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Claudin6 Suppresses Migration and Invasion via Blocking Smads/Snail/MMP2/9 Pathway in MCF-7 and SKBR-3 Cell Lines

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Abstract

Background: We have previously reported that CLDN6-mediated SB431542 suppresses EMT, migration and invasion in breast cancer cells, but the mechanisms need to be further studied. Our previous experiments verified that SB431542 a SMADs signaling pathway inhibitor inhibits invasion, migration and EMT, and down-regulates Snail, MMP2 and MMP9 expression in breast cancer cells. Knocking down CLDN6 reversed the effects of SB431542. In order to further study the mechanisms responsible for this effect, the following experiments were carried out.

Methods: 1- CLDN6 was knocked down by shRNA in MCF-7 and SKBR-3 cell lines, which had been pretreated with SB431542, The cells were incubated with MMP2/9 Inhibitor I selective antagonists of MMP-2/MMP-9. 2- Snail was knocked down by shRNA in MCF-7 and SKBR-3 cell lines. The levels of MMP2 and MMP9 were examined by RT-PCR and Western blot. The expression of E-cadherin and N-cadherin were analyzed by Western blot and Immunofluorescence Microscopy. Vimentin expression was detected by Western blot. Migration and invasion were analyzed by Wound Healing Assay and Matrigel Invasion Assay.

Results: MMP2/9 Inhibitor I reversed the effects of Knocking down CLDN6 on downregulation of E-cadherin, up-regulation of N-cadherin and Vimentin, facilitation of migration and invasion. In MCF-7-shSnail and SKBR-3-shSnail cells, migration and invasion were inhibited, E-cadherin was up regulated, MMP2, MMP9, N-cadherin and Vimentin were downregulated.

Conclusions: The experiment demonstrates that Claudin6 suppresses epithelial-mesenchymal transition, migration and invasion via blocking SMADs/Snail/MMP2/9 pathway in MCF-7 and SKBR-3 cell lines.

Keywords: CLDN6; Snail; MMP2; MMP9; SMADs

Introduction

Breast cancer is a main cause of cancer-associated death in women [1]. Recurrence and metastasis are the primary reasons for breast cancer-associated patient death [2]. CLDN6 is a member of the claudins family, which plays an important role in the biology of many neoplastic diseases. Its effects are often tissue specific [3-6]. EMT is one of the most important steps during cancer progression [7,8]. Cancer cells grow in situ initially. Following loss of polarity and changes in morphology, the malignant cells exit from their place of origin and invade adjacent local tissue or metastasize into distant organs through the blood vessels and continue to grow [9,10]. The epithelial markers are downregulated and the interstitial markers are upregulated [11]. The process of EMT is regulated by EMT associated transcription factors Twist, Snail, Zeb1 [12-17], and signaling pathways including TGF-β and Wnt pathway [18]. In our previous experiments, it is found that CLDN6-mediated SB blocked EMT, invasion and migration [19].

Snail is a zinc Finger structure transcription factor, one of the key regulators that promotes EMT, migration and invasion in many types of malignant tumors including head and neck squamous cell carcinoma (HNSCC) [20], lung cancer [21], hepatocellular carcinoma [22], Snail can regulate the expression of Matrix Metalloproteinases (MMPs) in numerous cancers [23-25]. MMPs degrade the extracellular matrix (ECM) to facilitate the metastatic process [26].

Our previous experiment demonstrated that CLDN6-mediated SB inhibited invasion, migration and EMT in breast cancer cells, as well as the downregulation of Snail, MMP2 and MMP9 [19,27]. In our present experiments, we have verified the hypothesis that CLDN6-mediated SB inhibits invasion, migration, and EMT via down regulation of snail/MMP2/9 in MCF-7 and SKBR-3 cell lines.

Materials and Methods

Cell culture and reagents

The human breast cancer cell lines MCF-7 and SKBR-3 were bought from the Cell Bank of the Chinese Academy of Sciences and cultured in DMEM (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (Hyclone, Logan, UT, USA). All the cells were put in a humidified incubator with 5% CO_2 at 37°C. SB431542 was obtained from Sigma (St. Louis, MO, USA).

Transient transfection with short hairpin RNA

Transient transfection CLDN6: Cells were transfected with short hairpin RNA and Lipofectamine 2000 (Invitrogen), following the instructions. shRNA targeting CLDN6 (5’-GTGCAAGGTGTACGACTCA-3’) and a negative control shRNA were obtained from Shanghai GeneChem Co. Ltd.

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Transient transfection snail: Following the method of Ding et al. [28], shRNA (containing sense and antisense sequences linked by a hairpin loop: TTCAAGAGA) was designed and constructed into the vector pGCsilencer TM U6/Neo/GFP (GeneChem, China). The MCF-7 and SKBR-3 cells were then transfected with SuperFect®. Transfection Reagent (QIAGEN, USA). The negative control cell line was generated by treating cells with the vector pGCsilencer TM U6/Neo/GFP constructed with oligonucleotides, which have no homology to the human gene. The control group was the untreated MCF-7 and SKBR-3cell lines.

Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription-polymerase chain reactions were carried out as described previously. Total RNA was extracted from the cloned cells using TRIzol (Invitrogen, USA) following the manufacturer's instructions. One microgram of total RNA was reverse-transcribed to synthesize cDNA using the MMuLVreverse transcriptase (TaKaRa, Japan) for 60 min at 42℃, 0.5ug cDNA was used for PCR. SNAIL, MMP2 and MMP9 were amplified along with GAPDH as an endogenous control following the instructions of Premix LA Taq Kit (TaKaRa, Japan). The PCR reaction conditions and the primer sequences are listed in Table 1. After electrophoresis, the gel was captured and analyzed by an imaging system (Syngene, Cambridge, UK).

Western blotting analysis

Western blotting analyses were carried out as described previously [29]. Anti-MMP2, MMP9, N-cadherin, and Vimentin antibodies were obtained from Abcam (Cambridge, UK), an anti-E-cadherin antibody was purchased from Bioworld Technology (Dublin, OH, USA), the anti-β-actin antibody was from Santa Cruz (Santa Cruz, CA, USA). The blots were imaged and analyzed using an ECL Western blotting system (GE, Fairfield, CT, USA).

Immunofluorescence microscopy

An immunofluorescence assay was carried out to evaluate the expression of these targets as previously described [30]. Cells were cultured with primary antibodies against E-cadherin diluted to 1:400 and N-cadherin diluted at 1:250 at 4℃ overnight. The secondary antibody was Alexa Fluor 647 anti-mouse IgG diluted at 1:200 (Cell Signaling Technology, Beverly, MA, USA). Cells were observed with a fluorescence microscope (Olympus, Tokyo, Japan).

Wound healing assays

1×10^6 cells were cultured overnight in 60 mm dishes, allowed reaching confluency, and a wound was introduced. Images were captured using a microscope at 0 and 24 hr after wounding to calculate the width of the wounded area. The relative migration distance was calculated as $(W_0-W_{24})/W_0\times100\%$.

Matrigel invasion assays

Matrigel invasion assays were carried out using transwells containing 8.0 μm pore membranes (Corning, Lowell, MA, USA). MCF-7 and SKBR-3 cells were inoculated into the upper chamber of the Trans well, and 48 hr later, the chambers were washed twice with phosphate buffer solution. The filter side of the upper chamber was cleaned with a cotton swab, and then the membrane was cut out of the insert. Cells were fixed by methanol and stained by 5% Giemsa at room temperature for half an hour. We counted the cells in each field (five fields/membrane) on the membrane to quantify the result of the assay.

Statistical analysis

All computations were performed using the SPSS version 22.0 for Windows. Unpaired t-tests were carried out to analyze data. The data are presented as means ± standard error from at least three independent experiments. $p < 0.05$ was considered statistically significant.

Results

CLDN6-mediated effects of the suppression of SMADs signaling pathway inhibited invasion, migration, and EMT via downregulation of MMP2/9 in MCF-7 and SKBR-3 cells

Our previous experiments demonstrated that CLDN6-mediated effects of the suppression of SMADs signaling pathway inhibited invasion, migration, and EMT in MCF-7 and SKBR-3 cells. These results also showed that CLDN6 mediates suppression of SMADs signaling pathway to regulate morphology, transepithelial resistance, and colony formation, which are associated with EMT, invasion and migration. However, the molecular mechanisms were not very clear. To address this problem, we evaluated the expression of MMP2 and MMP9 by RT-PCR and Western blot. In MCF-7and SKBR-3 cells, MMP2 and MMP9 were found to be downregulated following treatment with the SMADs signaling pathway inhibitor SB431542. CLDN6 was knocked down after cells were treated with SB431542, and the downregulation of MMP2 and MMP9 was blocked. The results were Similar as our previous reproered [27]. The accelerating effect of knocking down CLDN6 on migration and invasion were reversed by MMP2/9 Inhibitor I (Figures 1a and 1b), The effect of knocking down CLDN6 on up-regulation of EMT associated genes N-cadherin and Vimentin and effect on downregulation of E-cadherin were blocked by MMP2/9 Inhibitor I (Figure 1c and 1d). Thus, we conclude that SB431542/CLDN6 inhibited EMT, migration and invasion by downregulation of MMP2 and MMP9 in MCF-7 and SKBR-3 cells.

Snail knockdown downregulated MMP2/9 and inhibited EMT, migration and invasion in breast cancer cells

Snail was knocked down in MCF-7 and SKBR-3 cells. The levels of Snail in MCF-7-shSnail and SKBR-3-shSnail cells were decreased significantly compared with control groups (Figure 2a). In MCF-7 cells, MMP2 and MMP9 were significantly downregulated in knockdown groups (Figure 2b). Similar results were obtained in SKBR-3 cells (not shown). Similarly, migration and invasion were also inhibited in knockdown groups (Figure 3a and 3b), as well as suppression of

Table 1: Primers and information for RT-PCR.

Primer Name	Primer Sequence	Length (bp)	Annealing Temp (℃)	Cycles
GAPDH	5'-TGTTGCCATCAATGACCCCTT-3' 5'-CTCCACGACGTACTCAGCG-3'	178	56	25
SNAIL	5'-GCCTAGCGAGTGGTTCTTCTG-3' 5'-TAGGGCTGCTGGAAGGTAAA-3'	203	56	30
MMP2	5'-TCTCCGACATTGACCTTGGC-3' 5'-CAGGGTGCTGGCTGAGTAGATC-3'	302	60	30
MMP9	5'-TTGACAGCGACAAGAAGTGG-3' 5'-GCCATTCACGTTCGTCCTTAT-3'	180	58	30

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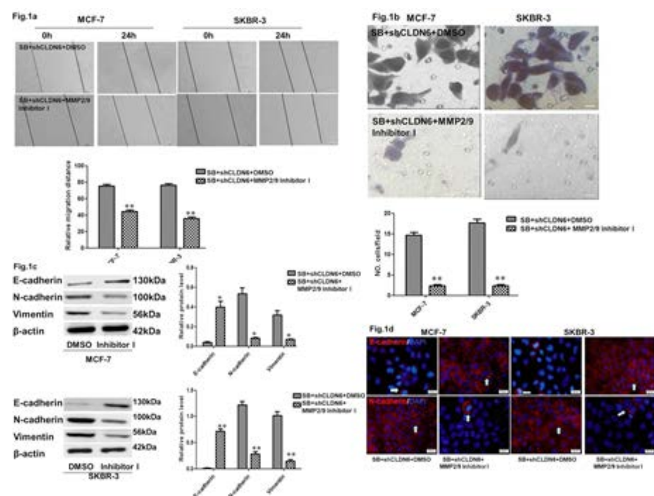

Figure 1: SB431542/CLDN6 inhibits EMT, migration and invasion in breast cancer cells via downregulation of MMP2 and MMP9. (a) Images of wound-healing assays for SB+MCF-7-shCLDN6, and SB+SKBR-3-shCLDN6 cells treated with DMSO or MMP2/9 Inhibitor I to evaluate their migration rate into the cell-free area (bar, 100 μm). (b) Matrigel invasion assay. Cells that invaded through the Matrigel were stained with Giemsa. All results were presented as the average of cells counted in 5 fields per condition (bar, 50 μm). (c) Western blotting analysis was used to determine the expression of E-cadherin and N-cadherin, and vimentin in cells. (* $p < 0.05$ and ** $p < 0.01$ are considered statistically significant and highly statistically significant, respectively). Bars represent mean±SE (n = 3). (d) Immunofluorescence analysis of EMT marker expression in cells. Representative immunofluorescence images (200×) generated using anti-E-cadherin and anti-N-cadherin primary in breast cancer cells. Blue, cell nuclei. Red, localization of E-cadherin and N-cadherin, indicated by white arrows (bar, 20 μm).

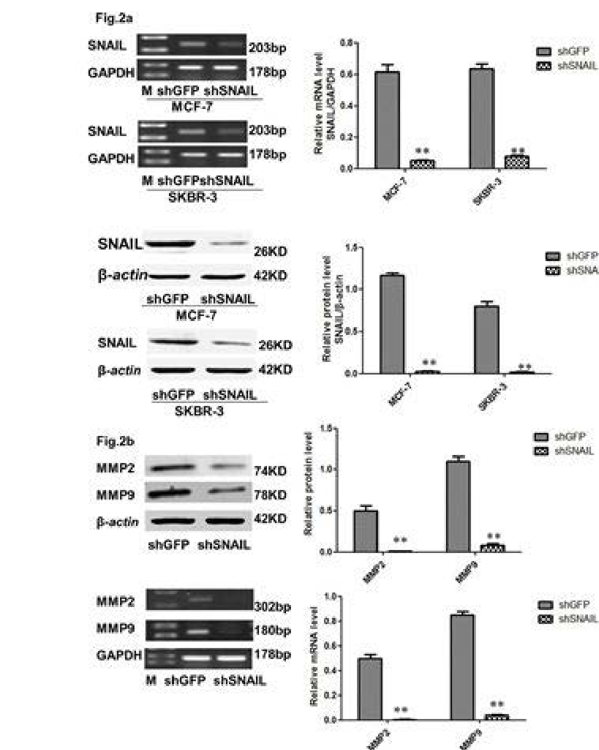

Figure 2: Snail knockdown downregulates the expression of MMP2 and MMP9. (a) RT-PCR and Western blot expression analyses of Snail in MCF-7-shGFP, MCF-7-shSnail, SKBR-3-shGFP, and SKBR-3-shSnail cells (b) RT-PCR and western blot expression analyses of MMP2 and MMP9 in MCF-7-shGFP and MCF-7-shSnail cells. “M” stands for marker.

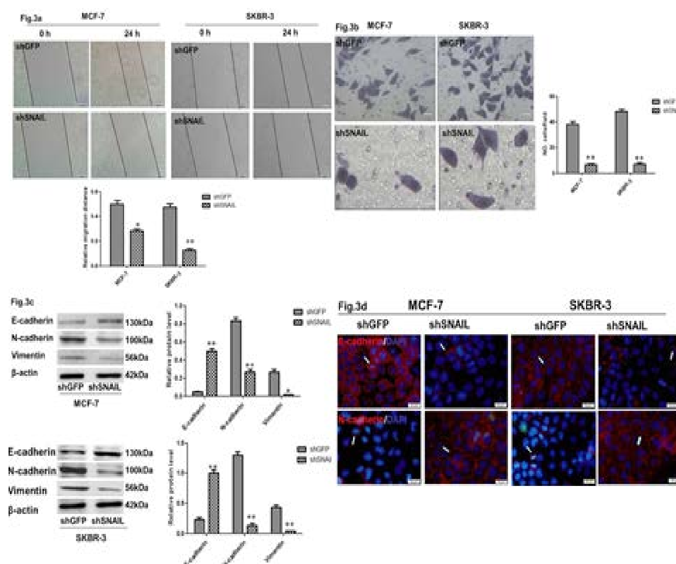

Figure 3: Snail knockdown inhibits EMT, migration and invasion in breast cancer cells. (a) Images of wound-healing assays for MCF-7-shGFP, MCF-7-shSnail, SKBR-3-shGFP, and SKBR-3-shSnail cells to evaluate their migration rate into the cell-free area (bar, 100 μm). (b) Matrigel invasion assay cells invaded through the Matrigel were stained with Giemsa. All results are presented as the average of cells counted in 5fields per condition (bar, 50 μm). (c) Western blotting analysis was used to detect the expression of E-cadherin, N-cadherin, and vimentin in MCF-7-shGFP, MCF-7-shSnail, SKBR-3-shGFP, and SKBR-3-shSnail cells. (d) Immunofluorescence analysis of the expression levels of EMT markers in MCF-7-shGFP, MCF-7-shSnail, SKBR-3-shGFP, and SKBR-3-shSnail cells.

expression of the EMT-associated genes N-cadherin and Vimentin, and enhancement of the level of E-cadherin (Figure 3c and 3d).

Discussion

The junctions between cells include tight junctions, adherens junctions, desmosomes and gap junctions [31]. Tight junction possesses the barrier function, fence function, and signal transfer function [32,33]. Claudins maintain the construction and function of tight junctions [34].

Claudins are expressed abnormally in numerous epithelial-derived cancers [35]. Our previous experiments confirmed that CLDN6 mediated inhibition of SMADs signaling pathway to regulate EMT-associated gene expression including upregulation of E-cadherin, downregulation of N-cadherin, Vimentin, Snail, MMP2 and MMP9, inhibition of migration and invasion in breast cancer cells. However, the molecular mechanism remains unclear. To further address this problem, we conducted the experiments in this study. In MCF-7 and SKBR-3 cells, the results showed that SB431542 downregulated the level of MMP2 and MMP9. Knocking down CLDN6 blocked the effects of SB431542 on downregulation of MMP2 and MMP9. MMP2/9 Inhibitor I blocked the effects of knocking down CLDN6 on upregulation of N-cadherin, Vimentin, down-regulation E-cadherin, facilitation of invasion and migration. The finding suggests that CLDN6-mediated effects of the suppression of SMADs signaling pathway inhibited invasion, migration, and EMT via downregulation of MMP2/9 in MCF-7 and SKBR-3 cells.

Combining current and previous studies [19], the results indicate that knocking down CLDN6 upregulates the level of Snail, MMP2 and MMP9. Our current findings suggest that MMP2 and MMP9 were downregulated in the Snail knocked down groups. Consistent with our data, other studies have also indicated MMP9 or MMP2 can be induced

by Snail in tumors [36-39]. However, this is in contrast to the results of Heta Merikallio's study [40]. The expressions of N-cadherin, Vimentin were downregulated and E-cadherin was upregulation, migration and invasion were inhibited in the Snail knocked down groups.

Conclusion

In summary, we confirm for the first time that SMADs-down-regulated CLDN6 promotes EMT, migration and invasion via Snail/MMP2/9 in MCF-7 and SKBR-3 cells. We suggest that SMADs /Snail/MMP2/9 axis might represent a therapeutic target for breast cancer, and CLDN6 might play an important role in the therapy of breast cancer.

Authors' contributions

Liping Wang carried out part of the experiments, participated in the design of the study, performed the statistical analysis, and drafted the manuscript. Ting Liu, Libo Jiang, Qingyang Bai, Shuqin Wu, Xiuwen Yu, Tian Wu, Junping Wang and Xiaojie Zhang carried out most of experiments, and helped draft the manuscript. Hongmei Li, and Kun Zhao assisted with the experiments, and helped to edit the paper. All authors have read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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