Received: 1 September 2020 | Revised: 30 September 2020 | Accepted: 3 October 2020

DOI: 10.1002/jcla.23635

RESEARCH ARTICLE

WILEY

Actin-binding protein Anillin promotes the progression of gastric cancer in vitro and in mice

Huanxia Jia[1] | Fang Yu[1] | Baoyu Li[2] | Zhenya Gao[1]

[1]School of Medicine, Xuchang University, Xuchang, China

[2]Department of General Surgery, The Secondary Hospital of Tianjin Medical University, Tianjin, China

Correspondence
Zhenya Gao, School of Medicine Xuchang University, Xuchang 461000, China.
Email: zhygao1221@126.com.

Abstract

Background: To detect the expression levels of actin-binding protein anillin (ANLN) in human gastric cancer (GC) tissues and explore the possible involvement of ANLN in GC cell proliferation, migration, and invasion.

Methods: The bioinformation analysis was performed in TCGA database to explore the expression of ANLN in human GC tissues and the difference of ANLN expression between multiple types of cancers. IHC assays and clinical pathological analysis were performed to confirm ANLN expression and its correlation with clinical features of GC patients. Colony formation, CCK-8, wound closure, and transwell assays were performed to detect its effects on GC cell proliferation, migration, and invasion in vitro. Tumor growth was also measured using a xenograft animal model.

Results: We found the high expression of ANLN in human GC tissues based on the results from TCGA database and IHC staining. We further noticed ANLN depletion resulted in the inhibition of GC cell proliferation, migration, and invasion. Our data further confirmed that ANLN contributed to tumor growth of GC cells in vivo.

Conclusions: We confirmed the involvement of ANLN in GC progression and thought ANLN could serve as a promising therapeutic target for GC.

KEYWORDS

anillin (ANLN), gastric cancer (GC), invasion, migration, proliferation

1 | INTRODUCTION

As the fourth most common form of cancer, gastric cancer (GC) is difficult to treat due to its molecular heterogeneity.[1,2] In decades, the prognosis remains poor in patients with advanced gastric cancer, and gastric cancer became the second most common cause of cancer-related death after lung cancer.[3] Despite the advances in chemoradiotherapy, gastrectomy, and endoscopic treatment, targeted therapy is still thought as the first and promising treatment choice for advanced GC.[4-6] However, the current therapeutic targets for gastric cancer and related targeted therapeutic drugs are still unable to meet clinical needs, and many subtypes of gastric cancer cannot be effectively treated. Therefore, the identification of novel GC-related proteins is important for developing therapeutic targets that could therefore improve clinical care.

Anillin (ANLN) is a conserved actin-binding protein, which functions in cytoskeletal dynamics during cell division.[7,8] The N-terminal of ANLN is actin- and myosin-binding domains and C-terminal is an anillin homology domain.[9] Apart for these domains, ANLN also contains PH domain binding to RhoA, Ect2, and Septins responsible for the assembly, maintenance, and ingression of the cleavage furrow.[10,11] Importantly, ANLN was also essential for correct assembly

of the cleavage furrow and cytokinesis.[8] Notably, ANLN has been implicated to be a marker of poor prognosis and involved in aggressive cancer phenotypes.[12,13]

ANLN was associated with the cancer progression and metastasis, and was upregulated in multiple types of cancer.[14-18] As the role of ANLN in cell cycle regulation, ANLN serves an important role in breast and pancreatic cancer invasion.[19-21] In human non-small cell lung cancer cells and breast cancer cells, loss of ANLN is associated with inhibition of cell proliferation.[14,15,18] Meanwhile, high expression of ANLN in human lung cancer tissue predicted poor survival rates in patients.[15,22] Although the close association of ANLN in cancer progression has been widely noticed, the role of ANLN in GC is still obscure.

In this study, we found the high expression of ANLN in human GC tissues, according to the results from TCGA database and the IHC staining. We further noticed ANLN depletion resulted in the inhibition of GC cell proliferation, migration, and invasion. Our data further confirmed that ANLN contributed to tumor growth of GC cells in vivo. We therefore thought ANLN could serve as a promising therapeutic target for GC.

2 | METHODS

2.1 | Antibodies, plasmids, and primers

Antibodies used in this study were listed as below. Rabbit Anti-Anillin antibody [CL0303] (For IHC assays, 1:200 dilution; for Immunoblot assays, 1:1000 dilution, ab211872, Abcam) and mouse anti-β-actin (1:10 000 dilution, ab8226, Abcam). The primer used for quantitative detection of ANLN is as follows: forward, 5′-CATCTCTGCCCCCTCTGCTGA-3′; reverse, 5′-GGATGACCTTGCCCACAGCCT-3′; The quantitative PCR primer sequences of GAPDH are as follows: 5′-CGACCACTTTGTCAA GCTCA-3′ and reverse, 5′-GGTTGAGCACAGGGTACTTTATT-3′.

Pre-designed shRNA constructs targeting ANLN were inserted into pAV-U6-GFP plasmid. ANLN shRNA was listed as follows: 5′-AAGGTCTATGACTCATGCTAAGC-3′.

2.2 | Immunohistochemistry

A total of 62 gastric cancer tissues and the corresponding adjacent normal tissues (5 mm from the tumor tissues) in the current study were collected from patients receiving surgical therapy without receiving chemoradiotherapy at our hospital between August 2015 and May 2018. Additionally, the gastric tissue was obtained with fully consent of patients. To detect the expression levels of ANLN in human GC tissues, immunohistochemistry (IHC) assays were performed. Briefly, gastric tumor tissues were excised and embedded and then cut into 5-μm slices, and then fixed it with 4% paraformaldehyde (PFA) at room temperature for 30 minutes and subsequently blocked with 2% BSA for 1 hour. After that, slices were subsequently incubated with ANLN antibodies at room temperature for 2 hours. Then, the sections were incubated with biotinylated secondary antibody for 1 hour. The images were captured with a microscope.

2.3 | Cell culture and transfection

Human gastric cancer cell lines MGC-803 and SGC-7901 cells were obtained from American Type Culture Collection (ATCC) and cultured in RPMI 1640 (Gibco) containing 10% fatal bovine serum (FBS) at 37°C in a humidified incubator of 5% CO^2. Lipofectamine 2000 (11668019, Invitrogen) was utilized for transfection of the shRNA plasmids into MGC-803 and SGC-7901 cells. Then, MGC-803 cells were cultured with 1 mg/mL puromycin (Sigma) to select stably transfected ANLN shRNA cells and used for the animal assays.

2.4 | Quantitative PCR assays

Total RNA were extracted by Trizol (Invitrogen, USA) reagent from human GC cells. Then, reverse transcribed mRNA into cDNA by reverse transcriptase (M1701, Promega). Quantitative PCR was performed via a SYBR Ex Taq kit (Takara), and the relative expression levels of ANLN were quantified to GAPDH level.

2.5 | Immunoblot assays

Total protein extracted from cells was separated by SDS-PAGE, and then transferred protein onto the polyvinylidene fluoride (PVDF) membranes. After blocking, the membranes were incubated with indicated primary antibodies at room temperature for 2 hours. And then, the PVDF membranes were conjugated with secondary antibodies for 1 hour. Signals were detected using an electrochemiluminescence (ECL) kit.

2.6 | Colony formation assays

Approximately 1000 cells were seed into a 6-well culture plate for 24 hours. After incubating for 2 weeks, fixed the cells with PFA, stained with 0.2% crystal violet buffer for 30 minutes, and washed with PBS, then photographed the colony and quantified the colony numbers.

2.7 | CCK-8 assay

GC cells with control or ANLN shRNA plasmids transfection were seeded into 96-well plates and incubated for 48 hours. GC cells were subjected to CCK-8 solutions (Dojindo Molecular Technologies) for 2 hours at 37°C. Absorbance of each well was measured by a microplate reader at 450 nm.

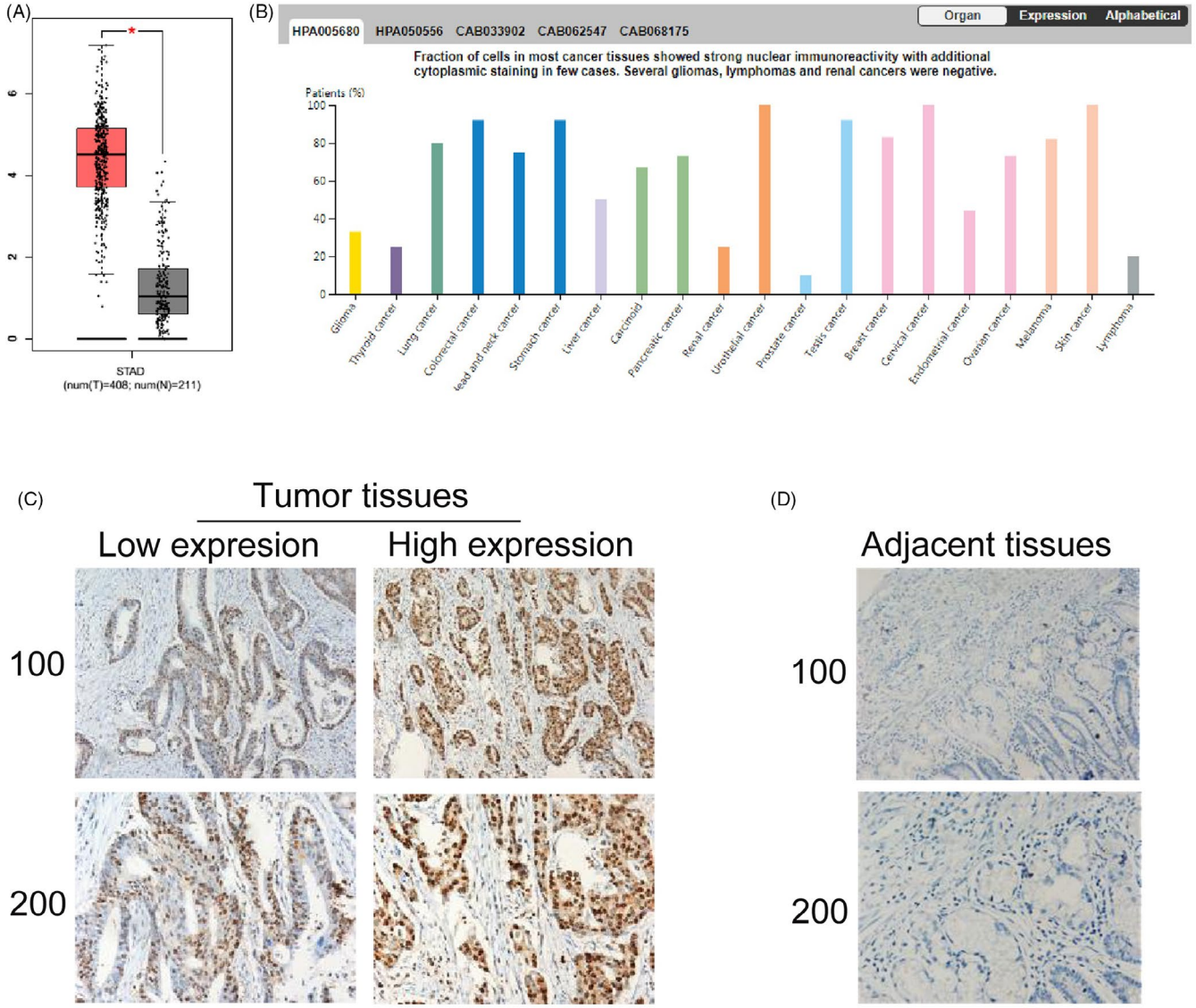

FIGURE 1 ANLN is high expression in human GC tissues. A, ANLN level in GC tissues and normal tissues analyzed from bioinformatic analysis. (For tumor tissues and normal tissues, n = 408 and 211 respectively, from TCGA dataset). B, ANLN expression levels were shown in most types of cancers. ANLN expression level in GC tissue samples (C) and normal tissues (D) identified by IHC assays (100× and 200× magnification, respectively)

2.8 | Wound healing assays

Both MGC-803 and SGC-7901 cells with control or ANLN shRNA plasmids transfection were maintained overnight, and mechanical lesion was made. Subsequently, cancer cells were washed and the complete culture medium was added. Photographs were taken, respectively, and the relative extent of wound healing was analyzed.

2.9 | Transwell assays

MGC-803 and SGC-7901 cells with control or ANLN shRNA plasmids transfection were re-suspended in DMEM culture medium only. Matrigel (20% in DMEM culture medium) was placed on the upper chambers of filters (8.0 μm membrane pores) and incubated at 37°C for 30 minutes. The complete medium was added into the bottom chamber. Cells were seeded into the upper chambers and induced to migrate toward the bottom chambers containing complete medium. After incubation, removed cells in the top chamber, fixed the remaining cells, and stained with 0.2% crystal violet buffer for 30 minutes. Then, photographed the cells and quantified the relative cell number.

2.10 | Tumor growth assays

All animal study in this study were approved by ethics of our hospital. MGC-803 cells with or without ANLN stable knockdown were subcutaneously injected into nude mice to induce tumor formation. After 14 days, tumors were excised every week until 4 weeks, and the volume of each tumor was measured. After 29 days, all tumors were isolated from mice and photographed.

2.11 | Statistics

GraphPad 6.0 was performed for data analysis. All data displayed in this study were representative of at least three independent experiments. And statistics were displayed as mean ± SEM. Student's *t* test was used for statistical comparisons. * indicates $P < .05$, and ** indicates $P < .01$.

3 | RESULTS

3.1 | Bioinformatic analysis indicates that ANLN is enhanced in GC patients

To analyze the expression level of ANLN in human GC patients and the corresponding normal tissues, we performed bioinformatic analysis. We observed a sharp induction of ANLN in tumor tissues (n = 408) compared to the normal tissues (n = 211) (Figure 1A, $P < .05$), according to the TCGA database. Importantly, ANLN was detected in most cancer tissues such as lung cancer, colorectal cancer, and skin cancer (Figure 1B). However, in several gliomas, lymphomas, and renal cancer, ANLN staining was negative (Figure 1B). These data suggest that ANLN was enhanced in GC tissues and ANLN expression could be widely observed in most cancer types.

3.2 | ANLN is highly expressed in human GC tissues compared with normal tissues

To identify the expression levels of ANLN in patients with GC, immunochemistry (IHC) assays were performed. We found moderate or strong ANLN staining in GC tissues (images displayed in 100× or 200× magnification), while negative staining in adjacent normal tissues (Figure 1C,D). We therefore demonstrated the high expression level of ANLN in human GC tissues.

Then, we further evaluated the clinical pathological features of GC patients with high or low ANLN expression in tumor tissues. According to the staining results, 32 showed low ANLN expression and the remaining showed high expression (Table 1). However, no correlations were found between ANLN expression and patient age ($P = .691$), gender ($P = .829$), and tumor grade ($P = .065$, Table 1). Notably, we found the obvious correlations between ANLN expression and tumor size ($P = .016$*) and pTNM stage ($P = .008$*, Table 1). Therefore, we demonstrated ANLN expression was correlated with the clinical features of patients with gastric cancer.

3.3 | ANLN is downregulated in MGC-803 and SGC-7901 cells after ANLN shRNA transfection

Given the abnormal upregulation of ANLN in GC patients, we wanted to seek the function of ANLN in GC cells. Human gastric cancer cell line MGC-803 and SGC-7901 cells were used as in vitro model of gastric cancer. ANLN shRNA were generated to specifically downregulate ANLN expression level. As detected by quantitative PCR assays, the mRNA level of ANLN decreased after transfection of indicated shRNA (Figure 2A). Similarly, the protein level of ANLN determined through immunoblot assays was decreased caused by

TABLE 1 Relationships of ANLN and clinicopathological characteristics in 62 patients with gastric cancer (* $p < 0.05$)

Feature	All n = 62	ANLN expression: Low n = 32	ANLN expression: High n = 30	χ^2	*P*
Age (y)					
<55	44	22	22	0.158	.691
≥55	18	10	8		
Gender					
Male	36	19	17	0.047	.829
Female	26	13	13		
Tumor grade					
Low	26	17	9	3.401	.065
High	36	15	21		
Tumor size					
<5 cm	24	17	7	5.792	.016*
≥5 cm	38	15	23		
pTNM stage					
I-II	18	14	4	6.953	.008*
III-IV	44	18	26		

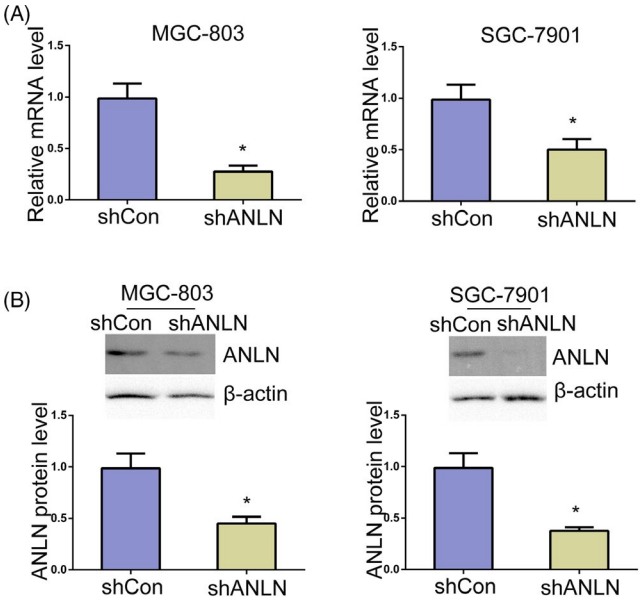

FIGURE 2 ANLN is downregulated in MGC-803 and SGC-7901 cells after ANLN shRNA transfection. Quantitative PCR analysis A, and immunoblot images B, verified downregulation of ANLN in MGC-803 and SGC-7901 cells transfected with ANLN shRNA plasmids. *$P < .05$

the transfection of ANLN shRNA (Figure 2B). The knockdown efficiency of ANLN shRNA plasmids was confirmed.

3.4 | ANLN depletion promotes reduction of GC cell proliferation, migration, and invasion

As the silencing efficiency was fully confirmed, the effects of ANLN knockdown on cell proliferation were assessed. GC cells transfected with control or ANLN depletion were used for colony formation assays. As shown in Figure 3A, ANLN depletion led to a significant reduction in colony number in both MGC-803 and SGC-7901 cells (Figure 3A). Also, the same treatment cells were utilized for detecting proliferation capacity by CCK-8 assays. We observed that ANLN-depleted MGC-803 and SGC-7901 cells proliferation rates were suppressed markedly which was consistent with the previous results (Figure 3B). For migration assessment, wound healing assays were conducted. After ANLN ablation, the closure of mechanical lesion was inhibited in both MGC-803 and SGC-7901 cells (Figure 3C). To detect the importance of ANLN in invasion, we conducted transwell assay. ANLN ablation induced relatively lower cell number of invasion suggesting the significant blockage of GC cell invasion (Figure 3D). Taken together, our data suggested that ANLN affected the proliferation, invasion, and migration of GC cells in vitro.

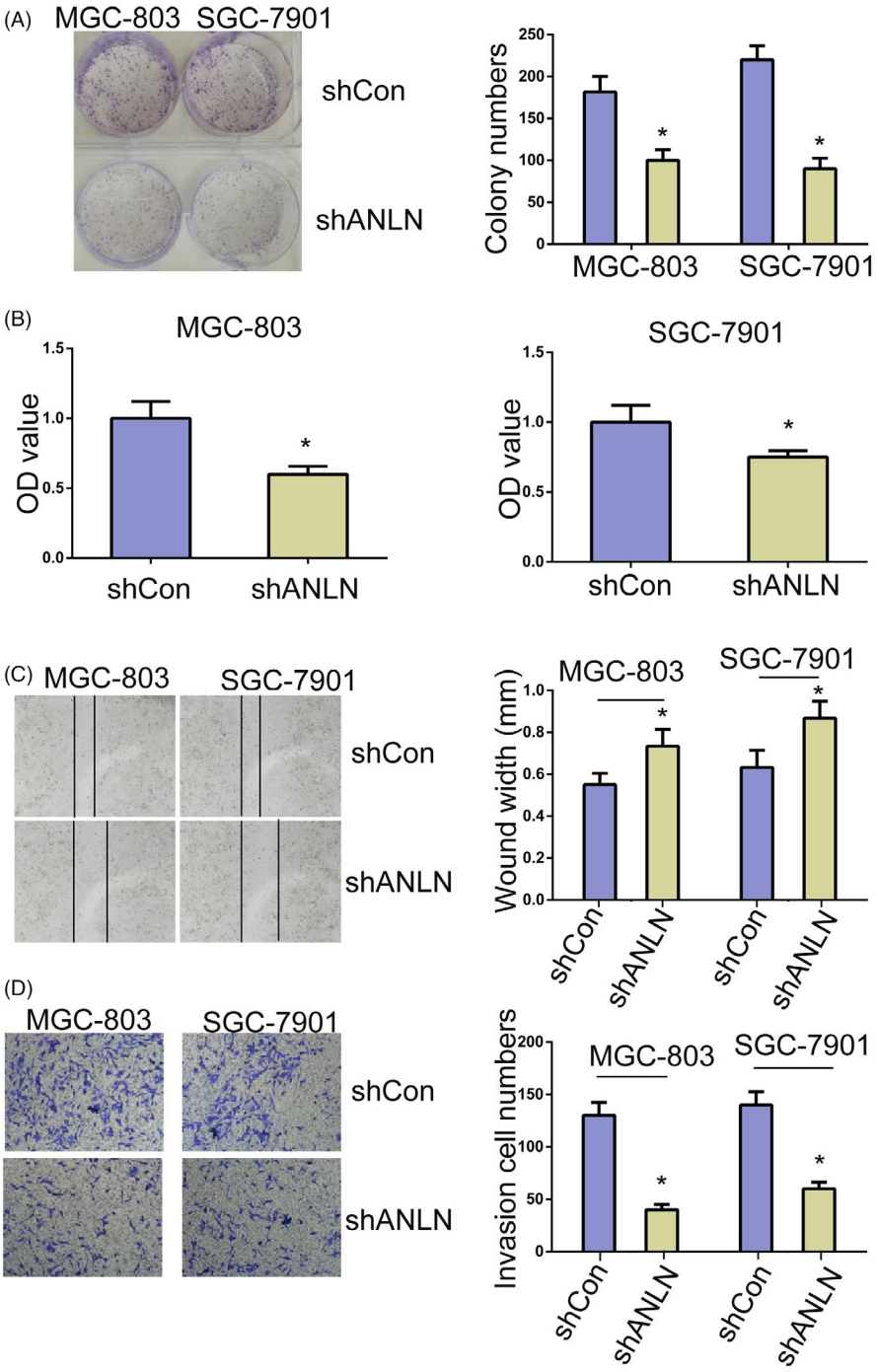

FIGURE 3 ANLN depletion promotes reduction of GC cell proliferation, migration, and invasion. A and B) Colony formation (A) assays and CCK-8 assays (B) were performed to evaluate the proliferative ability of MGC-803 and SGC-7901 cell transfected with negative control (NC) or ANLN shRNA plasmids. C and D, Scratch assays (C) and transwell assays (D) were conducted to evaluate cell migration and cell invasion after ANLN knockdown. *$P < .05$

3.5 | ANLN depletion inhibits tumor formation in vivo

As ANLN was essential for tumor cell proliferation, migration, and invasion, we speculated ANLN was functional in tumor formation in vivo. To further identify this hypothesis, we performed the xenograft model of GC cells in BALB/c nude mice using MGC-803 cells transfected with control shRNA or ANLN shRNA. MGC-803 cells with or without ANLN ablation were injected into BALB/c nude mice subcutaneously. After injection for 2 weeks, the tumor growth was monitored every 3 days until 29 days (n = 6) and tumor volume was measured and analyzed. Consistently, we observed smaller tumor in mice with ANLN depletion. Representative image of tumor in each group was shown in Figure 4A. The expression level of ANLN was further examined in tumor of each group. The tumor of mice receiving ANLN shRNA showed less level of ANLN using immunoblot (Figure 4B). Taken together, we performed animal assays and found the depletion of ANLN in gastric cancer cells inhibited the tumor formation in mice, and therefore demonstrated that ANLN depletion inhibited tumor growth of gastric cancer cells in vivo.

4 | DISCUSSION

Gastric cancer continues to be a major health problem with around 1 million new GC cases and more than 700 000 deaths annually in the world, which accounts for 10% of all cancer deaths. The main therapeutic strategies of GC include surgery and chemotherapeutic regimens. Given the prognosis of GC is often diagnosed at advanced stages when this regular therapy is invalid, advances in diagnosis and novel strategies against GC need to be developed and established to improve the patients' prognosis and treatment.

Interestingly, we found significant high expression of ANLN in GC patients by performing bioinformation analysis based on the TCGA database. We also noticed that ANLN expression could be detected in many tumor type suggesting the potential importance of ANLN in tumor progression. Therefore, we assumed that ANLN might have a specific function in GC.

Since ANLN was associated with the cancer progression and metastasis, and was upregulated in multiple types of cancer.[14-17] We sought to detect the expression level of ANLN in samples collected from GC patients in our hospital. Consistently, upregulation of ANLN was detected in our samples via IHC assays. Similarly, ANLN was identified to be a potential prognostic factor in bladder urothelial carcinoma by transcriptome sequencing.[23] Also, ANLN was found enhanced in breast cancer patients.[19] These studies, consistent with our observation, confirmed ANLN was abnormal high expression in GC and many tumor types.

Through colony formation and CCK-8 assays, decreased proliferation ability was observed in ANLN-depleted GC cells. Furthermore, we also noticed suppressed migration and invasion of GC cells after ANLN knockdown through wound closure assays and transwell assays. These results suggested that ANLN ablation inhibited tumor growth, migration, and invasion in GC cells. These results also indicated the important function of ANLN in GC progression. Similarly, in breast cancer and anaplastic thyroid carcinoma, ANLN promoted cell proliferation and cell cycle progression.[24,25] In lung adenocarcinoma, ANLN depletion by lentivirus also inhibited the migration of breast cancer cells in vitro.[14,26] Together, we and other investigators supposed ANLN promoted cancer cell proliferation, migration, and invasion and could be a potential target for cancer treatment. The mechanism underlying ANLN function in tumor progression needs further investigation.

As to the possible signaling pathway related to ANLN function, previous studies mentioned that ANLN affected cancer progression through Wnt/β-catenin pathway in gastric cancer[27] and through PI3K/AKT pathway in human lung carcinogenesis.[27,28] In this study, whether ANLN induced GC progression via Wnt/β-catenin pathway remained to be discovered in future study.

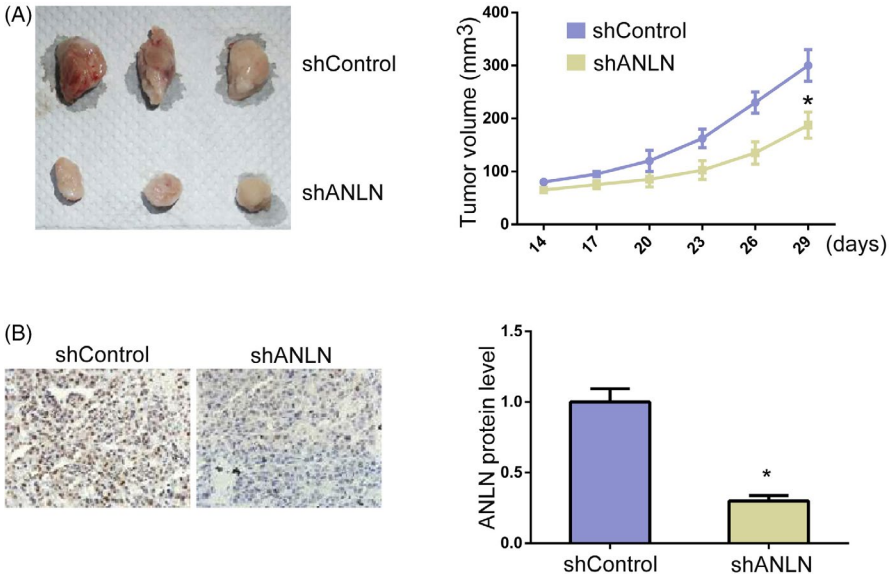

FIGURE 4 ANLN depletion inhibits tumor formation in vivo. A, Tumor growth curve of control or ANLN stably depletion MGC-803 cells were injected into nude mice, and tumor volume was evaluated each week (n = 6) (left). Representative images of isolated tumor from each group. B, ANLN expression level in excised xenograft tumor tissues was analyzed by IHC analysis. $^{*}P < .05$ and $^{**}P < .01$

Notably, ANLN was shown to be directly regulated by antitumor miR-217 and knockdown of ANLN significantly inhibited cancer cell aggressiveness in PDAC cells.[18] In addition, ANLN was reported to be and regulated by miR-15a/miR-16-1 in HBV-related hepatocellular carcinoma and required for tumor growth.[29,30] Our data confirmed the involvement of ANLN in the regulation tumor formation, migration, and invasion. Moreover, ANLN promoted the tumor growth of gastric cancer cells in vitro. We supposed inhibitor targeting ANLN could possibly have therapeutic effect for GC.

ETHICAL APPROVAL

All procedures performed in the current study were approved by the Ethics Committee of the School of Medicine Xuchang University. Written informed consent was obtained from all patients or their families.

ORCID

Zhenya Gao https://orcid.org/0000-0003-0134-9944

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How to cite this article: Jia H, Yu F, Li B, Gao Z. Actin-binding protein Anillin promotes the progression of gastric cancer in vitro and in mice. *J Clin Lab Anal*. 2021;35:e23635. https://doi.org/10.1002/jcla.23635