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DDR1 promotes migration and invasion of breast cancer by modulating the Src-FAK signaling

Qing HAN[1,2], Fei XIAO[1], Lili MA[1], Jinmei ZHOU[1], Lan WANG[1], Huang CHENG[1], Jingjing ZHU[1], Fuli YAO[3], Jianxin LYU[1,*], Linyong DU[1,*]

[1]Key Laboratory of Laboratory Medicine, Ministry of Education, School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou, China; [2]Department of Clinical Laboratory, The Second Hospital of Nanjing, Nanjing University of Chinese Medicine, Nanjing, China; [3]Department of Biochemistry and Molecular Biology, College of Preclinical Medicine, Southwest Medical University, Luzhou, China

**Correspondence: jxlu313@163.com; dulinyong@qq.com*

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Breast cancer is the most commonly diagnosed cancer among women, causing 15% of patient deaths. The metastasis of breast cancer cells is the leading cause of death for patients. Several studies have shown that Discoidin Domain Receptor 1 (DDR1) was highly expressed in breast cancer and could influence tumor cell behaviors. However, the specific role of DDR1 in breast cancer metastasis is still elusive. In this study, we uncovered that DDR1 is significantly increased in breast cancer and inversely correlated with the prognosis of patients. Knockdown of DDR1 suppressed the migration and invasion of breast cancer cells. Additionally, overexpression of DDR1 enhanced the metastatic capacity of cancer cells. Immunoblotting revealed that activation of Src and FAK, which are involved in cancer cell metastasis, were correlated with the expression level of DDR1. Co-immunoprecipitation experiments showed that DDR1 could bind to Src and FAK. Finally, the inhibition of FAK and Src could attenuate DDR1 enhanced migration ability of breast cancer cells. In summary, our study revealed that DDR1 was highly expressed in breast cancer and negatively correlated with the prognosis of breast cancer patients. DDR1 facilitates migration and invasion in breast cancer cells via activation of the Src-FAK signaling. Accordingly, blocking DDR1/Src/FAK axis is a promising therapeutic strategy for breast cancer treatment.

Key words: DDR1, breast cancer, Src, FAK, migration and invasion

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer death in women [1]. Although surgical resection, chemotherapy, and targeted therapy have been clinically applied to breast cancer treatment, the 5-year overall survival rate is still low due to disease recurrence and metastasis. Therefore, it is of great significance to further understand the molecular mechanisms of breast cancer metastasis, so as to find an effective treatment for the patients.

Discoidin Domain Receptor 1 (DDR1) is a unique set of receptor tyrosine kinases (RTKs) and have two members, DDR1 and DDR2. DDR1 is a type I transmembrane glycoprotein that is characterized by the presence of six distinct protein domains: a discoidin (DS) domain, a DS-like domain, an extracellular juxtamembrane (EJXM) region, a transmembrane (TM) segment, a long intracellular juxtamembrane (IJXM) region, and an intracellular kinase domain (KD) [2]. Accumulated studies have shown that DDR1 was upregulated in breast cancer, ovarian cancer, lung cancer, prostate cancer, and other cancers [3]. In addition, DDR1 is involved in the regulation of tumorigenesis and progression, including cell proliferation, epithelial-to-mesenchymal transition, migration, and invasion [4–6]. Thus, DDR1 is a promising target in tumor treatment. However, owing to the various factors such as structure specificity of DDR1, cell/tissue type specificity, and interaction with other molecules, the current research cannot fully define the network involved in the DDR1 signaling. Thus, elucidating the involved mechanism and role of DDR1 in breast cancer progression will be helpful for exploring novel therapeutic strategies in the treatment.

Src tyrosine kinase (Src) is a member of the Src family kinases (SFKs). SFKs are comprised of an amino-terminal membrane localization signal, also known as the Src homology 4 or SH4 domain, a poorly conserved unique domain, an SH3 domain, an SH2 domain, a tyrosine kinase domain, and a regulatory sequence [7]. Src normally remains inactive in normal cells, but its activity is altered in tumor cells [8, 9]. It has been reported that Src kinase activity had a rise of 4–20-fold in breast cancer tissues than in normal tissues, while Src protein expression was not changed [10].

Additionally, some studies suggest that Src was closely related to the growth and metastasis of breast cancer cells [11–13]. Src can bind to the cell membrane through N-myristoylation sites and thus interact with various cell surface receptors like integrins, epidermal growth factor receptor (EGFR) [14].

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that localizes to cellular focal adhesions and associates with a number of other proteins such as integrin [15]. FAK is involved in cellular processes such as adhesion, growth, apoptosis, and metastasis [16, 17]. Agochiya et al. found that elevated FAK expression was associated with the malignant potential and poor clinical prognosis of breast cancer [18]. Meanwhile, FAK acts as a substrate of Src, and the focal adhesion kinetics of FAK and Src deficient cells were dramatically decreased, indicating that Src and FAK were key molecules to promote cell migration and integrin-mediated adhesion [19, 20]. In 1990, Kanner et al. reported an interaction between FAK and Src in v-Src transformed cells [21]. The FAK autophosphorylation provides a high-affinity binding site for the SH2 domain of Src [22]. Moreover, the proline-rich sequence in upstream of FAK autophosphorylation site is a high-affinity binding site of the SH3 domain of Src [23]. Thus, the interaction between FAK and Src is mediated by the two sites and can promote the phosphorylation of FAK at Y397 [24]. Although activation of the Src-FAK signaling has been proved to promote proliferation and metastasis in breast cancer, hepatocellular carcinoma, bladder cancer, and other cancers [25–28], it remains unclear whether DDR1 regulates the migration and invasion by modulating the Src-FAK signaling in breast cancer.

In this study, we examined DDR1 mRNA levels in breast cancer tissues and protein levels in breast cancer cells. We also explored the functional role of DDR1 in breast cancer cell lines *in vitro*. Our results revealed that upregulation of DDR1 promotes the migration and invasion of breast cancer cells by the Src-FAK signaling. Our findings suggest that the upregulation of DDR1 plays a key part in the progression of breast cancer and targeting the DDR1/Src/FAK pathway is a novel method for blocking breast cancer metastasis.

Materials and methods

Antibodies and reagents. The DDR1 (5583), phospho-DDR1 (Tyr792, 11994), FAK (3285), phospho-FAK (Tyr397, 8556), Src (2109), phospho-Src (Tyr416, 2101) antibodies, and normal Rabbit IgG (2729) were purchased from Cell Signaling Technology (Danvers, MA, USA). The β-actin (AA128) antibody was obtained from Beyotime (Shanghai, China). FAK inhibitor Defactinib (HY-12289A) and Src inhibitor Dasatinib (HY-10181) were purchased from MedChemExpress (MedchemExpress, Monmouth Junction, NJ, USA).

Cell culture. Human breast cancer cell lines (MCF7, T47D, BT474, and BT549), normal human breast epithelial cell line (MCF10A), and 293T17 were obtained from the Cell Bank of the Shanghai Chinese Academy of Sciences (Shanghai, China). Breast cancer cell lines (SKBR3, HCC1954, MDA-MB-231, and SUM149PT) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, USA) supplemented with 10% fetal bovine serum (FBS; Lonsera, Uruguay), excluding the HCC1954 and SUM149PT cell lines. HCC1954 cell line was cultured in RPMI1640 (Sigma, USA) supplemented with 10% FBS. SUM149PT cell line was maintained in DMEM/F12 medium (Sigma, USA) supplemented with 5% FBS, 5 μg/ml insulin (Sigma, USA), and 1 μg/ml hydrocortisone (Sigma, USA). MCF10A cell line was cultured in the DMEM/F12 medium supplemented with 5% horse serum (Invitrogen), 20 ng/ml EGF (Peprotech), 100 ng/ml cholera toxin (Sigma), 10 μg/ml insulin (Sigma), and 0.5 μg/ml hydrocortisone (Sigma). Cells were incubated at 37 °C in a humidified incubator containing 5% CO_2.

Plasmids. Human DDR1 was cloned into the pCDH-SFB vector (pCDH/Flag-DDR1). The primers for expression plasmid construction were as follows: human DDR1, 5'-CTAGCTAGCATGGGACCAGAGGCCCTGTC-3' (forward) and 5'-ATAGTTTAGCGGCGCTCACTTGTCATCGTCGTCCTTGTAATCCACCGTGTTGAGTGCATCCT-3' (reverse).

DDR1 short-hairpin RNA (shRNA) lentiviral plasmids (pLKO.1/DDR1-sh798, pLKO.1/DDR1-sh800) and the random (control) shRNA (pLKO.1/NS) were designed and constructed by GeneChem Corporation (GeneChem, Shanghai, China). Human DDR1 shRNA oligonucleotides were used as follows: 5'-TACCTCAACGACTCCACCTAT-3' for shRNA798 and 5'-CAGGAGGTGATCTCAGGCAAT-3' for shRNA800.

Lentivirus infection. 293T17 cells were plated at a density of 2.5×10^7/10 cm^2 dish. Lipofectamine 3000 was used per the manufacturer's instructions (Invitrogen). The supernatant was collected 72 h post-transfection. Target cells were pretreated with 8 μg/ml polybrene (Sigma) at 30% confluence and then were infected with corresponding viruses. Stable cells expressing shDDR1 were selected with puromycin (Takara, Japan) post-infection.

Immunoblot analysis. Extraction of proteins from breast cancer cells was performed by using lysis buffer (1× SDS, 62.5 mM Tris-HCl [pH 6.8], 2% SDS, 0.002% bromophenol blue, 50 mM DTT, 10% glycerol). An aliquot of 20 μg denatured protein from each sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, blocked with 5% non-fat dried milk for 2 h at room temperature, and incubated overnight at 4 °C with the primary antibodies as indicated. The membranes were washed four times with Tris-buffered saline/0.05% Tween-20 solution (TBST), incubated with secondary antibody (horseradish peroxidase-conjugated, goat antibodies to rabbit or mouse IgG, 1:5000 dilution; Beyotime, Shanghai, China)

for 1 h at room temperature, washed three times with TBST, and developed by using chemiluminescence (ECL) reagent (Beyotime). Membranes were evaluated with ChemiDoc MP Imaging System (Bio-Rad, USA) and were analyzed using the Image Lab 5.0 software (Bio-Rad).

Proliferation, migration, and invasion assays. Cell growth was assessed by using Cell Counting Kit (CCK)-8 (Dojindo, Kumamoto, Japan), according to the manufacturer's instructions. Briefly, cells (2×10^3/well) were seeded into 96-well plates with 100 µl in the medium containing 10% FBS. Absorbance at OD450 was measured for 5 consecutive days (0, 24, 48, 72, and 96 h) and used to plot cell growth curves. Migration and invasion assays were performed using 24-well Transwell chambers with 8 µm pore polycarbonate membrane inserts (Corning, USA). For migration assays, cells (MCF7: 8×10^4, HCC1954: 2×10^4, MDA-MB-231: 2×10^4) were seeded in the upper chamber in a 200 µl serum-free medium, while 600 µl medium with 20% FBS were placed into the lower chambers as a chemoattractant. After a certain time (MCF7: 48 h, HCC1954: 24 h, MDA-MB-231: 24 h), the chamber was fixed with 4% paraformaldehyde and stained with 0.5% crystal violet for 15 min, respectively. Cells permeating the subsurface of the filter membrane were counted in five randomly selected fields using a microscope (Eclipse TS100; magnification, ×10; Nikon Corporation, Tokyo, Japan). Cell invasion assays were performed with the Transwell system as in the cell migration assay, except that trans coated filters in 24-well plates were incubated with diluted Matrigel (40 µl/chamber, 1:10 dilution; BD Biosciences, Bedford, MA, USA) for 6 h, and the upper chambers were hydrated 100 µl of serum-free medium for 30 min at 37 °C. The rest of the procedures were the same as the cell migration assay as described above, except those cells (MCF7: 10×10^4, HCC1954: 2×10^4, MDA-MB-231: 4×10^4) were seeded into the upper chamber.

Wound healing assay. Cells were grown in 12-well plates at 80–90% confluency. A linear wound was scratched with a 200 µl sterile pipette tip across the monolayers. After washing with PBS to remove cell debris, adherent cells were incubated in a medium with 2% FBS. Wounded monolayers were photographed every 12 h for 24 h at ×100 magnification under a microscope (Nikon, Tokyo, Japan). The percentage of wound closure was calculated as follows: percentage of wound closure = 1 – (widtht/width0) × 100%.

Inhibition experiments. To evaluate the inhibitory activity of FAK or Src inhibitor (Defactinib or Dasatinib), cells were seeded into 12-well plates and incubated overnight. Then Defactinib or Dasatinib was added at indicated concentrations. After 24 h, proteins were extracted for western blot. Cell migration assay was performed as described above except that inhibitor-treated media (Dasatinib: 10 nM, Defactinib:100 nM) were used in both the upper and lower chambers, and serum was added to the lower chamber only.

Immunoprecipitation. Breast cells were grown to 80–90% confluency and washed three times with ice-cold PBS before being lysed in IP lysis buffer (Thermo Fisher Scientific, USA). Then the lysates were incubated with antibodies or flag beads overnight at 4 °C. Pierce Protein A/G Agarose beads were added and incubated with the lysates for 3 h at 4 °C. The beads were collected and washed with lysis buffer three times. The precipitated proteins were eluted and denatured in 1× SDS loading buffer and heated at 100 °C for 5 min. After that, the samples were analyzed by western blot.

Statistical analysis. Statistical analysis was performed with SPSS17.0 software (Chicago, IL, USA). Quantitative data are presented as means ± SD. Unpaired two-tailed Student's t-test was used to compare data between the two groups. One-way ANOVA with Bonferroni's multiple comparison test correction was used to analyze data among multiple groups. A p-value <0.05 was considered statistically significant (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001), otherwise not significant (ns).

Results

Abnormal expression of DDR1 in breast cancer. To identify the DDR levels in breast cancer, we analyzed DDR1 and DDR2 mRNA levels in 1,108 breast cancer tissues and 113 adjacent normal tissues in TCGA database. The mRNA levels of DDR1 were significantly higher in breast cancer tissues than in adjacent normal tissues. By contrast, DDR2 levels were higher in adjacent normal tissues (Figure 1A). We next examined the protein levels of DDR1 and DDR2 in eight breast cancer cell lines (MCF7, T47D, BT474, SKBR3, HCC1954, SUM149, BT549, MDA-MB-231) and normal breast epithelial cell line MCF10A (Figure 1B). The protein levels of DDR1 were significantly higher than those of DDR2 in breast cancer cells. In addition, Figure 1B also showed that DDR1 protein levels were not related to the breast cancer subtype. Compared with DDR2, DDR1 may play a dominant role in the occurrence and development of breast cancer, that's why we focused the role of DDR1 in this study. We analyzed the correlation between DDR1 and the prognosis of breast cancer patients by the Kaplan-Meier Plotter online tool. DDR1 mRNA level was negatively correlated with the prognosis of breast cancer patients (Figure 1C).

DDR1 facilitates the migration and invasion of breast cancer cells. Previous studies have shown that DDR1 has different effects due to the complexity of the DDR1 signal network. DDR1 signals have high cell/tissue type specificity and dependence. For exploring the role of DDR1 in breast cancer cells, we knocked down DDR1 in breast cancer cell lines with high DDR1 levels (MCF7 and HCC1954) by two independent shRNAs, and overexpressed DDR1 in breast cancer cell line with low endogenous DDR1 levels (MDA-MB-231). The p-DDR1 (Tyr792) level was positively correlated with the DDR1 protein level (Figure 2A). And then, the CCK-8 cell viability assay was used to assess the effect of DDR1 on cell proliferation. Neither DDR1 knockdown nor overexpression affected the growth kinetics of MCF-7 and

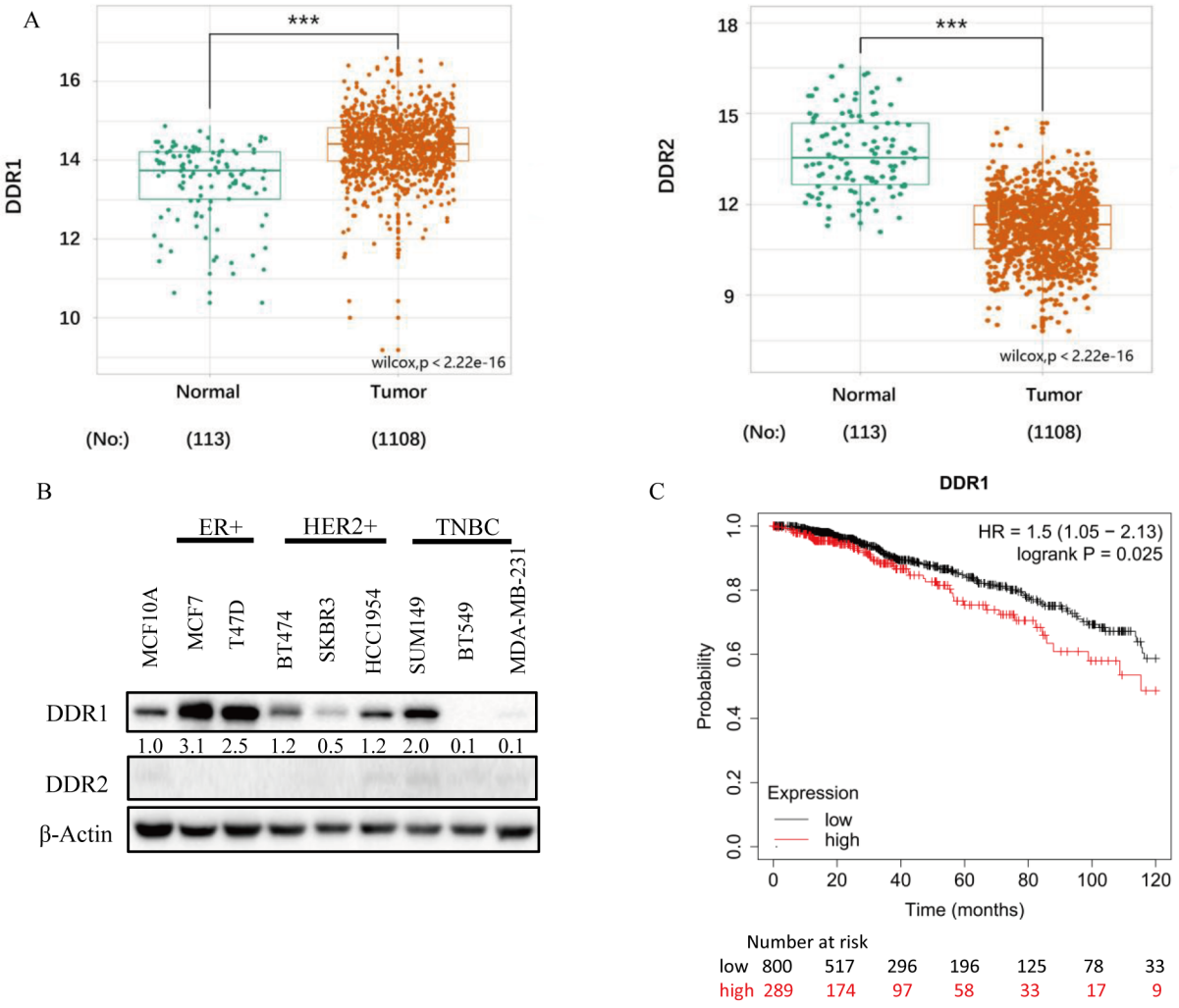

Figure 1. Abnormal expression of DDR1 in breast cancer. A) Comparison of DDR1 and DDR2 transcriptional levels in 1,108 breast cancer tissues and 113 adjacent normal tissues in TCGA database. B) Detection of DDR1 and DDR2 protein levels in breast cancer cell lines and normal breast epithelial cells by western blot. Abbreviations: ER+ – estrogen receptor-positive breast cancer; HER2+ – positive breast cancer; TNBC – triple-negative breast cancer. C) Analysis of the correlation between DDR1 expression level and prognosis of breast cancer patients by the Kaplan-Meier Plotter online tool.

HCC1954 cells, or MDA-MB-231 cells (Figure 2B). Transwell migration and invasion assays were performed to detect the effects of DDR1 levels on migratory behaviors (Figures 2C, 2D). Knockdown of DDR1 in MCF7 and HCC1954 cells could inhibit the migration and invasion in comparison to the control group (Figure 2C). Conversely, the migration and invasion of the -MB-231 cell line with overexpression of DDR1 were notably increased (Figure 2D). Wound-healing assay was performed to quantify the DDR1 overexpression on MDA-MB-231 cells migration ability to further verify the reliability of the results (Figure 2E). Taken together, DDR1 could promote the migration and invasion of breast cancer cells without causing proliferation.

Inhibitors of Src and FAK selectively suppress the migration of breast cancer cells with high expression of DDR1. Based on role of DDR1 in breast cancer cells migration and invasion, we wondered which molecules mediate the effect of DDR1. It has been reported that Src is highly activated in breast cancer and is closely related to its metastasis. Furthermore, FAK is a tyrosine kinase that also plays a key role in tumor migration and invasion. Therefore, we selected Dasatinib (Src inhibitor) and Defactinib (FAK inhibitor) to evaluate their efficacy on migration of MDA-MB-231 cells with overexpression of DDR1. After being treated with Src inhibitor, migration of MDA-MB-231, which overexpressed DDR1, was significantly inhibited, while the control group was not changed significantly (Figure 3A). Subsequently, and similar result was shown in MDA-MB-231 cell overexpressing DDR1 or control vector, which were treated with FAK inhibitor (Figure 3C). Meanwhile, Figures 3B and 3D

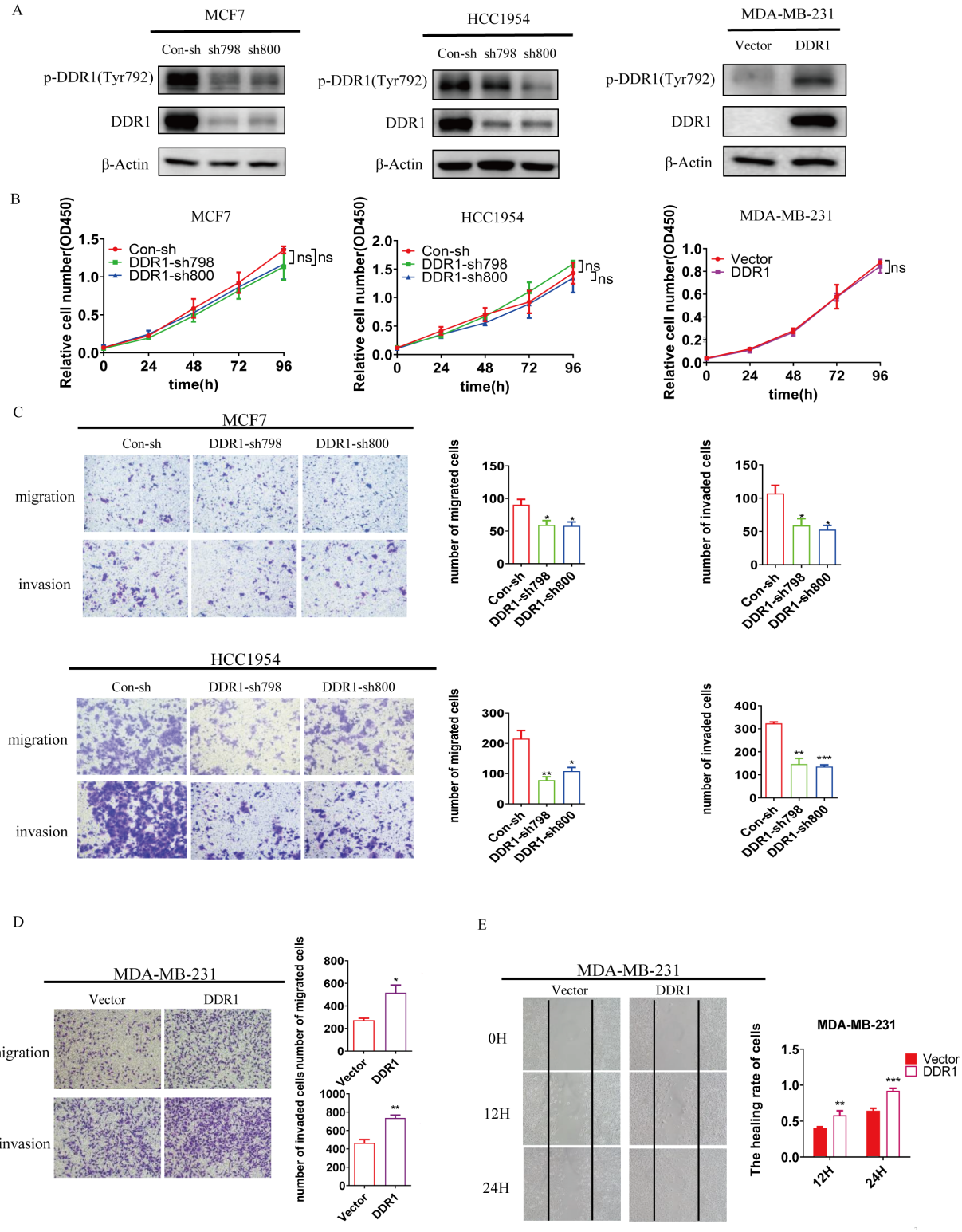

Figure 2. DDR1 facilitates the migration and invasion of breast cancer cells. A) Validation of DDR1 and p-DDR1 (Tyr792) expression levels by western blot. MCF7 and H1954 cells expressing control-shRNA (con-sh) and two different DDR1-shRNAs (sh798and sh800), and MDA-MB-231 cells with vector alone and DDR1 overexpression. B) Overexpression or knockdown of DDR1 does not affect the growth of breast cancer cell lines. C–D) DDR1 knockdown reduces and DDR1 overexpression promotes migration and invasion in breast cancer cells. E) Wound-healing assay was performed to quantify the DDR1 overexpression MDA-MB-231 cells' and control cells' migration ability. *p<0.05, **p<0.01, ***p<0.001

revealed that two inhibitors could significantly block its own targets activation.

Ectopic expression of DDR1 activates the Src-FAK signaling in breast cancer cells. Previous studies showed that Src and FAK mediated the regulation of DDR1 on the migration and invasion of breast cancer cells. To investigate the specific mechanism of DDR1 in breast cancer cells, we detected the levels of phosphorylation and protein of Src and FAK in DDR1 knockdown and overexpression cells. Knockdown of DDR1 significantly reduced phosphorylation levels of Src and FAK (Figure 4A). Overexpression of DDR1 increased their phosphorylation levels (Figure 4B). Some reports revealed that Src could mediate the movement of tumor cells by activating FAK [19]. Interestingly, other papers displayed that FAK was upstream of the signal and could activate Src to promote the growth and metastasis of hepatocellular carcinoma [29]. Aiming to verify the relationship between Src and FAK, we detected p-Src (Tyr416) and p-FAK (Tyr397) in the MDA-MB-231 cell line with overexpression of DDR1 after using Src and FAK inhibitors (Dasatinib; Defactinib) (Figure 4C). From the graph, we found that DDR1-stimulated FAK phosphorylation was blocked by Dasatinib, while Defactinib cannot impede Src phosphorylation, confirming that FAK is a downstream target of DDR1/Src. Additionally, it was observed that total FAK levels slightly increased in the Dasatinib-treated cells. Both Src and FAK could bind to RTKs and be activated by RTKs, while whether DDR1 could bind to FAK and Src was not elucidated in breast cancer cells. Thus, an endogenous co-immunoprecipitation assay was performed in MCF7 cells. The result showed that endogenous DDR1 can interact with Src and FAK (Figures 4D, 4F). To further validate the interaction between them, an exogenous co-immunoprecipitation assay was completed in MDA-MB-231 cells transfected with pcDH/Flag-DDR1 (Figures 4E, 4G).

Discussion

DDRs have been associated with a variety of diseases, including cardiovascular diseases [30, 31] and various cancers [32–38]. However, there are only a few studies showing the expression and function of DDRs in breast cancer. In this

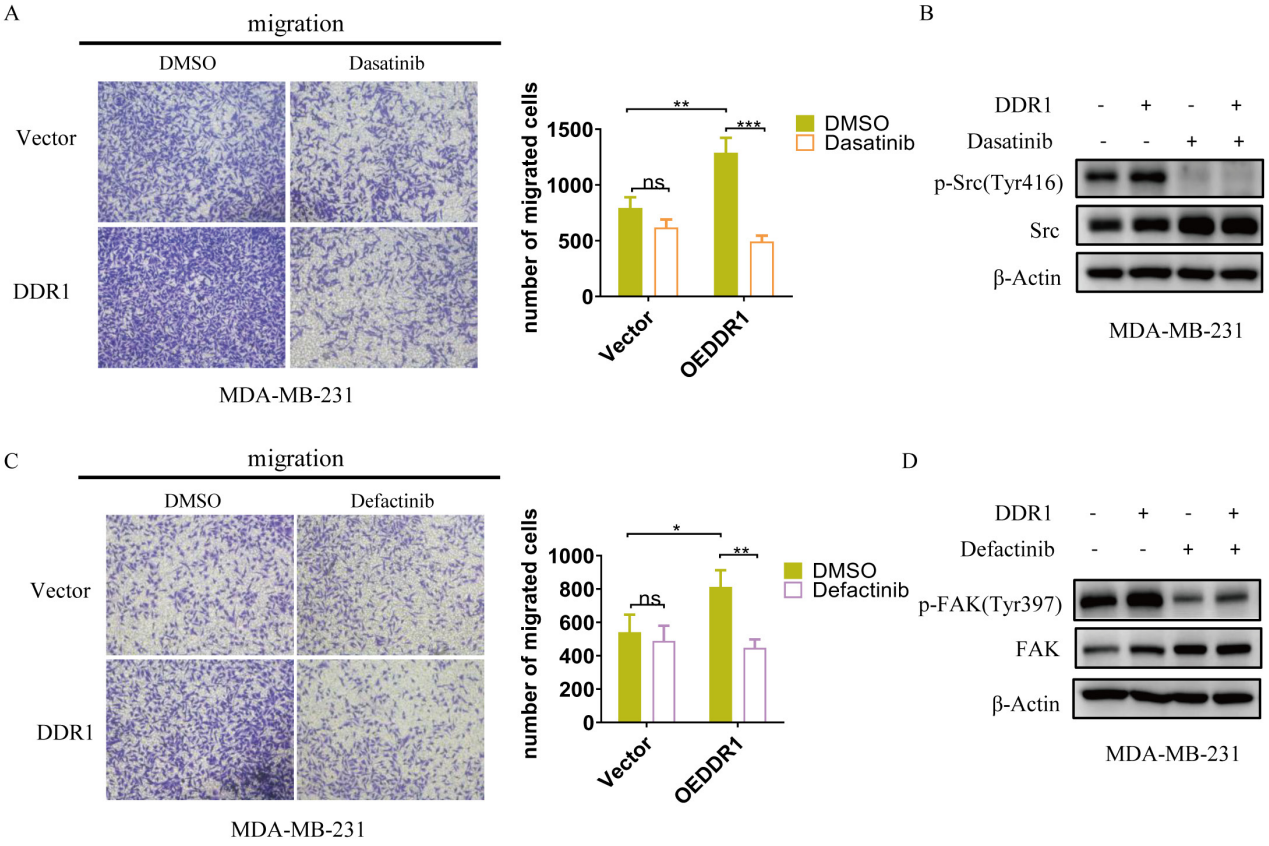

Figure 3. Inhibitors of Src and FAK selectively suppress the migration of breast cancer cells with high expression of DDR1. A) Transwell assay was performed to detect migration capacity in DDR1 overexpression MDA-MB-231 cells, which were pretreated with Dasatinib (10 nM). B) Identification of the inhibitory effect of Dasatinib. C) Transwell assay was performed to detect migration capacity in DDR1 overexpression MDA-MB-231 cells, which were pretreated with Defactinib (100 nM). D) Identification of the inhibitory effect of Defactinib. *p<0.05, **p<0.01, ***p<0.001

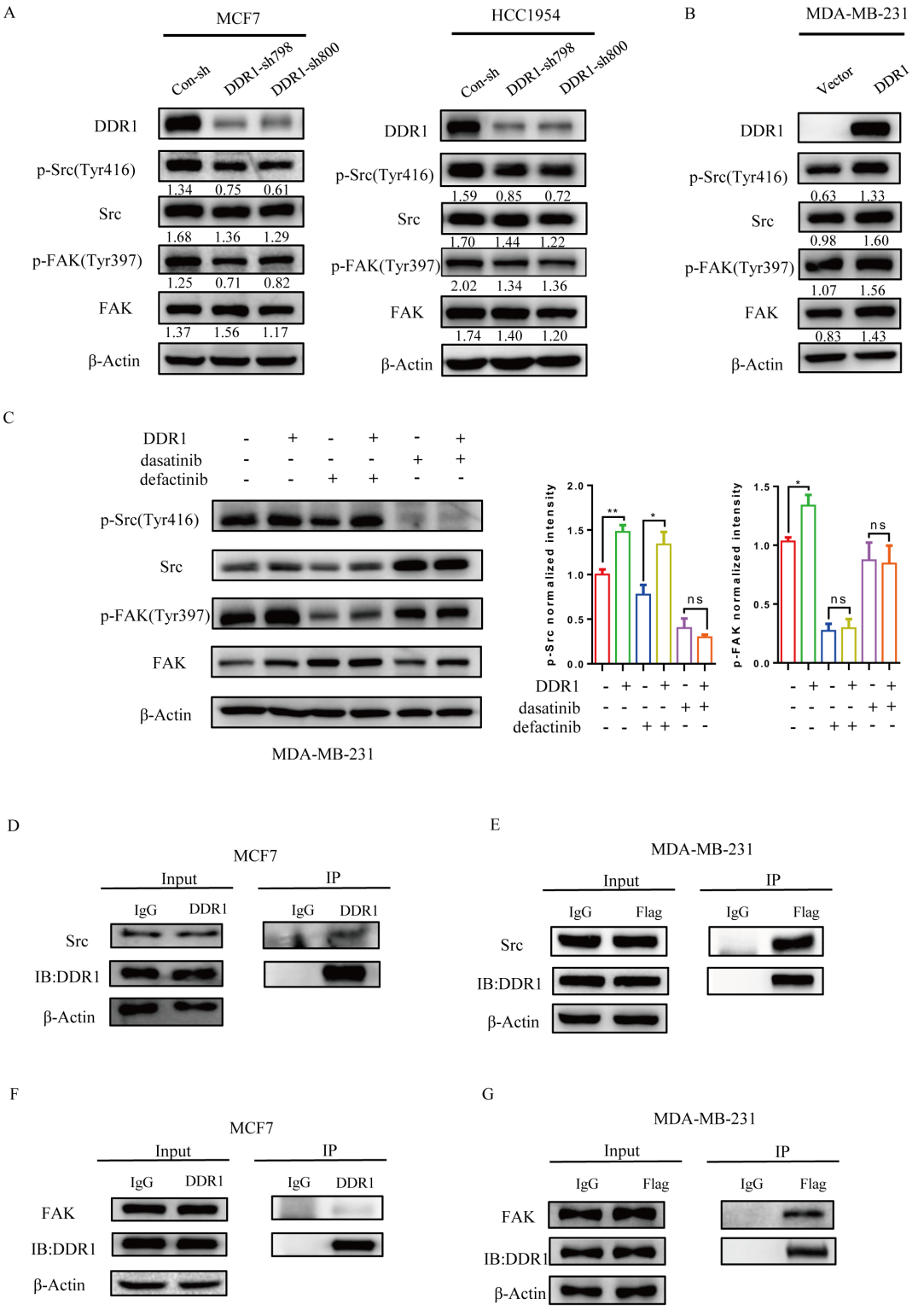

Figure 4. Ectopic expression of DDR1 activates the Src-FAK signaling in breast cancer cells. A) p-Src (Tyr416) and p-FAK (Tyr397) levels were decreased in DDR1 knockdown MCF7 or HCC1954 cell lines. B) p-Src (Tyr416) and p-FAK (Tyr397) levels were elevated in DDR1 overexpression MDA-MB-231 cells. C) Western blot assay was performed to detect p-Src (Tyr416) and p-FAK (Tyr397) levels in DDR1 overexpression MDA-MB-231 cells, which were pretreated with Src inhibitor (Dasatinib) and FAK inhibitor (Defactinib) and the graphs panels are shown. *p<0.05, **p<0.01, ***p<0.001. D–F) Endogenous DDR1 can interact with Src and FAK. E–G) Exogenous DDR1 can interact with Src and FAK.

study, we analyzed mRNA levels of DDRs in breast cancer tissues in TCGA database and found that mRNA levels of DDR1 in breast cancer tissues were increased, by comparing with adjacent tissues. Subsequently, analyzing the protein levels of DDR1 and DDR2 in breast cancer cell lines, we also demonstrated that DDR1 was significantly higher than DDR2. These results are consistent with a previous study [39]. Kaplan-Meier Plotter showed that DDR1 mRNA level is adversely correlated with the prognosis of breast cancer patients. Although DDR1 has been reported to associate with the progression of breast cancer, the effects remain controversial. Castro-Sanchez et al. reported that native type IV collagen induces cell migration through a CD9 and DDR1-dependent pathway in MDA-MB-231 breast cancer cells [40]. However, the promoting cell migration by DDR1 was dependent on its regulation of tyrosine protein kinase (Syk) activity in MDA-MB-468 breast cancer cells [41]. In addition, DDR1 regulates cell spreading and motility by associating with myosin IIA in MCF-7 cells [42]. However, Koh et al. revealed that overexpression of DDR1 can inhibit the migration ability of HS587T breast cancer cells in a 3D culture system which contained type I collagen [43]. Additionally, DDR1 can inhibit cell migration only when it is co-expressed with dopamine and cAMP-regulated neuronal phosphoprotein 32 (DARPP-32) in MDA-MB-231 breast cancer cells [44].

Thus, the role of DDR1 in breast cancer progression is still controversial. In the present study, we found that DDR1 showed a relatively low expression level in the MDA-MB-231 cell line, which was consistent with other studies [43, 44]. Thus, we knocked down DDR1 in MCF7 and HCC1954 cell lines, which have considerable expression level of DDR1, and overexpressed DDR1 in the MDA-MB-231 cell line, respectively. We found that p-DDR1 (Tyr792) levels positively correlate with protein levels of DDR1. Although some studies showed that DDR1 activation needs to bind with collagen [45, 46], it has also been found that the biological function of DDR1 is not entirely dependent on the extracellular signal stimulation of collagen. Chen et al. showed that the DDR1 phosphorylation level is related to DDR1 protein level and not responsive to collagen stimulation in oral squamous cell carcinoma [47]. Our study also supported that the activation of DDR1 was not entirely dependent on stimulation of collagen. Our study revealed that DDR1 promotes the migration and invasion of breast cancer cells without affecting cell proliferation. HCC1954 cell line has low adhesion ability and the migration ability of MCF7 cells is extremely weak after scratch treatment [12], that's why we did not present the results of a wound-healing assay in cell lines with knockdown of DDR1.

Numerous of studies have shown that both Src and FAK were involved in the metastasis of tumor [12–14, 16], we wondered whether inhibiting Src or FAK activity was an effective method to retard migration and invasion of breast cancer cells with high expression of DDR1. Indeed, the migration of MDA-MB-231, which overexpressed with DDR1, was greatly attenuated by Src or FAK inhibitor, suggesting that Src and FAK were involved in DDR1 enhanced migration of breast cancer cells. Furthermore, activation of Src and FAK was positively correlated with expression level of DDR1. Inhibition experiment demonstrated that Src was the upstream signal of FAK in breast cancer DDR1 signaling. As shown in Figure 4C, p-FAK (Tyr397) was significantly decreased after treatment with the Src inhibitor (Dasatinib) in MDA-MB-231 breast cancer cells. Additionally, it was observed that Dasatinib could regulate the total FAK levels as well. As for why Dasatinib affects FAK protein levels, we speculated that Dasatinib use may affect FAK protein degradation [48]. To investigate the specific mechanism, co-immunoprecipitation assays were performed in MCF7 and DDR1 overexpression MDA-MB-231 cells and suggested that DDR1 can interact with Src and FAK. One study has shown that native type IV collagen induces MMP-2 and MMP-9 production and tumor invasion through a DDR1 and Src-dependent pathway [49]. We examined the effect of DDR1 on the expression of MMP-2 and MMP-9 and revealed that DDR1 did not influence the MMP-2 and MMP-9 expression (data not shown), indicating that DDR1 could exerts its function in cancer invasion not via MMP-2 and MMP-9.

In summary, our study revealed a novel pathway regulating the migration and invasion of breast cancer cells in which DDR1 can interact with Src and FAK, resulting in the activation of Src-FAK signaling and promoting breast cancer migration and invasion (Figure 5). This study suggested that

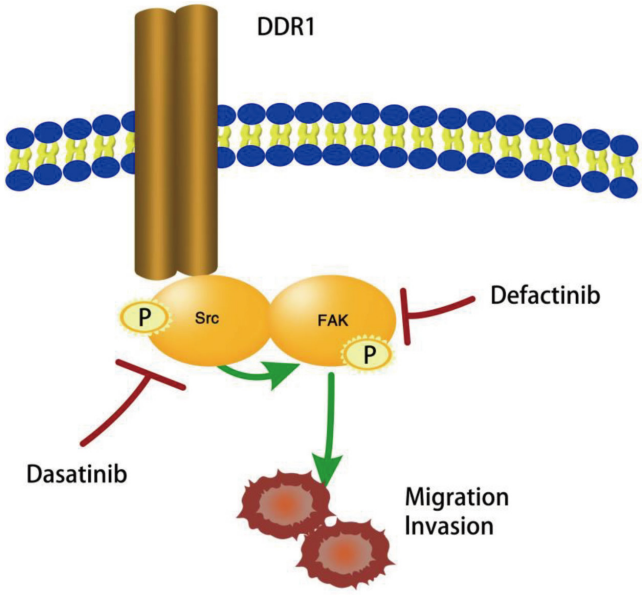

Figure 5. Schematic diagram of DDR1 promoting the migration and invasion of breast cancer cells. DDR1 interacts with Src and FAK and activates the FAK-Src signaling to promote the migration and invasion of breast cancer cells.

blocking DDR1/Src/FAK is a novel therapeutic strategy for breast cancer treatment according to the molecular mechanism of DDR1 in breast cancer metastasis.

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