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Original Article

Claudin 19 inhibits the malignant potential of breast cancer cells by modulating extracellular matrix-associated UBE2C/Wnt signaling

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Abstract: Claudin proteins are a major component of the tight junctions between cells, which are involved in a variety of human diseases, including cancer. This study aimed to investigate the functional role of claudin 19 (CLDN19) in human breast cancer progression. Here, we firstly found that CLDN19 was downregulated in breast tumor tissues than normal control, and loss of CLDN19 predicted poor patient survival in patients with breast cancer, by utilizing the Cancer Genome Atlas Program (TCGA) dataset analysis. To further validate the tumor suppressive effects of CLDN19, we established CLDN19 overexpressed MDA-MB-231 and T47D cells. And overexpression of CLDN19 resulted in suppression of cell growth/migration in breast cancer cells cultured in 3D environment or *in vivo*. Mechanistically, we demonstrated that CLDN19 downregulated ubiquitin conjugating enzyme E2 C (UBE2C) expression, which further suppressed Wnt/β-catenin pro-survival signaling pathway activation induced by extracellular matrix (ECM), in 3D environment or *in vivo*. Altogether, our study revealed a tumor suppressive role of CLDN19, which hindered ECM/UBE2C/Wnt signaling activation in breast cancer, and offered novel insight for tumor diagnosis and targeted therapy.

Keywords: Claudin 19, extracellular matrix, UBE2C, breast cancer, Wnt signaling pathway

Introduction

The local environment of tumor cells plays essential roles in regulating cancer progression [1]. The major component of local environment is ECM, which was a complex network composed of multidomain macromolecules organized in a tissue specific manner [2]. Abnormal cancer-associated ECM is an integral feature of tumor tissues and affect cancer progression by influencing cellular transformation [3]. For instance, elevated expression of collagens, the major component in ECM, promoted stem-like phenotypes of tumor cells, and is predictive of a poor outcome in patients with breast cancer [4, 5]. Extracellular fibronectin and fibrinogen can also predispose a tumor tissue to malignancy [6, 7]. Thus, understanding the composition of ECM and how their components affect tumor progression could help explore the mechanism underlying cancer development.

Tumor cells frequently express different cell adhesion molecules which guarantee anchorage, and support for cell-to-cell or -ECM junctions [8]. Claudins, a group of membrane proteins, are key components within tight junctions, and they are tightly involved in homo- and heteromeric interactions between adjacent cells [9]. In recent years, those proteins attracted increasing attention and have been implicated in diverse diseases. Claudins not only participated in paracellular junctions but also are essential for cell proliferation/differentiation [10]. The differential expression of claudins has also been studied in malignant tumors [11]. Claudin-1, 2 and 4 are the most studied claudins in cancer, however, their roles (especially claudin 1) as either tumor promoter or suppressor remains controversial [12]. Increasing expression of claudin 1 and 4 has been reported to promoted tumor growth and invasion in gastric and ovarian cancer [13, 14], whereas a claudin 1 negative phenotype predicts poor prognosis in breast cancer [15]. In some cancers, claudins also served as epithelial-mesenchymal transition promoter and mediated pro-survival signaling activation to stimulated neo-

plasia [16]. Thus, the roles of claudins in tumor progression has yet to be further investigated, and understanding the mechanism of claudins-ECM-associated tumor behaviors might help explore innovative therapeutic targets for clinical tumor therapy.

In the present study, we demonstrated a suppressive role of CLDN19 in breast tumor progression. We clarified that CLDN19 was downregulated in breast tumor tissues, and negatively correlated with poor prognosis in patients by utilizing the breast carcinoma database in TCGA. Next, we predicted function and mechanisms of CLDN19, and found that CLDN19 exerted a suppressive role to inhibit breast tumor progression *in vivo*, in which CLDN19 suppressed UBC2E/Wnt/β-catenin activation induced by ECM. Overall, during breast cancer development, CLDN19 served as a tumor suppressor to retard breast tumor development via ECM/UBE2C/Wnt signaling, which described novel insight into the role of claudins in breast cancer progression.

Materials and methods

Cell culture

Human breast cancer cell lines MDA-MB-231 and T47D were purchased from American Type Culture Collection (USA). Cells were cultured and maintained in Roswell Park Memorial Institute-1640 medium supplemented with 10% fetal bovine serum (Thermo Fisher, USA) under standard conditions. CLDN19 overexpressed MDA-MB-231 and T47D cell lines were purchased from Cyagen Biotech (China) and determined by western blotting. For 3D Matrigel culture, tumor cells were seeded in Matrigel Basement Membrane Matrix (356237, Corning, USA). Matrigel Basement Membrane Matrix was mixed with culture media at a concentration of 5 mg/ml. Cells were seeded at a density of 5,000 cells per well. Spheroid formation was assessed on day 3. Spheroid images were pictured daily for colony growth analysis by Image J 6.0 software.

Patient information

Expression of CLDN19 at mRNA in 1097 breast tumor tissues and 114 normal was obtained from http://ualcan.path.uab.edu/index.html. Transcriptome map and survival information of 816 breast cancer patients were obtained from https://www.cbioportal.org/. 22 clinical breast tissues were obtained from the Fifth People's Hospital of Huai'an City, and divided into metastatic (Mec, n=11) and non-metastatic (Non-M, n=11) groups according to follow-up visit. All patients agreed to participate in the study and informed with written consent. All experiments were performed according to the Declaration of Helsinki. Ethical review was granted by the Ethics Committee of the Fifth People's Hospital of Huai'an City.

RNA interference

Transfection of MDA-MB-231 or T47D cells with siRNA targeting UBE2C was performed using Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher, USA) at a final siRNA concentration of 20 nM according to protocol. The target UEB2C and CDC20 siRNA sequences were obtained from Ruibo Biotech (China): UBE2C, siRNA#1, 5'-GAAGTACCTGCAAGAAACCTACTCA-3' and siRNA#2, 5'-CAGCAGGAGCTGATGACCCTCATG-3'. CDC20, siRNA#1, 5'-TCGCATCTGGAATGTGTGCT-3' and siRNA#2, 5'-CCAGAAGGCUACCAGAA-3'.

Cell proliferation

MDA-MB-231 or T47D cells were seeded into each well of a 96-well plate at a density of 2×10^3 cells in 100 μl culture medium. The medium was then replaced with 100 μl fresh medium with 10% CCK-8 reagent after 0, 24, 48 and 72 hours. Then the cells were incubated for 3 hours at 37°C. The absorbance was measured at 450 nm using a Bio-rad micro-plate reader (Bio-rad, USA). All experiments were performed in triplicate.

Cell migration

MDA-MB-231 and T47D cells with a density of 1×10^4 in serum-free culture media were seeded in Transwell chambers with the Matrigel membrane covered. Cell migration assay was performed with the condition media containing 10% fetal calf serum. After 24 hours, migrating cells in chambers were stained with crystal violet and counted. All experiments were performed in triplicate.

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from MDA-MB-231/T47D cells using the TRIzol Reagent (Thermo Fisher, USA). 1 μg RNA was converted into cDNA

using PrimeScript RT Reagent kit (Takara, Japan). Real-time PCR analysis involved 1 ng cDNA, 10 μl SYBR Green PCR Master Mix (Thermo Fisher, USA) and 2 μM primers in the ABI StepOne Real-Time PCR System (Applied Biosystems, USA). The PCR primer sequences of *AKT1*, *Wnt3A*, *SOX2*, *STAT3*, *NOTCH1*, *ERK1, CDC20, UBC2E* and *MAPK1* were synthesized by Sangon Biotech (China).

Western blotting

Protein extracts from MDA-MB-231 and T47D cells were quantified and 20 μg proteins were subjected to SDS-PAGE on 12% SDS-acrylamide gels (Biyuntian Biotech, China). Separated proteins were transferred to PVDF membranes (Millipore, USA) and then blocked by nonfat-dried milk. Primary antibodies for Wnt1 (ab15251, Abcam, UK), Wnt2 (ab227859, Abcam, UK), Wnt3A (ab219412, Abcam, UK), β-catenin (ab32572, Abcam, UK), CLDN19 (ABIN3183937, 4A Biotech, China) and UBE2C (ab252940, Abcam, UK) were used for western blot analysis, and incubated at a 1:1000 dilution, followed by incubation with respective secondary antibodies.

Immunostaining

Sections of clinical specimens were treated with 2.5% H_2O_2 and blocked with 5% bovine serum albumin for 30 minutes at room temperature. Samples were incubated with primary antibodies: anti-Wnt3A (ab219412, Abcam, UK), anti-CLDN19 (ABIN3183937, 4A Biotech, China), anti-UBE2C (ab252940, Abcam, UK), anti-collagen I (ab138492, Abcam, UK), anti-fibrinogen (ab281924, Abcam, UK), anti-fibronectin (ab3413, Abcam, UK), anti-CDC20 (ab185814, Abcam, UK) and anti-laminin (ab11575, Abcam, UK) overnight at 4°C. Then samples were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature, visualized and analyzed under optical microscope (Leica, Germany) for immunohistochemical method and confocal microscope (OLYMPUS, Germany) for immunofluorescence. Protein expression was quantified by Image-Pro Plus 5.0 software.

Animal protocols

Female severe combined immunodeficiency mice (8 weeks) were purchased from Huafukang (China) and maintained in a specific pathogen-free facility. 1×10^6 vector or CLDN19 overexpressed MDA-MB-231 cells were subcutaneously injected into mice (n=5 in each group). Tumor volume and survival information were recorded daily. On day 20, part of mice was sacrificed for tumor tissues isolated. The protein level of UBE2C, Wnt3A, β-catenin, collagen I, fibronectin, fibrinogen and laminin in tumor tissues was examined by western blotting or immunostaining. Meanwhile, 1×10^6 tumor cells were isolated and injected into mice by tail vein injection. Those mice were sacrificed on day 25 for pulmonary metastasis analysis. The calculation formula of tumor volume: tumor volume = length × width^2/2. All animal experiments were performed according to the guidelines of the Institute Ethics Committee of the Fifth People's Hospital of Huai'an City.

Statistical analysis

All experimental data were analyzed using Graphpad 5.0. The data are presented as mean ± SEM. The differences between two groups were compared using an independent sample t test. Comparisons among multiple groups were analyzed using one-way ANOVA, followed by Tukey's post hoc test. The Kaplan-Meier estimator was performed to evaluate the overall survival of patients or animals. Each experiment was performed for at least three independent times. Statistical significance was considered at $P < 0.05$.

Results

CLDN19 was downregulated and negatively correlated with poor prognosis in breast cancer

To perform clinically relevant assessment of the role of CLDN19 in breast cancer progression, we analyzed its transcriptome expression in 1097 breast tumor tissues in comparison with 114 normal tissues by utilizing TCGA dataset. Remarkably, CLDN19 was downregulated in breast tumor tissues compared to normal tissues (**Figure 1A**). However, no significant difference was found in CLDN19 expression among patients in different clinical stages (stage I-IV, **Figure 1B**). We next explored whether increased CLDN19 expression had an influence on overall survival of breast cancer patients by divided cohort (n=816) into CLDN19-high and CLDN19-

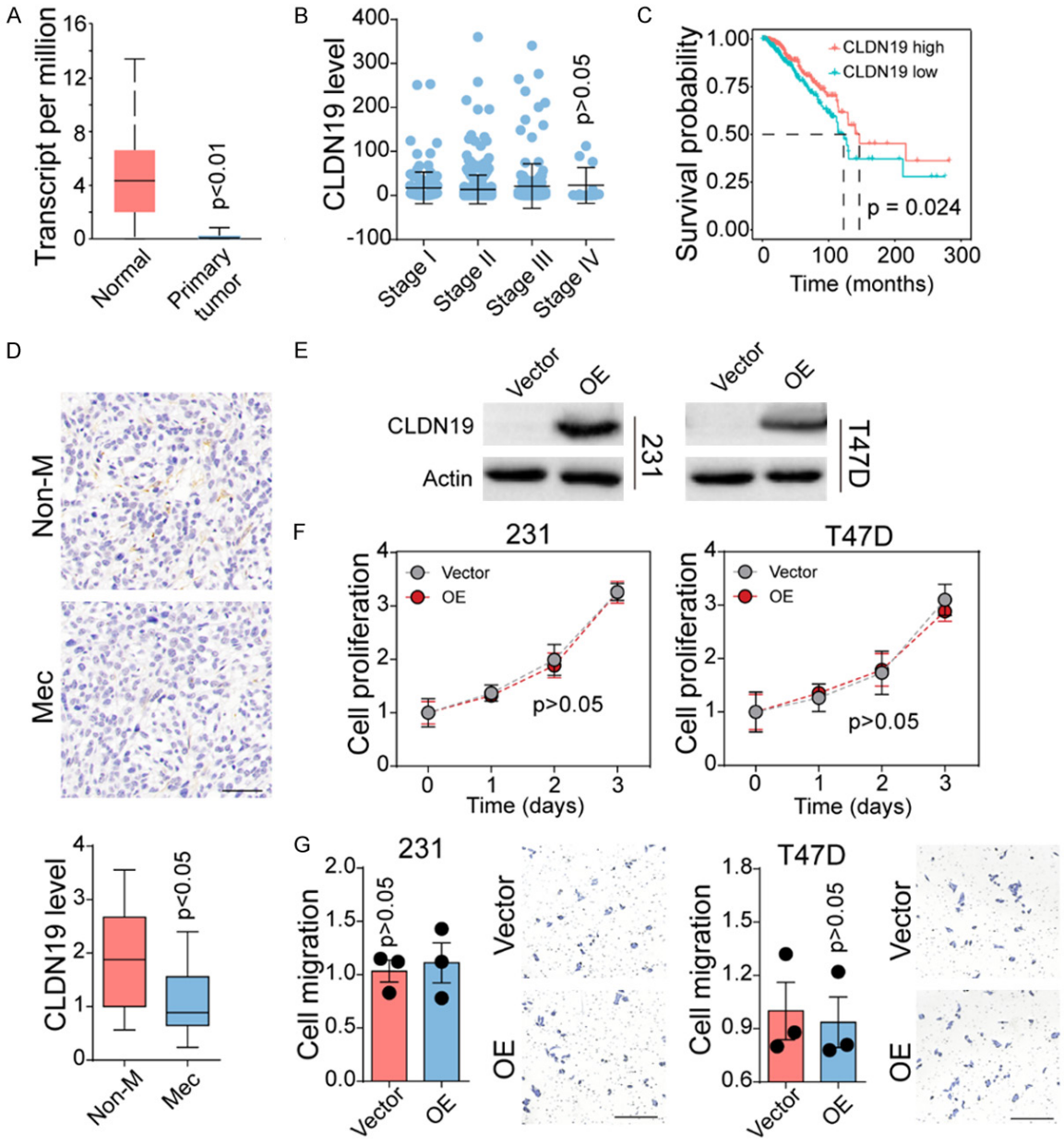

Figure 1. CLDN19 was downregulated in breast cancer. (A) MRNA expression of CLDN19 in normal tissues (n=114) and breast tumor tissues (n=1097) derived from TCGA data. (B) MRNA expression of CLDN19 in 816 breast tumor tissues with different stages derived from TCGA data. (C) Kaplan-Meier overall survival curve was shown according to high (n=407) and low (n=409) expression of CLDN19 in breast cancer patients derived from TCGA data. (D) CLDN19 protein level of non-metastatic (n=11) and metastatic (n=11) breast tumor tissues was quantified by immunohistochemical staining. The scale bar was 100 μm. (E) Western blotting of CLDN19 in vector and CLDN19 overexpressed MDA-MB-231 and T47D cells. (F, G) Cell proliferation (F) and migration (G) of vector and CLDN19 overexpressed MDA-MB-231 and T47D cells. The scale bar in Transwell was 100 μm.

low groups. Patients with high level of CLDN19 displayed a remarkably longer survival time than those in CLDN19-low group (**Figure 1C**). To further validate the role of CLDN19, tumor tissues from 22 clinical patients with breast cancer were collected and divided into non-metastatic and metastatic groups by follow-up visit. We observed a prominent decrease in CLDN19 expression in tumor tissues derived from metastatic patients in comparison with non-metastatic group (**Figure 1D**). Those results indicated that CLDN19 served as a suppressive role in

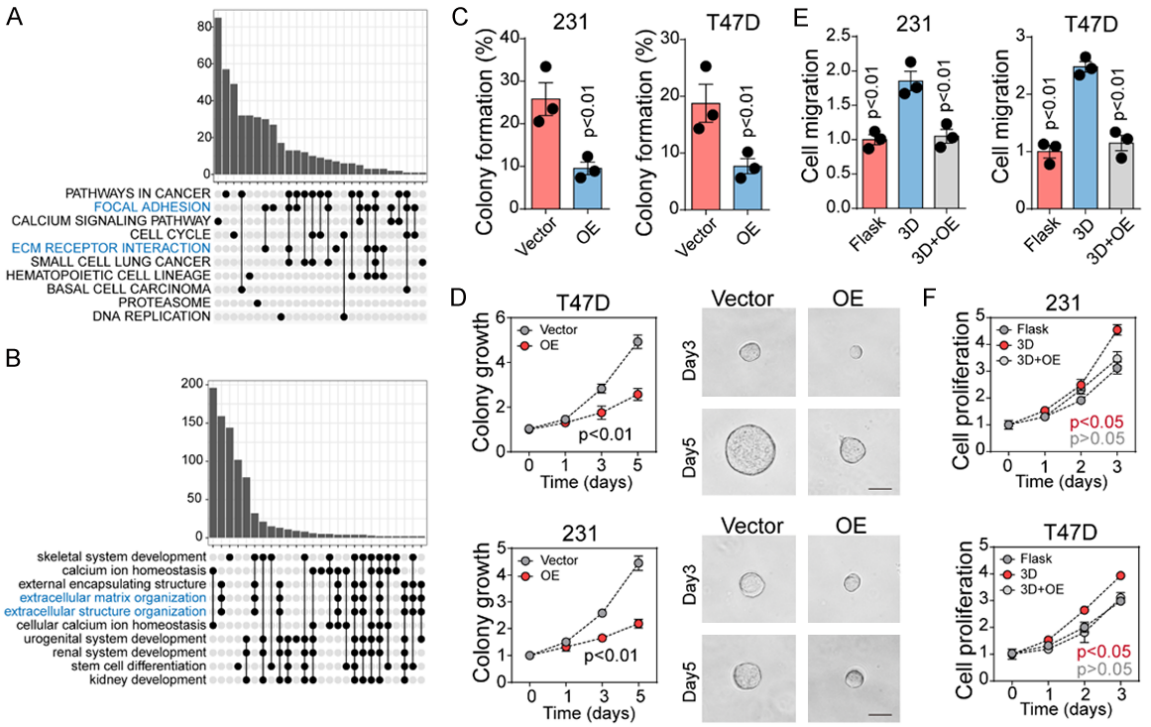

Figure 2. Excessive CLDN19 suppressed breast cancer cell growth in 3D microenvironment. (A, B) GO (A) and KEGG (B) enrichment of major signaling pathways in high-(n=407) and low-(n=409) CLDN19 breast cancer patients derived from TCGA data. (C, D) Colony formation (C) and growth (D) of vector and CLDN19 overexpressed MDA-MB-231 and T47D cells in Matrigel. The scale bar was 100 μm. (E, F) Vector and CLDN19 overexpressed MDA-MB-231 and T47D cells were seeded in flask or 3D Matrigel for 3 days. Then cells were isolated and cell proliferation/migration was determined.

breast cancer. To further validate our hypothesis, CLDN19 was overexpressed in breast cancer cell lines MDA-MB-231 and T47D (**Figure 1E**), and cell proliferation/migration was then determined. However, no significant alterations of cell proliferation (**Figure 1F**) or migration (**Figure 1G**) were found in CLDN19 overexpressed group, indicating that CLDN19 might participate in breast cancer progression regulation in an indirect manner.

Excessive CLDN19 suppressed breast cancer cell growth in 3D microenvironment

Next, we sought to elucidate the molecular pathway driven by CLDN19 to modulate breast cancer progression. We analyzed the mRNA expression profile of CLDN19-high patients in comparison with CLCN19-low patients by utilizing TCGA dataset. Differentially expressed genes were identified and enrichment analysis was performed. The CLDN19 involved main pathways for differentially expressed genes were determined. Notably, GO and KEGG enrichment analysis indicated that CLDN19 directly participated in modulating ECM signaling and cell focal adhesion process (**Figure 2A** and **2B**). As previously reported, the 3D extracellular matrix and focal adhesion kinase signaling could regulate cancer stem cell function, resulting in sustained tumor development *in vivo* [17, 18]. Thus, we supposed that excessive CLDN19 expression disrupted extracellular matrix-induced pro-survival signaling in breast cancer cells, and suppressed tumor progression in an ECM-induced 3D microenvironment. To validate our hypothesis, we seeded vector and CLDN19 overexpressed MDA-MB-231/T47D cells in 3D Matrigel, which has been reported to support cancer stem cells selection and amplification through 3D/ECM signaling. Intriguingly, reduced colony formation (**Figure 2C**) and growth (**Figure 2D**) were found in CLDN19 overexpressed MDA-MB-231 and T47D cells. Furthermore, we isolated tumor cells from 3D Matrigel and evaluated cell proliferation/migration again. CLDN19 overexpression suppressed the proliferative/migrative

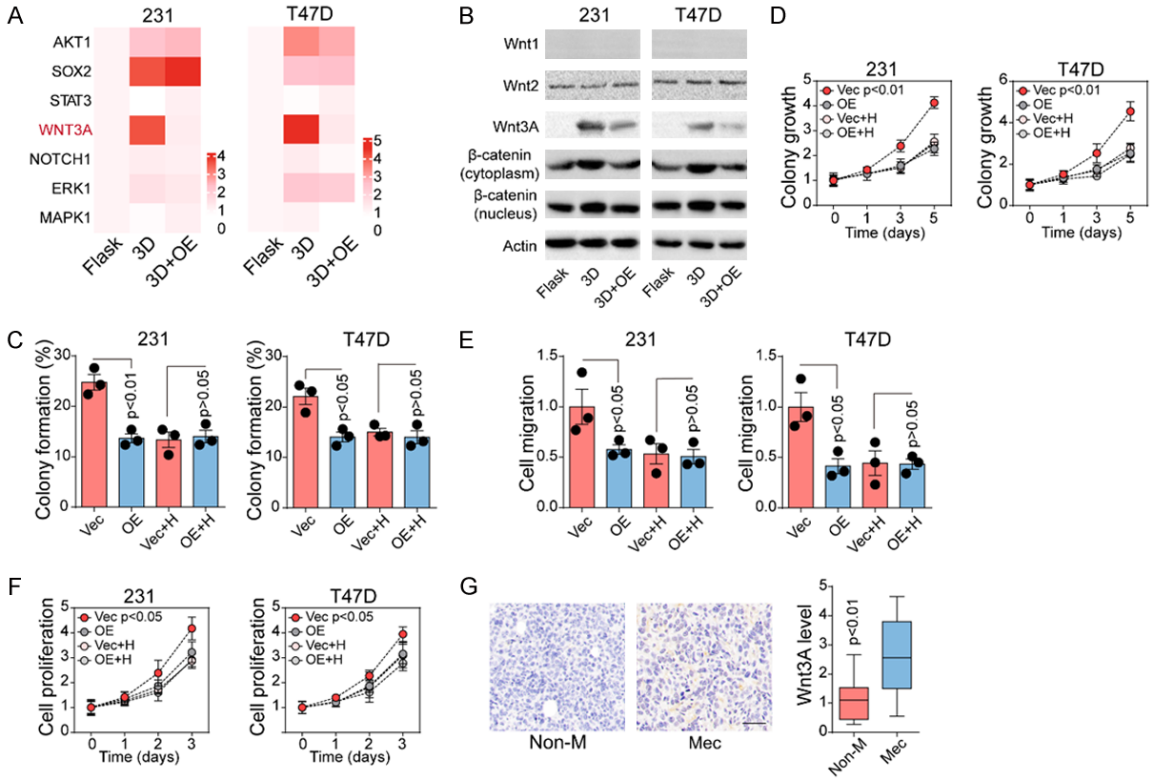

Figure 3. CLDN19 suppressed Wnt signaling activation in 3D microenvironment. A. Heatmap of mRNA expression of *AKT1*, *Wnt3A*, *SOX2*, *STAT3*, *NOTCH1*, *ERK1* and *MAPK1* in flask or 3D Matrigel cultured MDA-MB-231 and T47D cells (vector or CLDN19 overexpression). B. Western blotting of Wnt1, Wnt2, Wnt3A and β-catenin (in cytoplasm and nucleus) in flask or 3D Matrigel cultured MDA-MB-231 and T47D cells (vector or CLDN19 overexpression). C, D. Colony formation and growth of vector and CLDN19 overexpressed MDA-MB-231/T47D cells in 3D Matrigel (treated with PBS or 20 nM HLY78). E, F. Vector or CLDN19 overexpressed MDA-MB-231/T47D cells were seeded in 3D Matrigel (containing 20 nM HLY78 or not) for 3 days. Then cells were isolated and cell proliferation/migration was determined. G. Wnt3A protein level of non-metastatic (n=11) and metastatic (n=11) breast tumor tissues was quantified by immunohistochemical staining. The scale bar was 100 μm.

characteristics-induced by 3D Matrigel (**Figure 2E** and **2F**). Taken together, those data suggested that CLDN19 disrupted ECM-induced pro-survival signaling, resulting in a tumor suppressive effect.

CLDN19 suppressed Wnt signaling activation in 3D microenvironment

Motivated by prior results that CLDN19 disrupted ECM-associated pro-survival signaling, we next sought to explore the mechanism underlying CLDN19-ECM-breast cancer progression. Compelling studies have provided evidence that compounds in ECM, including collagen and fibronectin, could mediate pro-survival signaling pathways activation in tumor cells [19, 20]. Thus, we examined mRNA levels of major pro-survival signaling molecules, including AKT1, Wnt3A, SOX2, STAT3, NOTCH1, ERK1 and MAPK1 in MDA-MB-231 and T47D cells. Notably, elevated expression of Wnt3A was found in Matrigel-cultured tumor cells in comparison with flask-cultured group, whereas CLDN19 overexpression suppressed Wnt3A upregulation (**Figure 3A**). Accordingly, elevated expression of Wnt 3A and β-catenin (nucleus) at protein level was found in 3D cultured cells, whereas CLDN19 inhibited the activation of Wnt signaling, which was examined by means of western blotting (**Figure 3B**), indicating that CLDN19 might suppress Wnt signaling activation to influence ECM associated tumor progression. To further clarify the role of Wnt signaling, Wnt/β-catenin inhibitor HLY78 was added into the supernatant of 3D culture system. Accordingly, HLY78 suppressed colony formation and growth of 3D Matrigel cultured MDA-MB-231 and T47D, whereas limited effects were found in CLDN19 overexpressed group (**Figure 3C** and **3D**). Meanwhile, blockade of Wnt signaling

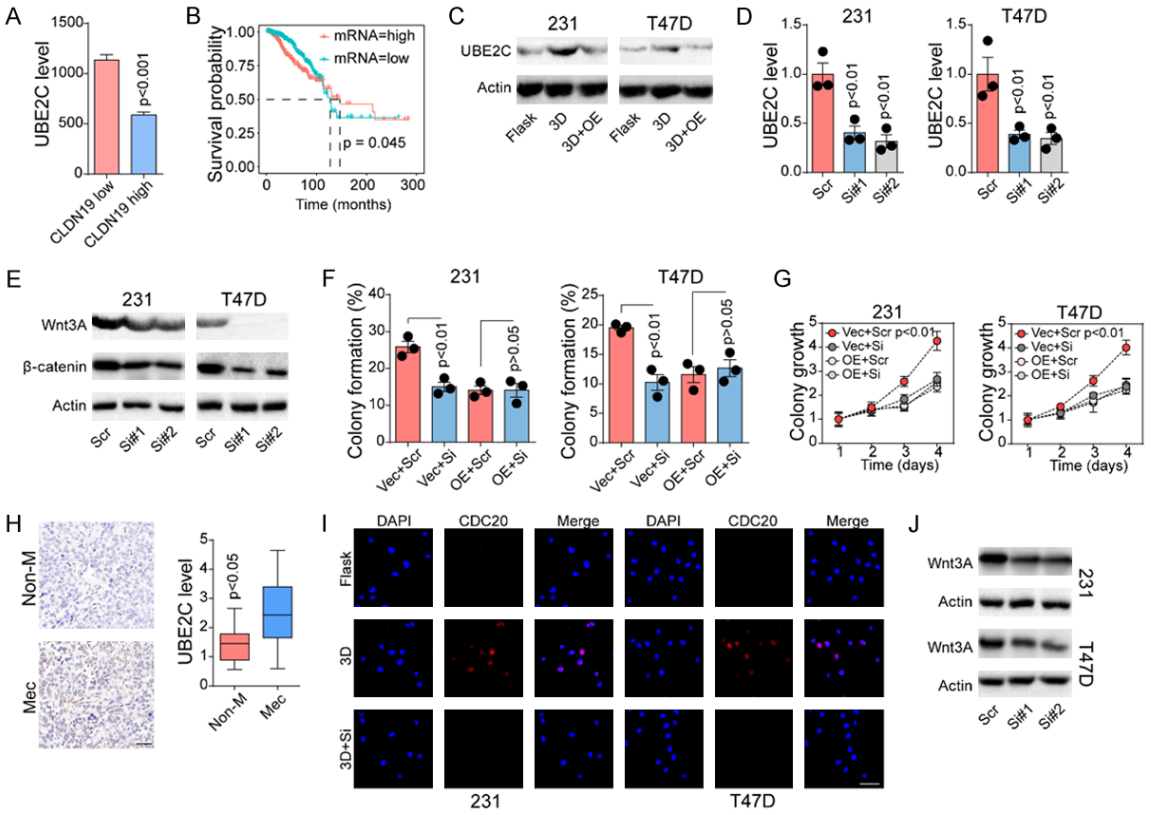

Figure 4. LDN19 downregulated UBE2C to regulate Wnt signaling pathway. A. MRNA level of UBE2C in CDLN19-high patients (n=407) in comparison to CLDN19-low patients (n=409), derived from TCGA data. B. Kaplan-Meier overall survival curve was shown according to high (n=408) and low (n=408) expression of UBE2C in breast cancer patients derived from TCGA data. C. Western blotting of UBE2C in vector or CLDN19 overexpressed MDA-MB-231/T47D cells cultured in flask or 3D Matrigel. D. MRNA level of UBE2C in MDA-MB-231/T47D cells treated with scramble or UBE2C siRNA. E. Western blotting of Wnt3A and β-catenin in 3D Matrigel cultured MDA-MB-231/T47D cells, treated with scramble or UBE2C siRNA. F, G. Colony formation and growth of MDA-MB-231/T47D cells treated with scramble or UBE2C siRNA in 3D Matrigel. H. UBE2C protein level of non-metastatic (n=11) and metastatic (n=11) breast tumor tissues was quantified by immunohistochemical staining. The scale bar was 100 μm. I. Immunostaining of CDC20 in flask or 3D cultured MDA-MB-231/T47D cells (scramble or UEB2C siRNA treatment). The scale bar was 50 μm. J. Western blotting of Wnt3A and β-actin in 3D cultured MDA-MB-231/T47D cells treated with scramble or CDC20 siRNA.

by HLY78 suppressed cell proliferation and migration induced by Matrigel, and had no obvious influence on CLDN19 overexpressed MDA-MB-231 and T47D cells (**Figure 3E** and **3F**). Next, we performed immunostaining analysis of Wnt3A in 22 clinical specimens and obtained similar results that tumor tissues derived from metastatic patients displayed elevated expression of Wnt3A in comparison with non-metastatic group (**Figure 3G**). Collectively, those results revealed that CLDN19 suppressed Wnt signaling activation in ECM microenvironment.

CLDN19 downregulated UBE2C to regulate Wnt signaling pathway

To further elucidate how CLDN19 inhibited Wnt signaling in breast cancer, we analyzed the upregulated/downregulated genes in CLDN19-high patients in comparison with LDN19-low group. Intriguingly, we observed reduced UBE-2C expression at mRNA level in CLDN19-high patients (**Figure 4A**), which has been demonstrated to promote Wnt signaling activation in gastric cancer [21]. Indeed, patients with high UBE2C expression exhibited a shortened survival time compared to low-UBE2C group (**Figure 4B**). Meanwhile, CLDN19 overexpression suppressed UBE2C expression in Matrigel cultured MDA-MB-231 and T47D cells (**Figure 4C**). And suppression of UBE2C by siRNA (**Figure 4D**) inhibited Wnt-β-catenin activation in Matrigel cultured cells (**Figure 4E**). Silence of UBE2C also hampered breast cancer cell colony formation and growth in Matrigel, whereas no difference was found in CLDN19 overex-

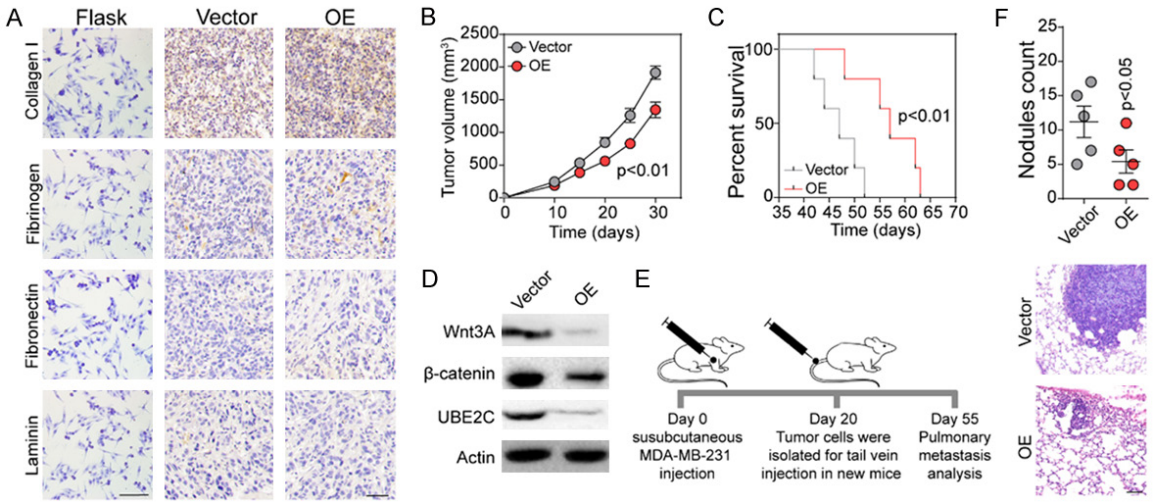

Figure 5. CLDN19 modulated tumor growth *in vivo*. A. Immunostaining of collagen I, fibrinogen, fibronectin and laminin in flask cultured MDA-MB-231 cells or tumor tissues derived from vector/CLDN19 overexpressed MDA-MB-231-bearing mice. The scale bar was 100 μm. B. Tumor growth curve of vector/CLDN19 overexpressed MDA-MB-231-bearing mice. C. Kaplan-Meier overall survival curve was shown in vector/CLDN19 overexpressed MDA-MB-231-bearing mice. D. Western blotting of UBE2C, Wnt3A and β-catenin in tumor tissues derived from vector/CLDN19 overexpressed MDA-MB-231-bearing mice. E. Tumor cells were isolated from subcutaneous vector/CLDN19 overexpressed MDA-MB-231-bearing mice (day 20), then injected into immunodeficient mice by tail vein injection. Lung tissues were harvested on day 25 for metastasis analysis. F. H&E staining of lung tissues and pulmonary metastatic tumor modules counting in vector/CLDN19 overexpressed MDA-MB-231-bearing mice. The scale bar was 200 μm.

pressed cells (**Figure 4F** and **4G**). Next, we further examined the expression of UBE2C in 22 clinical specimens, in which metastatic patients exhibited increased level of UBE2C in comparison with non-metastatic group (**Figure 4H**). Next, we wondered how UBE2C modulated Wnt signaling pathway in breast cancer. We screened the protein-to-protein interaction of UBE2C in String database, and found that UBE2C contracted with CDC20, which has been reported to promote Wnt signaling activation to mediate cell cycle control [22]. Thus, we further examined the expression of CDC20 in MDA-MB-231 and T47D cells. Indeed, 3D culture upregulated CDC20 expression in MDA-MB-231/T47D cells, and inhibition of UBE2C suppressed CDC20 expression in 3D culture system (**Figure 4I**). Silence of CDC20 by siRNA interference also suppressed Wnt3A expression in MDA-MB-231 and T47D cells cultured in 3D gels (**Figure 4J**), indicating that UBE2C regulated Wnt signaling through CDC20. Collectively, all these results indicated that CLDN19 downregulated UBE2C to suppress Wnt signaling pathway activation in breast cancer.

CLDN19 modulated tumor growth in vivo

Stemming from our *in vitro* results, we next became interested in exploring the influence of CLDN19 on breast tumor growth *in vivo*. To do this, vector and CLDN19 overexpressed MDA-MB-231 cells were subcutaneously inoculated into immunodeficient mice, and tumor volume was recorded. Given the essential role of ECM in CLDN19 associated tumor progression, we firstly examined the expression of ECM compounds, including collagen I, fibrinogen, fibronectin and laminin in tumor tissues derived from those mice. In line with our hypothesis, abundant extracellular collagen I and fibrinogen were found in tumor tissues (day 20, **Figure 5A**). Subsequently, we further explored the influence of CLDN19 on tumor progression *in vivo*, and found that vector MDA-MB-231 cells formed more rapidly growing tumors in comparison with CLDN19 overexpressed group (**Figure 5B**). Meanwhile, vector MDA-MB-231-bearing mice displayed a longer survival time than CLDN19 overexpressed group (**Figure 5C**), indicating the tumor suppressive role of CLDN19 *in vivo*. In line with the higher expression of CLDN19, reduced expression of UBE2C, Wnt3A and β-catenin was found in CLDN19 overexpressed MDA-MB-231-bearing mice (**Figure 5D**). Next, we sought to evaluate the influence of CLDN19 on cancer metastasis *in vivo*, we isolated vector and CLDN19 overexpressed MDA-MB-231 cells from those tumor-bearing mice, which were injected into immunodeficient

mice by tail vein injection. After 25 days, mice were sacrificed and metastatic pulmonary tumor nodules were counted (**Figure 5E**). As excepted, excessive CDLN19 hampered the pulmonary metastatic potential of MDA-MB-231 cells *in vivo* (**Figure 5F**). Together, our experiments suggested that CLDN19 played an essential role in suppressing breast tumor growth *in vivo*.

Discussion

This study described mechanistic insight into the observation that CLDN19 served as a tumor suppresser in breast cancer. Claudins expression, especially claudin 1 and 2, has been observed in most breast carcinomas [23]; however, the expression and role of CLDN19 in carcinomas remain unclear in recent years. Roles for claudins in both promoting and suppressing cancer growth have recently been reported [12]. Indeed, the elevated claudin-2 expression level has been shown to facilitate colorectal cancer progression [24], and high claudin-2 levels was found fibrolamellar hepatocellular carcinomas [25]. However, in breast cancer, claudin-2 expression is downregulated in invasive breast carcinomas [26], and patients with low claudin-1 exhibited a longer survival time in comparison with high claudin-1 patients [27]. In this investigation, we firstly found that CLDN19 expression was downregulated in human breast cancer tissues. Additionally, on the basis of TCGA dataset, we found that low level of CLDN19 expression in breast cancer patients was strongly corrected with metastatic potential and poor survival in patients. Those results indicated the potential of CLDN19 to serve as prognostic indicator for breast cancer progression.

To elucidate how CLDN19 affects the behavior of breast cancer cells, we established CLDN19 overexpressed MDA-MB-231 and T47D cells. Intriguingly, although expression levels of CLDN19 had a negative correlation with prognosis in patients, modulation of CLDN19 expression did not affect cell proliferation and migration in MDA-MB-231 or T47D cells. However, we further injected vector or CLDN19 overexpressed MDA-MB-231 cells into immunodeficient mice, and found slowed tumor growth curve in CLDN19 overexpressed tumor-bearing mice, suggesting that CLDN19 might participate in breast tumor progression regulation indirectly. Tight junction proteins participated in a variety of cellular processes that regulate cell differentiation and transformation [28]. Notably, ECM was enriched in tumor tissues and has been reported to modulates tumor cell differentiation and transformation to induce tumorigenic potential [29]. This reminded us the potential role of claudins in ECM associated tumor progression. Here, we further seeded vector or CLDN19 overexpressed tumor cells in 3D Matrigel, and found CLDN19 overexpressed cells displayed reduced colony formation capability and migrative potential in the presence of extracellular components. More importantly, CLDN19 overexpressed tumor cells displayed weakened proliferative characteristics and metastatic potential in the presence of ECM (collagen and fibrinogen) *in vivo*. Our study firstly indicated that excessive CLDN19 could suppress ECM-associated tumor progression *in vivo*, inhibiting tumor cell growth and contributing to prolonged survival time in tumor-bearing mice.

Claudins are tight junction membrane proteins that expressed in epithelia and a variety of cancer cells [30]. And aberrant expression of claudins in tumor cells is involved in the activation of several pro-survival signaling pathways, including β-catenin/TCF, ERK/MAPK and IL-6/STAT3 signaling [31-33]. The Wnt/β-catenin signaling pathway comprises a family of proteins that play essential roles in embryonic development and adult tissue homeostasis [34]. Increasing evidence suggested that upregulation of Wnt/β-catenin signaling promoted cancer stem cells proliferation, contributing in sustained tumor growth and distant metastasis in several cancer types, including colorectal cancer, lung cancer, and breast cancer [35]. Additionally, ECM proteins, including collagen, have been reported to module Wnt/β-catenin signaling activation to stimulate tumorigenic potential of cancer cells, resulting in enhanced tumor stemness [36]. Our study further confirmed that 3D ECM could mediate Wnt signaling upregulation in CLDN19 low breast cancer cells. Excessive CLDN19 reduced Wnt3A expression at protein level, resulting in the tumor suppressive effects in breast cancer *in vivo*. UBE2C is a member of the anaphase promoting complex/cyclosome, which facilitates the degradation of several target proteins [37]. Recently, compelling investigations suggested that UBE2C was overexpressed in many tumor

tissues and displayed higher expression in patients with advanced cancer [38, 39]. Intriguingly, UBE2C has been reported to induce epithelial-mesenchymal transition to promote gastric cancer cell metastasis through Wnt/β-catenin and PI3K/Akt signaling [21]. In this study, we found that CLDN19 downregulated UBE2C to suppress Wnt/β-catenin signaling activation, which further disclosed the mechanism underlying UBE2C-induced tumor progression, and clarified the role of UBE2C in ECM-associated tumor behaviors.

Based on the above results, our study illustrated that: (1) The expression of CLDN19 was negatively correlated with the poor prognosis and could be potentially translated into a prognostic indicator for breast cancer patients. (2) CLDN19 suppressed the proliferative properties and migratory ability of breast cancer cells in 3D environment and *in vivo*. (3) The tumor suppressive effects of CLDN19 mainly depended on Wnt/β-catenin signaling pathway, induced by extracellular matrix proteins. (4) CLDN19 suppressed UBE2C to downregulated Wnt/β-catenin signaling. (5) Overexpression of CLDN19 hampered tumor growth and pulmonary metastasis in tumor-bearing mice, which pointed to a potential target for breast cancer management.

In summary, our study suggests that CLDN19 has a suppressive role in breast cancer development. By modulating CLDN19 expression, we found that deficiency of CLDN19 is necessary for tumor cells to induce the ECM-regulating pro-survival signaling pathway (UBE2C/Wnt signals) activation. Our findings raise the possibility that CLDN19 might be exploited as a potential diagnostic indicator for breast cancer progression.

Acknowledgements

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The study was approved by the Ethics Committee of the Fifth People's Hospital of Huai'an City. All samples collection and processing were carried out respecting the Declaration of Helsinki. All patients signed informed consent prior to tumor tissues collection treatment, including allowing their data to be used for further research. All experiments were per-formed under the monitor of the Ethics Committee of the Fifth People's Hospital of Huai'an City.

Disclosure of conflict of interest

None.

Abbreviations

CLDN19, Claudin 19; TCGA, The Cancer Genome Atlas Program; UBE2C, Ubiquitin Conjugating Enzyme E2 C; ECM, Extracellular Matrix.

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