Nilotinib, a Discoidin domain receptor 1 (DDR1) inhibitor, induces apoptosis and inhibits migration in breast cancer

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Abstract

Background: Overexpression of Discoidin domain receptor 1(DDR1) is known to enhance the malignancy of breast cancer considerably. This study reports the identification of a potent DDR1 inhibitor, Nilotinib for the treatment of breast cancer. Methods: MTT assay was used to evaluate the inhibitory activity of Nilotinib and meantime we used flow cytometry to evaluate the pro-apoptotic activity of Nilotinib against MCF-7 and MDA-MB-231. Expression of DDR1 was manipulated in MCF-7 cell lines with low level DDR1 expression by transfecting with plasmids containing by shRNA. The effect of DDR1 or treatment with Nilotinib on cell migration was assayed. The expression of p-DDR1, DDR1, p-ERK1/2, ERK1/2 and E-cadherin、Vimentin, Snail1 were detected by western blot and immuno-fluorescent staining . Results: Nilotinib against MCF-7 (IC 50 =0.403 μM) and MDA-MB-231 (IC 50 =0.819 μM) and also indicated induced apoptotic cell death after co-cuturing with Nilotinib (500 nM), apoptosis rate is 29.60 % ± 2.19% and 18.75 % ± 2.30% respective ly. Moreover, Nilotinib effectually blocked cellular migration of MCF-7. Interestingly, Knock-down DDR1 could significantly block the migration of breast cancer, meantime the sensitivity of Nilotinib to MCF-7 was reduced. Conclusion: Targeting DDR1 therapeutically could potentially affect survival and influence metabolism in breast cancer, and Nilotinib could as a candidate for the treatment of breast cancer.

Background

Breast cancer is the most common type of malignancy in women and is the second leading cause of female cancer mortality in the world[1-3]. Recent reports have revealed that breast cancer is responsible for 6% of the total 7.5 million cancer mortalities worldwide[3, 4]. Although numerous advanced diagnostic and therapeutic methods have been used in the treatment of breast cancer, but its metastasis is still the primary cause of death[5, 6].

Numerous studies have demonstrated the critical role played by collagen in cellular proliferation, adhesion, migration, and invasion in breast carcinoma[7, 8]. Discoidin Domain Receptors (DDRs) are a family of receptor tyrosine kinases (RTKs), which are activated in response to collagen, and are composed of two highly homologous subtypes, DDR1 and DDR2[9, 10]. DDR1 is known to play a vital role in breast cancer metastases[11]. Overexpression of DDR1 is associated with poor prognosis of breast cancer, sometimes even lethal, in the patients[12, 13]. Activation of DDR1 by collagen induces MAPK signaling pathway, thus promoting the proliferation of malignant breast cancer cells[14, 15]. However, DDR1 is also known to stabilize E-cadherin and facilitate cellular migration by suppressing actomyosin at the sites of cell-cell contact[16, 17]. Thus, blocking the phosphorylation of DDR1, resulting in its reduced expression might be an effective strategy for the treatment of breast cancer.

Several reports have verified that Nilotinib is a potent DDR1 inhibitor. Clinical studies have also approved its use as a BCR-ABL kinase inhibitor[18-20]. It has been reported that Nilotinib can treat Chronic Myeloid Leukemia (CML) by inhibiting DDR1 phosphorylation[21]. Additionally, Nilotinib has been identified as a brand-new method for the treatment of metastatic colorectal cancer by the inhibition of DDR1-BCR

signaling pathway[20, 22]. However, there are no reports on the evaluation of Nilotinib for the treatment of breast cancer. In the present study, our main aim is to illuminate the biological role of the DDR1 signaling pathway in breast carcinoma and to evaluate the inhibitory activity of Nilotinib against breast cancer

Methods

Cell culture

Human breast cancer cell lines MCF-7 and MDA-MB-231(obtained from ATCC) were grown in DMEM (GIBCO) containing 10% FBS (GIBCO). The cell lines were maintained at 37°C in a 5% CO_2 incubator.

MTT Assay

MCF-7, MDA-MB-231, and MCF-7 SH*DDR1* were separately cultured in 10% FBS growth medium in 96-well plates (5000 cells/well) overnight. The cells were treated with various concentrations of Nilotinib (n=11; 14.5-30,000 nM) and cultured in 10% FBS medium for 48 h in triplicates. The control cells were treated with dimethyl sulfoxide (DMSO) only. Next, tetrazolium dye (MTT) solution (5 mg/mL, 20 µL/well) was added to each well. After incubation for 4 h at 37°C, the supernatant was aspirated, and the generated formazan crystals were dissolved in 150 µL of DMSO, and the absorbance was recorded spectrophotometrically at 490 nm using an enzyme-linked immunosorbent assay plate reader. The data were calculated using Graph Pad Prism version 5.0. The fitting of the IC_{50} values was done using a non-linear regression model with a sigmoidal dose-response.

Cell Apoptosis Assay

MCF-7 and MDA-MB-231 (3×10^5) cells were seeded into 6-well plates overnight. Fresh growth media with Nilotinib (500 nM) as well as medium with 1‰ DMSO (control) was added to the plates. After 48 h, the growth medium was collected, and cells were trypsinized and collected with the corresponding medium. After centrifugation at 1000 × g at 4 ºC for 3 min, the supernatant was removed completely, and the cells were washed twice with cold PBS. Then, 100 µL of 1× binding buffer, 5 µL PI (PI, BD) and 5 µL annexin-V (FITC-Annexin V, BD) were added. The cells were then gently vortex-mixed and incubated for 15 min at room temperature in the dark and 1× binding buffer was used for dilution to 500 µL. The cells were then stained with PI, and Annexin-V alone as a positive control. The samples were measured using a BD Accuri™ C6 flow cytometer (Becton Dickinson), and the data were processed using FlowJo 7.6.1.

Western blotting

Protein extracts were obtained using the KEYGEN total protein extraction kit (Nanjing, China). The concentration of protein in the supernatant fractions were determined using a BCA Protein Assay Kit (Pierce). Sixty micrograms of protein per sample were loaded and separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), followed by electrophoretic transfer onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After blocking with 5% skim milk in Tris-buffered saline containing 0.05% Tween-20, the membrane was first incubated with the indicated primary antibody at 4°C overnight. Signals were detected using an ECL+TM Western blotting system (Bio-Rad, Hercules, CA). Primary antibodies used were: antiphospho-DDR1 (cst#11994), anti-DDR1, anti-ERK1/2, antiphospho-ERK□E-Cadherin, Vimentin, Snail 1, and ant-GAPDH (Cell Signaling Technology).

Wound-healing assay

MCF7 and MCF-7 SH*DDR1* cells were seeded in 6-well plates and incubated at 37°C overnight. After reaching 100% confluence, a straight line of similar width was scratched across the monolayer using a 10-μL pipette tip, for each sample. After washing with PBS to remove non-adherent cells, cells were then treated with the indicated concentrations of Nilotinib and incubated for 24 h. When the wound in the DMSO control was healed, the image from the original scratch line was captured by Leica DFC 3000 G.

Immunofluorescence staining

Cells were seeded in a confocal dish, fixed with ice-cold 4% paraformaldehyde and incubated with 0.5% bovine serum albumin in phosphate-buffered saline (PBS) with 0.1% Triton X-100 for 30 min. After washing, cells were incubated with anti-DDR1 antibody before rinsing with PBS, then immunostained with secondary antibodies and stained with 4',6-diamidino-2-phenylindole (DAPI) (2 mg/ml). Fluorescence images were captured using a confocal microscope (Leica TCS SP2, Germany).

Lentiviral production and transduction

For viral creation, *DDR1* shRNA was designed based on the *DDR1* mRNA sequence (GenBank accession no. NM-001954), The shrna#1 sequence was GTGTGGCTCGCTTTCTGCAGT, and shrna#2 sequence was GGACTATATGGAGCCTGAGAA. Each of these sequences were cloned into a plko.1 lentiviral vector. The digestion analysis of restriction endonuclease confirmed the recombinant vector, and all inserted sequences were verified by DNA sequencing. Lentiviruses were developed by triple transfection of 80% confluent 293T cells with *DDR1* shRNA-expressing vector and the virion-packaging elements (pVSVG-I and pCMVΔR8.92) using Lipofectamine 2000 (Invitrogen). They were harvested in a serum-free medium after 48 hours and filtered through a 0.45mm filter (Millipore, Bedford, MA). Retroviruses harboring shRNA sequence were transduced into MCF-7. After incubation for 48 hours, the transduced cells were

positively selected in puromycin (1μg/mL), passaged, harvested, and named MCF-7 SH*DDR1*#1 and MCF-7 SH*DDR1*#2.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 5 software. Differences were analyzed using one-way analysis of variance followed by Tukey's post hoc test for multiple comparisons and Student's t-test for two comparisons. Data were presented as the means ± SD. *, ** and *** indicates $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Results

Nilotinib manifests anti-proliferation and pro-apoptosis activity in breast cancer.

Cytotoxicity of Nilotinib against breast cancer cell lines was assessed by the MTT assay. The breast cancer cells were exposed to different concentrations of Nilotinib for 48 h. As shown in Figure 1A, the cell viability of MCF-7 and MDA-MB-231 were prominently decreased by Nilotinib in a dose-dependent manner, with IC_{50} values of 0.403 μmol/L and 0.819 μmol/L, respectively. Also, we investigated whether Nilotinib inhibited the colony formation capability of breast cancer cells, a part of the tumor proliferation process. It was evident that Nilotinib effectively inhibited the growth capacity of MCF-7. As shown in Figure 1B, the MCF-7 cells exhibited a high sensitivity to the Nilotinib treatment even at a low concentration of 0.5 μmol/L.

Then we measured the apoptotic cell death by Nilotinib with Annexin V-FITC/PI staining. As shown in Figure 1C and 1D, the rate of apoptosis in MCF-7 and MDA-MB-231, when treated with Nilotinib was 29.6% and 18.8% respectively. This indicated that Nilotinib could promote cellular apoptosis in both MCF-7 and MDA-MB-231 cell lines.

Nilotinib inhibits breast cancer migration

To further characterize Nilotinib, its effect on cellular migration was evaluated through a wound healing assay in the breast cancer cell line (MCF-7). Significant blocking of the cellular migration was observed at three concentrations (50 nM, 100 nM, and 500 nM) of Nilotinib as compared to the DMSO group (Figure 2A). All the chosen concentrations exhibited low levels of cytotoxicity. Therefore, Nilotinib inhibited wound healing mainly by the inhibition of cellular migration instead of blocking cell proliferation.

Vimentin is an intermediate cell filament protein that is commonly considered as a mesenchymal marker, Snail1 plays an important role in embryonic development, tumor invasion, and metastasis, and E-cadherin is as an epithelial marker. After the wound-healing assay, total protein of the cells was collected, and the level of expression of E-cadherin, Vimentin, and Snail1 was evaluated. As shown in Figure 2B and

2C, after treatment with Nilotinib, levels of E-cadherin increased, Vimentin was almost not expressed in cells, and the expression of Snail1 was inhibited. This implied that Nilotinib could reverse EMT (epithelial-mesenchymal transition) in breast cancer.

Nilotinib targeted DDR1 and inhibited the phosphorylation of DDR1, MAPK signaling pathway

All the experimental results indicated towards the inhibitory potential of Nilotinib towards breast cancer, but the mechanism of action was still unknown. Previous reports have revealed Nilotinib to be a potent inhibitor of DDR1. Therefore, the content of the phosphorylation of DDR1 protein was tested (Figure 3A and 3B). As expected, it was revealed that after treatment with Nilotinib, phosphorylation of DDR1 protein decreased in a dose-dependent manner. Further investigation along the downstream pathway revealed that the MAPK signaling pathway was blocked.

As shown in Figure 4, immunofluorescence was used to detect the level of expression of proteins in the phosphorylation of DDR1 in MCF-7 cells after treatment with Nilotinib. Three different concentrations of Nilotinib (500 nM, 1000 nM, and 5000 nM) were chosen, to further verify the inhibitory activity of Nilotinib on DDR1 phosphorylation. It was revealed that Nilotinib targeted DDR1 to inhibit its activity.

DDR1 is required for migration of breast cancer and Nilotinib targets DDR1 to inhibit proliferation in cancer breast.

To examine whether the targeted downregulation of DDR1 in MCF-7 cells affected its migration and proliferation, genetic knockdown of *DDR1* via lentiviral shRNA-mediated RNA interference was done. As shown in Figure 5A, levels of DDR1 protein was significantly decreased in MCF-7 SH*DDR1* as compared to MCF7-control. In order to investigate whether Nilotinib targeted DDR1 to inhibit the growth of MCF-7, both MCF-7 and MCF-7 SH*DDR1* were treated with Nilotinib. As shown in Figure 5B, Nilotinib had no effect on MCF-7 SH*DDR1*. Next, migration was compared between MCF-7 to MCF-7 SH*DDR1* via wound healing. Interestingly, knockdown *DDR1* could effectively block migration (Figure 5C). Then, the total cellular protein was collected, as shown in Figure 5D, and it was observed that the expression levels of E-cadherin increased, the expression levels of Vimentin, and Snail1 decreased. These results are consistent with the results from treatment with Nilotinib.

Discussion

Metastasis is a sequential process that allows cancer cells to move from the primary tumor site and grow elsewhere in the human body[5, 23]. This is still the leading cause of death in patients with breast cancer[24, 25]. DDR1, a kind of receptor tyrosine kinase, is overexpressed in several malignant tumors and plays a vital role in cancer progression and metastasis[11]. Nilotinib has been reported as a DDR1 inhibitor, and Jeitany Maya et al[20]. demonstrated an additional important DDR1 kinase-dependent

function in CRC metastasis formation. Moreover, they reported that pharmacological inhibition of DDR1 by Nilotinib also inhibits the metastatic behavior of the CRC cells. Thus, the focus of this study was to investigate whether Nilotinib, a DDR1 inhibitor can inhibit breast cancer cells.

This study revealed that Nilotinib effectively blocked the proliferation of breast cancer cells and facilitated apoptosis. Further, the effect of Nilotinib on the metabolism of breast cancer was assessed. Nilotinib showed a significant inhibitory effect on the migration of MCF-7 cells. The migratory ability of tumor cells reflects the metastatic potential of the tumor. Based on previous research reports, the impact of Nilotinib on inhibition of DDR1 was investigated. As expected, Nilotinib inhibited the phosphorylation of DDR1. Moreover, our results demonstrated that the downregulation of DDR1 by RNAi influenced the migration of breast cancer. This indicated the correlation between DDR1 and the metastatic ability of breast cancer cells. Subsequently, it was observed that Nilotinib did not inhibit MCF-7 SH*DDR1*. The results indicated the inhibition of MCF-7 by Nilotinib by targeting DDR1.

Conclusions

In a nutshell, DDR1 plays an essential role in the growth and migration of breast cancer. Nilotinib inhibited breast cancer by blocking the DDR1 signaling pathway. Therefore, targeting DDR1 therapeutically could potentially affect the survival and influence the metabolism of breast cancer, and Nilotinib could be a potential candidate for the treatment of breast cancer.

Declarations

Acknowledgments

Not applicable

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Authors' contributions

Contributions to the ideas and design of the study: SY and MY; Performed the experiments and analyzed the data: SW and CY; Prepared the figure: YX and AB; Drafted the manuscript, and finalized the manuscript: SW, TY and BL. All authors have read and approved the final manuscript.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The data supporting the conclusions of this report are included within the article.

Publisher's Note

Abbreviations

DDR1: Discoidin domain receptor 1;

RTK: receptor tyrosine kinase;

EMT: epithelial-mesenchymal transition

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Figures

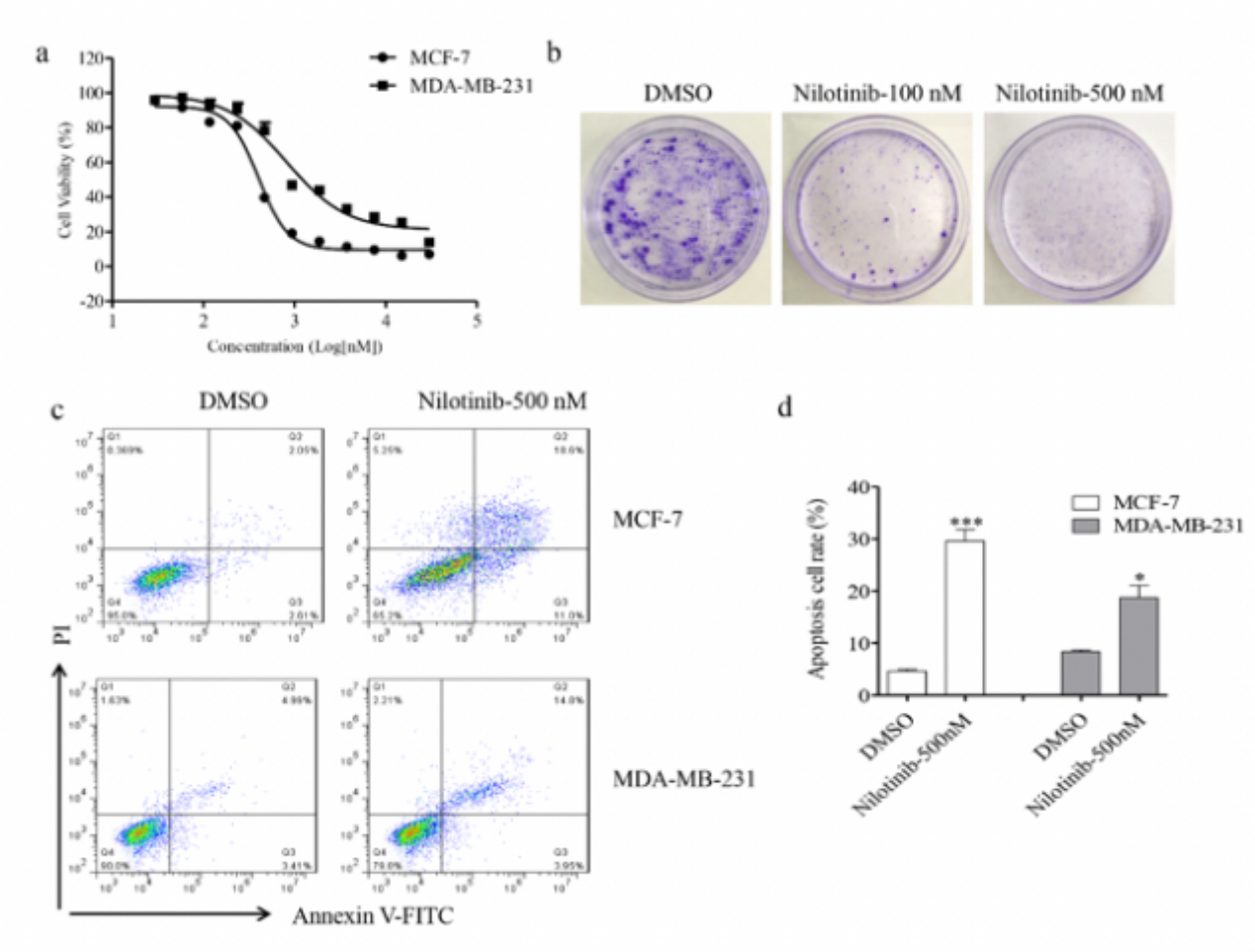

Figure 1

Nilotinib manifests anti-proliferation and pro-apoptosis activity in breast cancer. (A). MTT assay. The cells were treated with various concentrations of Nilotinib (n=11; 14.5-30,000 nM) and cultured in 10% FBS medium for 48 h in triplicates (B). Colony formation assay. The number of colonies were counted after MCF-7 cells were incubated with Nilotinib (100, 500nM) for a week, and stained with crystal violet. (C). Flow cytometry. (D) Effects of Nilotinib (500 nM) on apoptosis in MCF-7 and MDA-MB-231 cell lines. The figures are representative of more than three separate experiments. (*p<0.05, **p<0.01 and ***p<0.001 compared to DMSO group).

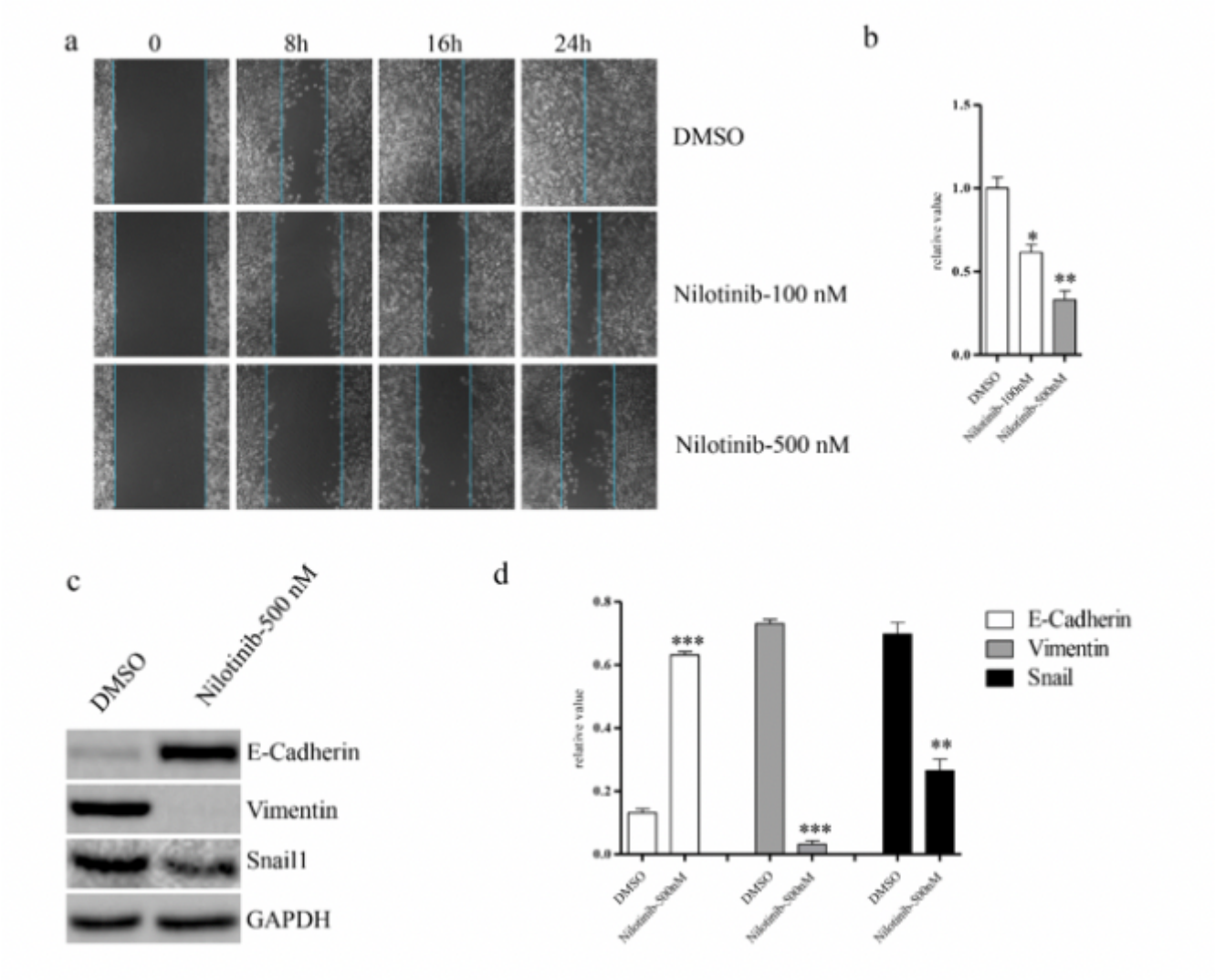

Figure 2

Nilotinib inhibits breast cancer migration. (A). Wound healing assay. MCF-7 cells were treated with two different concentrations of Nilotinib (100 and 500 nM) (B). The figures are representative of more than three separate experiments of (A). (C). Colony formation assay. The number of colonies were counted after MCF-7 cells were incubated with Nilotinib (100, 500nM) for a week and stained with crystal violet. (D). Western blotting. Effects of Nilotinib (500 nM) on protein expression levels of E-Cadherin, Vimentin, and Snail1 in MCF-7cell lines (D). The figures were representative of more than three separate experiments of (C). (*p<0.05, **p<0.01 and ***p<0.001 compared to DMSO group).

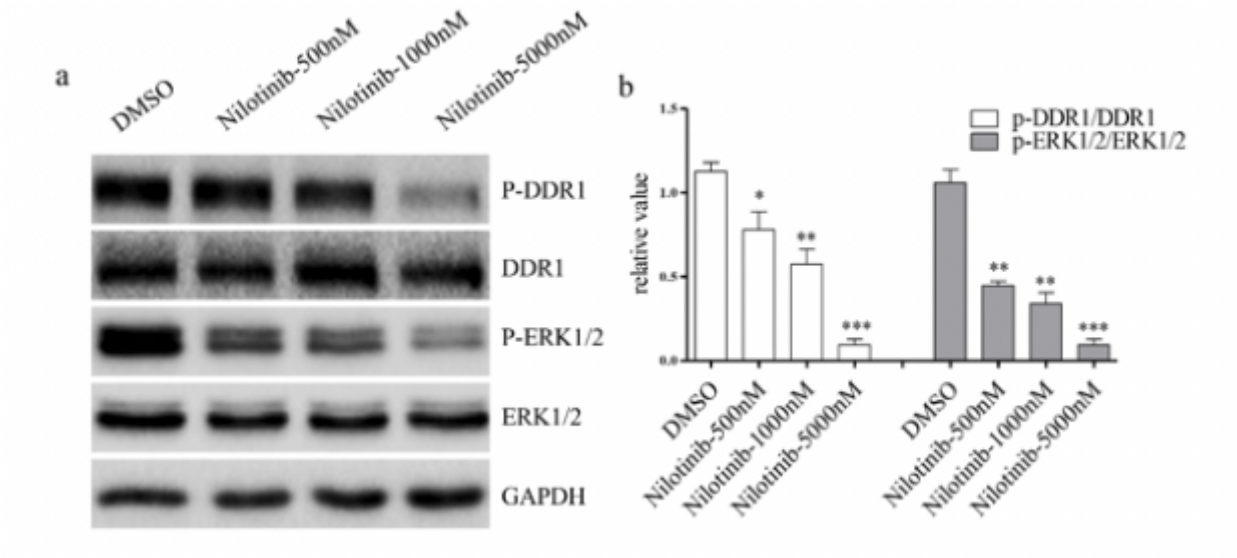

Figure 3

(A). Inhibitory effect of Nilotinib on DDR1 and its downstream ERK1/2 phosphorylation levels in MCF-7 cell lines. (B). The figures were representative of more than three separate experiments of (A) (*p<0.05, **p<0.01 and ***p<0.001 compared to DMSO group)

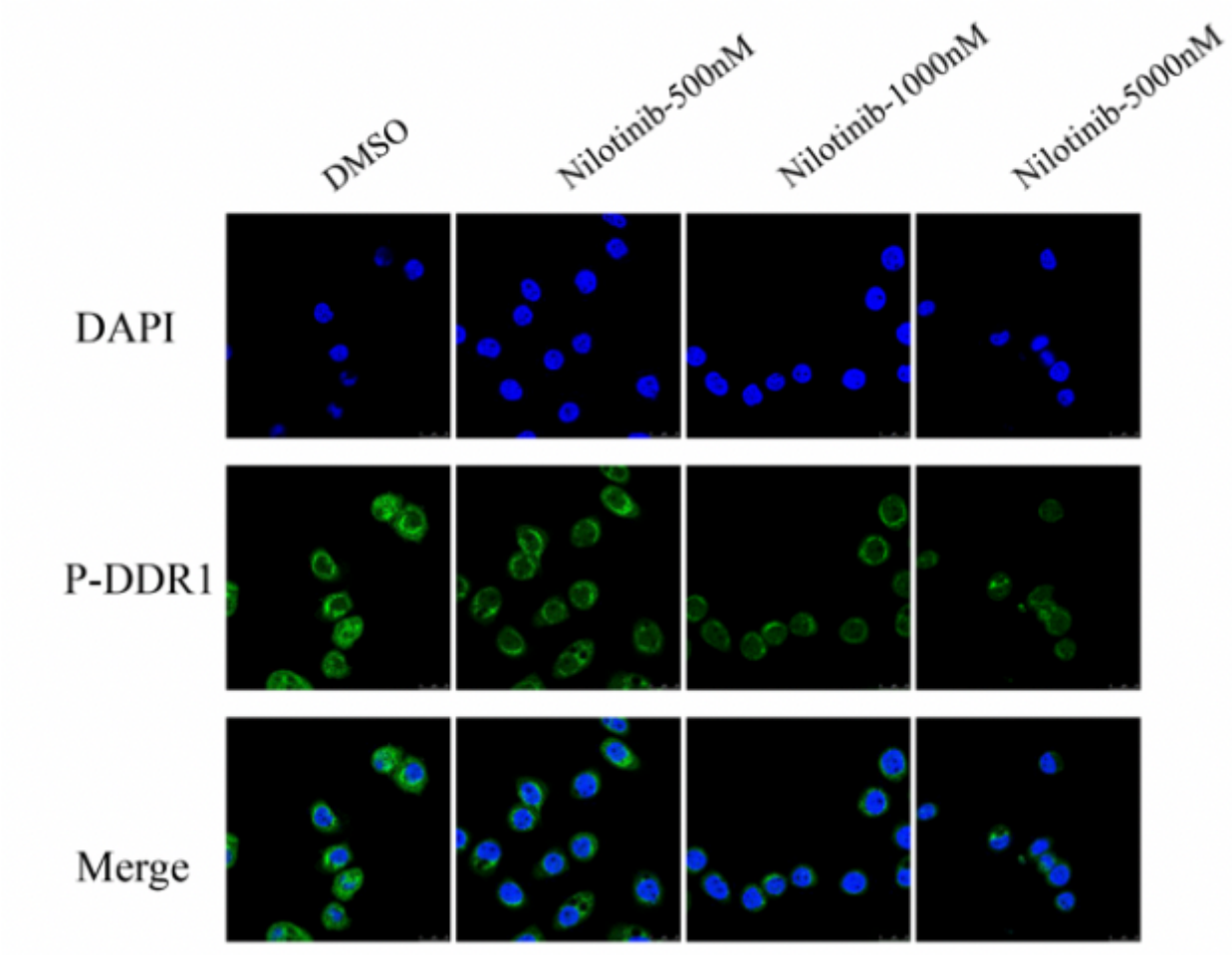

Figure 4

Immunofluorescence assay. MCF-7 cells were treated with DMSO, Nilotinib (0.5, 1 and 5 μM) for 1h, staining with p-DDR1 antibody.

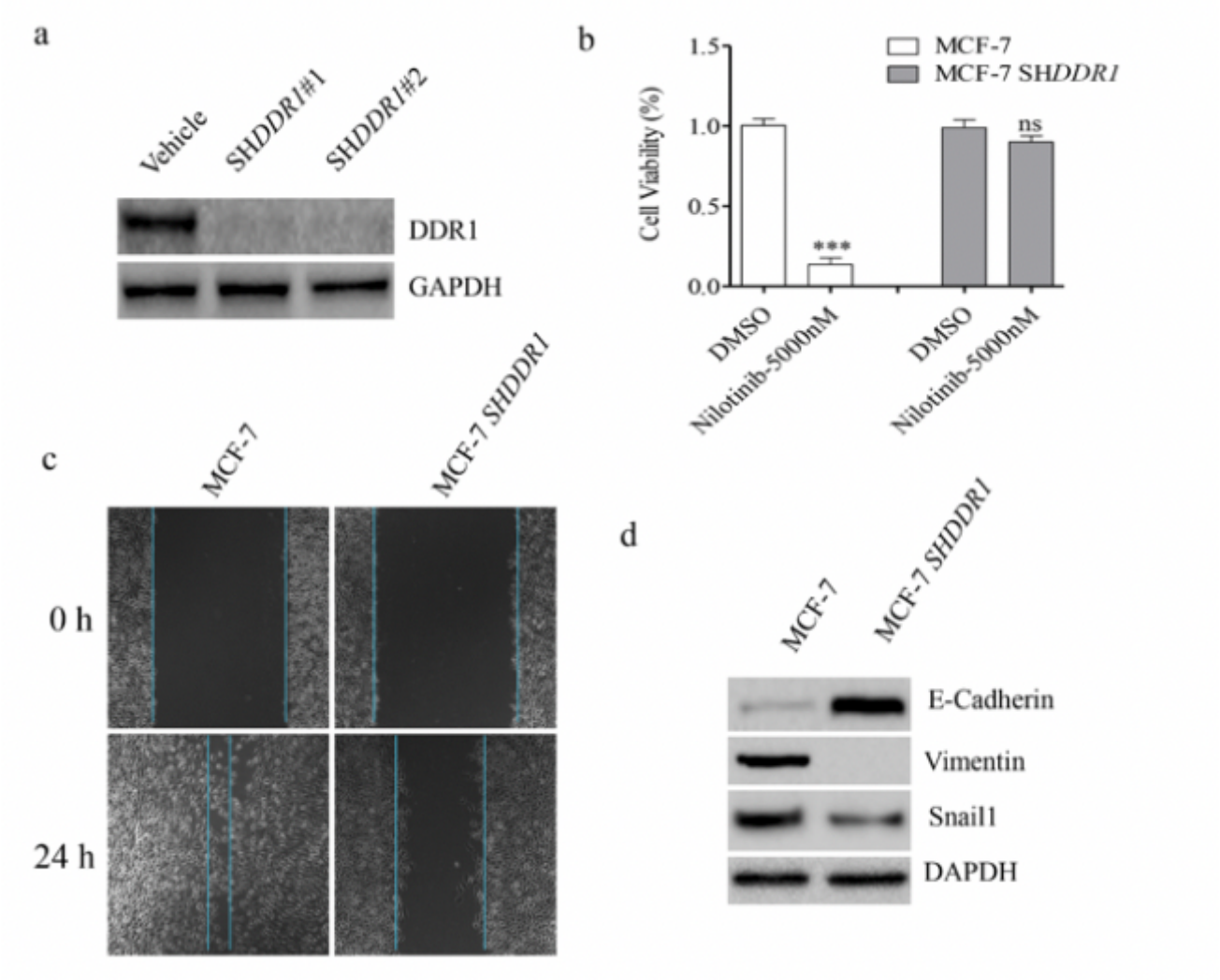

Figure 5

Effects of DDR1-shRNA on proliferation and migration of MCF-7. (A). Effects of DDR1-shRNA on expression of protein. (B). Comparison of the anti-proliferation ability of Nilotinib (500 nM) to MCF-F and MCF-7 SHDDR1 for 24h (C). The different migration ability between MCF-7 and MCF-7 SHDDR1. (D). Western blotting. Effects of DDR1-shRNA on protein expression levels of E-Cadherin, Vimentin, and Snail1 in MCF-7cell lines. (*p<0.05, **p<0.01 and ***p<0.001 compared to DMSO group).