test. A p-value less than 0.05 was considered as statistically significant.

Results

Expression of HAND2-AS1 in GC

We first explored the expression of HAND2-AS1 in GC tissues and normal tissues. As presented in Figure 1A, we found HAND2-AS1 expression level was significantly lower in GC tissues than in normal tissues. Furthermore, a similar result was observed in GC cells. As expected, we revealed that HAND2-AS1 was downregulated in GC cells compared with normal cell line (Figure 1B).

Overexpression of HAND2-AS1 Inhibits GC Cell Proliferation and Invasion but Promotes Apoptosis

The upregulation of HAND2-AS1 in GC cells transfected with pHAND2-AS1 transfection was explored by RT-qPCR (Figure 2A). CCK-8 assay was utilized to explore cell proliferation rate with pHAND2-AS1 transfection. We found the overexpression of HAND2-AS1 decreased cell proliferation ability of GC cells (Figure 2B). Colony formation assay was conducted to evaluate colony formation ability, and the results are presented in Figure 2C, which revealed colony formation ability was significantly suppressed by pHAND2-AS1. Moreover, flow cytometry assay revealed that cell apoptosis ability was significantly enhanced by pHAND2-AS1 transfection (Figure 2D).

Knockdown of HAND2-AS1 Promotes GC Cell Proliferation and Invasion but Inhibits Apoptosis

Furthermore, loss-of-function experiments were performed to fully understand the role of HAND2-AS1 in GC. The

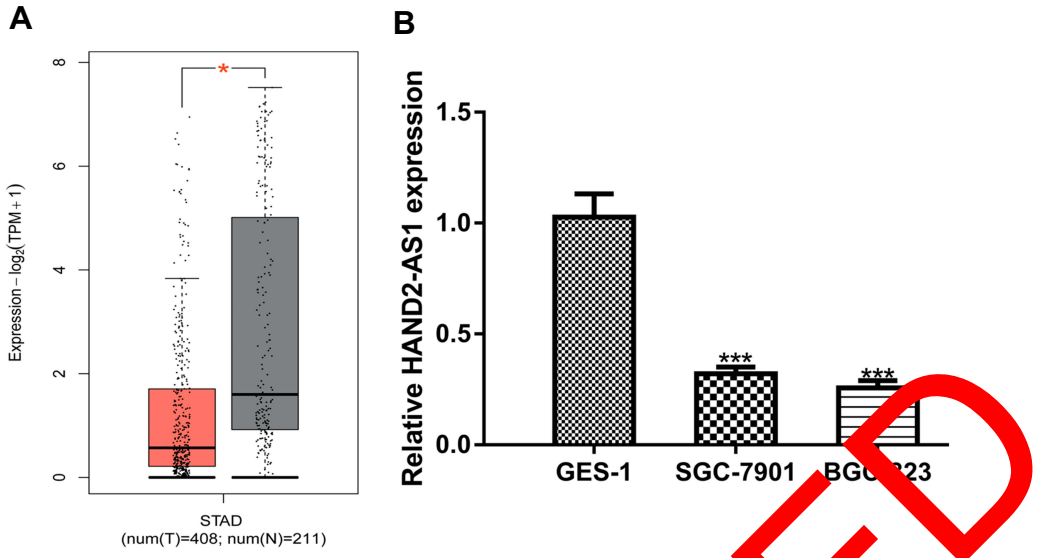

Figure 1 Downregulation of HAND2-AS1 was found in GC.
Notes: Downregulation of HAND2-AS1 was found in (**A**) GC tissues and normal tissues, and (**B**) GC cell li and a normal line. *P < 0.05; ***P < 0.001.
Abbreviations: HAND2-AS1, Heart and Neural Crest Derivatives Expressed 2 antisense RNA 1; GC, g cer.

introduction of si-HAND2-AS1 significantly decreased the expression level of HAND2-AS1 in GC cell compared with si-NC (Figure 3A). CCK-8 assay and colony formation assay revealed that cell proliferation ability and colony formation ability were significantly inhibited by si-HAND2-AS (Figure 3B and C). Furthermore, flow cytometry assay revealed that si-HAND2-AS1 transfection could cell apoptosis (Figure 3D).

miR-590-3p Was a Direct Target for HAND2-AS1

The ceRNA theory sugges that lncR can function as a miRNA sponge to regulate miRNA expression. Hence, we analyzed the potential miRNA target for HAND2-AS1 usi LncB Predict v.2, and we found miR-590-3p y bi with D2-AS1 (Figure 4A). Luciferase ctivity porter assay showed miR-590-3p mimic transf decreased luciferase activity in GC cells with wt-HA D2-AS1 transfection (Figure 4B). RIP assay showed HAND2-AS1 and miR-590-3p was enriched in anti-Ago2 groups compared with anti-IgG groups (Figure 4C). RNA pull-down assay showed HAND2-AS1 enrichment was significantly increased in bio-miR-590-3p-wt group (Figure 4D). Then, we explored the expression of miR-590-3p in GC tissues and cells. We showed that miR-590-3p expression level was significantly upregulated in both GC tumor tissues and cell lines compared with the normal tissues and cell line (Figure 4E and F).

KCNT2 Was a Direct Target for miR-590-3p

Subsequently, w analyzed the targets for miR-590-3p using Ta an, and we found KCNT2 was a putative tar for miR-590-3p (Figure 5A). Dual-luciferase activity reporter assay revealed that overexpression of miR-590-3p inhibited luciferase activity in GC cells with wt-KCNT2 expression (Figure 5B). Moreover, expression level of KCNT2 in GC tissues and cells was further explored. We showed KCNT2 expression level was significantly decreased in GC tissues and cell lines (Figure 5C and D).

HAND2-AS1 Regulates GC Cell Behaviors via Regulating miR-590-3p/KCNT2 Axis

At length, rescue experiments were performed to analyze whether HAND2-AS1 regulates GC cell behaviors via regulating the miR-590-3p/KCNT2 axis. We showed KCNT2 expression was enhanced by pKCNT2 but decreased by miR-590-3p mimic (Figure 6A). CCK-8 assay and colony formation assay revealed that cell proliferation ability and colony formation ability of GC cells were decreased by pKCNT2 but increased by miR-590-3p mimic (Figure 6B and C). The analysis of cell apoptosis rate revealed that cell apoptosis rate was increased by pKCNT2 but decreased by miR-590-3p mimic (Figure 6D). More importantly, we showed the introduction of pHAND2-AS1 or pKCNT2 could reverse the effects of miR-590-3p on GC cells (Figure 6B–D).

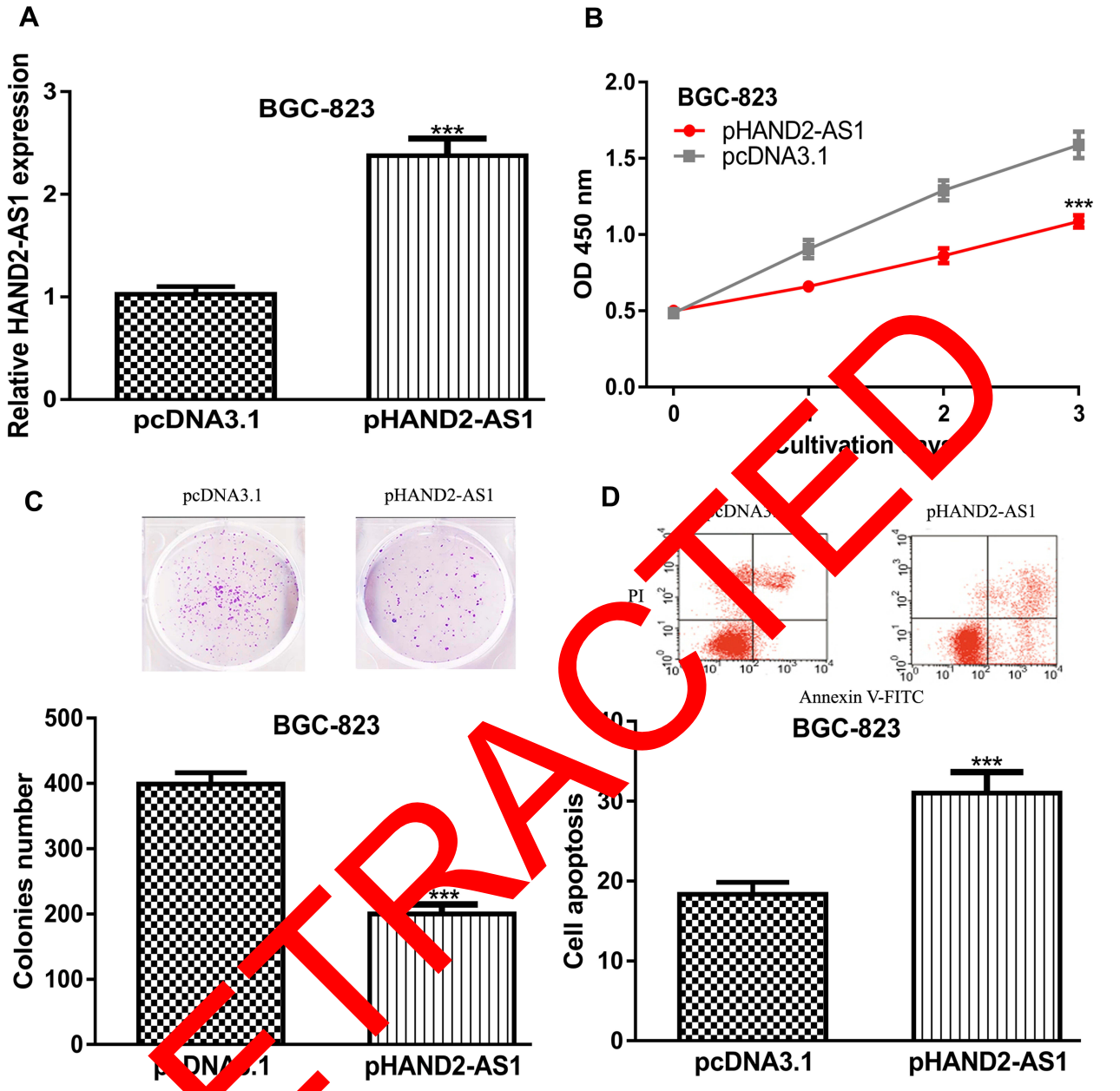

Figure 2 HAND2-AS1 overexpression inhibits GC cell proliferation and colony formation but promotes cell apoptosis.
Notes: (A) HAND2-AS1 expression, (B) Cell proliferation, (**C**) Colony formation, and (**D**) Cell apoptosis in GC cell with pHAND2-AS1 or pcNA3.1 transfection. ***P < 0.001.
Abbreviations: HAND2-AS1, Heart and Neural Crest Derivatives Expressed 2 antisense RNA 1; GC, gastric cancer.

Discussion

lncRNA has been reported to be a crucial mediator for tumorigenesis and the recent studies showed that their roles are partially mediated by ceRNA cross-talk.[12] In GC, the ceRNA network including lncRNA, miRNA, and message RNA was reported to play crucial roles in tumor initiation and progression.[13] lncRNAs were found to be abnormally expressed in multiple human cancers, however, the role of lncRNAs in GC remains to be defined. For instance, lncRNA TRPM2 antisense RNA (TRPM2-AS) expression in GC tumor tissues was found to be elevated by ELK1, and correlated with advanced tumor stages and poorer tumor stages.[14] In addition, it was revealed that TRPM2-AS could promote GC cell proliferation, migration, and invasion via regulating miR-195 and high-mobility group AT-hook 1.[14] Moreover, Gan et al[15] reported that lncRNA H19 had upregulated expression in

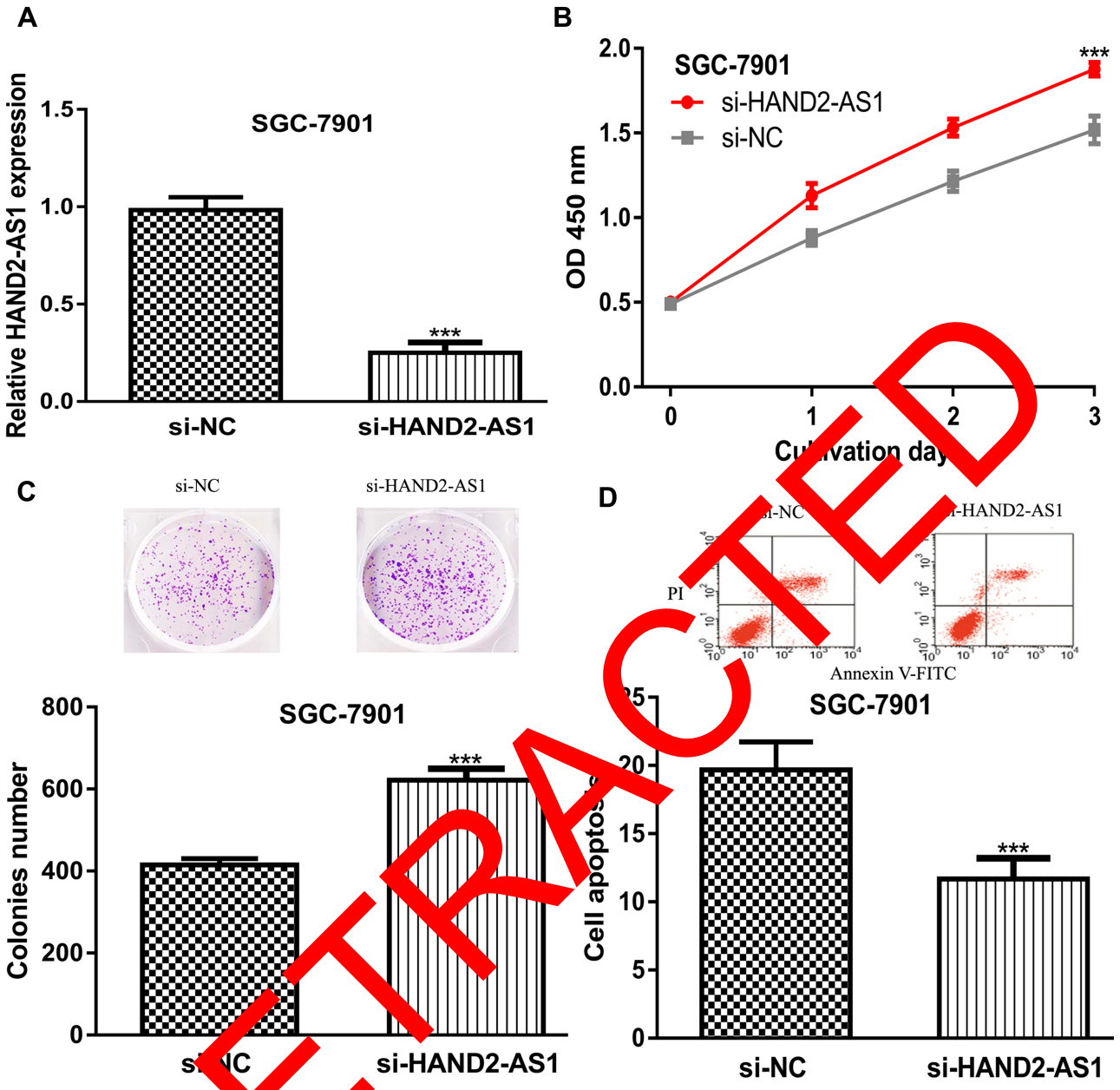

Figure 3 HAND2-AS1 knockdown promotes GC cell proliferation and colony formation but inhibits cell apoptosis.
Notes: (**A**) HAND2-AS1 expression. (**B**) Cell proliferation, (**C**) Colony formation, and (**D**) Cell apoptosis in GC cell with si-HAND2-AS1 or si-NC transfection. ***P < 0.001.
Abbreviations: HAND2-AS1, Heart and Neural Crest Derivatives Expressed 2 antisense RNA 1; GC, gastric cancer; si-NC, negative control small interfering RNA.

both GC tissues and cells. They also reported H19 functions as an oncogenic lncRNA to promote GC progression in vitro and in vivo via targeting the miR-22-3p/Snail1 axis.[15] Liu et al[16] revealed the upregulation status of lncRNA FLVCR1-AS1 in GC and its correlation with overall survival of cancer patients. Also, FLVCR1-AS1 was found to functions as ceRNA in regulating myc expression via sponging miR-155 to promote GC cell proliferation and invasion.[16]

In this study, through analysis of the expression of HAND2-AS1 in GC tumor tissues and normal tissues in GEPIA, we showed HAND2-AS1 was significantly decreased in tumor tissues compared with normal tissues. Moreover, we showed that HAND2-AS1 expression was lower in GC cells than in normal cell lines. Through gain and loss-of-function experiments, we showed the overexpression of HAND2-AS1 could inhibit GC cell proliferation

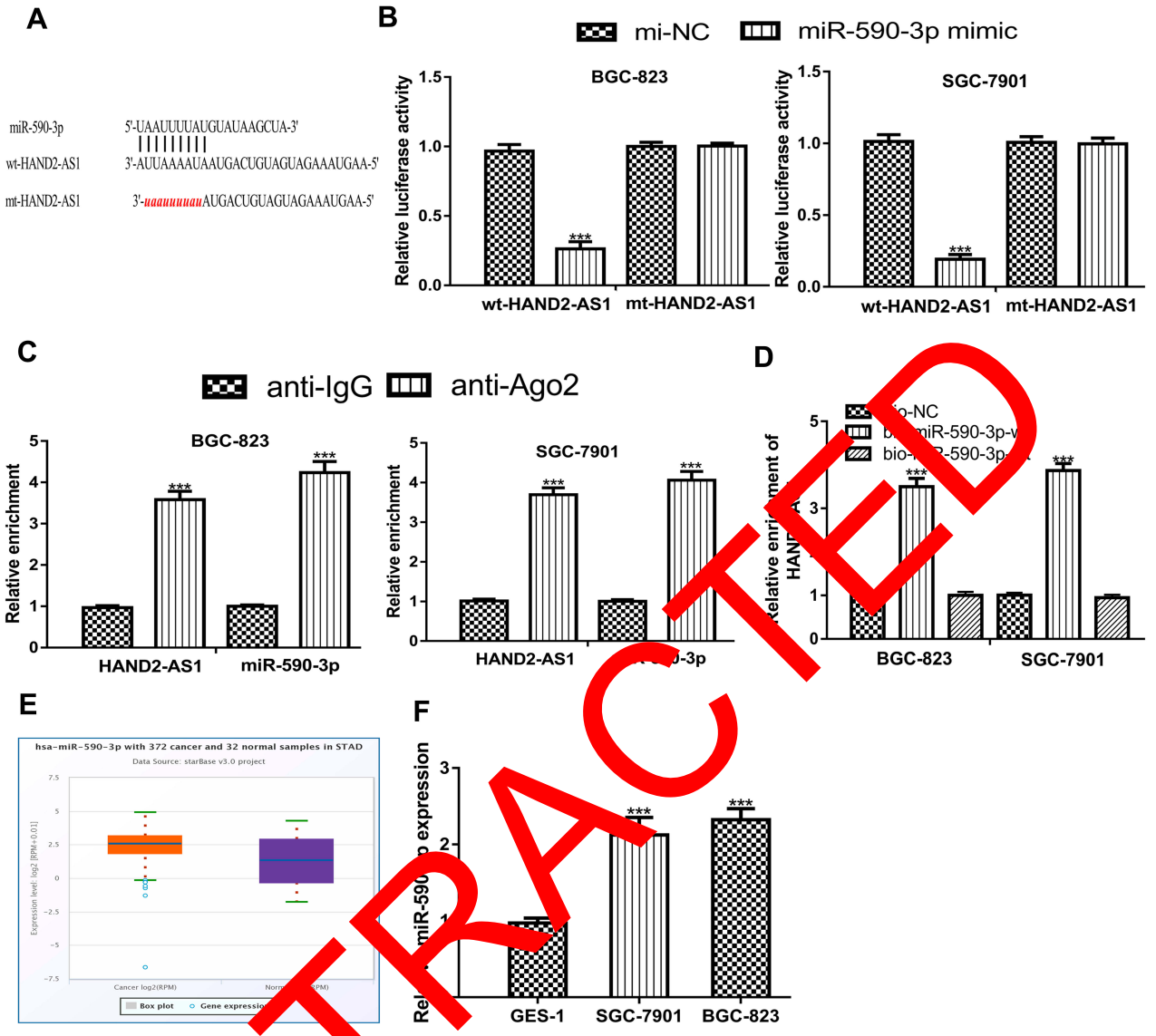

Figure 4 HAND2-AS1 direct b with miR-590-3p.
Notes: (**A**) The binding sit between HAND2-AS1 and iR-590-3p. (**B**) Luciferase activity reporter assay revealed the relative luciferase activity in cells with wt/mt HAND2-AS1 or miR-590 mimic/mi transfection. (**C**) RNA immunoprecipitation analysis for the enrichment of HAND2-AS1 and miR-590-3p in anti-Ago2 and anti-IgG groups. (**D**) HAND2-A indi o miR-590-3p analyzed by RNA pull-down assay. Expression of miR-590-3p in (**E**) GC tissues and normal tissues, and (**F**) GC cell lines and a normal cell line. ***P <
Abbreviations: AS1, H and N ral Crest Derivatives Expressed 2 antisense RNA 1; GC, gastric cancer; miR-590-3p, microRNA-590-3p; wt, wild-type; mt, mutant; mi-N negative ontrol mi go2, Argonaute 2.

and colony ormation but promote apoptosis, while the knockdown of AND2-AS1 caused the opposite effects on GC cell behaviors. Subsequently, ceRNA theory was employed to investigate the mechanisms behind HAND2-AS1-mediated GC cell behaviors. We showed that HAND2-AS1 may function as an miR-590-3p sponge to regulate KCNT2 expression. miR-590-3p is a miRNA reported to have elevated expression in colorectal cancer and predict the poor overall survival of cancer patients.[17] Also, miR-590-3p was demonstrated to function as an oncogenic miRNA in regulating colorectal cancer cell malignancy behaviors via targeting the Hippo pathway.[17] In colon cancer, miR-590-3p was found to have elevated expression in tumor tissues and cell lines.[18] Here, we have reported that miR-590-3p also had elevated expression in GC tumor tissues and cell lines. Also, we confirmed miR-590-3p was a direct target for HAND2-AS1 using dual-luciferase activity reporter assay, RIP assay, and RNA pull-down assay. Previously, we identified miR-338-5p as having decreased expression in GC stem cells, and its overexpression could

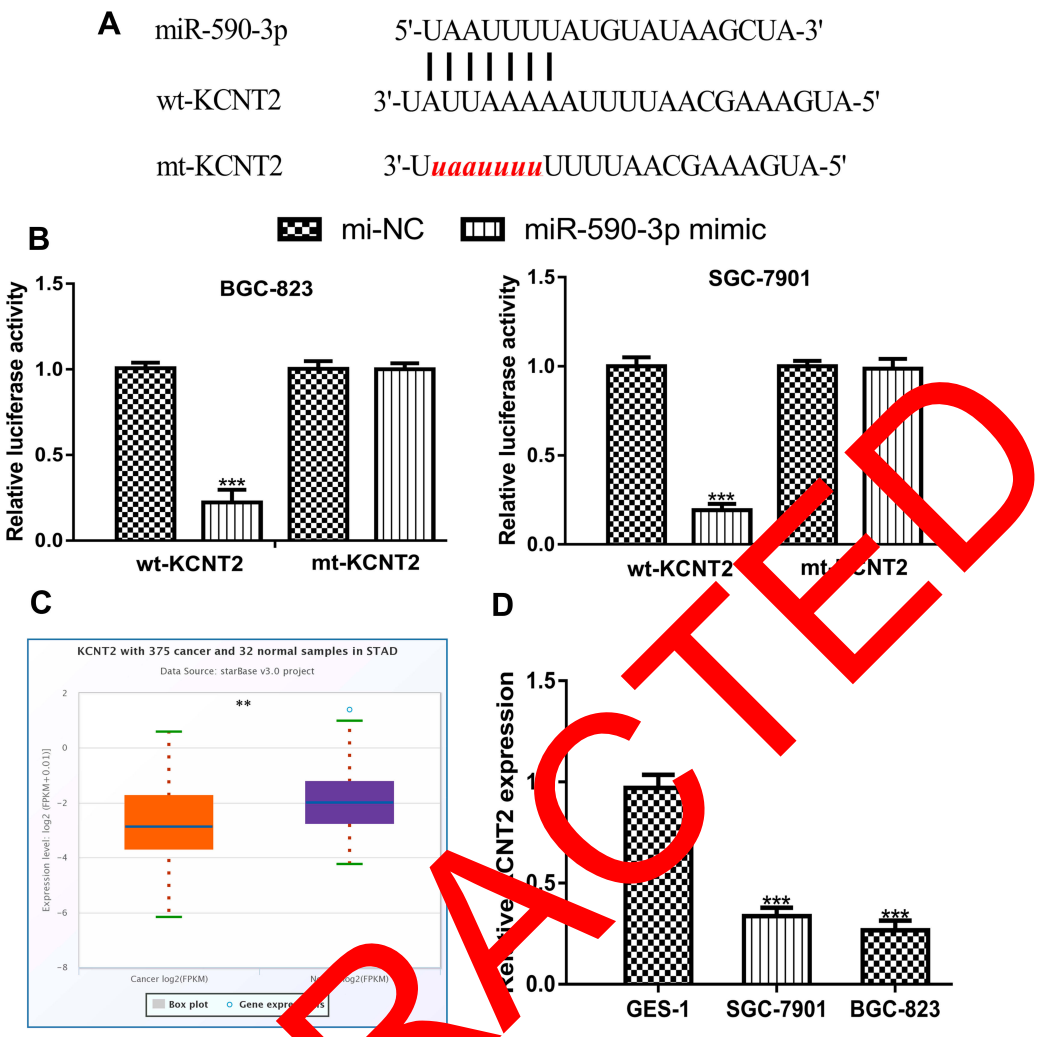

Figure 5 KCNT2 was a direct target for miR-590-3p.
Notes: (**A**) The binding site between KCNT2 and miR-590-3p. (**B**) Luciferase activity reporter assay revealed the relative luciferase activity in cells with wt/mt KCNT2 or miR-590-3p mimic/mi-NC transfection. Expression of KCNT2 in (**C**) GC tissues and normal tissues, and (**D**) GC cell lines and a normal cell line. **P < 0.01; ***P < 0.001.
Abbreviations: KCNT2, potassium sodium-activated channel subfamily T member 2; GC, gastric cancer; miR-590-3p, microRNA-590-3p; wt, wild-type; mt, mutant; mi-NC, negative control miRNA.

inhibit cell growth.[19] Although we showed an anticancer bioactive peptide-3 could exert its biological roles via regulating miR-338-5p, Here, we identified miR-590-3p was also able to regulate GC cell behaviors and was regulated by upstream lncRNA to exert biological roles.

KCNT2 was revealed to contain binding sites for Nuclear Factor-κB.[20] Yang et al[21] recently performed a bioinformatic-based study and revealed low KCNT2 level was associated with the worse survival of patients with melanoma.[21] We demonstrated that KCNT2 had decreased expression in tumor tissues and cell lines, and was a target of miR-590-3p. The roles of KCNT2 in GC development has not been reported until now. Here, we showed overexpression of KCNT2 could inhibit GC cell proliferation, colony formation and promote apoptosis. Moreover, functional assays revealed that HAND2-AS1 regulate GC cell behaviors via regulating miR-590-3p/KCNT2. Hence, we believe our work may advance our understanding of the mechanisms behind GC tumorigenesis. However, we should acknowledge the limitations of this work: 1) the lack of in vivo animal model results to strengthen the importance of HAND2-AS1/miR-590-3p/KCNT2 triplets in GC development; and 2) we did not investigate the upstream mechanism that is responsible for the elevated expression status of HAND2-AS1 in GC. There are studies showed epigenetic modifications can

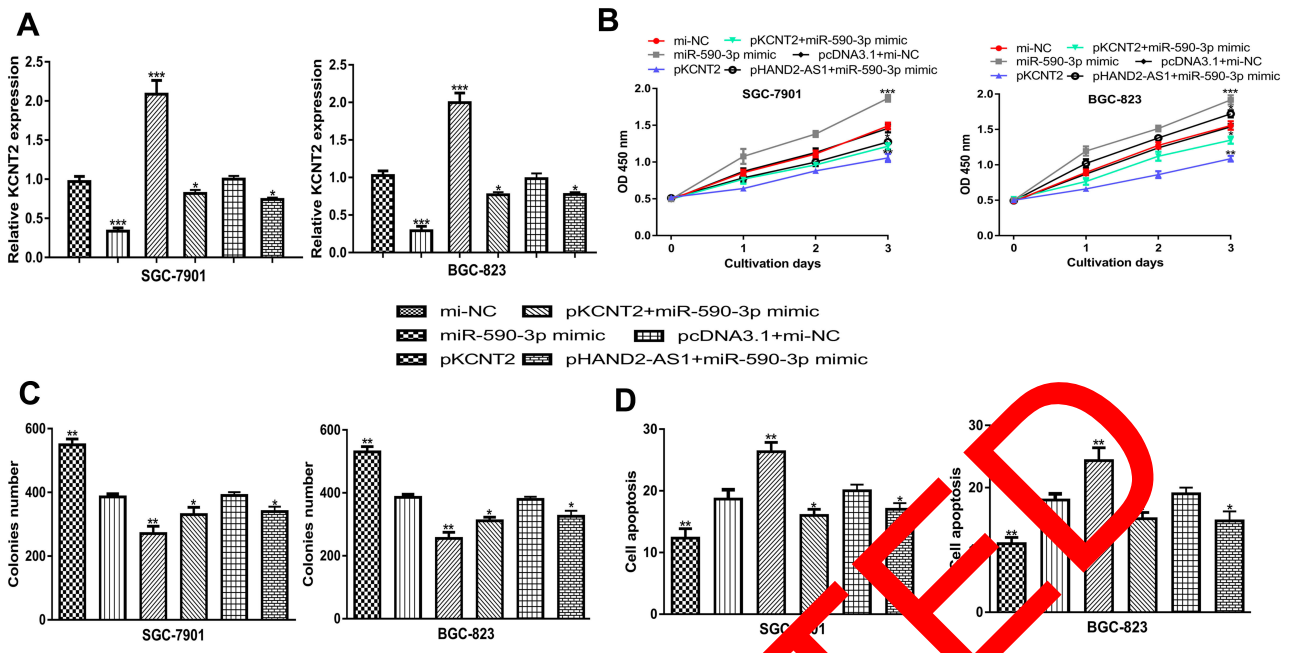

Figure 6 HAND2-AS1 regulates GC cell behaviors via regulating miR-590-3p/KCNT2 axis.
Notes: (**A**) KCNT2 expression, (**B**) Cell proliferation, (**C**) Colony formation, and (**D**) Cell apoptosis in GC cells with miR-590-3p mimic, pKCNT2, mi-NC, pcDNA3.1+mi-NC, miR-590-3p mimic+pKCNT2, and miR-590-3p mimic+pHAND2-AS1 transfection. *P < 0.05; **P < 0.01; ***P < 0.001.
Abbreviations: HAND2-AS1, Heart and Neural Crest Derivatives Expressed 2 antisense RNA 1; GC, gastric cancer; miR-590-3p, microRNA-590-3p; KCNT2, potassium sodium-activated channel subfamily T member 2; mi-NC, negative control miRNA.

affect lncRNA expression in cancers,[22,23] which may be a mechanism for the decreased expression of HAND2-AS1 and deserves to be deeply investigated in the future.

Conclusion

To sum up, our work presented here showed that HAND2-AS1 functions as oncogenic lncRNA to accelerate GC cell proliferation and colony formation but inhibit cell apoptosis via regulating KCNT2 through sponging miR-590-3p. Therefore, our results indicate that HAND2-AS1 may serve as a therapeutic target for GC.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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