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Kindlin-1 promotes gastric cancer cell motility through the Wnt/β-catenin signaling pathway

Jun-Ho Jang[1,9], Jiyoon Jung[2,9], Hyeon-Gu Kang[1,3,9], Woong Kim[3], Won-Jin Kim[1,3], Hana Lee[1,3], Ju Yeon Cho[4], Ran Hong[5], Jeong Won Kim[2], Joon-Yong Chung[6], Kyung-Hee Chun[7]✉ & Seok-Jun Kim[1,3,8]✉

Despite advances in gastric cancer diagnosis and treatment, its prognosis remains poor owing to aggressive tumor progression and metastasis. As understanding the relevant molecular mechanisms is essential to effectively improve patient outcomes, we elucidated the role of Kindlin-1 in gastric cancer progression and metastasis. Kindlin-1 expression was analyzed in 359 gastric cancer tissue samples provided by Kangnam Sacred Heart Hospital and publicly available GSE datasets. Kindlin-1 showed significantly higher expression in gastric cancer tissues than that in normal tissues, and high Kindlin-1 expression was associated with poor prognosis. Further, the mRNA and protein expression of Kindlin-1 were high in gastric cancer cell lines, where they were associated with increased proliferation, migration, and invasion. Our findings demonstrated that Kindlin-1 regulated epithelial–mesenchymal transition-related genes through interaction with activated Wnt/β-catenin signaling. Notably, Kindlin-1 enhanced β-catenin expression and promoted its nuclear translocation from the cytoplasm, increasing TCF4 transcriptional activity and inducing gastric cancer progression and metastasis. Overall, these findings demonstrate that Kindlin-1 is upregulated in gastric cancer and activates Wnt/β-catenin signaling to promote cell proliferation and motility.

Keywords Kindlin-1, β-catenin, Cell proliferation, Cell motility, Epithelial–mesenchymal transition, Gastric cancer

Abbreviations

IP	Immunoprecipitation
IHC	Immunohistochemistry
EGC	Early gastric cancer
AGC	Advanced gastric cancer
TMA	Tissue microarray
FFPE	Formalin-fixed and paraffin-embedded
DAB	3,3′-Dimaniobenzadine
IgG	Immunoglobulin G
IF	Immunofluorescence
ChIP	Chromatin immunoprecipitation
OS	Overall survival
EMT	Epithelial–mesenchymal transition

[1]Department of Integrative Biological Sciences and BK21 FOUR Educational Research Group for Age-Associated Disorder Control Technology, Chosun University, Gwangju 61452, Republic of Korea. [2]Department of Pathology, Kangnam Sacred Heart Hospital, Hallym University College of Medicine, Seoul 07441, Republic of Korea. [3]Institute of Well-Aging Medicare and Chosun University G-LAMP Project Group, Chosun University, Gwangju 61452, Republic of Korea. [4]Department of Internal Medicine, College of Medicine, Chosun University, Gwangju 61452, Republic of Korea. [5]Department of Pathology, College of Medicine, Chosun University, Gwangju 61452, Republic of Korea. [6]Molecular Imaging Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA. [7]Department of Biochemistry and Molecular Biology, Graduate School of Medical Science, Brain Korea 21 Project, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea. [8]Department of Biomedical Science, Chosun University, Gwangju 61452, Republic of Korea. [9]Jun-Ho Jang, Jiyoon Jung and Hyeon-Gu Kang contributed equally to this work. ✉email: khchun@yuhs.ac; heaven1472@chosun.ac.kr

TGF-β	Transforming growth factor beta
DFS	Disease-free survival
EGFR	Epidermal growth factor receptor

Gastric cancer is the fifth most common cancer and the fifth leading cause of cancer-related deaths worldwide[1]. Its incidence varies worldwide but is predominant in East Asia[2–4]. Although numerous cancer-related studies have been conducted over the past few decades, research on cancer types predominant in East Asia, such as gastric cancer, remains limited. The prognosis of metastatic gastric cancer is particularly poor, and the mechanisms underlying its metastasis remain unclear[5]. Therefore, identifying the genes related to gastric cancer progression and metastasis, as well as understanding the underlying molecular mechanisms, is essential for improving its diagnosis and treatment.

Kindlin-1 (KIND1, also known as FERMT1), a focal adhesion protein comprising ezrin, radisin, moesin, and a pleckstrin homology domain[6], is mainly expressed in keratinocytes and intestinal epithelial cells. It is involved in several important cellular functions, including proliferation, adhesion, and migration[7,8]. Mutations in Kindlin-1 cause Kindler syndrome, a genetic disorder that mainly affects the skin and intestine[9]. Kindlin-1 is overexpressed in many tumors, including breast[10,11], colon[12], pancreatic[13,14], non-small cell lung[15,16], gastric[17], nasopharyngeal[18], and oral squamous cell carcinomas[19]. Kindlin-1 is also involved in tumor proliferation, apoptosis, metastasis, and angiogenesis, and is thus considered an oncogene[20,21]. Although Kindlin-1 has been associated with poor prognosis in several cancers, its function and mechanism of action in gastric cancer remain unclear.

The canonical Wnt pathway, which maintains β-catenin stability and induces its nuclear translocation, is a highly conserved signaling pathway in mammals[22,23]. β-Catenin is a key transducer of Wnt/β-catenin signaling, and upon activation, it translocates to the nucleus where it binds to the transcription factors TCF/LEF to activate TCF/β-catenin-mediated Wnt target genes[24,25]. Activation of Wnt/β-catenin signaling triggers cell proliferation, survival, differentiation, organogenesis, tissue regeneration, and tumorigenesis[26–29]. Abnormal activation of Wnt/β-catenin signaling is characterized by excessive nuclear accumulation of β-catenin, a transcriptional regulator. β-Catenin promotes cancer progression and metastasis by interacting with or regulating various proteins[30–33]. Notably, hyperactivation of Wnt/β-catenin signaling is observed in more than 30% of gastric cancer cases[34–36]. Thus, understanding the mechanism underlying the abnormal activation of Wnt/β-catenin signaling in gastric cancer is important for developing relevant therapeutic strategies.

In this study, we investigated the role of Kindlin-1 in gastric cancer, hypothesizing its association with the hyperactivation of the Wnt/β-catenin signaling pathway. We examined the role of Kindlin-1 in cell proliferation and motility via Wnt/β-catenin signaling in gastric cancer cells, providing new insights into its role as a regulator of transcriptional activity within this pathway.

Materials and methods

Cell culture

Human gastric cancer cell lines (AGS, MKN28, SNU-601, SNU-638, and SNU-668) were purchased from the Korea Cell Line Bank (Seoul, Korea). GES-1 and YCC-2 cells were obtained from the Yonsei Cancer Center (Seoul, Korea). The cells were cultured in RPMI 1640 medium (Welgene, Gyeongsan, Korea) supplemented with 10% fetal bovine serum (FBS; Corning Costar, NY, USA) and 1% streptomycin/penicillin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) in an incubator at 37 °C with 5% CO_2.

siRNA transfection and plasmid construction

Transfection was performed using Lipofectamine RNAiMAX or Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The expression of Kindlin-1 and β-catenin was suppressed using human siRNAs purchased from Genolution Inc. (Seoul, Korea). Sequences of siRNAs were: KIND1 (Kindlin-1) siRNA #1 (5′-GAGAGUAUCUGGAGACCUUUU-3′), #2 (5′-CGACUUGAAUCC UAAAUAUUU-3′); CTNNB1 (β-catenin) siRNA #1 (5′- CAGUUAUGGUCCAUCAGCUUUCUAA-3′), #2 (5′- CGUGAGGGCUUACUGGCCAUCUUUA-3′). The human KIND1 coding sequence was cloned into a pcDNA3.1_Flag_Empty plasmid to generate a Kindlin-1 expression plasmid (pcDNA3.1_Flag_Kindlin-1). The Kindlin-1 coding sequence was amplified by PCR using primers containing EcoR1/Xba1 restriction enzyme sites (Supplementary Table S1).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using RNAiso Plus reagent (Takara, Shiga, Japan). The isolated mRNA was used to synthesize complementary DNA with a reverse transcription master mix (Toyobo, Osaka, Japan), followed by PCR using 2×TOPsimple DyeMIX-nTaq (Enzynomics, Daejeon, Korea). The amplified PCR products were evaluated using agarose gel electrophoresis. PCR primers used in this study are listed in Supplementary Table S1.

Western blotting analysis

Western blotting was performed as described previously[37]. The antibodies used were: anti-Kindlin-1 (Abcam, CAM, UK); anti-survivin and anti-GAPDH (Bioworld Technology, OH, USA); anti-GSK3β (Bethyl Lab, TX, USA); and anti-β-catenin, anti-vimentin, anti-E-cadherin, anti-N-cadherin, anti-COX-2, anti-cyclin D1, anti-fascin-1, anti-pGSK3β, anti-lamin A/C, and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Nuclear cytoplasmic fractionation assay

Whole cell lysates were fractionated into cytoplasmic and nuclear fractions using three different buffers, as detailed in a previous study[38]. Protein expression levels were determined using western blotting analysis.

Immunoprecipitation (IP) assay

Cell lysates were incubated with anti-Kindlin-1 or anti-β-catenin antibodies overnight at 4 °C on a rotator. Protein A/G Plus agarose beads (Santa Cruz Biotechnology) were added to each sample and incubated for an additional 2 h. The beads were precipitated by centrifugation at 350 × *g* for 5 min, and the supernatant was removed. Beads were washed thrice with cold 20% RIPA buffer. After adding 2 × SDS loading buffer, the samples were boiled for 5 min.

Tissue microarray and immunohistochemistry

A total of 359 gastric cancer samples (187 early- and 172 advanced-stage) were collected from patients enrolled at Kangnam Sacred Heart Hospital, Seoul, Republic of Korea, between 2010 and 2018. The Institutional Review Board of Kangnam Sacred Heart Hospital approved this study (2023–06-009), and informed consent was obtained from each patient. All procedures were conducted in accordance with the principles of the Declaration of Helsinki. Clinical data, including age, sex, survival status, and duration, were extracted from the medical records. Pathologic characteristics, including WHO grade, histologic type, pTNM classification, and the presence of lymphovascular and perineural invasion, were retrieved from pathology reports and slide reviews. TNM stage was coded according to the 8th American Joint Committee on Cancer Staging manual[39]. pT1 tumors were categorized as early gastric cancer (EGC), irrespective of lymph node metastasis, whereas advanced gastric cancer (AGC) included tumors beyond pT2 (Supplementary Table S2). Tissue microarrays (TMAs) were constructed by extracting two 1-mm diameter cores from each formalin-fixed and paraffin-embedded cancer specimen. Immunohistochemical staining was performed on 5-μm TMA sections using the Dako EnVision + System-HRP system (Dako, Carpinteria, CA, USA). Following deparaffinization, heat-induced antigen retrieval, and blocking, TMA sections were incubated with anti-Kindlin-1 rabbit polyclonal antibodies (#ab68041; dilution 1:5000; Abcam) or anti-β-catenin rabbit monoclonal antibodies (D13A1 clone; dilution 1:4000; Cell Signaling) for 30 min at 24 °C. Subsequently, the slides were visualized with DAB + substrate-chromogen, resulting in a brown precipitate at the antigen site, followed by light counterstaining with hematoxylin. Immunohistochemical staining images were assessed using Visiopharm software v6.9.1 (Visiopharm, Hørsholm, Denmark) as described previously[40]. The histoscore, ranging from 0 to 300, was calculated by multiplying the intensity with the percentage of positively-stained cells. The expression values of Kindlin-1 and β-catenin were dichotomized (high *vs.* low), with cutoff values set at 30.1 and 35.6, respectively. The control included IgG without the primary antibody.

Immunofluorescence assay

AGS and SNU-601 cells were seeded onto cell culture slides and transfected. After 48 h of incubation, the cells were fixed on slides with 3% formaldehyde and permeabilized with 0.5% Triton X-100. The slides were then incubated with primary antibodies diluted in 5% bovine serum albumin for 1.5 h at 24 °C. Subsequently, the sections were incubated with Alexa Fluor- or FITC-conjugated secondary antibodies (Invitrogen) for 1 h. The slides were mounted using VECTASHIELD mounting medium (Vector Laboratories Inc., Burlingame, CA) containing DAPI, and the cells on the chamber slides were imaged using a Zeiss LSM800 microscope (Zeiss, Jena, Germany).

Cell proliferation assay

Cell proliferation was assessed using the Quanti-Max™ WST-8 cell viability assay kit (BIOMAX, Seoul, Korea). AGS, YCC-2, SNU-601, and SNU-668 cells were seeded in 96-well plates (5×10^3 cells/well). The cells were transfected with siRNA or vector plasmid. After 48 h of transfection, WST-8 reagent was added to each well, and plates were incubated at 37 °C with 5% CO_2. Cell viability was measured at 450 nm at 30-min intervals for 2 h.

Transwell migration and invasion assays

Migration and invasion assays were performed on cells transfected for 24 h. After trypsinization, 2×10^4 cells in 200 μL FBS-free medium were added to the upper chamber of a Transwell chamber (Corning Costar, MA, USA) coated with 0.5 mg/mL collagen type I (BD Biosciences, Seoul, Korea) for migration assays. Matrigel (1:14)-coated filters were used for invasion assays. RPMI 1640 medium supplemented with 10% FBS and 1% antibiotics was added to the lower chamber, and plates were incubated for 18–20 h. The migrated and invaded cells were visualized and quantified after hematoxylin and eosin staining. For quantification, the cells were counted from five randomly selected areas per well using a widefield microscope.

TOPFlash/FOPFlash luciferase reporter assay

Cells were co-transfected with either TOPFlash or FOPFlash luciferase reporter plasmids along with Kindlin-1 expression plasmids or siRNAs. These plasmids were each co-transfected with the β-galactosidase expression plasmid vector, used as a control for transfection efficiency. After incubation for 24 h, cells were harvested, and luciferase activity was measured using a luciferase assay system and β-galactosidase enzyme assay system kit (Promega, Madison, WI, USA) following the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP) assay

Cells were cross-linked with 1% formaldehyde, harvested, and subjected to ChIP assays using the Pierce™ Agarose ChIP kit (Thermo Fisher Scientific), following the manufacturer's instructions. The antibodies used were anti-β-catenin (sc-7963, 1:200; Santa Cruz Biotechnology) and anti-DDDDK (M185-3L, 1:1000; MBL, MA, USA). Anti-mouse IgG (Santa Cruz Biotechnology) served as a negative control. The primers used are listed in Supplementary Table S1. PCR was performed using PrimeSTAR Max DNA polymerase (Takara).

Gene expression profile data and survival analysis

The available datasets (GSE13861, GSE63089, GSE64951, and GSE26899) for gene expression analysis of gastric cancer tissues were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). Survival analysis of patients with gastric cancer was conducted using the online resource, Kaplan–Meier Plotter (http://kmplot.com/analysis)[41]. The UALCAN database (https://ualcan.path.uab.edu/) was applied to predict the expression levels of FERMT1 in patients with stomach adenocarcinoma (STAD) and the association between FERMT1 expression and STAD prognosis.

Statistical analysis

All statistical analyses were performed on data obtained from at least three independent experiments. The results are presented as mean ± SD. Data were analyzed using Student's *t*-test, and $p < 0.05$ was considered statistically significant. The correlation between clinicopathological parameters and the expression levels of Kindlin-1 and β-catenin was assessed using the chi-square test. Kaplan–Meier survival analysis was performed using the log-rank test. Cox proportional hazards regression analysis was employed to assess the prognostic significance of Kindlin-1 and β-catenin levels. SPSS software (version 27.0; SPSS, Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) were used for clinical data analysis.

Results

Analysis of Kindlin-1 expression and survival rate in patients with gastric cancer from a public clinical database

Kindlin-1 is upregulated in various cancers; however, its expression in gastric cancer remains unclear. We investigated the expression of Kindlin-1 in gastric cancer tissues using GEO datasets and The Cancer Genome Atlas (TCGA) obtained from patients with gastric cancer (Fig. 1A–1E) and its effect on disease prognosis. The mRNA levels of Kindlin-1 were significantly increased in gastric cancer tissues compared with those in normal tissues. Further, the relationship between elevated Kindlin-1 expression and the survival rate of patients with gastric cancer was confirmed using Kaplan–Meier analysis (Fig. 1F–1H). For survival analysis, the best cutoff value for Kindlin-1 expression was determined using an online survival analysis tool[42]. The overall survival (OS), primary progression survival, and post-progression survival rates were significantly lower in the high Kindlin-1 expression group than those in the low expression group. Moreover, we confirmed that Kindlin-1 expression

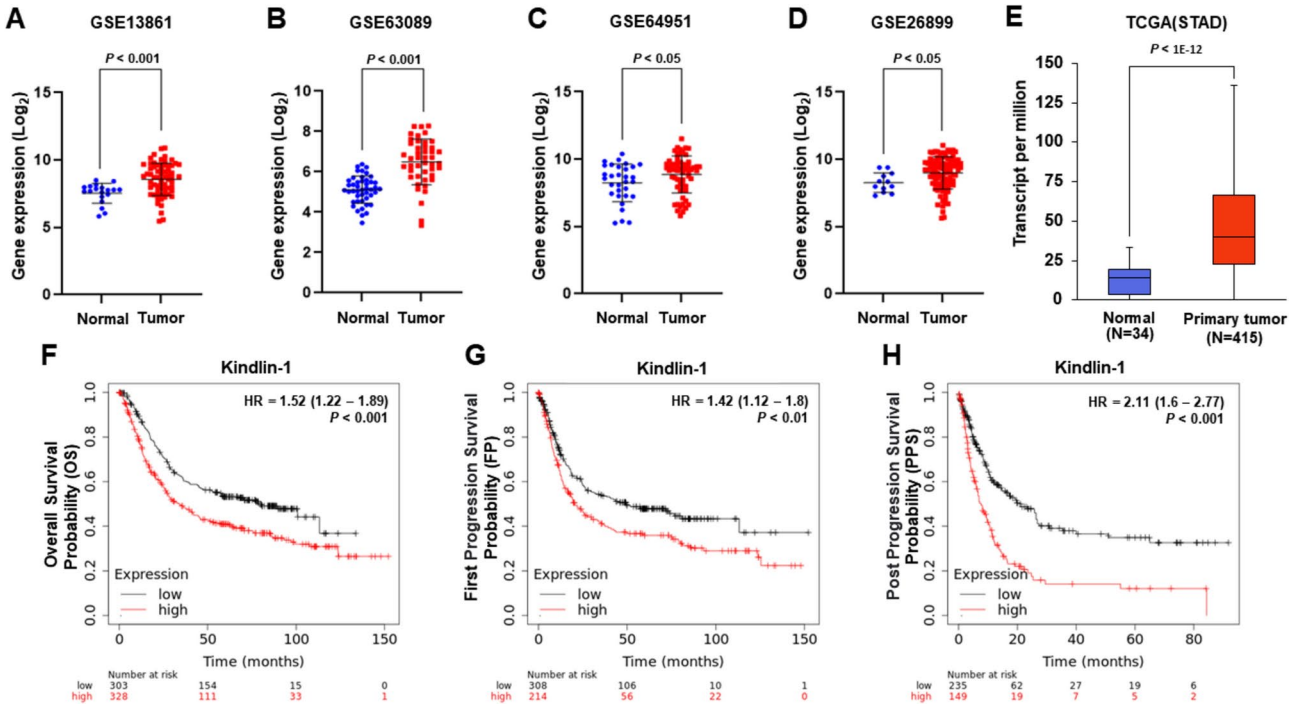

Fig. 1. Correlation between Kindlin-1 expression and survival in patients with gastric cancer based on the Gene Expression Omnibus (GEO) database and The Cancer Genome Atlas (TCGA). Kindlin-1 mRNA expression in gastric cancer tissues from the GEO database. Datasets are presented as scatter plots (**A**) GSE13861 (n = 84), (**B**) GSE63089 (n = 90), (**C**) GSE64951 (n = 94), (**D**) GSE26899 (n = 108). (**E**) Kindlin-1 expression levels in STAD were analyzed using data from the TCGA database (n = 449). *p*-values are calculated using Student's *t*-test. Kaplan–Meier survival plots show an association between Kindlin-1 levels and gastric cancer prognosis. (**F**) Overall survival (OS, n = 631), (**G**) first progression survival (FP, n = 522), and (**H**) post-progression survival (PPS, n = 384).

increases with stage, grade, and metastasis status using TCGA dataset (Supplementary Fig. S1). These results indicate that Kindlin-1 upregulation is associated with poor prognosis in patients with gastric cancer.

Silencing Kindlin-1 expression inhibits proliferation, invasion, and migration in gastric cancer cells

To investigate the role of Kindlin-1 in gastric cancer cells, we first confirmed its expression in normal gastric epithelial cells and six gastric cancer cell lines using RT-PCR and western blotting (Supplementary Fig. S2). Kindlin-1 expression was higher in all gastric cancer cells than in GES-1 cells. Among the gastric cancer cell lines, AGS and YCC-2 cells with relatively high loss-of-function expression were selected. Kindlin-1 expression was silenced using two siRNAs, and interference efficiency was measured using RT-PCR and western blotting (Fig. 2A). The mRNA and protein levels of Kindlin-1 were suppressed in AGS and YCC-2 cells following transfection with the two siRNAs. Kindlin-1-silenced cells were incubated for 48 h, and WST-8 assays were performed to confirm the effects on cell proliferation (Fig. 2B). Cell proliferation was significantly reduced after silencing Kindlin-1 in AGS and YCC-2 cells. Further, crystal violet staining of these cells allowed visualization of the decreased cell proliferation (Fig. 2C). Cell invasion and migration analyses were performed using Transwell assays (Fig. 2D, 2E). The results indicated that silencing Kindlin-1 expression inhibited the invasive and migratory abilities of gastric cancer cells.

Overexpressed Kindlin-1 promotes the proliferation, invasion, and migration of gastric cancer cells

For the gain-of-function analysis, we selected SNU-601 and SNU-668 cells, which showed relatively low expression of Kindlin-1 among the six gastric cancer cell lines (Supplementary Fig. S2), and transfected them with the Kindlin-1 expression vector. After 48 h, the overexpression efficiency for Kindlin-1 was determined using RT-PCR and western blotting (Fig. 3A). Overexpression of Kindlin-1 significantly increased cell proliferation compared with that of the control SNU-601 and SNU-668 cells (Fig. 3B). The increased cell proliferation was also visualized using crystal violet staining (Fig. 3C). In addition, overexpression of Kindlin-1 significantly enhanced cell invasion (Fig. 3D) and migration (Fig. 3E). These results suggested that Kindlin-1 mediated the regulation of biological functions such as proliferation and motility in gastric cancer cells.

Kindlin-1 regulates epithelial–mesenchymal transition (EMT) in gastric cancer cells

Enhanced cell motility is a specific feature of cancer and is associated with metastasis[43]. Therefore, we investigated whether Kindlin-1 is associated with EMT, the first step in cancer metastasis[44]. Western blotting confirmed

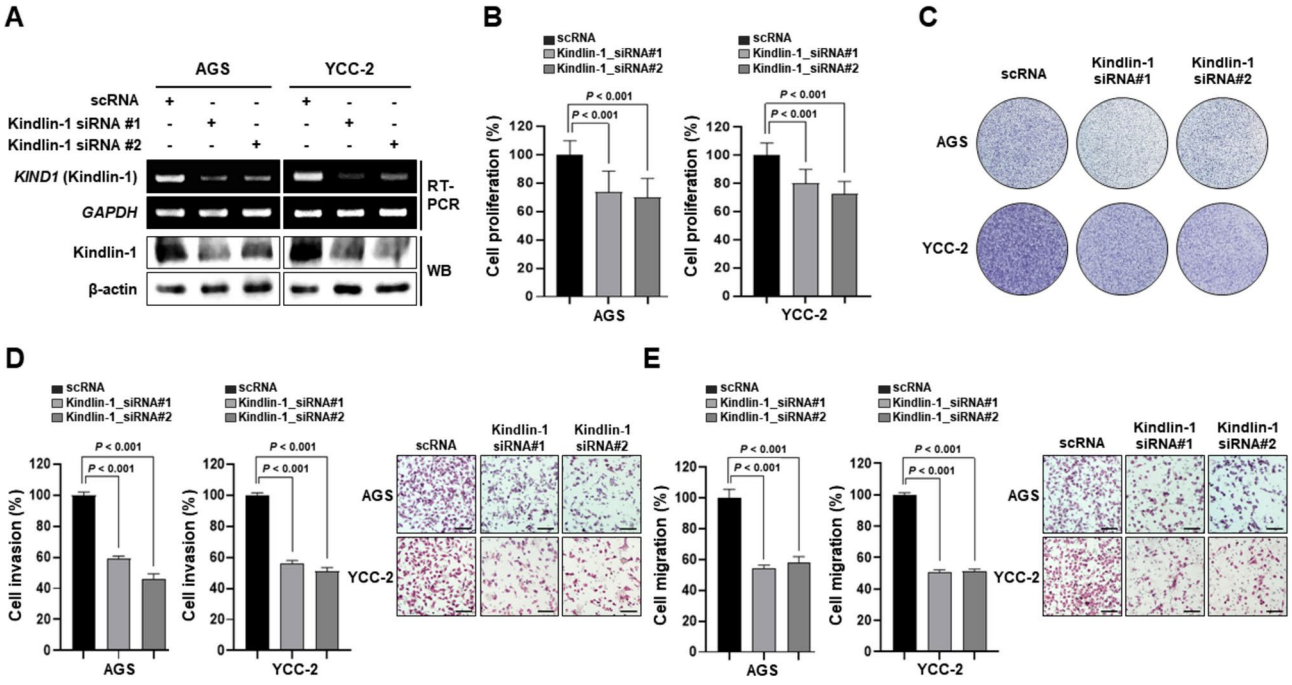

Fig. 2. Silencing of Kindlin-1 decreases proliferation, invasion, and migration in gastric cancer cells. AGS and YCC-2 cells are transfected with scrambled siRNA (scRNA) or Kindlin-1-specific siRNA (#1, #2) for 48 h. (**A**) Silencing efficiency of Kindlin-1 expression was confirmed using RT-PCR and western blotting, with GAPDH and β-actin serving as loading controls. (**B**) WST-8 assay and (**C**) crystal violet staining were performed to determine cell proliferation. A Transwell assay was conducted to determine the (**D**) invasion and (**E**) migration abilities of gastric cancer cells. The results are displayed as graphs (left) and images (right). Image magnification, × 100; scale bar, 100 μm. Data are presented as means ± SD (n = 5). *p*-values were calculated using Student's *t*-test.

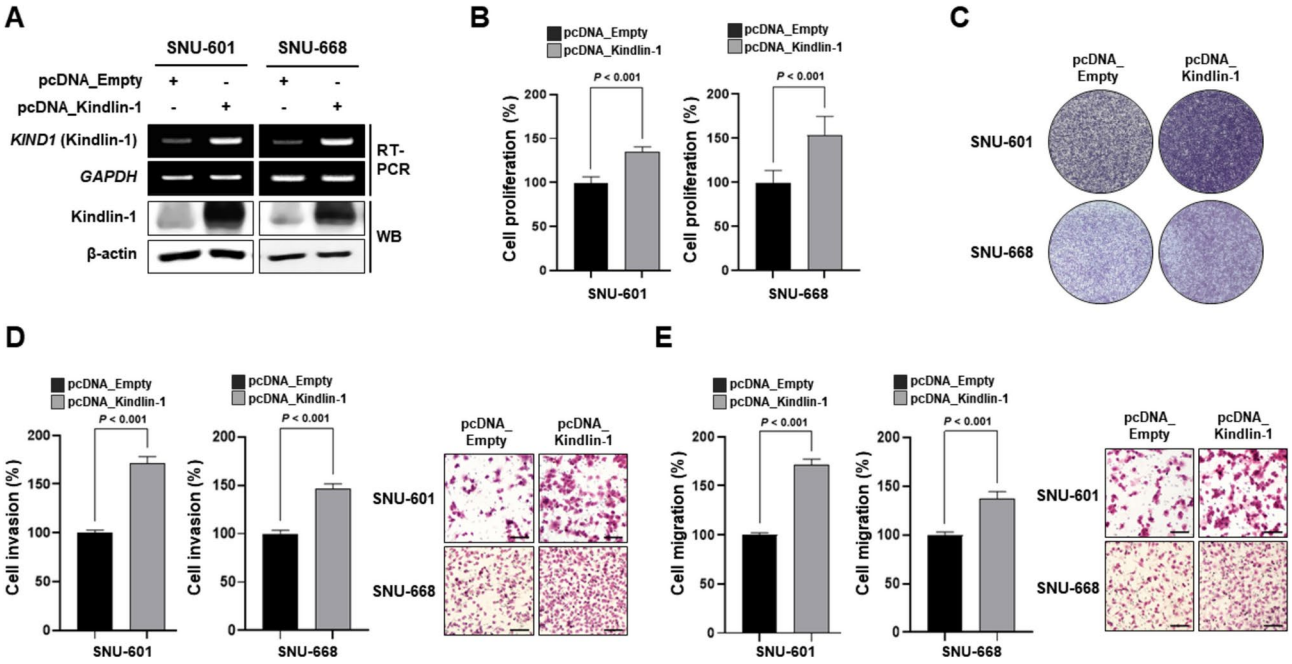

Fig. 3. Overexpression of Kindlin-1 increases proliferation, invasion, and migration in gastric cancer cells. SNU-601 and SNU-668 cells were transfected with pcDNA3.1_Flag_Empty (pcDNA_Empty) or pcDNA3.1_Flag_Kindlin-1 (pcDNA_Kindlin-1) for 48 h. (**A**) Overexpressed Kindlin-1 levels are confirmed using RT-PCR and western blotting analyses, with GAPDH and β-actin serving as loading controls. (**B**) WST-8 assay and (**C**) crystal violet staining were performed to determine cell proliferation. A Transwell assay was conducted to determine the (**D**) invasion and (**E**) migration abilities of gastric cancer cells. The results are displayed as graphs (left) and images (right). Image magnification, × 100; scale bar, 100 μm. Data are presented as means ± SD (n = 5). *p*-Values were calculated using Student's *t*-test.

that silencing Kindlin-1 suppressed the expression of EMT-related markers, whereas its overexpression induced EMT-related marker expression in gastric cancer cells. First, the expression of EMT-related markers was confirmed using western blotting of Kindlin-1-silenced AGS cells and Kindlin-1-overexpressing SNU-601 cells (Fig. 4A). The EMT-induction markers N-cadherin and vimentin were suppressed, whereas the EMT-inhibition marker E-cadherin was elevated by Kindlin-1 silencing. In contrast, overexpression of Kindlin-1 upregulated the expression of EMT-induction markers and downregulated the expression of EMT-inhibition markers. Immunofluorescence analysis showed similar results (Fig. 4B). Overall, we demonstrated that Kindlin-1 regulated the expression levels of EMT-related proteins. Next, we elucidated the molecular mechanisms by which Kindlin-1 exerted its effects. Dysregulation of several molecular signaling pathways, such as Wnt/β-catenin, Hedgehog, Notch, and TGF-β, plays an essential role in gastric tumorigenesis and metastasis[45]. As these signaling pathways are associated with EMT and are particularly hyperactivated in gastric cancer, we investigated their involvement in the cascade of processes leading to Kindlin-1-induced EMT[46]. Interestingly, Kindlin-1 regulated the mRNA expression of key molecules involved in several signaling pathways in gastric cancer cells (Fig. 4C). Furthermore, Kindlin-1 expression level showed a significant positive correlation with key molecules, except GLI1, during the analysis of datasets obtained from the GEO database (Fig. 4D, 4G) and TCGA dataset (Supplementary Fig. S3) for patients with gastric cancer. Overall, Kindlin-1 was involved in various signaling processes that induce EMT.

Kindlin-1 facilitates activation of the Wnt/β-catenin signaling pathway in gastric cancer cells

The Wnt/β-catenin signaling pathway is hyperactivated in approximately 30–50% of gastric cancer tissues[34–36]. In our datasets, the change in CTNNB1 (β-catenin) mRNA expression was the highest in Kindlin-1-regulated gastric cancer cells compared with that of other genes. Therefore, we focused on the activation of the Wnt/β-catenin signaling pathway by Kindlin-1 in gastric cancer. We observed a significant positive correlation between Kindlin-1 and β-catenin mRNA expression in patients with gastric cancer by analyzing RNA-seq data from TCGA (Fig. 5A, 5B). β-Catenin forms a complex with the transcription factor TCF4 and is directly involved in transcriptional activity[25]. Therefore, the reporter gene activity of TCF4 was confirmed using luciferase assays (Fig. 5C). In Kindlin-1-silenced AGS cells, reduced luciferase activity of TCF4 indicated inhibition of β-catenin activity. Conversely, increased luciferase activity was observed in Kindlin-1-overexpressing SNU-601 cells. Next, we examined whether Kindlin-1 affects β-catenin expression in gastric cancer cells, and conversely, whether β-catenin affects Kindlin-1 expression. (Fig. 5D, Supplementary Fig. S4). The results showed that Kindlin-1 regulated the expression of β-catenin as well as the phosphorylation of GSK3β (Ser9). GSK3β phosphorylation is a key event in the Wnt/β-catenin signaling pathway and determines the stability of β-catenin[47]. Our results confirmed the involvement of Kindlin-1 in the interaction between β-catenin and GSK3β to improve β-catenin

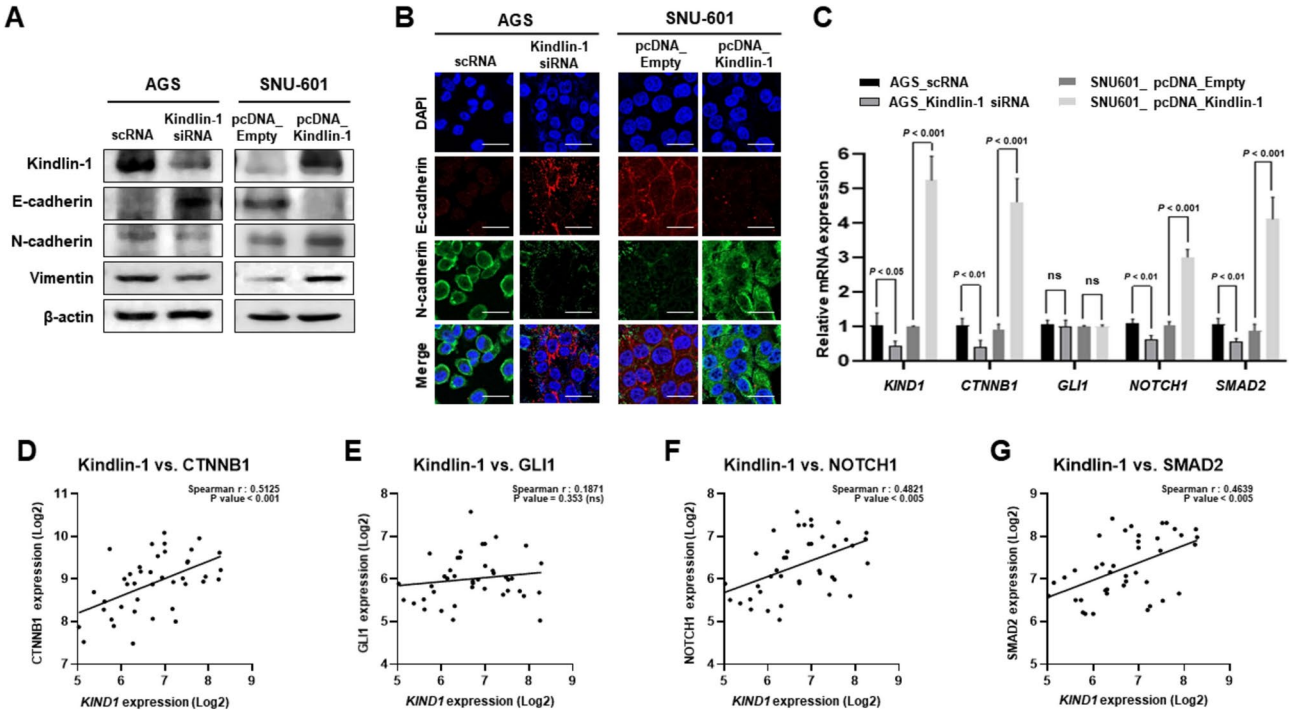

Fig. 4. Kindlin-1 regulates epithelial–mesenchymal transition (EMT) in gastric cancer. AGS cells were transfected with scRNA or Kindlin-1 siRNA #1 (Kindlin-1 siRNA). SNU-601 cells were transfected with pcDNA_Empty or pcDNA_Kindlin-1. (**A**) Protein expression of three EMT markers (E-cadherin, N-cadherin, and vimentin) were evaluated using western blotting analysis. (**B**) Immunofluorescence for N-cadherin (green) and E-cadherin (red) was performed and visualized using confocal microscopy. Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). Image magnification, × 400; scale bar, 20 μm. (**C**) mRNA levels of five genes (KIND1, CTNNB1, GLI1, NOTCH1, and SMAD2) were analyzed using qRT-PCR. Data are presented as mean ± SD (n = 3). *p*-Values were calculated using Student's *t*-test (**D**–**G**). Spearman correlation analysis indicates the relationship between KIND1 and four genes (CTNNB1, SMAD2, GLI1, and NOTCH1) in patients with gastric cancer based on the dataset obtained from a publicly available database, GSE63089 (n = 41).

stability (Supplementary Fig. S5). We also confirmed the expression of transcriptional target genes of TCF4 using RT-PCR and western blotting (Fig. 5E, 5F). Kindlin-1 silencing reduced the mRNA and protein levels of CCND1 (cyclinD1), PTGS2 (COX-2), BIRC5 (Survivin), and FSCN1 (Fascin-1). In contrast, Kindlin-1 overexpression increased the expression of these genes. Furthermore, the mRNA expression levels of cyclinD1 and fascin-1 increased by Kindlin-1 overexpression were regulated by β-catenin silencing (Supplementary Fig. S6). In addition, we examined the correlation of Kindlin-1 with β-catenin expression and that of its target genes (including Survivin, Cyclin D1, Fascin-1, and Cox-2) in publicly available datasets (Supplementary Fig. S7 and S8). Overall, these results showed that Kindlin-1 regulated the transcriptional activity of TCF4 and was related to the Wnt/β-catenin signaling pathway.

Correlation of Kindlin-1 and β-catenin expression with gastric cancer prognosis

Considering that Kindlin-1 activates Wnt/β-catenin signaling in gastric cancer cells, we aimed to determine the clinical relevance of Kindlin-1 and β-catenin expression in human cancer specimens (Fig. 6A). Among the 359 cases examined, high expression of Kindlin-1 (Kindlin-1^{high}) and β-catenin (β-catenin^{high}) were observed in 148 (41.2%) and 128 cases (35.7%), respectively. Kindlin-1^{high}, β-catenin^{high}, and Kindlin-1^{high}/β-catenin^{high} were more frequently observed in AGC than in EGC (χ^2 test; Fig. 6B). In addition, Kindlin-1 expression was strongly correlated with β-catenin expression (Fig. 6C). Both Kindlin-1^{high} and β-catenin^{high} were significantly associated with advanced cancer types, including poor differentiation, diffuse histologic type, high pT class, lymphovascular invasion, perineural invasion, lymph node metastasis, and advanced stage (Table 1). We investigated the association between the level of each protein and patient survival outcomes. Kindlin-1^{high} was significantly associated with poor disease-free survival (DFS) and OS compared to Kindlin-1^{low} (Fig. 6D). Similarly, patients with β-catenin^{high} showed significantly worse DFS and OS than those in patients with β-catenin^{low}. Furthermore, we analyzed the DFS and OS of patients across four groups categorized based on the combined expression patterns of Kindlin-1 and β-catenin: Kindlin-1^{low}/β-catenin^{low} (169 cases, 47.1%), Kindlin-1^{low}/β-catenin^{high} (42 cases, 11.7%), Kindlin-1^{high}/β-catenin^{low} (62 cases, 17.3%), and Kindlin-1^{high}/β-catenin^{high} (86 cases, 24.0%). Patients with Kindlin-1^{high}/β-catenin^{high} expression (DFS and OS rate of 73.3% and 72.3%, respectively) had a significantly worse outcome than patients with Kindlin-1^{low}/β-catenin^{low} expression (DFS and OS rate of

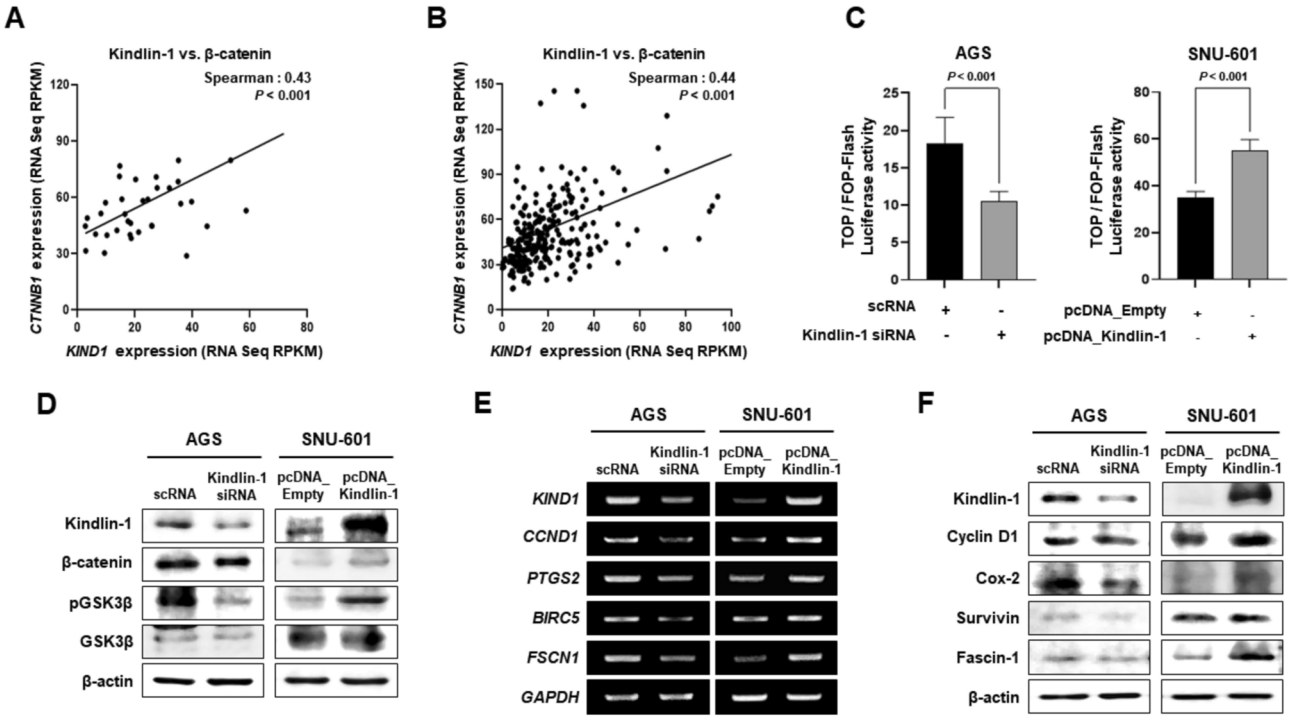

Fig. 5. Kindlin-1 is associated with Wnt/β-catenin signaling in gastric cancer. Spearman correlation test showing the relationship between Kindlin-1 and β-catenin in patients with gastric cancer. mRNA expression data were obtained from (**A**) The Cancer Genome Atlas (TCGA) (Firehose Legacy) RNA-seq RPKM (n = 36) and (**B**) TCGA (Nature 2014) RNA-seq V2 RSEM (n = 258). (**C**) Cells were transfected with TOPFlash (positive) or FOPFlash (negative) luciferase reporter plasmid. The TOPFlash/FOPFlash luciferase reporter assay assessed β-catenin/TCF4 transcriptional activity. Data are presented as means ± SD (n = 9). *p*-Values were calculated using Student's *t*-test. (**D**) GSK3β phosphorylation and β-catenin expression were detected using western blotting analysis. (**E**) mRNA and (**F**) protein expression levels for TCF4 target genes were detected using RT-PCR and western blotting, respectively.

91.1% and 90.9%, respectively). Significant differences in DFS rates were observed between Kindlin-1^{low}/β-catenin^{low} and Kindlin-1^{high}/β-catenin^{high} in a pairwise comparison. Similarly, significant differences in OS were observed between Kindlin-1^{low}/β-catenin^{low} and Kindlin-1^{high}/β-catenin^{high}, and between Kindlin-1^{high}/β-catenin^{low} and Kindlin-1^{high}/β-catenin^{high}. The Cox proportional hazard model demonstrated that Kindlin-1^{high} and β-catenin^{high} were associated with reduced DFS and OS in univariate survival analysis. Furthermore, Cox multivariate proportional hazard analysis revealed that lymph node metastasis was an independent prognostic factor for poor DFS and OS in patients with gastric cancer (Table 2). However, Kindlin-1^{high} and β-catenin^{high} showed a tendency toward shorter survival in multivariate survival analysis. Taken together, these data indicated that Kindlin-1 and β-catenin levels were closely linked with tumorigenesis and prognosis of gastric cancer.

Kindlin-1 interacts with β-catenin and causes its nuclear accumulation in gastric cancer cells

Finally, we explored how Kindlin-1 regulates the Wnt/β-catenin signaling pathway. β-Catenin is mainly present in the cytoplasm and translocates to the nucleus for transcriptional activation[12,25], interacting with various endogenous binding partners[31]. Therefore, we confirmed the interaction between Kindlin-1 and β-catenin using IP (Fig. 7A). Detection of Kindlin-1 in the IP for β-catenin indicated an interaction between the two molecules in gastric cancer cells. Next, nuclear fractionation was performed to verify Kindlin-1 involvement in β-catenin nuclear translocation (Fig. 7B, 7C). Kindlin-1 silencing reduced the nuclear accumulation of β-catenin in AGS cells. In Kindlin-1-overexpressing SNU-601 cells, both Kindlin-1 levels and β-catenin accumulation were increased in the nucleus. Nuclear accumulation of β-catenin is related to TCF4 transcriptional activation[24]. We therefore investigated whether the Kindlin-1/β-catenin complex could bind to the promoter region of CCND1, a TCF4 transcriptional target gene. We selected two TCF4 binding sites in the CCND1 promoter region. ChIP of the two TCF4 binding sites revealed the presence of Kindlin-1 and β-catenin (Fig. 7D). Taken together, our results showed that Kindlin-1, which was upregulated in gastric cancer, activated the Wnt/β-catenin signaling and promoted gastric cancer progression by regulating β-catenin expression or directly interacting with it to induce nuclear translocation (Fig. 7E).

Discussion

Despite advances in its diagnosis and treatment, gastric cancer remains one of the most threatening carcinomas worldwide. Kindlin-1 supports the progression and metastasis of various cancers and is considered an

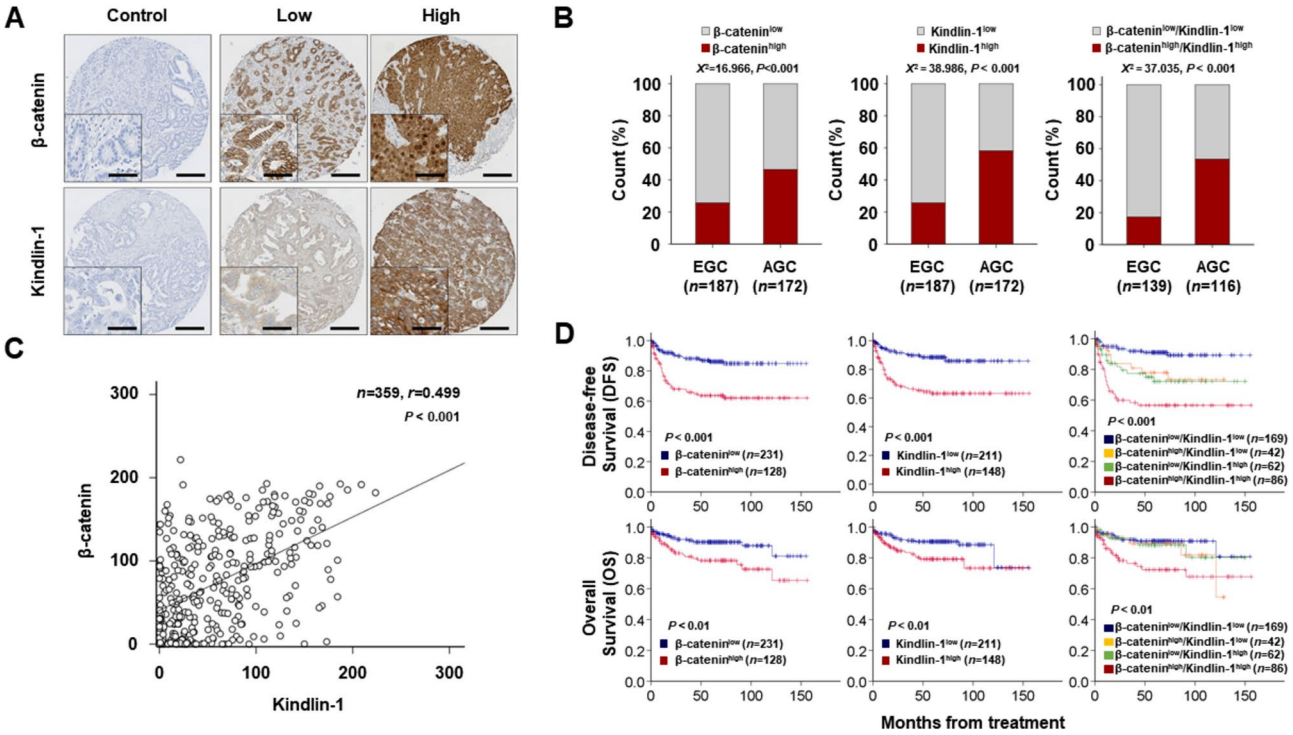

Fig. 6. Expression of Kindlin-1 and β-catenin in gastric cancer specimens correlates with disease stage and outcome. (**A**) Representative images of human gastric tumors with low and high expression levels of Kindlin-1 and β-catenin based on immunohistochemical staining. High-magnification images are shown in the inset. The scale bar represents 100 μm (50 μm in the inset). (**B**) Comparison of Kindlin-1 and β-catenin expression levels in early gastric cancer (EGC) and advanced gastric cancer (AGC). Kindlin-1^{high}, β-catenin^{high}, and Kindlin-1^{high}/β-catenin^{high} expression are more frequently observed in AGC than in EGC (all, $P<0.001$). (**C**) Correlation analysis between Kindlin-1 and β-catenin expression in gastric cancer. Kindlin-1 expression shows a significant positive correlation with β-catenin in gastric cancer (Spearman correlation $r=0.499$, $P<0.001$). (**D**) Kaplan–Meier survival plots for Kindlin-1 and β-catenin expression in patients with gastric cancer. Patients with Kindlin-1^{high} and β-catenin^{high} exhibit poorer DFS (log-rank, both $P<0.001$) and OS rates (log-rank, $P<0.01$ and $P<0.01$, respectively) compared with patients with Kindlin-1^{low} and β-catenin^{low} expression. Patients with Kindlin-1^{high}/β-catenin^{high} had the worst DFS (log-rank, $P<0.001$) and OS (log-rank, $P<0.01$) rates.

oncogene[20,21]. However, the mechanisms and biological functions of Kindlin-1 in gastric cancer have not yet been fully elucidated. Our study contributes to the understanding of biological functions that promote tumor progression via Kindlin-1, which is upregulated in gastric cancer. Moreover, we present the molecular mechanism by which Kindlin-1 activates the Wnt/β-catenin signaling in gastric cancer cells.

We investigated the expression of Kindlin-1 in patients with gastric cancer using four datasets from the publicly available GEO database. Kindlin-1 was upregulated in gastric cancer tissues compared with normal tissues. Additionally, Kaplan–Meier analysis showed that high Kindlin-1 expression was associated with poor prognosis, consistent with the findings of previous studies on the association between Kindlin-1 expression and gastric cancer prognosis[48]. Furthermore, we verified that silencing Kindlin-1 expression significantly decreased cell proliferation, migration, and invasion, whereas Kindlin-1 overexpression increased these malignant properties in gastric cancer cells. In addition, silencing or overexpression of Kindlin-1 modulated the EMT markers in gastric cancer cells; activation of EMT is a critical step in the metastatic cascade[49]. This indicates that Kindlin-1 is involved in gastric cancer cell proliferation and motility.

Next, we analyzed the molecular signaling mechanism of Kindlin-1, which plays an important role in gastric cancer progression and metastasis. Dysregulation of several signaling transduction pathways, such as Wnt/β-catenin, Hedgehog, Notch, and TGF-β, contributes to the initiation, development, and metastasis of gastric cancer[44]. Indeed, we identified alterations in key molecules associated with several signaling pathways, such as Wnt/β-catenin, Notch, and TGF-β signaling, in gastric cancer cells with altered Kindlin-1 expression. Further, the mRNA expression of β-catenin showed the most notable change. Previous studies have provided substantial evidence that the Wnt/β-catenin signaling pathway is particularly hyperactivated in gastric cancer and plays a key role in its progression and development[34–36].

We also confirmed the relationship between Kindlin-1 and the Wnt/β-catenin pathway. Overexpression of Kindlin-1 enhanced β-catenin/TCF transcriptional activity, as confirmed by luciferase reporter assays. Furthermore, Kindlin-1 expression regulated the levels of transcriptional target genes within the Wnt/β-catenin pathway. Analysis of datasets obtained for patients with gastric cancer revealed a positive correlation

Characteristics	Kindlin-1			β-catenin		
	Low (%) n = 211	High (%) n = 148	*p*-value	Low (%) n = 231	High (%) n = 128	*p*-value
Age (years)	63.52 ± 10.06	62.71 ± 11.63	0.481	63.60 ± 10.09	62.38 ± 11.82	0.325
Sex			0.157			0.184
Male	149 (70.6)	94 (63.5)		162 (70.1)	81 (63.3)	
Female	62 (29.4)	54 (36.5)		69 (29.9)	47 (36.7)	
WHO grade			< 0.001*			< 0.001*
Well	40 (19.0)	4 (2.7)		38 (16.5)	6 (4.7)	
Moderate	103 (48.8)	42 (28.4)		113 (48.9)	32 (25.0)	
Poor	68 (32.2)	102 (68.9)		80 (34.6)	90 (70.3)	
Histologic type			< 0.001*			< 0.001*
Intestinal	200 (94.8)	123 (83.1)		223 (96.5)	100 (78.1)	
Diffuse	11 (5.2)	25 (16.9)		8 (3.5)	28 (21.9)	
Depth of invasion			< 0.001*			< 0.001*
EGC	139 (65.9)	48 (32.4)		139 (60.2)	48 (37.5)	
AGC	72 (34.1)	100 (67.6)		92 (39.8)	80 (62.5)	
pT classification			< 0.001*			< 0.001*
pT1	139 (65.9)	48 (32.4)		139 (60.2)	48 (37.5)	
pT2	20 (9.5)	17 (11.5)		25 (10.8)	12 (9.4)	
pT3	20 (9.5)	30 (20.3)		31 (13.4)	19 (14.8)	
pT4	32 (15.1)	53 (35.8)		36 (15.6)	49 (38.3)	
LVI			< 0.001*			< 0.001*
Absent	154 (73.0)	71 (48.0)		164 (71.0)	61 (47.7)	
Present	57 (27.0)	77 (52.0)		67 (29.0)	67 (52.3)	
Perineural invasion			< 0.001*			< 0.001*
Absent	167 (79.1)	88 (59.5)		179 (77.5)	76 (59.4)	
Present	44 (20.9)	60 (40.5)		52 (22.5)	52 (40.6)	
LN metastasis[a]			< 0.001*			< 0.001*
Absent	138 (66.0)	68 (45.9)		156 (68.1)	50 (39.1)	
Present	71 (34.0)	80 (54.1)		73 (31.9)	78 (60.9)	
Distant metastasis			0.074			0.087
Absent	206 (97.6)	139 (93.9)		225 (97.4)	120 (93.8)	
Present	5 (2.4)	9 (6.1)		6 (2.6)	8 (6.3)	
Stage[a]			< 0.001*			< 0.001*
I	140 (67.0)	61 (41.2)		151 (65.9)	50 (39.1)	
II	27 (12.9)	16 (10.8)		29 (12.7)	14 (10.9)	
III	37 (17.7)	62 (41.9)		43 (18.8)	56 (43.8)	
IV	5 (2.4)	9 (6.1)		6 (2.6)	8 (6.2)	

Table 1. Association of Kindlin-1 and β-catenin expression with clinicopathologic characteristics in gastric cancer. LN, lymph node; EGC, early gastric cancer; AGC, advanced gastric cancer; LVI, lymphovascular invasion. [a]Calculated with only 357 cases with available information. *$p < 0.05$ denotes significance.

between the gene expression levels of Kindlin-1 and β-catenin. Finally, we confirmed that Kindlin-1 interacted with β-catenin, facilitating its nuclear translocation and subsequent activation of transcriptional target genes. Depletion or overexpression of Kindlin-1 modulated β-catenin expression and its nuclear accumulation in gastric cancer cells. Overall, these results indicate that overexpressed Kindlin-1 interacts with β-catenin and activates Wnt/β-catenin signaling pathway. Notably, in gastric cancer, more than 30–50% of cases show activation of the Wnt/β-catenin pathway; therefore, selection and optimization of partner proteins are important for developing diagnosis and therapeutic strategies[50]. These results show that Kindlin-1 can be used as a partner molecule.

Kindlin-1 is implicated in multiple signaling pathways beyond Wnt/β-catenin. The importance of epidermal growth factor receptor (EGFR) signaling in gastric cancer has recently been revealed[51,52]. Despite numerous studies, clinical trials on EGFR targeting have not been successful[53]. Kindlin-1 acts as a downstream effector of the EGFR signaling pathway[54,55] and protects EGFR from the effects of EGFR inhibitors. The discovery of crosstalk between Kindlin-1 and the Wnt/β-catenin pathway may provide positive clues for the clinical success of targeted therapies such as EGFR inhibitors[56]. Therefore, further studies on upstream mechanisms regulating Kindlin-1 expression are warranted.

Variables	Disease-free survival		Overall survival	
	Univariate HR (95% CI), *p*-value	Multivariate HR (95% CI), *p*-value	Univariate HR (95% CI), *p*-value	Multivariate HR (95% CI), *p*-value
Age (years)	1.017 (0.989–1.046), 0.229		1.018 (0.990–1.047), 0.209	
Sex	1.113 (0.610–2.028), 0.727		1.083 (0.594–1.974), 0.795	
Histologic type	2.299 (1.113–4.752), 0.025	1.326 (0.580–3.032), 0.504	2.375 (1.149–4.908), 0.020*	1.523 (0.668–3.469), 0.317
EGC/AGC	6.336 (3.061–13.117), < 0.001*	2.390 (0.892–6.404), 0.083	6.149 (2.972–12.721), < 0.001*	2.338 (0.873–6.256), 0.091
Lymphovascular invasion	4.767 (2.599–8.746), < 0.001*	0.977 (0.410–2.330), 0.958	4.667 (2.550–8.544), < 0.001*	1.008 (0.427–2.381), 0.985
Perineural invasion	4.792 (2.686–8.547), < 0.001*	1.835 (0.829–4.063), 0.135	4.732 (2.654–8.439), < 0.001*	1.729 (0.781–3.827), 0.177
Lymph node metastasis	10.817 (4.820–24.277), < 0.001*	4.768 (1.776–12.804), 0.002*	10.892 (4.853–24.443), < 0.001*	4.946 (1.847–13.240), 0.001*
Distant metastasis	5.263 (1.850–14.969), 0.002*	2.030 (0.674–6.117), 0.208	5.037 (1.776–14.283), 0.002*	1.928 (0.641–5.802), 0.243
Kindlin-1^{high}	2.091 (1.181–3.700), 0.011*	1.089 (0.559–2.123), 0.802	2.039 (1.151–3.610), 0.015*	1.047 (0.534–2.055), 0.893
β-Catenin^{high}	2.327 (1.317–4.112), 0.004*	1.392 (0.716–2.709), 0.330	2.317 (1.312–4.094), 0.004*	1.327 (0.680–2.588), 0.407

Table 2. Univariate and multivariate analyses of disease-free and overall survival in patients with gastric cancer. EGC, early gastric cancer; AGC, advanced gastric cancer; HR, hazard ratio; CI, confidence interval. *$p < 0.05$ denotes significance.

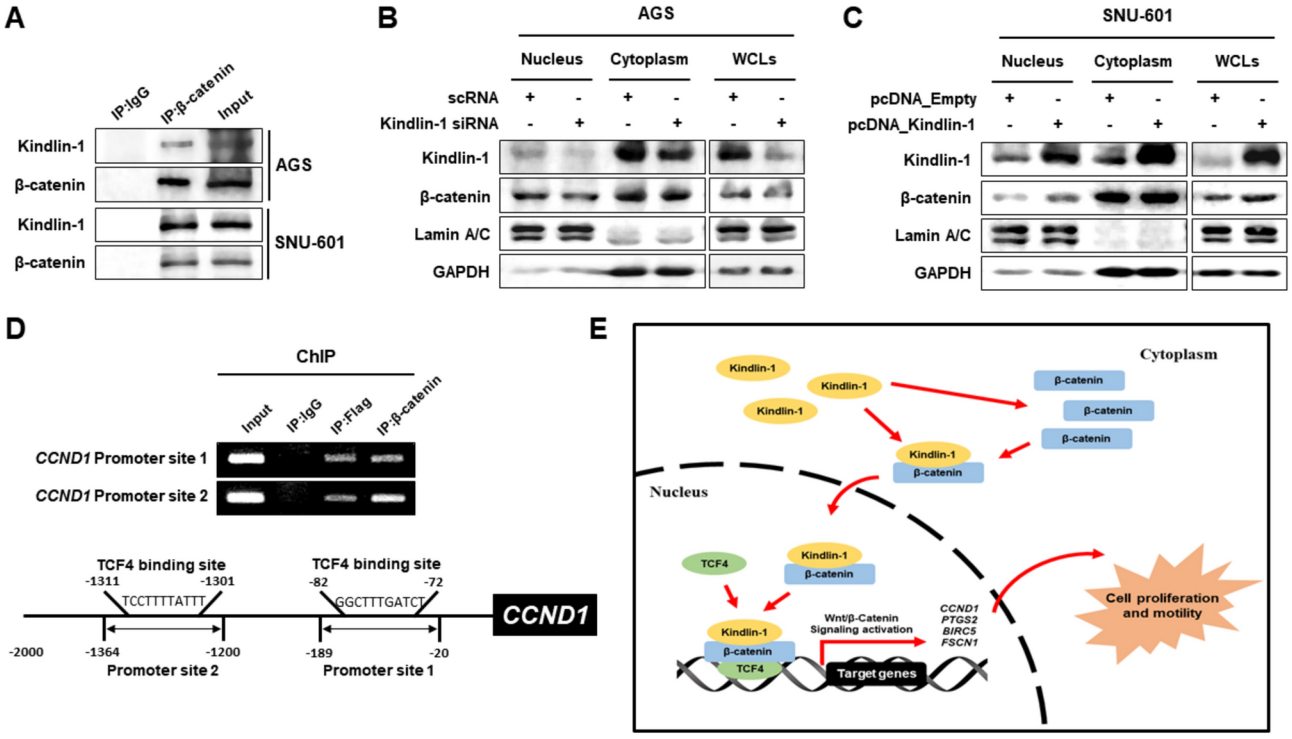

Fig. 7. Kindlin-1 enhances the nuclear accumulation of β-catenin in gastric cancer cells. (**A**) Immunoprecipitation analysis using anti-β-catenin to detect Kindlin-1 interaction with β-catenin in AGS and SNU-601 cells. Anti-IgG was used as a negative control. Nuclear fractionation was performed in (**B**) Kindlin-1-silenced AGS cells and (**C**) Kindlin-1-overexpressing SNU-601 cells. Kindlin-1 and β-catenin expression levels were detected using western blotting analysis. Lamin A/C and GAPDH were used as markers for the nucleus and cytoplasm, respectively. (**D**) SNU-601 cells were transfected with pcDNA_Kindlin-1 plasmid, and IP was performed with anti-β-catenin and anti-Flag antibodies. DNA fragments for the interacting promoter site 1 (− 189 bp to − 20 bp) and site 2 (-1364 bp to − 1200 bp) were detected using RT-PCR. Total genomic DNA in the input lane was used as a qualitative control for PCR. Anti-IgG was used as a negative control. (**E**) Schematic diagram showing the molecular mechanism underlying Kindlin-1 involvement in the Wnt/β-catenin signaling pathway leading to gastric cancer progression. Kindlin-1 upregulates β-catenin or interacts directly with it, facilitating its nuclear accumulation, resulting in activation of the Wnt/β-catenin pathway and increased proliferation and motility of gastric cancer cells.

In conclusion, our study elucidates the molecular mechanisms through which Kindlin-1 regulates β-catenin expression or interacts with it to promote gastric cancer progression and metastasis via activation of the Wnt/β-catenin signaling pathway. These results suggest that Kindlin-1 is a promising therapeutic target against gastric cancer progression and metastasis.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Received: 15 October 2024; Accepted: 9 January 2025
Published online: 20 January 2025

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Acknowledgements

This work was supported by grants from the National Research Foundation of Korea, funded by the Korean government (Grant No. RS-2023-00244707), Global-Learning & Academic Research Institution for Master's and PhD students, and Postdocs (LAMP) Program of the National Research Foundation of Korea, funded by the Ministry of Education (Grant No. RS-2023-00285353).

Author contributions

J-H Jang, J Jung, H-G Kang, W Kim, W-J Kim, H Lee, and J Y Cho performed the experiments, analyzed and interpreted the data, and drafted the manuscript. J Jung, R Hong, J W Kim, and J-Y Chung performed the experiments related to cancer tissues. J Y Cho, J-Y Chung, K-H Chun and S-J Kim contributed to the manuscript writing. J-H Jang, J Jung, H-G Kang, K-H Chun, and S-J Kim designed the experiments, interpreted the data, supervised all aspects of the study, handled funding, and wrote and revised the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-86220-7.

Correspondence and requests for materials should be addressed to K.-H.C. or S.-J.K.