ORIGINAL RESEARCH

Long Noncoding RNA *NEAT1* Upregulates Survivin and Facilitates Gallbladder Cancer Progression by Sponging microRNA-335

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Background: Gallbladder cancer (GBC) is the most common cancer of the biliary tract, but molecularly targeted therapies are not available for GBC. Loss of microRNA (miR)-335 expression may be a useful predictor of clinical outcomes and the reversal of its loss of expression may be a useful treatment strategy for GBC. In this study, we investigated whether a long noncoding RNA, nuclear paraspeckle assembly transcript 1 (*NEAT1*) sponges *miR-335* in GBC cells.

Materials and Methods: Quantitative reverse transcription-polymerase chain reaction (qRT-PCR), Western blotting, and immunohistochemistry were used to determine the expression of *miR-335; NEAT1*; survivin; and *Ki67* in GBC cell lines (GBC-SD and SGC-996) and tissue samples from patients (n = 25). Cell Counting Kit-8, colony-formation, and Transwell migration and invasion assays were performed to measure cell proliferation, migration, and invasion. Bioinformatic analysis and dual-luciferase reporter assays were utilized to analyze correlativity.

Results: *miR-335* overexpression resulted in inhibition of GBC cell proliferation and invasion. In addition, knockdown of *NEAT1* resulted in downregulation of survivin expression. As *NEAT1* competitively "sponges" *miR-335, NEAT1* knockdown resulted in inhibited GBC cell proliferation and invasion in vitro and GBC tumor growth in vivo. Furthermore, *NEAT1* was found to be upregulated in GBC samples, and its expression was inversely correlated with *miR-335* levels, but positively correlated with survivin levels.

Conclusion: These findings indicate that *NEAT1* promotes survivin expression by functioning as a competitive endogenous RNA for *miR-335* in GBC cells; thus, we have identified a potential biomarker and target for GBC diagnosis and therapy.

Keywords: long noncoding RNA, *NEAT1*, *miR-335-5p*, competitive endogenous RNA, survivin, gallbladder cancer

Introduction

Globally gallbladder cancer (GBC) is the most common cancer of the biliary tract and the fifth most common digestive tract cancer.[1] GBC is uncommon in developed countries, but is more common in certain developing countries. It is characterized by a poor prognosis and usually a late diagnosis, leading to adverse treatment outcomes.[2] Currently, complete surgical resection of the tumor is the most effective treatment for GBC, but by the time patients—most—are diagnosed with GBC, the optimal time for surgery has passed.[3] In recent years, with rapid developments in

tumor biology and sequencing technologies, the molecular pathogenesis of GBC has been elucidated in detail. Therefore, identification of new molecular targets and the development of new therapeutic strategies against GBC are urgently needed.

MicroRNAs (miRs, miRNAs) are small noncoding single-stranded RNAs that bind to the 3′ untranslated regions of their target mRNAs to suppress protein expression.[4] Increasing evidence indicates that miRNAs play key roles in the progression of many human cancers, by acting as oncogenic RNAs or tumor suppressors.[5] *miR-335* is reported to be a tumor suppressor that is downregulated in many cancers, including thyroid cancer, lung cancer, osteosarcoma, and neuroblastoma.[6–9] Recently, the expression of *miR-335* was found to be significantly lower in GBC tissues, but its involvement in GBC and the associated molecular mechanisms remain unclear.

Long noncoding RNAs (lncRNAs) are noncoding RNAs that are longer than 200 nucleotides in length; they often show dysregulated expression in cancers and play an important role in the initiation and/or progression of cancers.[10] In GBC, the lncRNA *TUG1* promotes cell proliferation and metastasis by downregulating *miR-300*, and the lncRNA *MINCR* promotes GBC progression by stimulating EZH2 expression.[11,12] The lncRNA, *MALAT1*, facilitates growth and inhibits senescence by antagonizing ABI3BP in GBC cells.[13] Another lncRNA, *PVT1* promotes tumor progression by regulating the *miR-143*/HK2 axis in GBC.[3] Accumulating evidence suggests that the expression of the lncRNA, nuclear paraspeckle assembly transcript 1 (*NEAT1*), is dysregulated in many human cancers, and that this closely correlates with poor prognosis.[14] *NEAT1* promotes the progression of colon cancer by sponging *miR-495-3p* and activating CDK6.[15] It performs an oncogenic function in triple-negative breast cancer, by promoting the development of chemoresistance and cancer stemness.[14] Nevertheless, it is not known whether the aberrant expression of *NEAT1* in GBC is associated with the progression of malignancy. Importantly, the mechanism by which *NEAT1* exerts its oncogenic effect remains to be identified.

In this study, we first showed that overexpression of *miR-335* inhibited GBC cell proliferation and invasion. Next, we demonstrated that *NEAT1* upregulated survivin by competitively "sponging" *miR-335*, and this enhanced GBC cell proliferation and invasion in vitro and promoted GBC tumor growth in vivo; thus, we have identified a potential biomarker and/or target for GBC diagnosis and therapy.

Materials and Methods
Clinical Samples and Cell Lines
A total of 25 human GBC samples (Supplementary Table 1) and corresponding normal gallbladder tissue samples were obtained from the Department of Biliary II, Shanghai Eastern Hepatobiliary Surgery Hospital, Naval Military Medical University (Shanghai, China), after obtaining written informed consent. This study was performed according to an established protocol approved by the Ethics Committee of the Shanghai Eastern Hepatobiliary Surgery Hospital, Naval Military Medical University. Tissue samples were collected during surgical resection and were immediately frozen in liquid nitrogen. GBC cell lines, GBC-SD and SGC-996 were purchased from Guangzhou Gino Bio Co., Ltd. (Guangzhou, China), and the human gallbladder epithelium cell line, H69, was acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.

RNA Extraction and qRT-PCR Assays
Total RNA was extracted using TRIzol reagent (Life Technologies Inc., Carlsbad, CA, USA), according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using the Superscript III Reverse Transcription Reagent (Life Technologies). qRT-PCR analysis was performed using SYBR Green chemistry on an ABI7500 real-time PCR detection system (Applied Biosystems, Foster City, CA, USA). β-Actin or *U6* small nuclear RNA served as the endogenous control. Each experiment was performed in triplicate, and the $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression.

Western Blotting
For Western blotting, proteins in cell lysates were separated on SDS-polyacrylamide gel by electrophoresis, and electrotransferred onto a polyvinylidene difluoride membrane, which was then sequentially probed with primary and secondary antibodies. Signals were detected by chemiluminescence reagents (ECL Kit, Pierce Biotechnology, Waltham, MA, USA) and imaged on a Tanon-5200 Chemiluminescent Imaging System (Bio-tanon, Shanghai, China). Anti-survivin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-β-actin (Proteintech, Wuhan, China) antibodies were employed for Western blotting.

Oligonucleotides and Transfection

An *miR-335* mimic, an *miR-335* inhibitor, and their negative controls (NC mimic and NC inhibitor, respectively) were purchased from Ribobio (Guangzhou, China). siRNAs specifically targeting *NEAT1* (si*NEAT1*) and a scrambled control siRNA (siNC) were synthesized by GenePharma (Shanghai, China). All transfections were performed using Lipofectamine 2000 (Life Technologies).

Lentivirus Vector Construction and Infection

A lentiviral vector encoding a short hairpin RNA (shRNA) specific for *NEAT1* (designated as shNEAT1) and its negative control (designated as shNC), as well as a lentivirus expressing anti-*miR-335* (designated anti-*miR-335*) and its negative control (anti-NC) were purchased from Hanbio (Shanghai, China). All transfections were performed using Lipofectamine 2000 (Life Technologies).

Luciferase Reporter Assay

The plasmids, pmirGLO-NEAT1-wt or pmirGLO-NEAT1-mt, carrying wild-type or mutant binding sites for *miR-335*, respectively, were cotransfected with either the *miR-335* or NC mimic, into HEK293T cells via Lipofectamine-mediated gene transfer. Survivin-wt and -mt 3′-UTRs were constructed and transfected into cells along with the *miR-335* or NC mimic, respectively. Firefly and Renilla luciferase activities were measured 48 h after transfection, according to the manufacturer's instructions. The ratio of the luminescence from the firefly luciferase to that of Renilla luciferase was calculated as the relative luciferase activity.

Cell Counting Kit-8 Assay

Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8) assay at 1, 2, 3, and 4 days after transfection. Cells were seeded in a 96-well plate at a density of 1500 cells/well and 10 µL of CCK-8 was added to 90 µL of cell culture medium per well. The cells were then incubated for 2 h and cell viability was determined by measuring the absorbance at 450 nm.

Colony-Formation Assay

For colony-formation assays, 1000 cells were seeded in each well of a 6-well plate and incubated at 37°C for 2 weeks. The colonies were fixed and stained with a solution containing 0.1% crystal violet and 20% methanol and were then counted. All stainings were performed in triplicate.

Cell Migration and Invasion Assays

Cell migration was assessed in a 24-well Transwell chemotaxis chamber (BD Biosciences, Franklin Lakes, NJ). Cells were seeded at a density of 4×10^4 in the upper chamber a Transwell plate containing a non-coated membrane for the migration assay. For the invasion assays, cells were seeded at a density of 4×10^4 in the upper chamber of a Transwell plate containing a Matrigel-coated membrane. After several hours of incubation at 37°C, cells that migrated or invaded were fixed with 20% methanol and stained with crystal violet (0.1%). These cells were then counted and imaged using an inverted microscope (Olympus, Tokyo, Japan).

Tumor Xenograft Experiments

GBC-SD cells stably expressing a control shRNA, sh*NEAT1*, an *miR-335* inhibitor, or sh*NEAT1* + an *miR-335* inhibitor were subcutaneously injected into either side of the flank of 6-week-old nude mice (n = 4 mice per group). Tumor volumes were calculated ($1/2 \times$ length $\times$ width^2) on a weekly basis. After 5 weeks, the mice were euthanized, and the tumors were excised and subjected to immunohistochemistry (IHC). All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the Naval Military Medical University.

IHC

After heat-induced epitope retrieval, paraffin-embedded sections were incubated with 3% H_2O_2 and blocked for another 60 min with 3% normal serum. Sections were then incubated with primary antibodies overnight at 4°C. An Elite ABC Staining Kit and DAB Peroxidase Substrate Kit (Vector Laboratories, Inc., Burlingame, CA, USA) were used to visualize antibody binding, according to the manufacturer's instructions. Anti-survivin (Abcam, Cambridge, UK) and anti-Ki67 (Cell Signaling Technology, Beverly, MA, USA) antibodies were used for IHC. ImageJ software was used to analyze IHC staining in randomly selected slides from four mice in each group (more than six representative fields in each slide were analyzed). Positive staining in each slide was scored and presented as relative expression level (the protein expression level in shNC mice was arbitrarily set at 1). Data are expressed as means ± SD.

Statistical Analysis

Results are presented as means ± SD. Statistical analysis was performed with Prism, version 5.0 (GraphPad, San

Diego, CA, USA), using the Mann–Whitney test to compare two groups and a one-way ANOVA, followed by Tukey's post hoc test to compare two or more groups. Correlation analysis was performed using Spearman correlation coefficient rank test. P values <0.05 were considered statistically significant.

Results

miR-335 Decreased GBC Cell Proliferation and Invasion

It has been demonstrated that *miR-335* levels are lower in GBC tumors than in that in nondysplastic gallbladder epithelia.[16] Nonetheless, the function of *miR-335* and its

mechanism of action in GBC remain unclear. Here, we determined the expression of *miR-335* in the human nontumorigenic biliary epithelial cell line, H69 and in GBC cell lines, SGC-996 and GBC-SD, by qRT-PCR. We found that *miR-335* was significantly underexpressed in GBC cell lines with respect to that in the normal biliary epithelial cell line (Figure 1A). Then, we overexpressed *miR-335* in SGC-996 and GBC-SD cells by transiently transfecting a *miR-335* mimic. qRT-PCR was performed to confirm *miR-335* overexpression in these cells (Figure 1B). A colony-formation assay showed that *miR-335* mimic-transfected cells formed significantly fewer and significantly smaller colonies than NC mimic-transfected cells (Figure 1C). CCK-8 assay
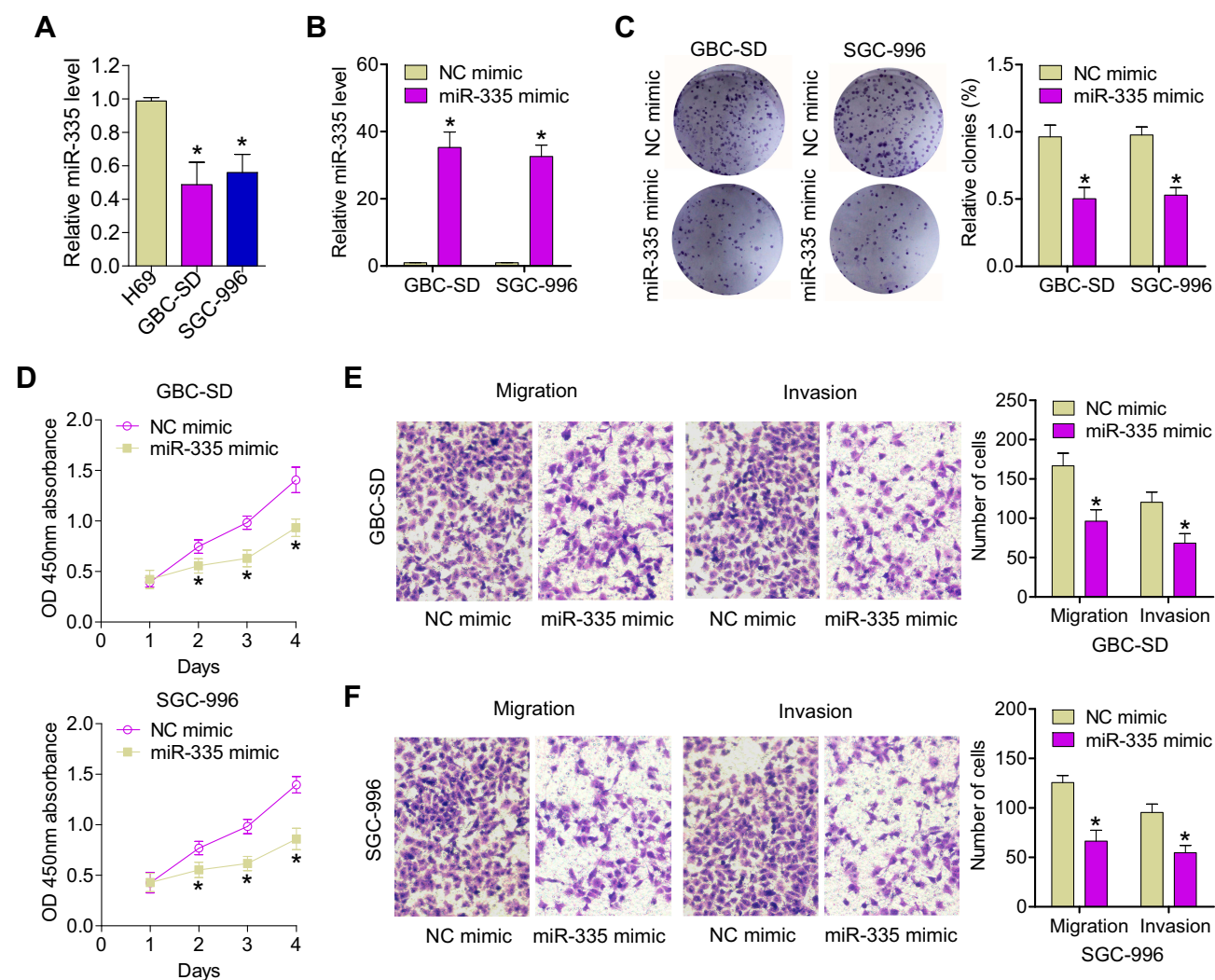
Figure 1 Overexpression of *miR-335* inhibits GBC cell proliferation and invasion. (**A**) The relative expression of *miR-335* in GBC cells (GBC-SD and SGC-996) and in the human nontumorigenic biliary epithelial cell line, H69, was determined by qRT-PCR. (**B**) The transfection efficiency of the *miR-335* mimic was evaluated by qRT-PCR. (**C**) Representative images and quantification of colony-formation assay data. (**D**) Changes in GBC cell proliferation after transfection with the *miR-335* mimic or the NC mimic were determined by CCK-8 assay. (**E**) Representative images and quantification of data from Transwell migration and invasion assays of GBC-SD cells transfected with the *miR-335* or NC mimics. (**F**) Representative images and quantification of data from Transwell migration and invasion assays of SGC-996 cells transfected with the *miR-335* or NC mimics. *P < 0.05.

showed that the viability of GBC cells transfected with the *miR-335* mimic was markedly lower than the viability of GBC cells transfected with the NC mimic (Figure 1D). Transwell migration and invasion assay demonstrated that transfection of the *miR-335* mimic into GBC cells clearly reduced their migratory and invasive abilities (Figure 1E). These results suggest that overexpression of *miR-335* markedly decreases the viability and the migration and invasiveness of GBC cells.

NEAT1 Upregulated Survivin Expression by Sponging *miR-335*

We next aimed to identify the molecular mechanisms responsible underlying *miR-335* downregulation in GBC. It has recently been reported that, in gastric cancer, *miR-335* expression is subject to post-transcriptional regulation by a competing endogenous RNA (ceRNA).[17] In this study, *NEAT1* was found to contain *miR-335*-binding elements and to function as a sponge for *miR-335*. We hypothesized

that *NEAT1* could regulate GBC progression by binding to *miR-335*. We first confirmed the binding of *miR-335* to the sites predicted by the online bioinformatic database, starBase 2.0 (Figure 2A). Dual reporter luciferase assays—performed in HEK293T cells—demonstrated that the *miR-335* mimic significantly decreased the luciferase activity of *NEAT1*-wt but not that of *NEAT1*-mt (Figure 2B), indicating the existence of *miR-335*-binding site(s) in *NEAT1*. Moreover, we found that *NEAT1* knockdown significantly increased *miR-335* levels in GBC-SD and SGC-996 cells (Figure 2C). Taken together, these results suggest that, in GBC cells, *NEAT1* can act as a sponge and reduce *miR-335* levels.

In addition, to elucidate the molecular mechanisms underlying the regulation of *miR-335* expression, we examined *miR-335* targets that were computationally predicted by the TargetScan algorithm. Among the high-scoring mRNA targets predicted for *miR-335*, survivin mRNA was selected for further research. Our previous study had shown that survivin is implicated in GBC, as
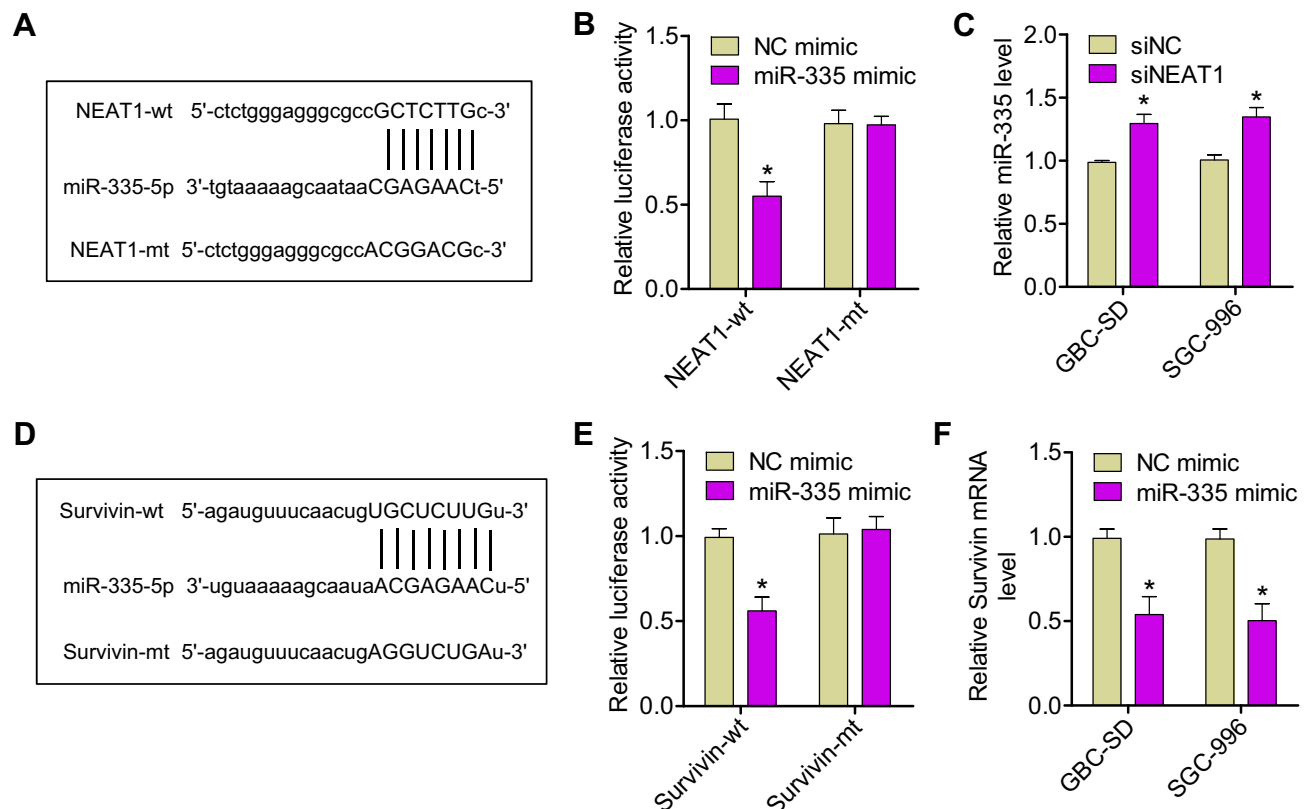
Figure 2 *NEAT1* promoted survivin expression by sponging *miR-335*. (**A**) The *miR-335* response element in *NEAT1*, as predicted by the starBase 2.0 algorithm. (**B**) Relative luciferase activity of HEK293T cells after cotransfection with either the *NEAT1*-wt or -mt reporter construct, along with either the *miR-335* or NC mimic. (**C**) Expression of *miR-335* was assessed by qRT-PCR in GBC-SD and SGC-996 cells transfected with either si*NEAT1* or siNC. (**D**) Bioinformatic analysis indicated that the 3′-UTR sequence of survivin was complementary to the seed sequence of *miR-335*. (**E**) Relative luciferase activity of HEK293T cells after cotransfection with either the survivin-wt or -mt 3′-UTR reporter gene, along with either the *miR-335* or NC mimic. (**F**) Expression of survivin mRNA was measured by qRT-PCR in GBC cells transfected with either the *miR-335* or NC mimic. *$P < 0.05$.

a critical regulator of tumor cell apoptosis.[18] Bioinformatic analysis showed that a sequence within the 3′-UTR of survivin mRNA was complementary to the seed sequence of *miR-335* (Figure 2D). The dual-luciferase reporter assay showed that cotransfection with the *miR-335* mimic and survivin-wt resulted in marked decrease in luciferase activity; however, cotransfection with the *miR-335* mimic and survivin-mt did not have any effect on luciferase activity (Figure 2E). Overexpression of *miR-335* in GBC cells significantly reduced the expression of survivin at mRNA and protein levels (Figure 2F and S1A). An online bioinformatic database predicted that *NEAT1* and survivin had the same putative binding site in *miR-335* (Figure 2A and D). Furthermore, *NEAT1* knockdown resulted in decreased luciferase activity in cells transfected with survivin-wt (Figure S1B). We next tested whether *NEAT1* regulates the expression of survivin in GBC cells in an *miR-335*-dependent manner. We found that *NEAT1* knockdown results in decreased expression of survivin at mRNA and protein levels in GBC cells (Figure S1C and D). These results indicated that *NEAT1* positively regulated survivin expression by sponging *miR-335* in GBC cells.

The *NEAT1*/*miR-335*/survivin axis affects GBC cell proliferation and invasion in vitro.

We next investigated whether *NEAT1* promoted the expression of survivin by targeting *miR-335* in GBC cells. Compared to its expression in control cells, survivin expression (mRNA and protein levels) was significantly lower in GBC-SD and SGC-996 cells transfected with a *NEAT1*-specific siRNA and was higher in cells transfected with an *miR-335* inhibitor. By contrast, in cells cotransfected with an *miR-335* inhibitor and si*NEAT1*, the upregulation of survivin by *NEAT1* was attenuated (Figure 3A). We continued to investigate the effects of the *NEAT1*/*miR-335*/survivin axis on the proliferation and invasiveness of GBC cells. CCK-8 and colony-formation assays showed that the viability and colony-formation ability of GBC cells were clearly reduced upon si*NEAT1* transfection and were increased by *miR-335* inhibitor transfection. These effects were attenuated by simultaneous cotransfection of the *miR-335* inhibitor and si*NEAT1* (Figure 3B and C). The knockdown of *NEAT1* decreased the number of migratory and invasive GBC cells, but this effect was reversed by cotransfection of the *miR-335* inhibitor (Figure 3D and E). These results showed that *NEAT1* promoted GBC proliferation and invasion by regulating the *miR-335*/survivin pathway.

The Oncogenic Activity of *NEAT1* Was, in Part, Mediated by Downregulation of *miR-335* in vivo

To verify our in vitro findings, we established an in vivo xenograft model in nude mice. Sh*NEAT1*, anti-*miR-335*, or both sh*NEAT1* and anti-*miR-335* were transfected into GBC-SD cells, which were then injected into nude mice. Tumors were allowed to form and grow for 5 weeks, during which tumor size and weight were measured at regular intervals. The results showed that *NEAT1* knockdown resulted in markedly reduced tumor volume, whereas anti-*miR-335* clearly promoted tumor growth (Figure 4A and B). However, double knockdown of *NEAT1* and *miR-335* had no significant impact on tumor formation (Figure 4A and B). Meanwhile, smaller tumors were observed in mice treated with sh*NEAT1* than in mice treated with shNC. Larger tumors were observed in mice treated with anti-*miR-335* than in those treated with shNC, but this effect was attenuated by simultaneous cotreatment with the *miR-335* inhibitor and sh*NEAT1* (Figure 4C). IHC showed lower expression of survivin and fewer Ki67-positive cells in tumors derived from sh*NEAT1*-transfected cells; this effect was significantly attenuated by anti-*miR-335* treatment (Figure 4D).

NEAT1 Was Upregulated in GBC Samples and Was Inversely Correlated with *miR-335* Levels

We next measured the levels of *NEAT1*, *miR-335*, and survivin in GBC tissue samples (n = 25) and normal gallbladder tissue samples (n = 25) by qRT-PCR. We found that *NEAT1* and survivin levels were significantly higher, and *miR-335* levels were significantly lower in GBC samples, compared with those in normal gallbladder tissue samples (Figure 5A–C). Moreover, we evaluated the correlations between *NEAT1* and *miR-335* levels, between *NEAT1* and survivin levels, and between *miR-335* and survivin levels in GBC samples. As shown in Figure 5D–F, *NEAT1* levels negatively correlated with *miR-335* levels, but positively correlated with survivin levels in GBC samples. Further, *miR-335* levels negatively correlated with survivin levels.

Discussion

A lot of evidence suggests that certain miRNAs target specific mRNAs related to the initiation and progression of GBC and are involved in regulating the proliferation
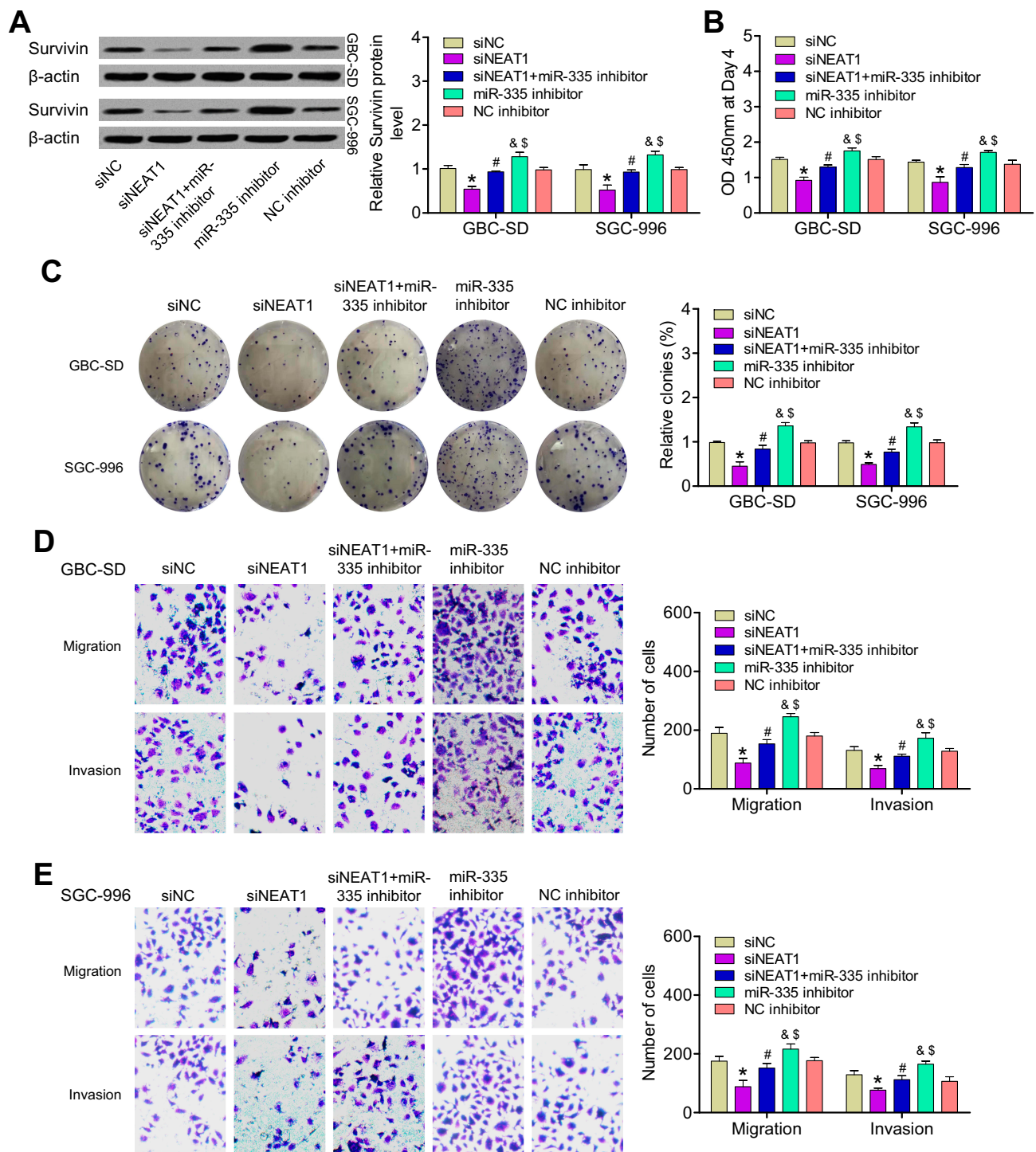
Figure 3 Influence of the *NEAT1/miR-335*/survivin axis on GBC cell proliferation and invasion in vitro. (**A**) Expression of survivin protein was determined in GBC-SD and SGC-996 cells transfected with siNC, si*NEAT1*, or an *miR-335* inhibitor or cotransfected with an *miR-335* inhibitor and si*NEAT1*. (**B**) Changes in cell proliferation in the above-mentioned GBC cell groups were determined by CCK-8 assay. (**C**) Quantification of colony-formation assay data from different groups of GBC cells. (**D, E**) Quantification of data from Transwell migration and invasion assays of different groups of GBC cells. *$P < 0.05$ vs the siNC group; #$P < 0.05$ vs the si*NEAT1* group; &$P < 0.05$ vs the si*NEAT1* + *miR-335* inhibitor group; $$P < 0.05$ vs the NC inhibitor group.

and invasiveness of GBC cells.[19,20] For example, *miR-139-5p* is associated with a poor prognosis and it controls glycolysis by downregulating PKM2 in GBC,[21] whereas *miRNA-30a-5p* inhibits GBC cell proliferation, migration,

and metastasis by targeting *E2F7* mRNA.[22] In the present study, we first identified *miR-335* as a key miRNA involved in GBC cell proliferation and invasion. We then explored the potential effects of lncRNAs on the
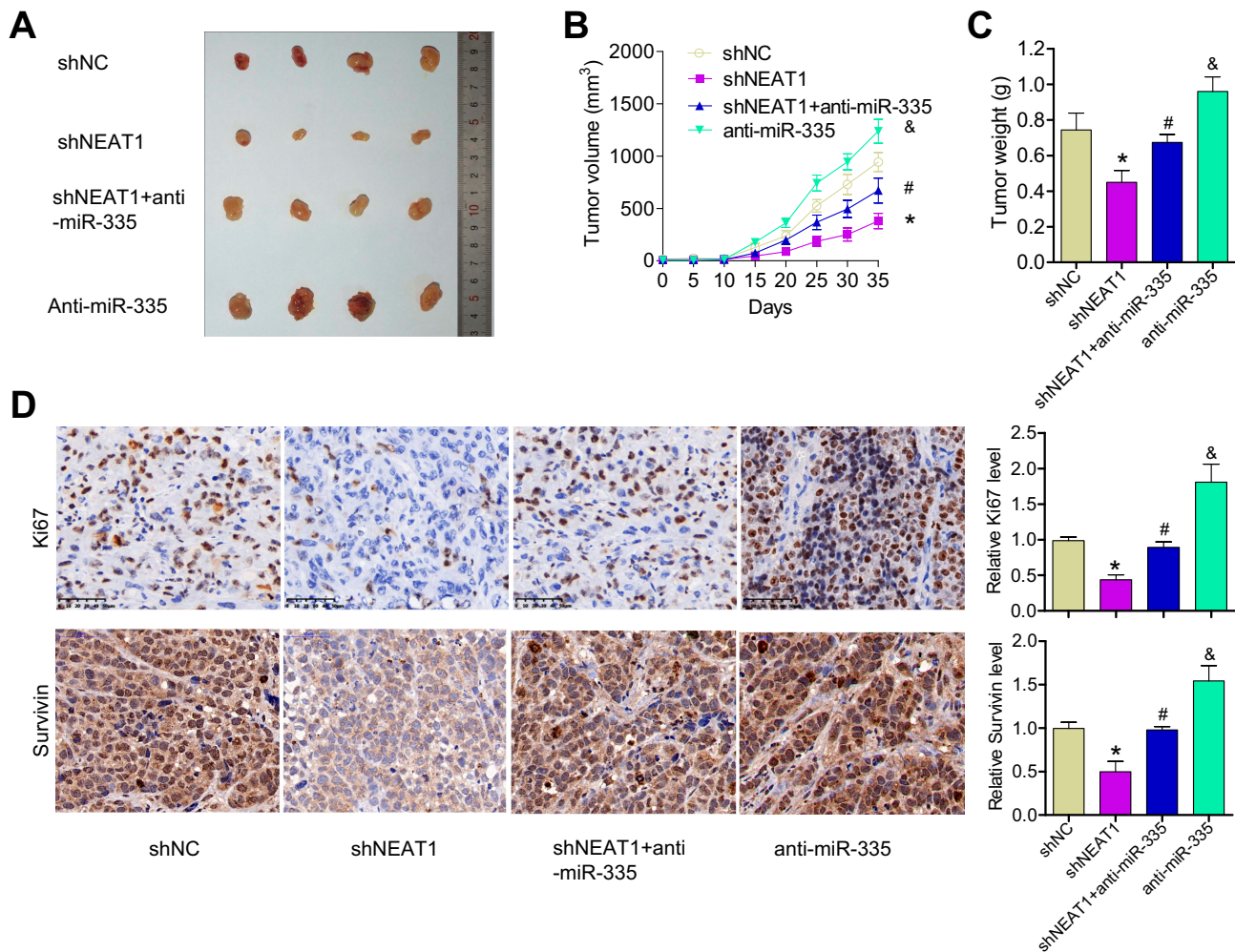
Figure 4 Knockdown of *NEAT1* inhibited tumor growth, in part through *miR-335* downregulation in vivo. (**A**) Representative image of xenograft tumor formation in nude mice injected with shNC, sh*NEAT1*, anti-*miR-335*, and sh*NEAT1* + anti-*miR-335* transfected cells (n = 4 per group). (**B**) Tumor volume growth curves. (**C**) Tumor weight. (**D**) IHC showing Ki67 and survivin levels in tumor. *$P < 0.05$ vs the siNC group; #$P < 0.05$ vs the si*NEAT1* group; &$P < 0.05$ vs the si*NEAT1* + *miR-335* inhibitor group.

regulatory functions of *miR-335* in GBC. We showed that *NEAT1* significantly reduced the activity of *miR-335* by sequestering *miR-335* molecules. Therefore, the following possible mechanism is proposed: *NEAT1* sponges *miR-335* to promote survivin expression, and by extension, GBC cell proliferation and invasion in vitro and tumor growth in vivo, thereby facilitating GBC progression.

LncRNAs are a newly identified class of noncoding RNAs that perform important functions, such as regulation of cell cycle, cell growth, migration, and apoptosis, and are involved in almost all physiological and pathological processes.[23,24] Several studies have uncovered the effectiveness of lncRNAs as miRNA sponges, affecting GBC initiation and progression as tumor suppressors or oncogenic RNAs. For example, *H19* regulates FOXM1 expression by competitively binding endogenous *miR-342-3p* in GBC;[25] *MALAT1* functions as a ceRNA by sponging *miR-*

363-3p, thereby regulating MCL-1 expression in GBC;[26] and the *MINCR*/*miR-26a-5p*/EZH2 axis is reported to be involved in the proliferation, invasion, and apoptosis of GBC cells.[11] In our study, we observed that *NEAT1* contained *miR-335*-binding elements, and an online bioinformatic database identified *NEAT1* as a potential sponge of *miR-335*. Our dual-luciferase reporter assay confirmed that *miR-335* binds to a site(s) within *NEAT1*. Further, *NEAT1* has been shown to be enriched in miRNA-binding sites and to function as a ceRNA that interacts with miRNAs; it is also known to play an essential role in tumor initiation and progression. *NEAT1* facilitates melanoma cell proliferation, migration, and invasion by regulating *miR-495-3p* and E2F3[27] and promotes colorectal cancer progression by competing with *SIRT1* mRNA for binding to *miR-34a* and by activating the Wnt/β-catenin signaling pathway.[28] In our study, we found that NEAT1 knockdown resulted in
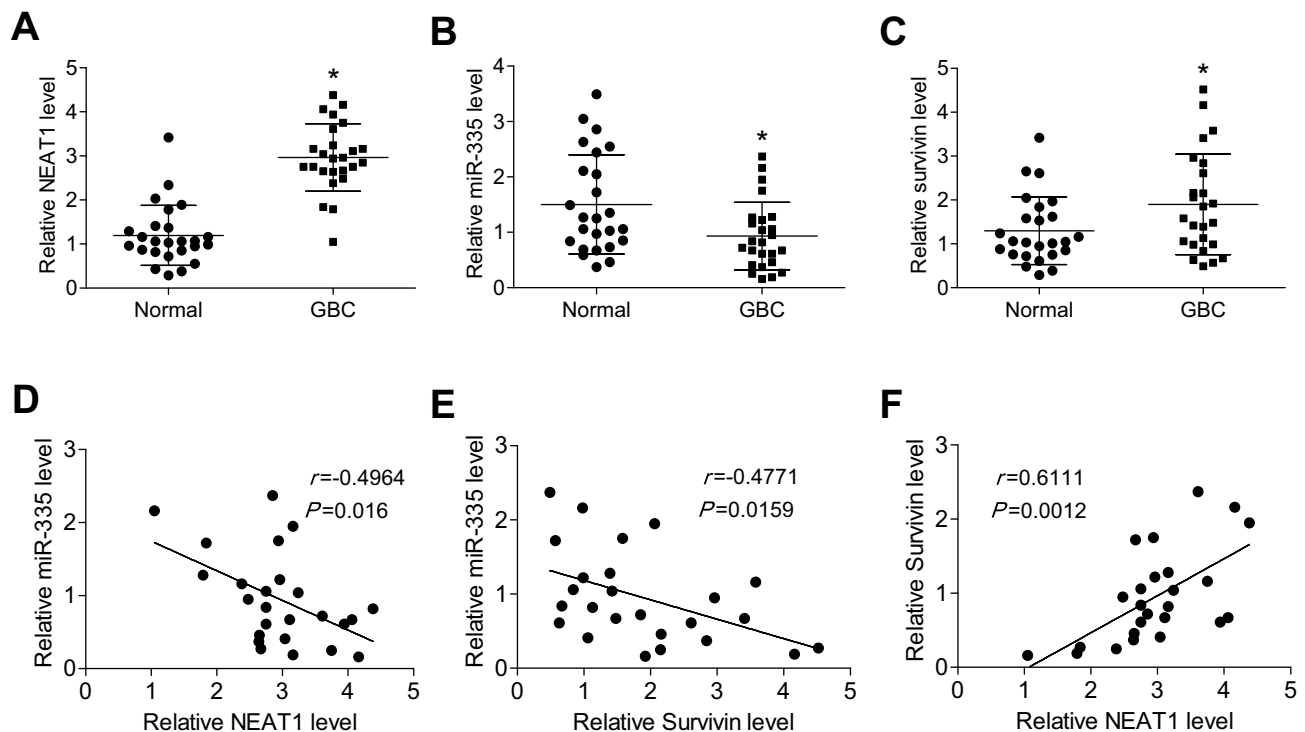
Figure 5 *NEAT1* was upregulated in GBC tissue samples, and its levels inversely correlated with miR-335 levels, but positively correlated with survivin levels. (**A**) The relative expression of *NEAT1* in human GBC samples and normal gallbladder tissue samples. The relative expression of *miR-335* (**B**) and survivin (**C**) in human GBC samples and normal gallbladder tissue samples (**D**) A significant inverse correlation was observed between *NEAT1* and *miR-335* levels in GBC samples. (**E**) A significant inverse correlation was observed between *miR-335* and survivin mRNA levels in GBC samples. (**F**) A significant positive correlation was observed between *NEAT1* and survivin mRNA levels in GBC samples. *$P < 0.05$.

a significant increase in *miR-335* levels in GBC-SD and SGC-996 cells. Thus, we can speculate that *NEAT1* serves as a ceRNA that interacts with *miR-335*, thereby influencing the expression of *miR-335* target mRNAs in GBC.

We examined computationally predicted targets of *miR-335* and found that survivin was a possible target. Survivin—also known as baculoviral inhibitor of apoptosis repeat-containing 5—is the newest member of a family of proteins that is involved in inhibiting apoptosis; it was recently suggested as a novel biomarker of cancer.[29,30] Overexpression of survivin in tumors is generally associated with poor prognosis and high drug resistance.[31] Our previous study implicated survivin as a critical regulator of tumor cell apoptosis in GBC.[18] In the present study, bioinformatic analysis showed that the 3′-UTR of survivin mRNA is complementary to the seed sequence of *miR-335*; dual-luciferase reporter assays confirmed the interaction between *miR-335* and survivin mRNA. Overexpression of *miR-335* resulted in significantly reduced survivin expression in GBC cells. Online bioinformatic database searches predicted that *NEAT1* and survivin shared almost the same putative binding site in *miR-335*. Survivin expression significantly decreased in GBC-SD and

SGC-996 cells after transfection with an *NEAT1*-specific shRNA, but increased after transfection with an *miR-335* inhibitor. By contrast, in cells cotransfected with an *miR-335* inhibitor and sh*NEAT1*, the stimulatory effect of *NEAT1* on survivin expression was attenuated. We demonstrated that the oncogenic activity of NEAT1 was, in part, mediated by the downregulation of *miR-335* in vitro and in vivo. Furthermore, *NEAT1* was found to be upregulated in GBC samples, and its expression was inversely correlated with *miR-335* levels, but positively correlated with survivin levels. These data indicated that *NEAT1* positively regulated survivin expression by sponging *miR-335* in GBC cells.

Several studies also have shown that plasma *NEAT1* and *miR-335* levels are abnormal in some cancers. For example, the plasma levels of *NEAT1* are higher in lymph node-negative patients, lymph node-positive patients, and particularly, in triple-negative patients, indicating that *NEAT1* dysregulation is associated with more aggressive tumors.[32] Downregulation of *miR-335* has also been demonstrated in samples from gastric cancer patients, and this phenomenon is inversely correlated with methylation of the promoter region, supporting the hypothesis that methylation of *miR-335* may serve as

a promising non-invasive treatment strategy for gastric cancer.[33] Plasma *NEAT1* levels may also be abnormal in GBC patients. In future experiments, we will collect multiple GBC plasma samples to measure *NEAT1* levels and analyze the correlation between *NEAT1* expression and GBC.

In conclusion, we found that *miR-335* overexpression results in inhibition of proliferation and invasion of GBC cells. In addition, we demonstrated that *NEAT1* promotes survivin expression by competitively sponging *miR-335*, thereby promoting GBC cell proliferation and invasion in vitro and GBC tumor growth in vivo. Moreover, *NEAT1* was found to be upregulated in GBC tissue samples, and to inversely correlate with *miR-335* levels, but positively correlate with survivin levels. These findings suggest that *NEAT1* is a potential biomarker and a target for GBC diagnosis and therapy.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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