**Overexpression of collagen type V α1 chain in human breast invasive ductal carcinoma is mediated by TGF-β1**

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**Abstract.** Collagen type V α1 chain (COL5A1) is a minor fibrillar collagen in mammals that co-polymerizes with type I collagen to adjust the diameter of collagen molecules. However, the function of COL5A1 in invasive ductal carcinoma (IDC) of the human breast remains unknown. In the present study, our group examined the expression of COL5A1 in IDC compared with its adjacent normal tissue and fibroadenoma of the breast. COL5A1 was revealed to be overexpressed in IDC compared with benign tumor and adjacent normal control tissues, and was associated with the expression of estrogen receptor and progesterone receptor. No association between COL5A1 expression and tumor size, lymph node metastasis, clinical stage, age, or Her2 expression was identified. High expression of COL5A1 mRNA was associated with distant metastasis free survival in patients with breast cancer. Knockdown of COL5A1 led to a decrease of cell viability, as detected by the WST-1 assay, and an inhibition of migration, as detected by wound healing and Transwell assays, respectively, in the breast cancer cell line MCF-7. The expression of COL5A1 in MCF-7 cells was downregulated by transforming growth factor (TGF)-β1, which was abolished in the presence of SB-431542, an inhibitor of TGF-β type I receptor. In conclusion, these data indicated that COL5A1 is overexpressed in IDC and regulated by TGF-β1, suggesting that an increase of COL5A1 reflects tumor progression and may serve as a novel biomarker and therapeutic target for the treatment of breast IDC.

**Introduction**

Breast cancer is one of the most commonly diagnosed cancers in females worldwide (1). The estimated new cases of female breast cancer and associated mortalities were 252,710 and 40,610, respectively, in the United States in 2017 (2) and 268,600 and 69,500, respectively, in China in 2015 (3). Among American women, there were an estimated 231,840 new cases of invasive breast cancer diagnosed in 2015 (4). The majority of breast cancer cases are histologically invasive ductal carcinoma (IDC; also known as infiltrating ductal carcinoma) (5), followed by the invasive lobular cancer subtype (6). Despite improvements to various treatment methods, there remains a lack of effective ways to prevent and cure this malignant disease. Therefore, identifying specific biomarkers and therapeutic targets of breast cancer at an early stage is of particular importance.

Collagen type V α1 chain (COL5A1) is a minor fibrillar collagen which has previously been reported to be associated with embryonic development, fibrillogenesis and Ehlers-Danlos syndrome (7). COL5A1 forms a heterotrimer (one α1 and two α2) (8) and usually co-polymerizes with type I collagen to adjust the diameter of the collagen molecules (9). Overexpression of the COL5A1 gene has been observed in certain pathological conditions, including inflammation and atherosclerosis (10). However, previous studies have suggested that COL5A1 may be involved in tumor initiation and progression in several types of malignant tumor. For example, the expression of COL5A1 is increased in tongue squamous cell carcinoma and ovarian cancer, and is associated with certain clinical characteristics (11,12). Furthermore, COL5A1 has been identified as a biomarker of human gastric cancer using gene expression profiling (13) and RNA-sequencing has revealed that it is associated with the extracellular matrix (ECM) degradation pathways in papillary thyroid carcinoma (14). More recently, comprehensive bioinformatics analysis has revealed that COL5A1 is one of the key genes between patients with inflammatory and non-inflammatory breast cancer (15). The collagen family is also a promising prognostic marker for...
patients with cancer (11) and contributes to cancer progression by participating in the ECM-receptor interaction pathway (16). Furthermore, in osteoblasts, COL5A1 has been revealed to be mediated by transforming growth factor-β (TGF-β) (17), a cytokine that participates in the invasive progression of breast cancer (18). However, the function of COL5A1 in IDC and whether COL5A1 is regulated by TGF-β in IDC remains unclear.

The present study was performed to detect the expression of COL5A1 in human IDC compared with its adjacent normal tissue and fibroadenoma of the breast. The loss-of-function of COL5A1 and the regulation of COL5A1 protein expression by TGF-β1 were also investigated in breast cancer cells.

Materials and methods

Human subjects and tissue sample preparation. Informed consent was obtained from patients, and the present study was approved by the Ethics Committee of Jinshan Hospital, Fudan University (Shanghai, China). A total of 180 paraffin-embedded breast tissue samples (90 cases of IDC and 90 cases of fibroadenoma) were collected at Jinshan Hospital between January 2010 and December 2015. The median age of patients was 54 years (range, 33-85 years). No patients underwent chemotherapy and radiotherapy prior to cytoreductive surgery. The IDC and fibroadenoma tissues were diagnosed by pathologists.

Immunohistochemical (IHC) staining and analysis. IHC analysis was performed as previously described (19). Briefly, the 4% paraformaldehyde-fixed, paraffin-embedded tissue specimens were sectioned (4 µm thick), deparaffinized in xylene and rehydrated in a descending alcohol series. Following blocking for 30 min at room temperature with 10% normal goat serum (Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China; cat. no. SP KIT-B2), the sections were incubated with monoclonal rabbit anti-COL5A1 (1:200 dilution; cat. no. SAB4500384, Sigma-Aldrich; Merck KGaA), with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:200 dilution; cat. no. L3012 and L3032; Signalway Antibody LLC, College Park, MD, USA) at 4˚C overnight and subsequently incubated with 10% normal goat serum (Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China; cat. no. SP KIT-B2), the sections were incubated with a primary antibody at 4˚C overnight and subsequently incubated with HRP-conjugated goat anti-rabbit or anti-mouse IgG (10 µg/ml rhTGF-β1; cat. no. 240-B; R&D Systems, Inc., Minneapolis, MN, USA) at 37˚C for 48 h. For the blocking assay, cells were pretreated with an inhibitor of TGF-β type I receptor kinase (10 µM SB-431542, cat. no. S4317-5MG; Sigma-Aldrich; Merck KGaA) at 37˚C for 30 min, followed by treatment of rhTGF-β1 at 37˚C for 48 h.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA in the tissues and cells was extracted using TRIzol reagent (cat. no. 9109; Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer’s protocol. Total RNA (1 µg) was reverse transcribed using a First Strand cDNA Synthesis kit (cat. no. 0489686601; Roche Diagnostics, Indianapolis, IN, USA). The primers were synthesized by BioTNT Co., Ltd. (Shanghai, China). The sequences of primers were as follows: human COL5A1 forward, 5’-CTCCCTGC TTTCCTTTATCCT-3’ and reverse, 5’-GAATGTGCTTTGGC TATCCTG-3’; human β-actin forward, 5’-AAGGTGACACGATCGTGGT-3’ and reverse, 5’-TGTGTTGACTTGAGG GAGG-3’. PCR amplification was performed on 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), using a SYBR-Green I Master kit (cat. no. 04707516001; Roche Diagnostics) with the following steps: 1 cycle of 95˚C for 10 min for denaturation and 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec for amplification. The 2-ΔΔCq method (22) was used to calculate the relative amount of COL5A1 normalized to the β-actin control using Sequence Detection Software v1.4 (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Cell culture and TGF-β1 administration. Human breast non-tumorous MCF-12A and cancerous MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). MCF-12A and MCF-7 cells were cultured in Dulbecco’s modified Eagle’s medium/F12 and RPMI-1640 media, respectively. Following incubation for 24 h in 6-well plates, the cells were treated with recombinant human TGF-β1 (10 ng/ml rhTGF-β1; cat. no. 240-B; R&D Systems, Inc., Minneapolis, MN, USA) at 37˚C for 48 h. For the blocking assay, cells were pretreated with an inhibitor of TGF-β type I receptor kinase (10 µM SB-431542, cat. no. S4317-5MG; Sigma-Aldrich; Merck KGaA) at 37˚C for 30 min, followed by treatment of rhTGF-β1 at 37˚C for 48 h.
using GIS ID Analysis Software v4.1.5 (Tanon Science and Technology Co., Ltd.).

Small interfering (si)RNA transfection. A total of 2x10^5 cells/well were plated into 6-well plates for 24 h, and then were transfected with 50 nM/well human COL5A1-siRNA (COL5A1-siR) or scramble, nonspecific control-siRNA (C-siR). The sequences of human COL5A1-siR were 5'-GGGAUUCUCAAGGUUATT-3' (sense) and 5'-UUAACCUUGGAUCCCTT-3' (antisense). The sequences of C-siR were 5'-UUCUCCGAACGUGUACUGTT-3' (sense) and 5'-AAGUUGACAGGUUCGGA GAATT-3' (antisense). GAPDH was used as a positive control with the sequences of 5'-UGACCUACUACAUUGGUUTT-3' (sense) and 5'-AACAUUGAGUUGAGGUATTT-3' (antisense). All siRNAs were purchased from Shanghai GenePharma Co., Ltd., (Shanghai, China). Transfection was performed using X-tremeGENE siRNA Transfection Reagent (cat. no. 4476093001; Roche Diagnostics).

Cell viability assay. Cells were seeded in a 96-well plate at a density of 3,000/well for 24 h, and then transfected with 0.5 µg/well human COL5A1 siRNA (COL5A1-siR) or control scramble siRNA (C-siR) and incubated for 24 to 48 h. Cell viability was measured using the WST-1 kit (cat. no. W201; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol. The optical density of each well was read at 450 nm using a plate reader (BioTek Instruments, Inc., Winooski, VT, USA). The experiment was repeated at least three times.

Wound healing assay. Following COL5A1-siR transfection, a scraping wound was made in the cell culture using a 1 ml pipette tip. Following removal of the detached cells and cellular debris by washing with culture medium, the attached cells were incubated in medium containing 10% fetal bovine serum (FBS; cat. no. 35-010-CV; Corning Life Sciences, Tewksbury, MA, USA) for 48 h. Photos were taken using an inverted microscope and the width of each gap was analyzed using MATLAB software version R2015b (The MathWorks, Inc., Natick, MA, USA).

Transwell invasion assay. Transwell chambers (cat. no. 3422; Corning Life Sciences) were inserted into 24-well plates with the addition of 0.1 ml of pre-warmed and diluted Matrigel Matrix (cat. no. 356234; Corning Life Sciences). Following gel formation for 1 h and hydration for 2 h at 37°C, the transfected cells (1 x 10^5 cells/well) were seeded into the upper chamber with serum-free medium. In the lower chamber, 0.5 ml culture medium with 10% FBS was added. Following cell culture at 37°C for 24 h, media in the upper and lower chambers were aspirated. Non-invaded cells on the inner surface of the membrane were removed with a cotton swab and the invaded cells on the outer surface of the membrane were fixed with 4% paraformaldehyde for 10 min and stained with crystal violet for 30 min at room temperature. The cells were observed using an inverted microscope and the survival plots revealed that high COL5A1 is overexpressed in human breast cancer. Positive staining of COL5A1 protein in ductal epithelial cells of breast tissue derived from patients with malignant IDC was observed by IHC (Fig. 1A). The positive rate of high COL5A1 protein expression was significantly increased in IDC tissues (malignant) compared with adjacent normal tissues (normal) and fibroadenoma tissues (benign; P<0.05) (Fig. 1B). Overexpression of COL5A1 at the mRNA and protein levels was further confirmed in IDC tissues by RT-qPCR (Fig. 1C) and western blot analysis (Fig. 1D and E).

COL5A1 expression is associated with the expression of ER and PR in patients with breast cancer. Next, our group examined whether the expression of COL5A1 protein was associated with the clinicopathological features of patients with IDC. The expression of COL5A1 protein was not significantly associated with the majority of clinicopathological features, including age (≥60 vs. <60), lymph node metastasis (yes vs. no), tumor size (≤2.0 vs. >2 cm), histological grades and clinical stages (all P>0.05) (Table I). However, through comparisons of COL5A1 protein expression with breast cancer-associated markers, expression of COL5A1 protein was revealed to be associated with the expression of ER and PR (P<0.05) (Fig. 2 and Table I), but was not associated with the expression of Her2 and Ki-67, a proliferation marker (Table I). Using bioinformatics analysis of the Oncomine database (www.oncomine.org), these results were confirmed to be consistent with data from public databases. A high level of COL5A1 mRNA expression was observed in invasive breast carcinomas compared with normal breast tissues in the microarray datasets from different groups with a fold change ≥2 (Fig. 3). There were fold changes of 5.043 (P<0.001; Fig. 3A) in the datasets of Finak (23), 3.585 (P<0.0001; Fig. 3B) in the datasets of Ma (24), 2.84 (P<0.0001; Fig. 3C) in the datasets of TCGA (http://tcga-data.nci.nih.gov/tcga/), 2.703 (P=0.001; Fig. 3D) in the datasets of Radvanyi (25), 2.178 (P<0.0001; Fig. 3E) in the datasets of Zhao (26), and 2.005 (P<0.0001; Fig. 3F) in the datasets of Curtiss (27), respectively. These data indicated that COL5A1 may be involved in IDC development.
Figure 1. Expression of COL5A1 in breast tissues. (A) Expression of COL5A1 protein in adjacent normal tissues (Normal), fibroadenomas (Benign) and invasive ductal carcinomas (Malignant) was detected by IHC using a specific antibody against COL5A1. The bottom panel shows amplified images from the square area of the upper panel. Original magnification, x100 and x400. (B) Quantitative analysis of the positive rate of COL5A1 protein expression in normal, benign, and malignant tissues (each group: n=90 cases). (C) Reverse transcription-quantitative polymerase chain reaction analysis of COL5A1 mRNA expression in normal, benign, and malignant tissues (n=3 each). (D) Western blot analysis of COL5A1 protein expression in normal, benign, and malignant tissues. Representative images are presented. (E) Histogram with the semi-quantification of the gels in D (n=3 each). *P<0.05, with comparisons indicated by lines. COL5A1, collagen type V α1 chain; IHC, immunohistochemistry.

Figure 2. Pathological H&E and immunohistochemistry staining of the low and high expression of COL5A1, ER, and PR. Representative images are shown. Original magnification, x200; scale bar, 100 µm. H&E, hematoxylin and eosin; COL5A1, collagen type V α1 chain; ER, estrogen receptor; PR, progesterone receptor.
expression of COL5A1 mRNA was associated with distant metastasis free survival in patients with breast cancer (Fig. 3G), but was not associated with overall survival (OS), relapse-free survival or progression-free survival.

Knockdown of COL5A1 inhibits breast cell viability, migration, and invasion. To determine the effect of COL5A1 on the biological functions of breast cells, a loss-of-function approach was applied. Following transfection with a specific siRNA for 24 h, a significant decrease of COL5A1 protein was observed in MCF-12A and MCF-7 cells (P<0.05; Fig. 4A and B). Cell viability was significantly decreased in MCF-7 cells (P<0.05) but not in MCF-12A cells (P>0.05) following COL5A1-siRNA transfection for 24, 48 and 72 h (Fig. 4C and D).

Next, the migration of cells was examined following COL5A1 knockdown. The wound healing assay revealed that there was no difference in the width of the wound between COL5A1-siRNA-transfected and control-siRNA (NC)-transfected MCF-12A cells (P>0.05; Fig. 5A). However, wound width was significantly decreased in MCF-7 cells following COL5A1-siRNA transfection compared with NC at 48 h (P<0.05; Fig. 5B). Furthermore, knockdown of COL5A1 did not affect MCF-12A cell invasion (Fig. 5C) but significantly decreased the number of invading MCF-7 cells in the Transwell assay (Fig. 5D). These data indicated that COL5A1-siRNA influenced breast cancer MCF-7 cell viability, migration, and invasion, but this did not occur in the non-tumorous cell line MCF-12A.

COL5A1 expression is regulated by the TGF-β signaling pathway in breast cancer cells. As the TGF-β signaling pathway is involved in breast tumorigenesis (29), our group subsequently investigated whether the TGF-β signaling pathway regulates COL5A1 expression. Using western blot analysis, COL5A1 protein expression was revealed to be significantly increased following treatment with 10 ng/ml TGF-β1 in MCF-7 cells at 24, 48 and 72 h (P<0.05; Fig. 6A). An increase in Smad2 phosphorylation, which is a TGF-β signaling transducer protein activated following TGF-β1 treatment, was also observed in TGF-β1-treated MCF-7 cells, suggesting that TGF-β signaling exists in these cells (Fig. 6A). TGF-β1-mediated COL5A1 expression was blocked by its type I receptor inhibitor SB-431542 (10 nM; Fig. 6B), indicating that COL5A1 is regulated by the TGF-β signaling pathway.

Discussion

The present study demonstrated the overexpression of COL5A1 in human IDC. Collagen is one of the main components of the ECM and functions in cell adhesion, migration, differentiation and tissue regeneration, and at least 28 types of collagen have been identified (30). Type V collagen is a minor fibrillar collagen and has three subtypes: COL5A1, COL5A2 and COL5A3. Despite it at a low level in certain tissues, COL5A1 has been implicated in biological and pathophysiological processes and serves an important function in ECM regulation and assembly (9).

Previous studies have revealed differential expression of COL5A1 in several types of cancer, including serous ovarian cancer, gastric cancer, meningioma, tongue squamous cell carcinoma and invasive bladder transition cell carcinoma (11-13,31,32). The present study focused on IDC, the most common histological type of breast cancer. The results clearly demonstrated that COL5A1 was overexpressed in malignant tumor IDCs compared with adjacent normal tissues and benign tumor fibroadenomas, suggesting that COL5A1 may have biological functions in the development of breast cancer. Bioinformatics analyses of the microarray table 1. Associations between COL5A1 and clinicopathological characteristics of patients with invasive ductal carcinoma (n=90).

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<th>High (n=44)</th>
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<th>P-value</th>
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*P<0.05. COL5A1, collagen type V α1 chain; ER, estrogen receptor; LN, lymph node; Her2, human epidermal growth factor receptor-2; n, number of case; PR, progesterone receptor.
Figure 3. Bioinformatics analyses of COL5A1 mRNA expression in breast tissues. Data were obtained from the microarray datasets of the Oncomine database (www.oncomine.org). High levels of COL5A1 mRNA expression were observed in invasive breast cancers compared with normal breast tissues in the microarray datasets of (A) Finak, (B) Ma, (C) TCGA, (D) Radvanyi, (E) Zhao and (F) Curtis, respectively.
data from the public datasets confirmed the observation that COL5A1 was overexpressed in malignant breast tumors, and may be associated with metastasis. Although no correlation was observed between COL5A1 expression and OS in patients

Figure 3. Continued. (G) Survival plot. Data were obtained from the Kaplan-Meier Plotter datasets (http://kmplot.com/analysis/). High expression of COL5A1 mRNA was associated with distant metastasis free survival in patients with breast cancer (Affymetrix ID: 212488_at). COL5A1, collagen type V α1 chain; TCGA, The Cancer Genome Atlas.

Figure 4. Detection of COL5A1 protein expression and cell viability. Knockdown of COL5A1 by siR was detected at the protein level by western blot analysis in (A) MCF-12A and (B) MCF-7 cells. Cell viability was detected by the WST-1 assay in (C) MCF-12A and (D) MCF-7 cells. Blank, no transfection control; C-siR, scramble siRNA control; COL5A1-siR, COL5A1 siRNA (n=3). *P<0.05 (COL5A1-siR vs. C-siR). COL5A1, collagen type V α1 chain; siR, small interfering RNA.
with breast cancer from the Kaplan-Meier Plotter database, an association between COL5A1 expression and poor OS of patients with ovarian cancer has been reported (12). In order to investigate whether the expression of COL5A1 is associated with OS, a large cohort study in patients with breast cancer is required in the future.

The present study demonstrated that increased COL5A1 expression in IDC was associated with estrogen receptor (ER) and progesterone receptor (PR) expression, indicating that COL5A1, like Her3 and Ki-67 (33,34), may serve a prognostic function in patients with IDC. Estrogen is a major driver of breast tumor cell growth (35) and ERα+ breast cancer is the leading cause of breast cancer-associated mortality (36). ER and PR, along with matrix metalloproteinase-2, have a prognostic value in patients with breast cancer (37). Indeed, the ER and PR status of human breast cancer represent important prognostic and predictive markers for human breast cancer (38,39). COL5A1 may also be a prognostic marker and an important contributor to tumor cell behavior as regulated by these hormone pathways. However, further studies are required in the future.

COL5A1 has a mutual interaction with other subtypes of collagen (9). Abnormal expression of COL5A1 is observed in a variety of diseases. Natural genetic mutation

Figure 5. Cell migration and invasion. The wound-healing assay shows cell migration following knockdown of COL5A1 in (A) MCF-12A and (B) MCF-7 cells. The Transwell assay shows cell invasion following knockdown of COL5A1 in (C) MCF-12A and (D) MCF-7 cells. NC, non-specific control of scramble siRNA; COL5A1-siR, COL5A1 siRNA (n=3). *P<0.05, with comparisons indicated by lines. COL5A1, collagen type V α1 chain; siR, small interfering RNA.
of the COL5A1 gene leads to defects in its expression and results in a congenital connective tissue dysplasia known as Ehlers-Danlos syndrome (40). In zebrafish embryos, COL5A1 is expressed in the spinal cord, somite, interstitial, cranial neural crest, and head cartilage (41). Knockout of COL5A1 causes severe dysfunction in the synthesis of collagen fibers (42,43). Notably, the present study demonstrated that knockdown of COL5A1 resulted in a decrease of the viability, migration, and invasion of breast cancer MCF-7 cells, but not of non-tumorous MCF-12A cells. As the expression of COL5A1 was relatively increased in MCF-7 cells compared with MCF-12A cells, an increase in its expression in cancerous cells may cause the change of cellular characteristics. Inhibiting collagen V has been demonstrated to lower the rate of growth, migration and invasion in 8701-BC breast cancer cells (44). These data indicate that COL5A1 is overexpressed in IDC and regulated by TGF-β1. An increase of COL5A1 reflects the breast tumor progression. COL5A1 may serve as a novel biomarker and become a therapeutic target for the treatment of human breast IDC.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.
Authors’ contributions

WR conducted experiments, and performed data analysis, figure generation and manuscript writing. YZ contributed to the collection of clinical samples and pathological diagnosis. LZ, QL, JZ performed part of the experiments and bioinformatics analyses. GX contributed to the experimental design, data analysis, figure generation and manuscript writing. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Jinshan Hospital, Fudan University (no. E-2017-11-01; Shanghai, China).

Consent for publication

Informed consent was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

References


