DIXDC1 Activates the Wnt Signaling Pathway and Promotes Gastric Cancer Cell Invasion and Metastasis

Cong Tan,1,2 Fan Qiao,3 Ping Wei,1,2 Yayun Chi,4 Weige Wang,1,2 Shujuan Ni,1,2 Qifeng Wang,1,2 Tongzhen Chen,1,2 Weiqi Sheng,1,2 Xiang Du,1,2* and Lei Wang1,2*

1Department of Pathology, Fudan University Shanghai Cancer Center, Shanghai, China
2Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, China
3Department of Cardiothoracic Surgery, Shanghai Hospital, Second Military Medical University, Shanghai, China
4Cancer institute, Fudan University Shanghai Cancer Center, Shanghai, China

DIXDC1 (Dishevelled-Axin domain containing 1) is a DIX (Dishevelled-Axin) domain-possessing protein that promotes colon cancer cell proliferation and increases the invasion and migration ability of non-small-cell lung cancer via the PI3K pathway. As a positive regulator of the Wnt/β-catenin pathway, the biological role of DIXDC1 in human gastric cancer and the relationship between DIXDC1 and the Wnt pathway are unclear. In the current study, the upregulation of DIXDC1 was detected in gastric cancer and was associated with advanced TNM stage cancer, lymph node metastasis, and poor prognosis. We also found that the overexpression of DIXDC1 could promote the invasion and migration of gastric cancer cells. The upregulation of MMPs and the downregulation of E-cadherin were found to be involved in the process. DIXDC1 enhanced β-catenin nuclear accumulation, which activated the Wnt pathway. Additionally, the inhibition of β-catenin in DIXDC1-overexpressing cells reversed the metastasis promotion effects of DIXDC1. These results demonstrate that the expression of DIXDC1 is associated with poor prognosis of gastric cancer patients and that DIXDC1 promotes gastric cancer invasion and metastasis through the activation of the Wnt pathway; E-cadherin and MMPs are also involved in this process. © 2015 Wiley Periodicals, Inc.

Key words: DIXDC1; the Wnt signaling pathway; gastric cancer; invasion; migration

INTRODUCTION

DIXDC1 (Dishevelled-Axin domain containing 1), the human homolog of Ccd1 (Coiled-coil-Dishevelled-Axin1), is a DIX (Dishevelled-Axin) domain-possessing protein that acts as a positive regulator of the Wnt/β-catenin pathway [1]. The DIX domain has been demonstrated to be important in connecting the Wnt-signaling factors Axin, Dishevelled, and β-catenin [2–4].

Recent studies have shown that DIXDC1 promotes the proliferation of nerve cells and neuronal differentiation by modulating GSK3-dependent β-catenin phosphorylation [5,6]. In the field of cancer research, the elevated expression of DIXDC1 was first found in colorectal cancer and has been demonstrated to promote the proliferation of colon cancer cells [7]. Recently, DIXDC1 was reported to increase the invasion and migration ability of non-small-cell lung cancer [8]. Both of the aforementioned studies revealed that DIXDC1 affected the biological role of human cancer through the PI3K/AKT pathway. It is not clear if the canonical Wnt/β-catenin pathway, which plays an important role in tumorigenesis [9], affects the biological role of cancer induced by DIXDC1. Therefore, there is a need to further understand the molecular mechanisms regulating DIXDC1-induced tumor cell proliferation and invasion.

Gastric cancer is one of the most common malignancies, especially in Asia. Dysregulation of the canonical Wnt/β-catenin pathway is involved in gastric tumorigenesis [10]; however, the mechanisms of this involvement are not yet understood. In this study, the biological role of DIXDC1 in human gastric cancer was investigated. Our results demonstrate that DIXDC1 is overexpressed in gastric cancer and that DIXDC1 promotes cancer cell metastasis. We observed that the expression of DIXDC1 led to increased protein levels of E-cadherin and matrix metalloproteinases (MMPs), both of which are related to the Wnt...
pathway [11,12]. DIXDC1 expression also enhanced the nuclear accumulation of β-catenin, the core factor of the Wnt pathway, suggesting the activation of the canonical Wnt pathway. Taken together, our data suggest that DIXDC1 promotes gastric cancer invasion and metastasis through the activation of the Wnt pathway and that E-cadherin and MMPs are involved in this process. A more thorough understanding of DIXDC1-induced gastric cancer cell invasion and metastasis may lead to a new target to control the metastasis of gastric cancer.

MATERIALS AND METHODS

Tissue Samples

All of the cases in this study were retrieved from the files of the Department of Pathology, Shanghai Cancer Center, China. The study was approved by the institutional review board in Shanghai Cancer Center, and consent was obtained from all of the patients, who were previously diagnosed with either gastric intraepithelial neoplasia (n = 28) or gastric cancer (n = 66). The patients had not undergone previous chemotherapy treatment. All of the patients were diagnosed by two experienced pathologists.

Immunohistochemical Staining

Serial sections of formalin-fixed and paraffin-embedded tissues were subjected to antigen retrieval by high pressure repair in 0.1 M citrate solution (pH 6.0) for 10 min and then incubated with a goat anti-DIXDC1 antibody (sc-109530, dilution 1:80, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. After the samples were incubated in SuperVision™ HRP-Conjugated Rabbit anti-Goat IgG (RGHL-50P, dilution 1:200, ICL, Newberg, OR) for 60 min at room temperature, the sections were developed in 0.05% diaminobenzidine containing 0.01% hydrogen peroxidase.

Two pathologists, who were blinded to the clinical outcomes, interpreted the results of the immunohistochemical staining. Cytoplasmic staining for the DIXDC1 protein was considered positive. The positive cases were further divided into weak, moderate, and strong based on the staining intensity. For statistical analysis, DIXDC1 expression was subdivided as negative and positive (weak, moderate, and strong) expression.

Cell Culture

Gastric cancer cell lines BGC-823, AGS, and HGC-27 were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). The human embryonic kidney cell line HEK293T was cultured in Dulbecco’s modified Eagle medium.

Lentiviral Vectors Construction and Establishment of DIXDC1 Stable Cell Line

Full-length DIXDC1 cDNAs (pcDNA-3.0-DIXDC1 plasmids containing full-length DIXDC1 cDNA were kindly provided by Dr. Wan-Guo Liu of Louisiana State University) were inserted into the MCS sites of lentiviral vectors. Lentivirus was produced by transfecting the vectors into HEK293T cells using a Lentivirus Packaging Kit (Sigma, St Louis, MO) according to the manufacturer’s instructions. The virus-containing supernatant was collected 48 h after transfection. Human gastric cancer (BGC-823) cells, as target cells, were incubated with the lentiviral supernatant for 24 h. Then, the infected BGC-823 cells were cultured in selective medium containing 800 μg/mL hygromycin (Roche Diagnostics, Mannheim, Germany) for 2–3 wk. The stable cells were designated as BGC-823-DIXDC1 cells and used for subsequent experiments.

Migration and Invasion Assays

A 24-well plate containing 8 mm-pore size chamber inserts (BD Biosciences, Franklin Lakes, NJ) was used to evaluate the migration and invasion of tumor cells. For the migration assay, 5 × 10⁴ cells were seeded in the upper chamber. For the invasion assay, the membrane was coated with Matrigel (BD Biosciences) to form a matrix barrier, and then 10⁵ cells were placed in the upper chamber. In each lower chamber, 500 μL of RPMI-1640 medium with 10% FBS was added. After 36 h of incubation at 37°C, the cells that had migrated through the pore were fixed and stained with a mixture of 20% methanol and 0.1% crystal violet for 0.5 h. Then, the cells were counted and photographed under an IX71 inverted microscope (Olympus, Tokyo, Japan).

Cell Counting Kit-8 assay

Stable transfected BGC-823-DIXDC1 cells and control cells were seeded in a 96-well plate at 5 × 10³ cells per well and cultured. A volume of 10 μL of CCK-8 (Cell Counting Kit-8, Beyotime, Jiangsu, China) solution was added to each well of the plate and incubated at 37°C for 4 h. The absorbance at 450 nm was measured to represent the cell viability.

Luciferase Reporter Assay

AGS cells were cultured in 96-well plates and transfected with 50 ng of the reporter plasmid (TOP flash or its negative control FOP) (Millipore, Billerica, MA), 50 ng of an expression vector (pcDNA-3.0-DIXDC1 or vector control), and 10 ng of Renilla. After 24 h of incubation, luciferase activity was detected using the dual-luciferase reporter assay system (PromeGa, Madison, WI).

Western Blot Analysis

Whole cell lysates were generated using RIPA lysis buffer (Abcam Inc., Cambridge, MA). Nuclear protein extraction strictly followed the manufacturer’s instructions (Takara Bio Inc.). Protein samples were separated using 10% SDS-PAGE and then transferred...
onto a polyvinylidene fluoride membrane. The membrane was incubated with a primary antibody at 4°C overnight followed by an HRP-conjugated secondary antibody for 2 h at room temperature. The immunoreactive bands were visualized using enhanced chemiluminescence with ECL reagents (Pierce, Rockford, IL). The following primary antibodies were used in the study: anti-DIXDC1 (Abcam Inc., Cambridge, MA), anti-β-catenin (BD Biosciences, Franklin Lakes, NJ), anti-β-actin, phosphor-β-catenin(Ser33/37/Thr41), and phosphor-β-catenin(Ser675) (Cell Signaling Technology, Danvers, MA) as well as antibodies to E-cadherin, MMP2, MMP7, and MMP9 (1:1000, Proteintech Group, Chicago, IL).

Co-Immunoprecipitation (Co-IP)

The DIXDC1 plasmid (as described above) was transiently cotransfected with the β-catenin plasmid into the AGS cells. Thirty-six hours after transfection, the cells were lysed with lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 15 mM MgCl2, 0.1% NP-40, and Protease Inhibitor Cocktail [Roche, Mennheim, Germany]) and then clarified by centrifugation. The supernatant was pre-cleared using M-agarose beads (Roche) for 4 h at 4°C and then incubated with 2 μg of the DIXDC1 antibody (or β-catenin antibody) and beads overnight at 4°C. The immunocomplexes were pelleted and washed three times using the lysis buffer. The immunocomplexes and cell lysate protein were separated on a 10% SDS-PAGE gel and blotted on polyvinylidene fluoride membrane.

Immunofluorescence

AGS cells were cultured in 24-well plates and transfected with pcDNA-3.0-DIXDC1 or the control vector. After 24 h of incubation, the cells were fixed with methanol and then blocked with 2% bovine serum albumin. Primary antibody incubations were performed overnight at 4°C (DIXDC1, dilution 1:100; β-catenin, dilution 1:200). Secondary antibody (dilution 1:500, Jackson ImmunoResearch Laboratories, West Grove, PA) incubations were performed at room temperature for 1 h. Fluorescence was monitored by inverted confocal laser microscopy (FV1000, Olympus, Japan).

Short Interference RNA

Negative-control siRNA and DIXDC1 siRNA were obtained from GenePharma (Shanghai GenePharma Co., Ltd, China). The sequence of the DIXDC1-targeted siRNA-duplex was as follows: sense, 5’-r(AUGCCUUAGCAGCAAGAU) dTdT-T3’; antisense, 5’-r(AUCUCUGCUGAAGGCAU) dCdC-3’. The HGC27 cells were transfected at ~30% confluence. The transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). The knockdown of DIXDC1 was confirmed by western blotting. The cells transfected with DIXDC1 siRNA or control siRNA were subjected to migration and invasion assays.

Statistical Analysis

The data are shown as the mean ± standard deviation (SD). Statistical analyses were performed using a two-tailed Student t-test unless otherwise specified (χ2 test). Survival curves were generated using the Kaplan–Meier method. Differences were considered statistically significant at P < 0.05.

RESULTS

DIXDC1 was Overexpressed in Gastric Cancer and Correlated With Poor Prognosis

To illuminate the role of DIXDC1 in the carcinogenesis of gastric cancer, we performed an immunohistochemical assay to analyze the effect of DIXDC1 expression on human gastric cancer and matched precancerous lesions. DIXDC1 protein was detected mainly in the cytoplasm of cancer cells. Positive staining of DIXDC1 was observed in the tumor cells of 37 of the 66 patients with gastric cancer (56.0%) and 8 of the 28 (28.6%) precancerous lesions. None of the normal gastric mucosa showed positive DIXDC1 staining (Figure 1A; P = 0.003). The correlation between DIXDC1 expression and the clinicopathological features of gastric cancer patients is summarized in Table 1. The overexpression of DIXDC1 was associated with more advanced TNM stage gastric cancer (P = 0.03) as well as lymphatic metastasis (P = 0.034).

All of the patients received follow up for at least 36 months. After 36 months, among the 66 gastric cancer patients, 36 were still alive, 19 succumbed to the disease, and 11 declined to continue participating in the study. The median survival time of the gastric cancer patients was 50 months. Overall survival (OS) was significantly worse in the gastric cancer patients with DIXDC1 positive expression than in the patients with negative DIXDC1 expression (P = 0.024, Figure 1B).

DIXDC1 Promoted the Migration and Invasion of Gastric Cancer Cells, the Upregulation of MMP and the Decrease of E-cadherin

To validate whether DIXDC1 affects the migration and invasion abilities of gastric cancer cells, stable DIXDC1 transfected clones were generated. A lentivirus vector expressing DIXDC1 was constructed and used to infect BGC-823 cells to establish the stable cell line. The BGC-823 cell line was chosen for the study because this cell line was determined to have relatively low levels of endogenous DIXDC1 protein expression (Figure 2A). The expression of DIXDC1 in the stable cell line was increased compared with the vector transfectant, as determined using Western blot analysis (Figure 2B). Compared with the negative control, the migratory ability of BGC-823 was largely
Figure 1. Expression of DIXDC1 in human gastric cancer tissues and the relationship between DIXDC1 expression and overall survival (OS). (A) Immunostaining using an anti-DIXDC1 antibody was performed on human gastric cancer and matched normal tissues. Representative photograph of DIXDC1 immunostaining: (a) negative in normal tissues (Magnification ×200); (b) weak positive in gastric cancer (Magnification ×200); (c) moderate positive in gastric cancer (Magnification ×400); (d) strong positive in gastric cancer (Magnification ×400). (B) Kaplan-Meier curve depict OS of gastric cancer patients with or without DIXDC1 expression. n = 66; 6: DIXDC1 negative, 1: DIXDC1 positive.
increased after DIXDC1 transfection, as assessed by a wound healing assay (Figure 2C). In the transwell assays (both with and without Matrigel), enhanced migratory and invasive abilities were observed in the BGC-823 cells along with the overexpression of DIXDC1 (Figure 2D). Besides, CCK8 assay was done in both DIXDC1 transfected BGC-823 cells and negative control. The results suggested that DIXDC1 has no effect on the proliferation of gastric cancer cells (Figure 2E, \(P = 0.2194\)), which excluded the proliferation effect of exogenous DIXDC1 for migration and invasion assays. To further confirm the findings that DIXDC1 increased the migratory and invasive abilities of gastric cancer cells, DIXDC1 siRNA was transfected into HGC-27 cells, in which high endogenous DIXDC1 protein expression was detected (Figure 2A). As shown in Figure 3A, DIXDC1 protein levels in the HGC-27 cells were dramatically reduced after transfection with DIXDC1 siRNA. The wound healing assay and the Matrigel Transwell invasion assay both showed that DIXDC1 siRNA significantly reduced the migratory and invasive abilities of HGC-27 cells (Figures 3B and C).

To identify metastasis-related molecules that are regulated by DIXDC1, western blot analysis was used to detect the expression of various MMPs and E-cadherin. DIXDC1-overexpressing BGC-823 cells had a significant increase in the levels of MMP-3 and MMP-7 and a decrease in the level of E-cadherin. No obvious difference was detected in the amount of MMP2 and MMP9 between the BGC-823-DIXDC1 and control cells (Figure 4).

### Table 1. Correlation of DIXDC1 Expression and Clinicopathologic Features in Gastric Cancer Patients

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DIXDC1 Actives the Canonical Wnt Signaling Pathway and Promotes \(\beta\)-catenin Accumulation

Previous findings prompted us to investigate the underlying molecular mechanism through which DIXDC1 promotes the progression of gastric cancer. MMPs are transcriptional targets of the canonical Wnt pathway [13,14]. The E-cadherin/\(\beta\)-catenin complex is formed at cell-to-cell junctions and is known to be involved in the Wnt pathway [15,16]. The canonical Wnt pathway is regulated by the stability of \(\beta\)-catenin, whereby stabilized \(\beta\)-catenin can activate the canonical Wnt pathway through the transcription of Wnt/\(\beta\)-catenin target genes [17]. The TOPFlash reporter assay was used to validate DIXDC1-induced activation of the canonical Wnt pathway in gastric cancer cells. The results showed that the activity of TOPFlash, a classic Wnt-responsive reporter, could be heightened by DIXDC1 in AGS gastric cancer cells compared with the control, confirming that the canonical Wnt pathway is activated by DIXDC1 expression (Figure 5A). Next, we measured the amount of nuclear \(\beta\)-catenin, which is an indicator of an active canonical Wnt pathway [18]. Nuclear proteins were extracted and then analyzed by Western blot. As shown in Figure 5B, the amount of \(\beta\)-catenin in the nucleus was significantly elevated in the BGC-823-DIXDC1 cells compared with the control cells, while the amount of \(\beta\)-catenin in the cytoplasm was slightly reduced in the BGC-823-DIXDC1 cells compared with the control cells; immunofluorescence confirmed these results (Figure 5C). Taken together, these data suggest that DIXDC1 increased the translocation of \(\beta\)-catenin to the nucleus, which led to the transcriptional upregulation of Wnt target genes, such as MMPs, resulting in the promotion of metastasis.

To further investigate whether the increased \(\beta\)-catenin protein levels in the nucleus of gastric cancer cells was attributed to the stabilization of \(\beta\)-catenin, we assessed the influence of DIXDC1 on the phosphorylation status of \(\beta\)-catenin. First, the formation of a complex between \(\beta\)-catenin and DIXDC1 was
Figure 2. DIXDC1 promoted gastric cancer cell migration and invasion. (A) Endogenous DIXDC1 protein expression in various gastric cancer cell lines; (B) DIXDC1 overexpression in a stable cell line; (C) Wound-healing assays: a monolayer of BGC-823-vector (A, C) and BGC-823-DIXDC1 (B, D) cells were scratched and incubated for 36 h. Then, the migration of cells into the space left by the scratch was photographed. Pictures were taken at the same magnification. Data represent three experiments. (D) Transwell migration and invasion assays of stable BGC-823-DIXDC1 gastric cancer cells and negative control cells. (E) CCK8 assay was done in both DIXDC1 transfected BGC-823 cells and negative control cells. The results are representative of at least three independent experiments. Statistical analysis was performed using Student's t-test.
detected. In an exogenous coimmunoprecipitation, β-catenin was found to be bound to DIXDC1. Moreover, DIXDC1 could be detected in the β-catenin precipitates in the in vitro binding assay. Thus, DIXDC1 and β-catenin interact in gastric cancer cells. Then, we tested the phosphorylation status of β-catenin. The results showed that the overexpression of DIXDC1 decreased β-catenin phosphorylation on Ser33/37/Thr41 (Figure 5E). The data also showed that DIXDC1 activated the Wnt pathway by decreasing the phosphorylation and subsequent accumulation of β-catenin.

The Wnt Signaling Pathway was Involved in DIXDC1-Induced Gastric Cancer Cell Migration and Invasion

To examine whether DIXDC1 increased the invasion ability of gastric cancer cells via the Wnt pathway, β-catenin, the molecule at the heart of the Wnt pathway, was knocked down by β-catenin siRNA. The expression of β-catenin in BGC-823-DIXDC1 cells transfected with β-catenin siRNA was significantly decreased compared with the control cells (Figure 6A). Additionally, compared with the BGC-823-DIXDC1 cells transfected with control siRNA, the invasion ability of the cells transfected with β-catenin siRNA was significantly decreased (Figure 6B). In addition, DIXDC1-overexpressing cells transfected with β-catenin siRNA invaded matrigel significantly less than the DIXDC1-uninduced cells (Figure 6B). This anti-invasive activity created by the knockdown of β-catenin in the DIXDC1-induced cells was different from that in the uninduced cells, which revealed the potential contribution of the Wnt pathway in DIXDC1-induced gastric cancer metastasis.

DISCUSSION

Although *Helicobacter pylori*-associated atrophic gastritis followed by intestinal metaplasia is recognized as the beginning of gastric neoplastic...
transformation, the progression into an invasive cancer is a multistep process, which remains largely unclear [19,20]. It has been reported that the activation of the Wnt pathway is one of the major causes of gastric cancer. Nuclear β-catenin accumulation, a hallmark of Wnt pathway activation, can be found in 30–50% of gastric cancer cases [10]. However, in gastric cancer, APC mutations are rarely detected, and the incidence of β-catenin mutations is less than 30% [21]. These observations suggest that there are other mechanism(s) besides APC or β-catenin mutations may be responsible for the activation of the Wnt pathway in gastric cancer patients.

DIXDC1 is a positive regulator of the Wnt pathway. The biological role of DIXDC1 in human cancer formation and progression has not been clarified. Currently, only two studies have shown that DIXDC1 plays a significant role in human cancer [7,8]. DIXDC1 promotes colon cancer cell proliferation and increases the invasion and migration ability of non-small-cell lung cancer via the PI3K pathway. In this study, we investigated the biologic role of DIXDC1 and the relationship between DIXDC1 and the Wnt pathway in gastric cancer.

Our data confirmed that DIXDC1 was significantly upregulated in gastric cancer compared with premalignant lesions, intraepithelial neoplasias, and normal gastric mucosa, which indicated that DIXDC1 plays an important role in gastric cancer development. The overexpression of DIXDC1 in gastric cancer was closely related to advanced TNM stage, lymph node metastasis, and poor prognosis. Cancer metastasis is a multistep process includes the migration, invasion of basement membranes, intravasation, and growth at different organ sites [22–24]. Understanding the mechanisms that drive the migration and invasion of these tumor cells is crucial to better understand metastasis. Our results indicate that the upregulation of DIXDC1 could increase the migration and invasion abilities of gastric cancer cells.

E-cadherin is a calcium-dependent transmembrane glycoprotein that is expressed in most epithelial cells. The loss of E-cadherin expression is known to be a marker of epithelial to mesenchymal transition (EMT) changes and is known to promote tumor invasion and metastasis [25]. The increased expression of MMP3, -7, and -9 has also been shown to be involved in promoting cancer metastasis [26–28]. To better understand the mechanisms that promote gastric cancer metastasis by DIXDC1, the protein levels of E-cadherin and various MMPs were analyzed using immunoblotting. Our data show that the increased migration and invasion abilities in DIXDC1-overexpressing gastric cancer cells might be facilitated by DIXDC1-mediated E-cadherin downregulation and the upregulation of MMP3 and MMP7. The intracellular domain of E-cadherin interacts with β-catenin. Together, the E-cadherin/β-catenin complex functions as an intercellular junction [29]. The expression of E-cadherin was also affected by the Wnt pathway [30]. MMPs are transcriptional targets of the canonical Wnt pathway [31,32]. Stabilized β-catenin can activate the canonical Wnt pathway through the transcription of Wnt/β-catenin target genes [33]. Thus, we asked if DIXDC1 promoted gastric cancer metastasis through the activation of the Wnt pathway and whether E-cadherin and MMPs were targeted in this process.

The TOPFlash reporter assay was used to demonstrate that DIXDC1 activated β-catenin-directed transcriptional activity. The luciferase assay results showed that DIXDC1 could increase TOPFlash activity, further indicating that the upregulation of MMP3 and MMP7 by DIXDC1 might be occurring through a Wnt/β-catenin-dependent mechanism. β-catenin, the key mediator of the Wnt pathway, is phosphorylated on Ser33/37/41 by GSK-3β followed by ubiquitination and degradation under normal conditions. When the Wnt pathway is activated, non-phosphorylated β-catenin is translocated to the Figure 4. Effects of DIXDC1 on the expression of MMPs and E-cadherin. Cell lysates were prepared from BGC-823-DIXDC1 and negative control cells. Proteins related to gastric cancer metastasis (MMPs and E-cadherin) were detected by western blot analysis. * indicates P < 0.05.

Molecular Carcinogenesis
Figure 5. DIXDC1 activates the Wnt signaling pathway and promotes β-catenin accumulation. (A) DIXDC1 increased TOPFlash reporter activation in AGS cells. AGS cells were transfected with TOPFlash together with pcDNA3.0-DIXDC1 or pcDNA3.0-vector. Columns: mean of three independent experiments; bars: SD. (B) DIXDC1 increased the protein level of nuclear β-catenin in gastric cancer cells. (C) Overexpression of DIXDC1 promoted the nuclear localization of β-catenin. (D) In AGS cells, an immunoprecipitation assay showed the in vitro interaction between DIXDC1 and β-catenin. (E) The effects of DIXDC1 on the protein level of phosphorylated β-catenin in BGC-823 cells. * indicates $P < 0.05$. 

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nucleus, where it triggers the transcription of target genes, such as MMPs [34–36]. However, the molecular mechanisms of β-catenin nuclear activation in Wnt pathway activation remain unclear. Some studies suggest that β-catenin shuttles from the cytoplasm to the nucleus by interacting with the nuclear pore complex [37] and FoxM1 [38]. In this study, DIXDC1 increased the translocation of β-catenin to the

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**Figure 6**. DIXDC1-induced gastric cancer cell invasion was inhibited by the knockdown of β-catenin. (A) Successful siRNA-mediated knockdown of β-catenin in BGC-823-DIXDC1 cells. Actin served as a loading control. (B) Transwell invasion assays of BGC-823-DIXDC1 and BGC-823-Vector cells transfected with β-catenin-siRNA or a negative control. Statistical analysis was performed using Student's t-test. * indicates P < 0.05.
nucleus of gastric cancer cells, as demonstrated by immunoblotting and immunofluorescence. To further explore the relationship between DIXDC1 and β-catenin in gastric cancer, a DIXDC1/β-catenin complex was detected. Then, the phosphorylation status of β-catenin was assessed. Our results show that DIXDC1 is bound to β-catenin and stabilizes β-catenin by decreasing the phosphorylation level of β-catenin on Ser33/37/41. β-catenin accumulation in the nucleus is often associated with the loss of E-cadherin expression, which induces an invasive phenotype in gastric cancer cells [16]. In the nucleus, β-catenin is also recruited to the promoters of Wnt target genes, such as MMPs, resulting in the promotion of metastasis in gastric cancer. In our study, after β-catenin was knocked down, the invasion ability of gastric cancer cells decreased, especially in the DIXDC1-overexpressing cells. These results indicate that the Wnt pathway is involved in DIXDC1-induced gastric cancer cell migration and invasion.

Taken together, the results of this study demonstrate that DIXDC1 is overexpressed in gastric cancer and that the upregulation of DIXDC1 is associated with advanced TNM stage, lymph node metastasis, and poor prognosis. DIXDC1 plays an important role in gastric cancer development. We also found that DIXDC1 promotes the migration of gastric cancer via the Wnt/β-catenin pathway. DIXDC1 enhances β-catenin nuclear localization by decreasing the phosphorylation level of β-catenin, leading to the downregulation of E-cadherin and the transcriptional upregulation of Wnt target genes, such as MMPs, resulting in the promotion of gastric cancer metastasis. To further investigate the mechanism of how DIXDC1 affects the phosphorylation of β-catenin, the phosphorylation level of GSK-3β should be addressed because β-catenin is normally phosphorylated by GSK-3β [9,39]. Collectively, our data suggest that DIXDC1 operates downstream of or parallel to GSK-3β, possibly at the level of β-catenin, and may be the molecule responsible for the activation of the Wnt pathway in gastric cancer.

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REFERENCES


