Research Square

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miR-367-3p enhanced gastric cancer progression by targeting Smad7 to regulate the transforming growth factor-1/Smad3 pathway

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Keywords: gastric cancer; miR-367-3p; Smad7; biomarker; prognosis

Posted Date: February 21st, 2020

DOI: https://doi.org/10.21203/rs.2.24158/v1

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Abstract

Several studies have shown that miR-367-3p can function so as to promote or suppress the development of many forms of cancer, but its specific role in gastric cancer (GC) is not fully characterized. In this study, we found that patient GC samples exhibited significantly elevated miR-367-3p expression relative to healthy para-cancerous tissues, and the expression of this miRNA was positively correlated with features of more aggressive disease lymph node metastasis (p=0.025) and depth of invasion (p = 0.047). When miR-367-3p was overexpressed, this led to increased growth, migration, and epithelial-mesenchymal transformation of GC cells, whereas inhibiting this miRNA resulted in the opposite phenotype. Luciferase reporter assays further confirmed the ability of miR-367-3p to target Smad7 and to inhibit its expression. As Smad7 functions to suppress TGF-β1/Smad3 signaling, this miRNA is thus able to enhance TGF-β1/Smad3 signaling, which in turn drives GC progression and feed forward enhancement of this signaling pathway. Together these findings thus offer valuable new insight into the role of miR-367-3p in GC.

Introduction

Gastric cancer (GC) is one of the deadliest forms of cancer in the world[1], and it is highly prevalent in China[2] where it is the second most common and third deadliest cancer subtype[3]. While there have been many complex and comprehensive analyses of GC conducted to date, the mechanisms governing the onset and progression of this disease remain incompletely understood, thus limiting patient access to efficacious treatment options.

microRNAs (miRNAs) are small RNAs that lack coding potential and yet are apple to suppress the expression of specific target genes. Different miRNAs can act to suppress or promote tumor development in a miRNA- and cancer-specific manner, with miRNA dysregulation having previously been detected in the context of GC[4–7]. Indeed, such altered miRNA expression patters are thought to be closely linked to the proliferative and metabolic activity of these cancer cells[8–10], consistent with the observation that miRNAs are abnormally expressed not just in GC but also in other cancers such as glioma and cancers of the lungs, liver, bladder, breast, and prostate[11–13]. Several recent studies have highlighted miR-367-3p as a miRNA that is dysregulated in a range of cancer types[14–19]. However, what role is played by miR-367-3p in GC development and progression has not been well documented. As such, in the present study we examined the functional relevance of miR-367-3p in GC and sought to unravel the underlying molecular mechanisms governing this relevance

Materials And Methods

Tissue specimens

A total of 45 pairs of GC patient tumor and normal para-cancerous tissue were obtained from patients that underwent surgery at the Shaoxing Hospital, Zhejiang University School of Medicine. Patients

enrolled in the present study had not undergone any preoperative systemic or local anti-tumor therapy. Following collection, pathological examination was used to confirm GC diagnosis, and samples were snap frozen prior to storage at -80 °C. All patients provided written informed consent to participate, and the Ethics Committee of Shaoxing Hospital, Zhejiang University School of Medicine approved all human studies. Patient demographics and clinical findings are listed in **Table 1**.

Cell culture

Both GC cell lines (HGC-27 and MKN-45) and the control GES-1 gastric epithelial line came from the Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured at 37˚C with 5% CO2 in RPMI-1640 containing 20% FBS and antibiotics (Gibco, USA). For miRNA transfection, GC cells in the exponential phase were transfected with 100 nM of miR-367-3p mimics, inhibitors, or appropriate scrambled control RNA constructs (GeneCopoeia Ltd., iGeneBio) using Lipofectamine 2000 (Invitrogen). After 48 h cells were used for downstream studies.

Luciferase reporter assay

Potential miR-367-3p target genes were identified using the TargetScan database (www.targetscan.org), highlighting Smad7 as a potential target. Based on this prediction, pmiR-RB-report plasmids (RiboBio Inc) were cloned so as to contain either a mutant or wild-type version of the 3’-UTR of Smad7. These plasmids were then transfected into cells together with appropriate miRNA mimics, inhibitors, or controls. After 48 h, a Dual luciferase reporter assay kit (Promega) was then used to analyze luciferase activity in these cells, with Renilla luciferase used for normalization purposes.

Western blotting

RIPA buffer (Beyotime Institute of Biotechnology) was used to lyse cells, after which a BCA kit (Thermo Fisher Scientific) was used to quantify protein levels and 20 μg of each sample was separated through 10% SDS-PAGE prior to transfer to a PVDF membrane. These blots were then blocked with 5% non-fat milk for 2 h, after which they were probed overnight with antibodies specific for GAPDH (1:2000; Bioworld Technology, Inc.), Smad7, Smad2, p-Smad2 (1: 2000), Smad3, and p-Smad3 at 4˚C. Next, blots were incubated for 1 h with HRP-conjugated goat anti-mouse or anti-rabbit (1:5,000; Bioworld Technology, Inc.) secondary antibodies, and were then washed prior to development via enhanced chemiluminescence (Thermo Fisher Scientific) together with a ChemiDoc Imaging Platform (Bio-Rad).

RT-qPCR

Cell line or paraffin-embedded tissue RNA was collected using a miRNeasy FFPE kit (Qiagen GmbH, Hilden, Germany) or a miRNeasy Mini kit (Qiagen) based on provided directions. Appropriate cDNA was generated using a miScript II RT kit (Qiagen) based on provided directions using 1ug of input RNA or miRNAs. Reverse transcription thermocycler settings were: 37°C for 60 min, 95°C for 5 min. For qRT-PCR reactions, the miScript SYBR Green PCR kit (Qiagen) or SYBR Green PCR Master mix (Qiagen) were used together with a 7500 Fast Real-Time PCR platform (Applied Biosystems, MA, USA) with the following

thermocycler settings: 95°C for 15 min; 40 cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s. For normalization purposes, relative GAPDH and U6 levels were quantified in appropriate samples. Triplicate samples were analyzed for each reaction. Primers are compiled in **Table 2**.

BrdU proliferation

A total of 10,000 - 20,000 of the indicated GC cell lines were added to wells of 96-well plates and were then used with a BrdU proliferation assay kit (Cell Signalling Technology) according to provided directions.

Transwell assay

For all migration/invasion assays, 12-well plates containing Transwell inserts (Corning, MA, USA) that had an 8 μm pore size were used. In migration assays, a total of 100,000 cells were resuspended in serum-free media and were added to the upper portion of this chamber for 8 h at 37 °C, after which cells remaining in the upper chamber were removed and those in the lower chamber were subjected to methanol fixation prior to staining using 0.04% crystal violet. Cells were then quantified. For invasion assays, the protocols were identical to those above, but the transwell inserts had first been coated using Matrigel (BD Biosciences, MA, USA), and plates were instead incubated for 24 h.

Statistical analysis

All results were analyzed with SPSS 17.0 (SPSS Inc., IL, USA), and are given as means ± SD. Data were compared via Student's t-tests and one-way ANOVAs as appropriate. The relationship between miR-367-3p expression and patient clinical findings was analyzed via chi-squared test. Receiver operating characteristic (ROC) curve analyses were used to assess the diagnostic and prognostic importance of miR-367-3p levels in GC patients. Survival outcomes were compared using Kaplan-Meier curves and compared via log-rank tests. To identify factors associated with patient outcomes, univariate regression analyses were performed and all significant factors were then incorporated into a multivariate analysis. Experiments were repeated in triplicate, with $P < 0.05$ as the significance threshold.

Results

GC samples exhibit elevated miR-367-3p expression

We began by exploring the relationship between miR-367-3p and GC by measuring the levels of this miRNA in GC patient samples. We found that miR-367-3p levels were markedly elevated in GC tumor samples relative to corresponding control samples (**Fig.1A**), and we further found that GC patients who had more advanced disease exhibited significant increases in miR-367-3p expression as compared to those in whom disease was less advanced (**Fig.1B**). Consistent with these results, miR-367-3p levels were significantly higher in the HGC-27 and MKN-45 GC cell lines relative to the control GSE-1 cell line (all $P<0.05$; **Fig.1C**). This thus suggested that miR-367-3p expression is enhanced in GC and that it may thus be linked to the progression of this disease.

miR-367-3p is associated with GC progression

We next extended our analyses to examine the relationship between miR-367-3p expression and GC progression via dividing patients into miR-367-3p-high and -low expressing groups (n = 23 and 22, respectively) based upon median expression levels of this miRNA. We then compared clinical findings in these patients, thus revealing a significant correlation between elevated expression of miR-367-3p and lymph node metastasis (p=0.025) and depth of invasion (p = 0.047) (**Table 1**).

GC cell proliferation, migratory and invasive activity is enhanced by miR-367-3p

To expand upon our patient findings, we conducted functional analyses of miR-367-3p in GC cell lines that had been transfected using mimics or inhibitors of this miRNA, leading to its corresponding overexpression or downregulation (**Fig.2A**). We found that the proliferation of MKN-45 and HGC-27 cells was enhanced upon miR-367-3p overexpression (**Fig.2B**), whereas inhibiting this miRNA impaired BrdU incorporation by these cells, suggesting that their proliferation was suppressed in the absence of this miRNA (**Fig.2C**). We next conducted Transwell-based assays which revealed that transfection of GC cells with a miR-367-3p mimic enhanced their migratory and invasive activity, whereas inhibition of this miRNA resulted in the opposite phenotypic changes relative to appropriate controls (**Fig.2D and E**). This therefore suggested that miR-367-3p can promote GC cell proliferation, migration and invasion.

miR-367-3p promoted EMT in GC cells

We next examined how miR-367-3p expression in GC cells related to EMT progression. We found that miR-367-3p mimic transfection resulted in the enhanced expression of mesenchymal markers (N-cadherin and vimentin) in MKN-45 and HGC-27 cells along with corresponding decreases in E-cadherin (**Fig.2F**). In contrast, inhibition of this miRNA led to increased E-cadherin levels and reduced vimentin and N-cadherin expression in HGC-27 and MKN-45 cells (**Fig.2F**). This thus indicated that miR-367-3p mediates GC cell EMT.

miR-367-3p inhibits the expression of Smad7

We next sought to identify putative miR-367-3p target genes using TargetScan, revealing Smad7 to be among the candidate targets. We then determined that miR-367-3p overexpression reduced Smad7 mRNA and protein levels, whereas miR-367-3p inhibition had the opposite effect relative to appropriate controls as measured via Western blotting and qRT-PCR (**Fig.3A and B**). To formally demonstrate the ability of this miRNA to directly target Smad7, we then conducted a luciferase reporter assay wherein cells were co-transfected with miR-367-3p mimics or inhibitors together with luciferase vectors containing either a wild type or mutant version of the 3'-UTR of Smad7. We found that overexpressing miR-367-3p led to suppression of the WT Smad7 3'-UTR vector luciferase activity, whereas miR-367-3p inhibition increased this activity. In contrast, no miRNA-dependent changes were observed for the vector in which this putative 3'-UTR binding sites had been mutated (**Fig.3C-E**). This thus indicated that miR-367-3p directly binds the Smad7 3'-UTR and suppressed its expression.

miR-367-3p targets Smad7 and thereby enhances TGF-β1/Smad3 activity in GC

We next assessed Smad7 expression ad it related to miR-367-3p in GC patient tissue samples, revealing that at the mRNA level Smad7 expression was decreased in GC tumors relative to paracancerous control tissues (**Fig.4A**), with the same also being true at the protein level (**Fig.4B**). To formally demonstrate that this miR-367-3p/Smad7 axis is important in GC, we next used the pcDNA3.1-Smad7 plasmid (pcDNA-Smad7) or an appropriate control vector to increase Smad7 expression in these GC cells which were then co-transfected using miR-367-3p mimic or control constructs (**Fig.4C**). In this system we found that Smad7 overexpression inhibited Smad2 and Smad3 phosphorylation, whereas miR-367-3p mimic transfection enhanced the phosphorylation of these proteins (**Fig.4D**). These results thus support the ability of miR-367-3p to target Smad7 and to thereby enhance TGF-β1/Smad3 activity within GC cells.

Discussion

GC is the fifth leading form of cancer globally, making it a key public health issue[20]. As there are no effective means of screening the general population for GC and it is often asymptomatic until the disease is advanced, many patients with GC are first diagnosed when the disease is already in its advanced stages. For these patients, surgery and chemotherapy are of limited value, and as a result the prognosis for these GC patients is poor. Recent studies have highlighted a role for DNA methylation and altered miRNA expression in GC development[21], and as such it is essential that novel biomarkers of GC be identified in order to facilitate the more rapid and reliable diagnosis of this disease.

Several studies have documented a key role for miRNA dysregulation in the development of cancers[22-26], with these miRNAs potentially functioning to promote or suppress oncogenesis in a miRNA- and cancer-specific manner[27, 28]. A number of cancers have to date been found to exhibit abnormal miR-367-3p expression, suggesting that this miRNA may be closely linked with key oncogenic processes [29-34] The importance of miR-367-3p in GC, however, remains incompletely understood. In our study we found that this miRNA was expressed at higher levels in GC patient tumors relative to paracancerous control tissues. In addition, we found miR-367-3p upregulation to correlate with features of more advanced GC including lymph node metastasis ($p=0.025$) and depth of invasion ($p = 0.047$), suggesting that this miRNA is associated with poorer patient outcomes. Through in vitro analyses we further found that miR-367-3p was able to promote GC cell proliferation, metastasis, and invasion, and that this miRNA functions at least in part via suppressing Smad7 expression and modulating Smad3 signaling. Together these results thus suggest a role for miR-367-3p in the development and progression of GC.

The EMT is a central mechanism in tumor metastasis wherein cells of an epithelial lineage acquire a more mesenchymal-like phenotype[35]. This transition is characterized by reduced E-cadherin expression and corresponding release from intercellular adhesion[36], thus leading to increased motility and migration of affected cells[37]. EMT has been shown to be closely linked with GC progression, facilitating the metastasis of tumors in affected patients[38]. Indeed, in GC patients that do not exhibit an EMT phenotype have better outcomes than do GC patients with such an EMT phenotype[39, 40]. Circulating

GC cells typically exhibit low levels of epithelial marker expression and high expression of mesenchymal markers, thus further supporting a role for EMT in GC metastasis[41]. In this report we found that overexpressing miR-367-3p led to a more robust EMT phenotype owing to E-cadherin downregulation and upregulation of both vimentin and N-cadherin in GC cells. Smad7 has been shown to suppress TGF-β signaling[42], which is among the most important signaling pathways for EMT regulation in tumor cells, and as such Smad7 activity modulates the ultimate metastatic progression of these cells[43]. In this study, we found Smad7 to be directly targeted by miR-367-3p such that overexpression of this miRNA in GC cells led to marked reductions in Smad7 expression. As such, the miR-367-3p/ Smad7 axis may be a viable therapeutic target for the treatment of GC.

In summary, our results provide novel evidence that miR-367-3p can function in an oncogenic manner in GC owing to its ability to inhibit Smad7, thus suggesting that this pathway may be a viable target for treating GC.

Declarations

Ethics approval and consent to participate

All patients provided written informed consent to participate, and the Ethics Committee of Shaoxing Hospital, Zhejiang University School of Medicine approved all human studies.

Consent for publication

All the authors were consent for publication

Availability of data and material

The datasets used and/or analyzed during the present study are available from the corresponding authors on reasonable request.

Competing interests

The author reports no conflicts of interest in this work.

Funding

This study was supported by Public Welfare Application Plan Project of Shaoxing (2018C30109), Chinese Society of Clinical Oncology (CSCO) Foundation Project (Y-HR2016-131)

Authors' contributions

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

Acknowledgements

Not applicable

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Tables

Table 1. Association between miR-367-3p expression and clinicopathological features of human GC

Clinical features	Total	miR-367-3p		*p*-vaule
		High	Low	
		(N=23)	(N=22)	
Age (years)				0.445
< 60	12	5	7	
≥ 60	33	18	15	
Gender				0.884
Male	23	12	11	
Female	22	11	11	
Tumor size (cm)				0.608
< 5	16	9	7	
≥ 5	29	14	15	
Differentiation grade				0.672
Well	13	6	7	
Moderate + Poor	32	17	15	
TNM stage				0.436
I + II	26	12	14	
III	19	11	8	
Depth of invasion				**0.025**
T1 + T2	19	6	13	
T3 + T4	26	17	9	
Lymph node metastasis				**0.047**
No	26	10	16	
Yes	19	13	6	
Distant metastasis				0.208
No	33	15	18	
Yes	12	8	4	
CEA, μg/ml				0.668
< 4.5	19	9	10	
≥ 4.5	26	14	12	
CA19-9, kU/L				0.672
< 40	32	17	15	
≥ 40	13	6	7	

CA19-9 carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; Pearson chi-square test was used for comparison between subgroups.

Table 2. The primers used for qRT-PCR analysis

	Primer sequence (5'- 3')
E-cadherin	F: AAGAAAACCCGAAGAGG
E-cadherin	R: CTGACTCAAGGTGCAGC
N-cadherin	F: TGACTCCCTGTTAGTGTTTGAC
N-cadherin	R: CCCAGTCGTTCAGGTAATCATAG
Vimentin	F: CCTGAACCTGAGGGAAACTAAT
Vimentin	R: CGTTGATAACCTGTCCATCTCT
Smad7	F: GCTATTCCAGAAGATGCTGTTC
Smad7	R: GTTGCTGAGCTGTTCTGATTTG
miR-367-3p	F:GTGGAGCTCTTGTTCGTCTC
miR-367-3p	R:TTCAGGGTGCAGAATTCATC
U6	F: GACCGAGTGTAGCAAGG
U6	R:GTTCTTCCGAGAACATATAC
GAPDH	F:AGAAGGCTGGGGCTCATTTG
GAPDH	R:AGGGGCCATCCACAGTCTTC

Figures

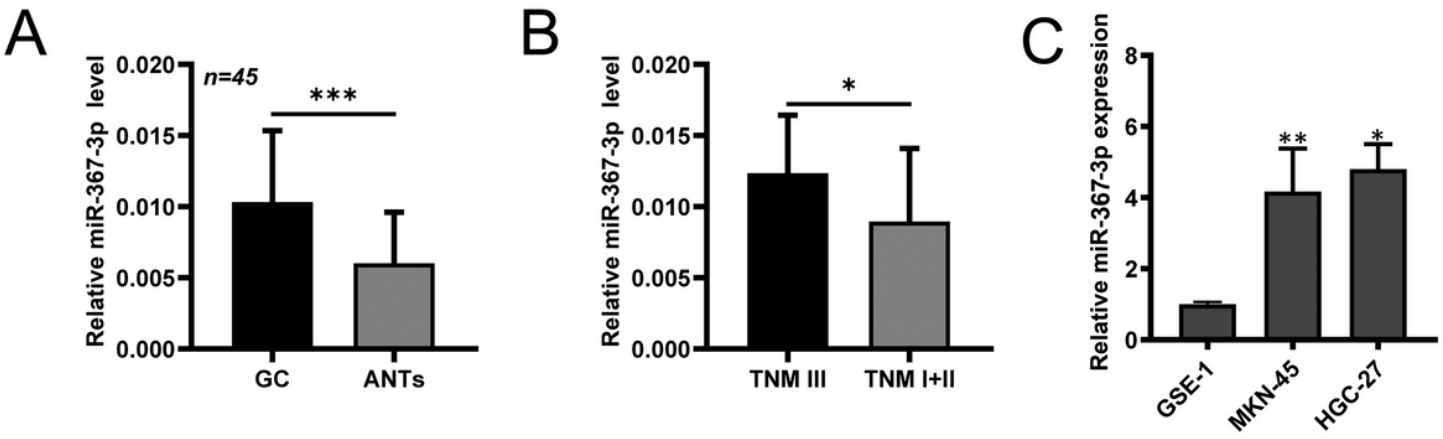

Figure 1

Elevated miR-367-3p expression GC (A) Expression of miR-367-3p was assessed via qRT-PCR in 45 pairs of GC tumors and paracancerous normal tissue. (B) miR-367-3p expression levels were higher in patients that had more advanced GC. (C) qRT-PCR was used to assess miR-367-3p levels in human GC and control cell lines. *P<0.05, **P<0.01, ***P<0.001.

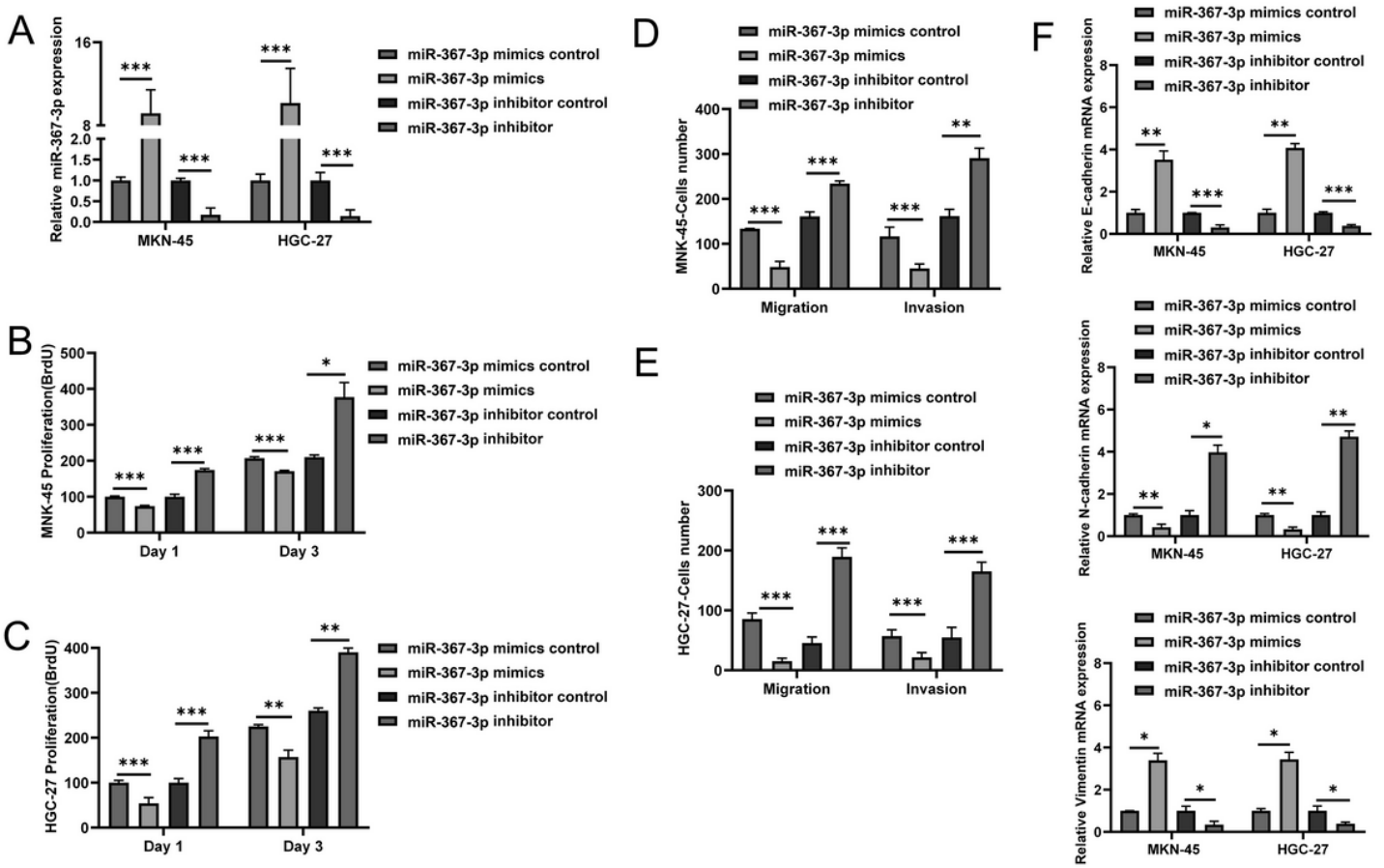

Figure 2

miR-367-3p promoted GC cell proliferation, migration, and invasion (A) Transfected miR-367-3p mimics or inhibitors and this miRNA level was quantified via qRT-PCR. (B, C) miR-401-3p affected GC cell proliferation was assessed based upon BrdU. (D and E) Overexpressing miR-367-3p led to improved GC cell migration and invasion in Transwell assays. (F) Inhibition of miR-367-3p led to increases in E-cadherin expression as well as reductions in N-cadherin and vimentin, whereas overexpressing this miRNA resulted in the opposite phenotype. *P<0.05, **P<0.01, ***P<0.001.

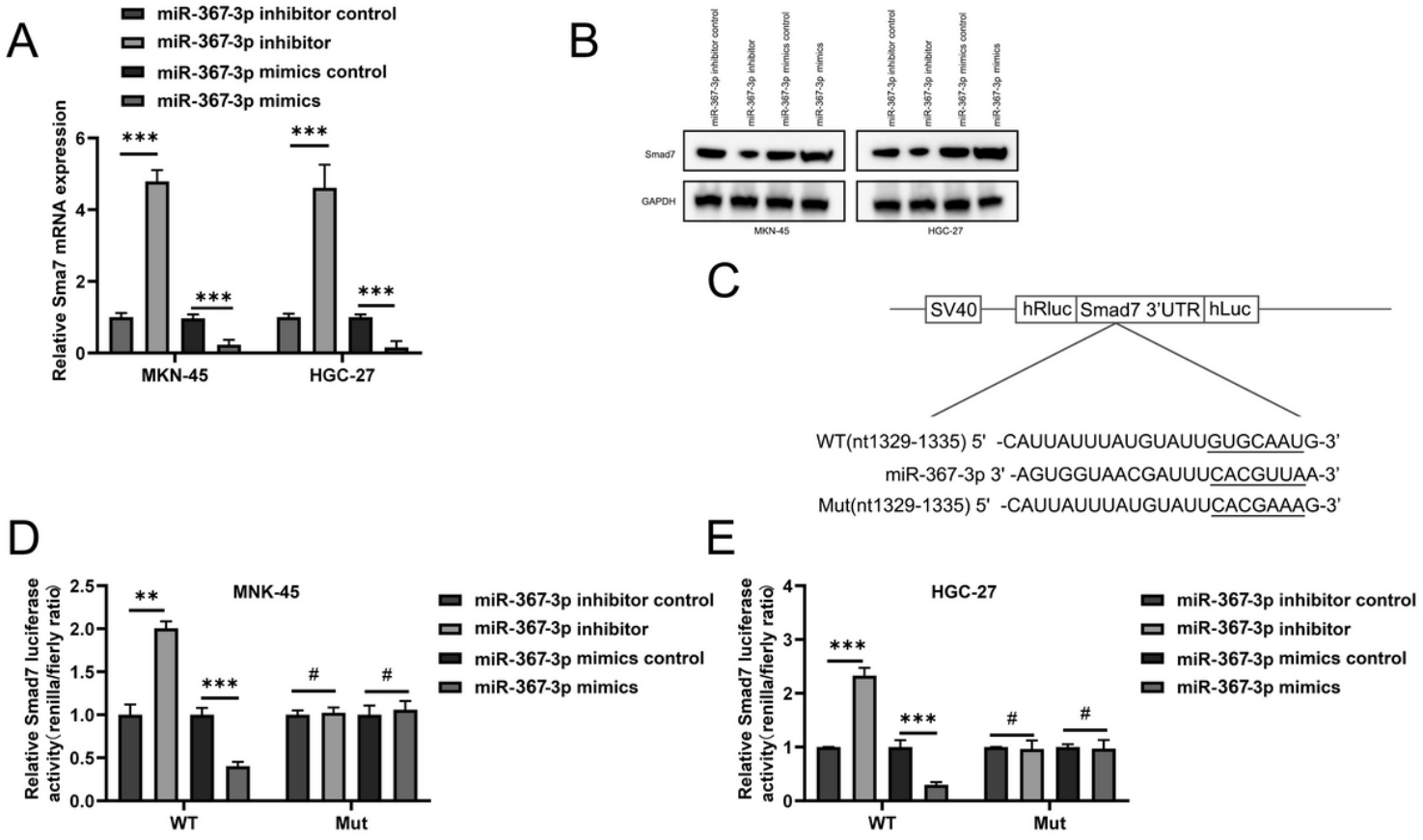

Figure 3

miR-367-3p inhibits the expression of Smad7 (A) Western blotting and (B) qRT-PCR confirmed Smad7 expression. (C) WT and mutant Smad7 3'-UTR constructs were generated as shown. A luciferase reporter assay revealed that miR-367-3p inhibition enhanced Smad7 3'-UTR-driven transcription, while miR-367-3p mimics had the opposite impact in both (D) MKN-45 and (E) HGC-27 cells. In contrast, miR-367-3p had no impact on luciferase activity for constructs bearing a mutant Smad7 3'-UTR. *P<0.05, **P<0.01, ***P<0.001.

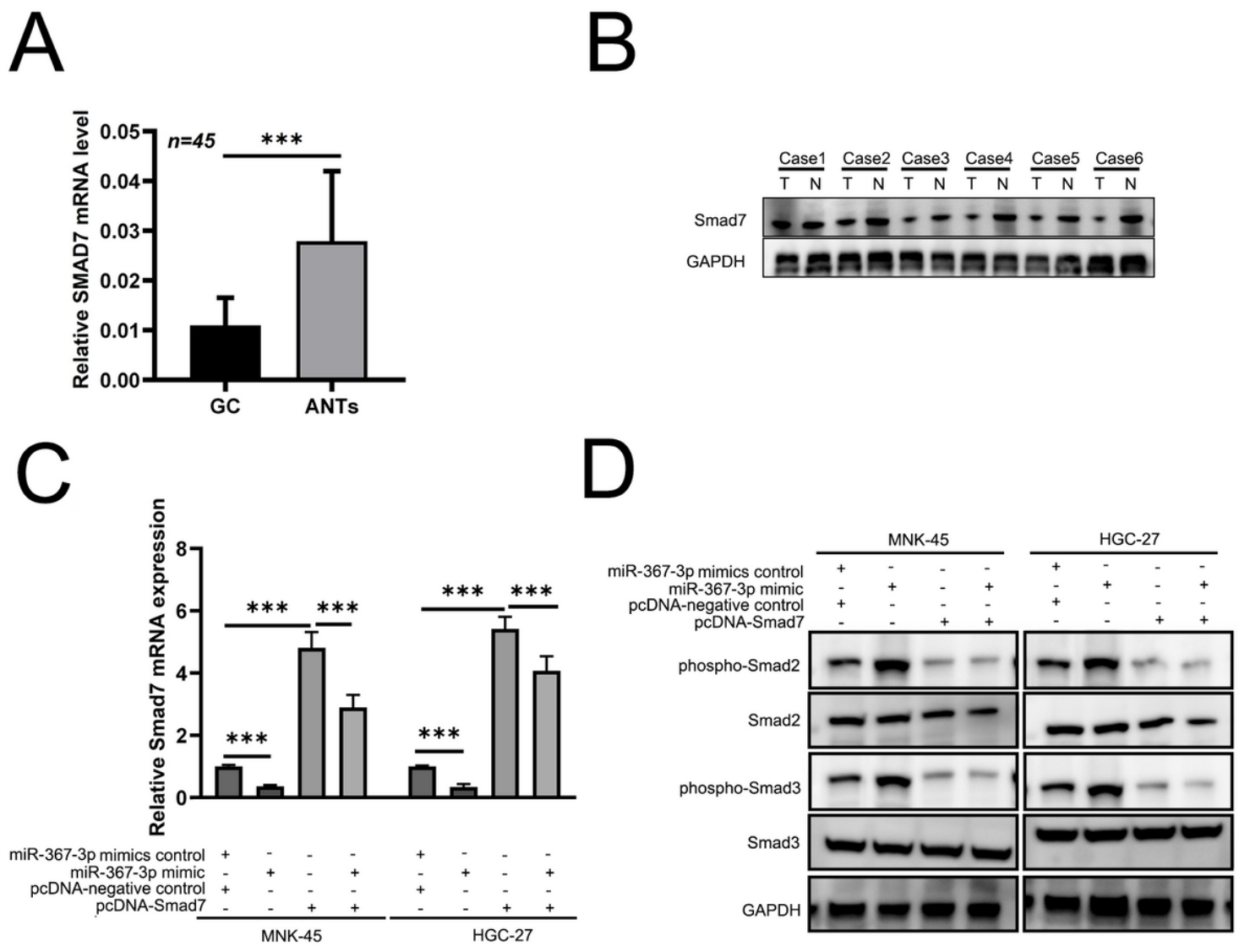

Figure 4

miR-367-3p targets Smad7 and thereby enhances TGF-β1/Smad3 activity The expression of Smad7 in GC was analyzed at the (A) mRNA and (B) protein levels. (C) Expression of Smad7 was assessed following transfection of cells using pcDNA-NC or pcDNA-Smad7. (D) Smad7, and both total and phospho-Smad2 and Smad3 protein levels were assessed in cells transfected using miR-367-3p mimics and/or pcDNA-Smad7. *P<0.05, **P<0.01, ***P<0.001.