LncRNA-HNF1A-AS1 functions as a competing endogenous RNA to activate PI3K/AKT signalling pathway by sponging miR-30b-3p in gastric cancer

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BACKGROUND: Accumulating evidence demonstrated that long noncoding RNAs (lncRNAs) played important regulatory roles in many cancer types. However, the role of lncRNAs in gastric cancer (GC) progression remains unclear.

METHODS: RT-qPCR assay was performed to detect the expression of HNF1A-AS1 in gastric cancer tissues and the non-tumourous gastric mucosa. Overexpression and RNA interference approaches were used to investigate the effects of HNF1A-AS1 on GC cells. Insight into competitive endogenous RNA (ceRNA) mechanisms was gained via bioinformatics analysis, luciferase assays and an RNA-binding protein immunoprecipitation (RIP) assay, RNA-FISH co-localisation analysis combined with microRNA (miRNA)-pulldown assay.

RESULTS: This study displayed that revealed expression of HNF1A-AS1 was associated with positive lymph node metastasis in GC. Moreover, HNF1A-AS1 significantly promoted gastric cancer invasion, metastasis, angiogenesis and lymphangiogenesis in vitro and in vivo. In addition, HNF1A-AS1 was demonstrated to function as a ceRNA for miR-30b-3p. HNF1A-AS1 abolished the function of the miRNA-30b-3p and resulted in the derepression of its target, PIK3CD, which is a core oncogene involved in the progression of GC.

CONCLUSION: This study demonstrated that HNF1A-AS1 worked as a ceRNA and promoted PI3K/AKT signalling pathway-mediated GC metastasis by sponging miR-30b-3p, offering novel insights of the metastasis mechanism in GC.
MATERIALS AND METHODS

Clinical samples
Sixty-seven samples of fresh GC tissue and six samples of non-tumourous gastric mucosa tissue were obtained from the Qilu Hospital, Shandong Provincial Hospital and the First Affiliated Hospital of Weifang Medical University. All samples were stored in liquid nitrogen. None of the patients had undergone treatment prior to surgery. The clinicopathological characteristics for all of the patients are shown in Table 1. The study was approved by the Research Ethics Committee of Shandong University.

Cell culture and transfection
Human gastric cancer cell lines (MKN-45 and BGC-823) were commercially obtained from the Shanghai Cancer Institute. Human umbilical vein endothelial cells (HUVECs) were generously provided by Dr. Hu (Shandong University). All cell lines were routinely maintained in RPMI 1640 supplemented with 10% foetal bovine serum. Human lymphatic endothelial cells (HLECs), generously provided by Dr. Li (Shandong University), were cultured in MEM (ScienCell, San Diego, CA, USA) with 10% FBS. Mycoplasma PCR testing of these cells was performed every month. Cells were grown for no more than 15 passages in total for any experiment. HNF1A-AS1 siRNA (si-HNF1A-AS1), and siRNA-negative control (si-NC) were synthesised by GenePharma (Shanghai, China). MKN-45 and BGC-823 were seeded on 6- or 12-well plates and transfected 48 h later using X-tremeGENE transfection reagent (Roche Applied Science, Indianapolis, IN, USA). Quantitative PCR was performed using SYBR Green (Roche Applied Science, Indianapolis, IN, USA). The expression level of each specific gene was normalised to that of GAPDH. The All-in-One™ miRNA qRT-PCR Detection Kit (Genecopoeia, USA) was used for miRNA RT-qPCR. The expression level of miR-30b-3p was detected using miR-30b-3p-specific primer (Genecopoeia). U6 small nuclear RNA was used as the internal control. The relative gene expression was calculated using the 2^(-ΔΔCt) method.

Plasmid construction
HNF1A-AS1 cDNA was subcloned into the pcDNA3.1 vector and named pcDNA3.1-HNF1A-AS1. Point mutations were introduced into the miR-30b-3p response elements in pcDNA3.1-HNF1A-AS1, producing a new plasmid designated as HNF1A-AS1-mut (miR-30b-3p). The HNF1A-AS1 vector and named pmiRGLO-HNF1A-AS1. The pmiRGLO-HNF1A-AS1 with point mutations in the miR-30b-3p response elements was named pmiRGLO-MUT-1. The 3' untranslated regions (3′-UTR) of PIK3CD and AKT3, containing the intact miR-30b-3p recognition sequences, respectively, were subcloned into the pmiRGLO vector and named pmiRGLO-PIK3CD or pmiRGLO-AKT3. The pmiRGLO-PIK3CD with point mutations in the miR-30b-3p response elements was named pmiRGLO-MUT-3. The 3′-UTR of PIK3CD was subcloned into the pcDNA3.1 vector and named pcDNA3.1-PIK3CD 3′-UTR.

Dual-luciferase reporter assay
MKN-45 and BGC-823 cells were co-transfected with wild- or mutant-type pmiRGLO plasmid and miRNA mimics using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Forty-eight hours after transfection, the relative luciferase activity was measured using the dual-luciferase reporter system as previously reported.15

Western blotting
The anti-PIK3CD antibody (1:500 dilution), anti-PIK3R1 antibody (1:1000 dilution), anti-AKT1 antibody (1:1000 dilution), anti-AKT2 antibody (1:300 dilution), anti-AKT3 antibody (1:500 dilution) and anti-β-actin antibody (1:1000 dilution) were incubated with polyvinylidene difluoride membranes at 4°C overnight. β-actin was used as an endogenous control. A previously described detailed procedure is available.16

Enzyme-linked immunosorbent assay (ELISA)
Cell culture supernatants of MKN-45 and BGC-823 cells that were transiently transfected with the pcDNA3.1-HNF1A-AS1 and pcDNA3.1 were collected and centrifuged at 3,000 rpm for 10 min. VEGF-C and VEGF-A secretion levels were detected via ELISA according to the manufacturer’s protocol (Multi Sciences Biotech, Co., Ltd, Hangzhou, China).

Table 1. The association between HNF1A-AS1 expression and clinicopathological parameters.

| Variables                      | Low expression of HNF1A-AS1 | High expression of HNF1A-AS1 | P-value |
|--------------------------------|-----------------------------|-----------------------------|---------|
| Tumour size                    |                             |                             | P = 0.2308 |
| <5 cm                          | 9                           | 14                          |         |
| >=5 cm                         | 24                          | 20                          |         |
| Age (years)                    |                             |                             | P = 0.7029 |
| <62                            | 14                          | 16                          |         |
| >=62                           | 19                          | 18                          |         |
| Gender                         |                             |                             | P = 0.4158 |
| Female                         | 6                           | 9                           |         |
| Male                           | 27                          | 25                          |         |
| Differentiation                |                             |                             | P = 0.1384 |
| Well differentiated            | 1                           | 0                           |         |
| Poorly                         | 17                          | 11                          |         |
| Moderately                     | 15                          | 23                          |         |
| Lymph node metastasis          |                             |                             | P = 0.0262 |
| Yes                            | 23                          | 31                          |         |
| No                             | 10                          | 3                           |         |
Migration and invasion in vitro assays of GC cells
The migration and invasion abilities of GC cells were observed in both Matrigel assay-coated and uncoated Transwell chambers. A detailed procedure is described in previous study.17

Migration, proliferation and tube-formation assay in HUVECs and HLECs
Migration, proliferation, and tube-formation assay in HUVECs and HLECs were performed as described in previous study.17,18

RNA-binding protein immunoprecipitation (RIP) assay
RNA immunoprecipitation was performed using the EZ-Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA) and the Argonaute2 (AgO2, Millipore) antibody in accordance with the manufacturer’s protocol. The cells were lysed using RIP lysis buffer. A 100-μl volume of whole-cell lysate was incubated with RIP buffer containing magnetic beads conjugated with human anti-AgO2 antibody (Millipore) and normal mouse immunoglobulin G (IgG, Millipore), which served as a negative control. Samples were incubated with Proteinase K buffer and immunoprecipitated RNA was then extracted. RT-qPCR analysis, using Fos and HNF1A-AS1 primers, was performed to identify the presence of binding targets in the co-precipitated RNAs.

Biotin-labelled pull-down assays
MKN-45 and BGC-823 cells were transfected with biontinylated miR-30b-3p and a scrambled control (GenePharma, Shanghai) and collected 48 h after transfection. The cell lysates were incubated with M-280 streptavidin magnetic beads (Invitrogen, San Diego, CA, USA) and 10 μl of yeast tRNA on a rotator at 4°C for 2 h. Add 750 μl of Trizol (Invitrogen, Carlsbad, CA) and 250 μl of water to the input, and the pull-down beads and the bound RNAs were purified. The 3′-biontin-labelled miR-30b-3p sequence is 5′-CUGGGAGGGAGGUUUACUUC-3′ BiO. The scrambled control miRNA sequence is 5′-UUCUCCGAAAGCU GUCACGUTT-3′ BiO.

Mouse tumour xenograft experiments
Four-week-old male Nu/nu athymic nude mice were obtained from the National Laboratory Animal Center (Weitonglihua Biotechnology, Beijing, China) and were maintained under SPF room conditions for feeding and observation. The mice were randomized divided into four groups. MKN-45 cells were transduced with lentivirus vector LV5-GFP-HNF1A-AS1 or LV5-GFP-Negative control (GenePharma Co., Ltd., Shanghai, China). The titre of lentivirus was 1 × 10^6 TU/ml. HNF1A-AS1 was initiated by the EF-1α promoter, with the full sequence map for LV5 shown in Supplementary Fig. 1a. A total of 2 × 10^5 LV5-HNF1A-AS1 or LV5-NC cells were then injected into the lateral tail vein or the axillary fossa of each mouse (n = 6 mice per group) on 18 September 2017. To ameliorate pain to the mice throughout experimental studies, nasal anaesthesia (isoflurane) was introduced. The benefits of isoflurane anaesthesia for animals are that the anaesthesia depth was easy to control, and isoflurane did not affect body metabolism. Mice were anaesthetised using an anaesthetic machine (Kodak, Rochester, USA) with MAC 1.6% isoflurane. All experiments were performed inside a biosafety cabinet during the animal’s light time cycle on the first floor of the Experimental Animal Room at Shandong University. The mice were killed by cervical dislocation, and tumour, lung, liver and kidney were isolated from the mice for further analysis. All of the animal experiments were conducted according to the Guidelines for Animal Health and Use (Ministry of Science and Technology, China, 2006). Animal experiments were approved by the Committee for Animal Protection and Utilisation of Shandong University.

Immunochemistry for CD-34
Immunohistochemical (IHC) analysis for CD-34 was performed to determine the amount of blood isolated from the mouse xenograft tumour tissues. Procedures were performed, and the results assessed as previously reported.19

RESULTS
High expression of HNF1A-AS1 is correlated with lymph node metastasis in GC patients
HNF1A-AS1 expression was measured in human GC tissues and non-tumourous gastric mucosa tissues. HNF1A-AS1 in gastric cancer has been found to be significantly overexpressed compared with human non-tumourous gastric mucosa tissue. Further upregulation of HNF1A-AS1 was observed in GC cases with lymph node metastasis (LNM) when compared with GC cases without LNM (Fig. 1a). The expression of HNF1A-AS1 was classified as high or low expression based on a median score. The clinicopathological assay revealed that higher HNF1A-AS1 expression in GC tissues was remarkably correlated with positive LNM (P = 0.0262), but there was no correlation between HNF1A-AS1 expression and tumour size (P = 0.2308), age (P = 0.7029), gender (P = 0.4158) or differentiation (P = 0.1384) (Table 1). A receiver-operating characteristic (ROC) curve assay was performed to evaluate whether HNF1A-AS1 expression could be used to distinguish between patients with LNM and those without LNM. The AUC value for HNF1A-AS1, distinguishing GC cases with LNM from those without LNM, was as high as 0.7650 (Fig. 1b, 95% confidence interval (CI) = 0.6177–0.9122, P = 0.0032). These results imply that HNF1A-AS1 expression is associated with LNM and may serve as a biomarker for predicting GC LNM.

HNF1A-AS1 promotes cell migration, invasion and metastasis in GC
To further explore the biological functions of HNF1A-AS1 in GC cells, GC cells were transfected with the pcDNA3.1-HNF1A-AS1 plasmid or siRNAs against HNF1A-AS1. The overexpression and knockdown efficiency of HNF1A-AS1 was detected 48 h after transfection (Fig. 1c, d, e, f). HNF1A-AS1 significantly promoted cell migration and invasion, while HNF1A-AS1 knockdown markedly suppressed cell migration and invasion (Fig. 1g, h). MKN-45 cells transfected with LV5-HNF1A-AS1/LV5-NC expressing green fluorescent protein/GFP (Fig. 1i) were injected into the axillary fossa of mice to investigate the functional roles of HNF1A-AS1 in vivo. RT-qPCR assay showed that HNF1A-AS1 was stably overexpressed in MKN-45 cells in vivo (Supplementary Fig. 1b). Local invasion occurred in all of the mice with LV5-HNF1A-AS1-transfected tumours. In contrast, only two of six LV5-NC mice developed local invasion; the other four mice had tumours that were well-encapsulated with non-invasive margins (Fig. 1j). Moreover, we observed that the number of CD-34-positive cells was higher in the LV5-HNF1A-AS1 group compared with the control group by IHC analysis (Fig. 1k), indicating that HNF1A-AS1 promoted angiogenesis in GC. In addition, metastatic cancer cells were observed in the lungs of mice in the HNF1A-AS1 group, which may be due to HNF1A-AS1-mediated angiogenesis in GC (Fig. 1j). In homogeneous metastatic models, mice in the LV5-HNF1A-AS1 group had a greater number of more metastatic loci in the lungs compared with mice in the LV5-NC group (14.6 ± 3.24 loci...
**HNF1A-AS1 promotes angiogenesis of HUVECs and lymphangiogenesis of HLEC**

Next, we explored the role of HNF1A-AS1 in GC angiogenesis and lymphangiogenesis in vitro. The Transwell migration assay revealed that the migration activity of HUVECs dramatically increased when cultured with conditioned medium from MKN-45 and BGC-823 cells transfected with pcDNA3.1-HNF1A-AS1 (Fig. 2a). In addition, HUVEC proliferation was significantly enhanced in the HNF1A-AS1 overexpression group (Fig. 2b).

Matrigel-based capillary tube-formation assays were subsequently performed to evaluate the role of HUVECs in tube formation. Tube-formation ability was increased in the HNF1A-AS1 overexpression group compared with the control group (Fig. 2c). In addition, our results showed that HNF1A-AS1 promoted HLEC tube formation compared with the control group (Fig. 2d). The above observations indicate that HNF1A-AS1 overexpression stimulates GC angiogenesis and lymphangiogenesis in vitro.

HNF1A-AS1 regulates PI3KCD, PIK3R1 and AKT3 expression

To better examine the detailed regulatory mechanism of HNF1A-AS1 in GC metastasis, the PI3K/AKT signalling pathway that was involved in cancer cell invasion, angiogenesis and lymphangiogenesis...
HNF1A-AS1 promotes HUVEC angiogenesis, HLEC lymphangiogenesis and regulates PIK3CD, PIK3R1 and AKT3 expression. a In independent experiments were performed, and data are presented as mean ± SD. Culture supernatant from the HNF1A-AS1-transfected groups rather than the negative control groups promoted the migration (b, x400) and proliferation (c, x100) capabilities of HUVECs and also enhanced tube-formation abilities of HLECs (d, x100). Three independent experiments were performed, and data are presented as mean ± SD. g Western blotting assays demonstrated that HNF1A-AS1 overexpression enhanced PIK3CD, PIK3R1 and AKT3 protein expression. Three independent experiments were performed, and data are presented as mean ± SD. h Western blotting assays demonstrated that knockdown of HNF1A-AS1 by siRNA decreased PIK3CD, PIK3R1 and AKT3 protein expression. Three independent experiments were performed, and data are presented as mean ± SD. i, j The secretion level of VEGF-A (i) and VEGF-C (j) significantly enhanced in the culture supernatant from the HNF1A-AS1-transfected MKN-45 cells, compared with the negative control groups, detected by ELISA. Three independent experiments were performed, and data are presented as mean ± SD. k, l The GC cell line MKN-45 cells were transfected with pcDNA3.1 or pcDNA3.1-HNF1A-AS1, and the PI3K/AKT inhibitor NVP-BKM-120 (1 μM) was then added; DMSO was added as a control. In pcDNA3.1-HNF1A-AS1-transfected GC cells, VEGF-A (k) and VEGF-C (l) protein levels decreased significantly with NVP-BKM-120 treatment compared with those of DMSO-treated GC cells. *P < 0.05, **P < 0.01, ***P < 0.001.
PI3K/AKT signalling pathway was highly expressed and promoted GC progression. Furthermore, PIK3CD, PIK3R1, and AKT3, rather than AKT1 and AKT2, were observed to be notably upregulated in the HNF1A-AS1 overexpression group (Fig. 2e, f, g; Supplementary Fig. 1c, d, e), and were downregulated in the HNF1A-AS1-knockdown group at both the mRNA and protein levels (Fig. 2h; Supplementary Fig. 1f, g, h, i, j). Recent studies showed that the activation of the PI3K/AKT pathway in tumour cells increases the secretion of VEGF-A and VEGF-C. VEGF-A exerts a pro-angiogenic role in human cancer, and VEGF-C is a well-known regulator of the lymphangiogenesis. Therefore, an ELISA assay was performed to demonstrate the effect of HNF1A-AS1 on VEGF-A and VEGF-C secretion. Interestingly, the VEGF-A and VEGF-C secretion levels in the supernatants isolated from MKN-45 and BGC-823 cells transfected with the HNF1A-AS1-overexpression vector were respectively significantly improved compared with control cells (Fig. 2i, j; Supplementary Fig. 1k), indicating that PI3K/AKT signalling pathway may be involved in HNF1A-AS1-induced VEGF-A and VEGF-C secretion. Next, we demonstrated the effect of a PI3K inhibitor (NVP-BKM120) on HNF1A-AS1-mediated VEGF-A and VEGF-C secretion. Our results showed that the secretion levels of VEGF-A and VEGF-C were decreased in LV5-HNF1A-AS1 GCs with NVP-BKM120 treatment (1 μM) compared with the control cells treated only with DMSO (Fig. 2k, l). A rescue experiment was also performed to confirm that HNF1A-AS1 exerts its biological functions through PI3K/AKT signalling. Interestingly, NVP-BKM120 treatment rescued the tube formation of HUVECs and HLECs, and the migration abilities of GCs enhanced by HNF1A-AS1. *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 3 The effect of PI3K inhibition on HNF1A-AS1-mediated biological functions. a–c The GC cell line MKN-45 cells were transfected with pcDNA3.1 or pcDNA3.1-HNF1A-AS1, and the PI3K/AKT inhibitor NVP-BKM-120 was then added; DMSO was added as a control. NVP-BKM120 (PI3K inhibitor) treatment rescued the tube formation of HUVECs (a, ×40) and HLECs (b, ×40), and migration abilities (c, ×100) of GCs enhanced by HNF1A-AS1. *P < 0.05, **P < 0.01, ***P < 0.001.
HNF1A-AS1 functions as a ceRNA with miR-30b-3p

Recently, accumulating evidence has demonstrated that lncRNAs can function as ceRNAs to sponge miRNAs, resulting in decreased miRNA expression in the cytoplasm and modulating the derepression of miRNA targets at the post-transcriptional level. HNF1A-AS1 was found to be distributed in both the nucleus and cytoplasm of gastric cancer cells via RNA-FISH (Fig. 4a, b). To evaluate whether HNF1A-AS1 functions as ceRNA, miRNAs were predicted to be bound to HNF1A-AS1 and PIK3CD, PIK3R1 or AKT3, using programmes, such as RegRNA, miRBase, miRwalk and targetscan. Based on these data and the previous reports about the candidate miRNAs function and the number of predicted target sites, we chose eight cancer-related or tumour-suppressing miRNAs for further investigation, including miR-7-5p, miR-18a, miR-122, miR-149, miR-494, miR-636, miR-93 and miR-30b-3p. MiR-30b-3p decreased luciferase activity in pmirGLO-HNF1A-AS1 (Fig. 4c, d), but not in MUT-1 (Fig. 4e, f), indicating that the miRNA can directly bind to HNF1A-AS1 through their respective miRNA-binding sites (Supplementary Fig. 1). In addition, HNF1A-AS1 overexpression decreased miR-30b-3p expression (Fig. 4g, h), while HNF1A-AS1 knockdown markedly enhanced miR-30b-3p expression (Fig. 4i). Overexpression of HNF1A-AS1-Mut (miR-30b-3p) did not decrease the expression of miR-30b-3p (Fig. 4j, k).

Interestingly, miR-30b-3p notably decreased HNF1A-AS1 expression (Fig. 4l, m). It has been proposed that miRNAs directly regulate their targets and induce RNA degradation and/ or translational repression through the formation of RNA-induced silencing complexes (RISCs) containing the Ago2 protein, a key component of RISC. To investigate whether HNF1A-AS1 interacts with RISC, RIP experiments were performed with BGC-823 cell extracts using antibodies against Ago2. The endogenous HNF1A-AS1 was specifically enriched in Ago2 immunoprecipitate compared with control IgG immunoprecipitates from BGC-823 cell extracts. The expression level of HNF1A-AS1 in Ago2 immunoprecipitates relative to control IgG immunoprecipitates was markedly elevated in the biotin-labelled miR-30b-3p-captured fraction compared with the negative control, indicating that HNF1A-AS1 functions as a ceRNA with miR-30b-3p.
suggesting that miR-30b-3p interacts with HNF1A-AS1 directly and in a sequence-specific manner.

Next, we explored the effects of HNF1A-AS1 with miR-30b-3p-mutated binding sites on GC cell migration and invasion abilities. Our data showed that HNF1A-AS1 with miR-30b-3p-mutated binding sites exerted only a weaker effect on GC migration and invasion abilities compared with the wild-type HNF1A-AS1 (Fig. 5d, e). In addition, a rescue experiment was then performed to explore whether HNF1A-AS1 exerts biological functions through miRNA. The migration and invasion abilities were enhanced in
the HNF1A-AS1-overexpression group; however, the increased migration and invasion activities promoted by HNF1A-AS1 were reversed by miR-30b-3p mimics (Fig. 5f, g), suggesting that HNF1A-AS1 exerts its tumour-oncogenic effects by repressing miRNAs in GC cells. Taken together, the above results indicate that HNF1A-AS1 acts as a ceRNA with miR-30b-3p.

MiR-30b-3p exerts tumour-suppressive functions through regulation of PI3K/AKT signalling
To ascertain the biological functions of miR-30b-3p in GC cells, miR-30b-3p mimics were transfected into MKN-45 and BGC-823 cells (Supplementary Fig. 1m, n). MiR-30b-3p inhibited GC cell migration and invasion activities (Fig. 5h, i). The potential fundamental roles of miR-30b-3p in angiogenesis were then evaluated. The migration (Fig. 5j) and tube-formation abilities (Fig. 5k) of HUVECs were inhibited when cultured with conditioned medium from MKN-45 and BGC-823 cells transfected with miR-30b-3p mimics.

MiR-30b-3p was then investigated to determine whether it targeted PIK3CD, PIK3R1 and AKT3. Bioinformatic programmes (miRwalk and targetscan) revealed binding sites for miR-30-3p in the 3′-UTR of PIK3CD and AKT3, but not PIK3R1 (Fig. 6a). Luciferase assays demonstrated that the miR-30b-3p suppressed the luciferase activity of pmirGLO-PIK3CD-WT (Fig. 6b, c), but not pmirGLO-PIK3CD-Mut (miR-30b-3p) (Fig. 6d, e). Subsequently, miR-30b-3p significantly reduced PIK3CD protein levels (Fig. 6f), suggesting that PIK3CD is a direct target gene of the miRNA. A biotin-labelled pull-down assay was performed to detect whether miR-30b-3p could directly interact with PIK3CD. Our result showed that the level of PIK3CD 3′-UTR was markedly elevated in the biotin-labelled miR-30b-3p-captured fraction compared with that in the negative control (Fig. 5b, c), suggesting that miR-30b-3p interacted with PIK3CD 3′-UTR in a sequence-specific manner. In addition, neither miRNA induced a significant decrease in the luciferase activity of pmirGLO-AKT3 (Fig. 6g, h), indicating that AKT3 is not a direct target gene of miR-30b-3p. However, miR-30b-3p did decrease the AKT3 protein levels (Fig. 6f). AKT3 has previously been reported to function as a downstream gene in the PI3K signalling pathway, indicating that miR-30b-3p may indirectly reduce the AKT3 protein level, due to a decrease in PIK3CD.

As demonstrated, HNF1A-AS1 enhanced PIK3CD transcripts and protein levels. In order to examine whether HNF1A-AS1-mediated upregulation of PIK3CD relied on miRNAs, the miR-30b-3p-binding site was mutated, and luciferase activity assays were performed. As expected, overexpression of HNF1A-AS1, but not HNF1A-AS1 (miR-30b-3p-mut), increased the luciferase activity of pmirGLO-PIK3CD. Ectopic expression of miR-30b-3p diminished this upregulation (Fig. 6i, j), suggesting an essential role for HNF1A-AS1 in regulating PIK3CD by competitively binding to miRNAs. MiRNA pull-down assays were further performed to detect the competitive relationship between HNF1A-AS1 and PIK3CD. Our data indicated that the level of PIK3CD 3′-UTR was reduced in the biotin-labelled miR-30b-3p and pcDNA3.1-HNF1A-AS1-captured fraction (Fig. 6k, l). The level of HNF1A-AS1 was markedly decreased in the pull-down product isolated from the cells transfected with biotin-labelled miR-30b-3p and pcDNA3.1-PIK3CD 3′-UTR (Fig. 6m, n). Taken together, these results revealed that HNF1A-AS1 functioned as a ceRNA by competitively binding to miR-30b-3p to upregulate PIK3CD expression.

Moreover, expression of PIK3CD and miR-30b-3p was detected in mouse xenograft tumours. Overexpression of HNF1A-AS1 decreased the expression of miR-30b-3p, while promoting PIK3CD protein expression, confirming that HNF1A-AS1 functions as a ceRNA by competitively binding to miR-30b-3p to upregulate the expression of PIK3CD in vivo (Fig. 6o, p). Taken together, HNF1A-AS1 exhibited oncogenic properties by activating PI3K/AKT signalling through competitive binding to miR-30b-3p.

**DISCUSSION**
In this study, we showed that the expression of HNF1A-AS1 in patients with LNM was significantly higher than in patients without LNM. Moreover, ROC curve analysis revealed that HNF1A-AS1 expression could be used to distinguish between cases with LNM and those without LNM, and thus it could be used as a biomarker to predict LNM in GC. Moreover, HNF1A-AS1 enhanced migration and invasion abilities in vitro, which is consistent with previous reports, indicating that HNF1A-AS1 is a crucial oncogene in GC. Furthermore, we found that HNF1A-AS1 enhanced GC invasion, metastasis and angiogenesis in vivo. In addition, HNF1A-AS1 increased HUVEC and HLEC tube formation in vitro. It is known that angiogenesis promotes the initial development of primary malignant tumours, and is closely connected with infiltration and metastasis of cancer cells, and that new lymphatic vessels formed through lymphangiogenesis are responsible for cancer metastasis. Thus, we concluded that HNF1A-AS1 enhanced GC metastasis maybe due to its effect on angiogenesis and lymphangiogenesis.

To further explore the regulation mechanism of HNF1A-AS1 in GC metastasis, five genes, such as PIK3CD, PIK3R1, AKT1, AKT2 and AKT3, were chosen based on RT-qPCR and western blot results. HNF1A-AS1 increased the mRNA and protein expression of PIK3CD, AKT3 and PIK3CD, rather than AKT1 and AKT2, suggesting that HNF1A-AS1 exerts its oncogenic role by upregulating PIK3CD, PIK3R1 and AKT3. Moreover, HNF1A-AS1 increased the VEGF-A and VEGF-C secretion, and both are downstream of the PI3K/AKT signalling pathway. Furthermore, the PI3K inhibitor (NVP-BKM120) treatment rescued the tube formation of HUVECs and HLECs, and the migration abilities of GCs were enhanced by HNF1A-AS1, indicating that HNF1A-AS1-induced biological functions might be mediated by PI3K/AKT signalling pathway. Many IncRNAs have recently been demonstrated to act as ceRNAs by competitively binding miRNA- responsive elements and impacting the expression levels of miRNA targets. For example, LncRNA PTAR competitively binds to miR-101-3p to regulate ZEB1 expression, and thus promote EMT and invasion–metastasis in serous ovarian cancer. LncRNA UICLM acts as a ceRNA by
sponging miR-215 to regulate ZEB2 expression and promote colorectal cancer liver metastasis.43 RNA-FISH assays showed that HNF1A-AS1 was located in both the cytoplasm and nucleus. HNF1A-AS1 was hypothesised to function as a ceRNA by upregulating the expression of PIK3CD, PIK3R1 and AKT3. Bioinformatic analyses, luciferase assays, biotin-labelled miRNA pull-down assays and RIP assays were used to explore potential interactions between HNF1A-AS1 and miRNAs, and to confirm the direct binding capabilities of the predicted miRNA-responsive elements to the full-length HNF1A-AS1 transcript. As expected, miR-30b-3p was predicted to show complementary base pairing with HNF1A-AS1 and PIK3CD or AKT3. MiR-30b-3p significantly reduced the luciferase activity of the HNF1A-AS1 WT reporter vector. In addition, RIP assays indicated that endogenous HNF1A-
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