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Original Article

High expression of COL6A1 correlates with poor prognosis in patients with breast cancer

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Abstract: Background: Breast cancer refers to one of the most common types of malignant cancer and accounts for the second leading malignant tumor in females. The overexpression of collagen type VI alpha 1 chain (COL6A1) has been associated with poor prognosis in patients with multiple solid malignant tumors. Aim: The present study aimed to investigate the clinical and prognostic significance of COL6A1 in breast cancer. Method: Tissue samples were obtained from 232 patients with breast cancer who underwent surgical resection between 2003 and 2007. Immunohistochemical COL6A1 expression pattern was performed. Statistical correlations between COL6A1 expression, clinicopathological characteristics, and prognosis were evaluated. Real-time polymerase chain reaction (PCR) and western blotting analyses were performed to confirm COL6A1 expression in patient-derived breast cancer and adjacent normal tissue. Result: Of 232 tissues from patients with breast cancer, 224 (96.6%) were positive for COL6A1. COL6A1 protein expression was significantly associated with postsurgical pathological T phase (P = 0.002) and N phase (P = 0.001). COL6A1 expression was negatively correlated with overall survival and recurrence-free survival. The mean COL6A1 mRNA (P = 0.021) and protein levels (P = 0.001) were significantly higher in breast cancer tissues compared with adjacent normal tissue. Conclusion: Our results suggest that overexpression of COL6A1 can be found in patients with higher T classification, N classification, or poor prognosis including death or recurrence. COL6A1 expression may be valuable for the prognostic evaluation of breast cancer.

Keywords: COL6A1, prognosis, breast cancer

Introduction

Breast cancer is one of the most common types of malignant cancer and is the second leading female malignant tumor, affecting over 20% of women worldwide, and 38% of women in China [1, 2]. Moreover, the incidence of breast cancer is increasing in recent decades [3]. Thus, it is a major cause of mortality that threatens the health of women [4]. Great improvements on traditional treatments, such as surgery and chemotherapy, have improved the quality of life of breast cancer patients. Significantly, adjuvant therapy such as endocrine therapy (e.g., tamoxifen, fulvestrant), which is frequently used following surgical treatment, plays an important role in reducing the postoperative recurrence and prolonging the survival rate [5]. Despite recent advances in diagnosis and treatment strategies, the prognosis for advanced and metastatic breast cancer patients remains poor. The clinical resistance, such as recurrence, metastasis, and even death is the cruel reality [6, 7]. It is well known that tumor progression and distant metastases, which are markedly influenced by the proliferative nature of the disease, are still the primary issues affecting the clinical outcome of patients with breast cancer [8, 9]. Therefore, it is essential and necessary to explore potential biomarkers and the underlying molecular mechanisms involved in tumor progression and distant metastasis, to facilitate improvement in the clinical outcome of patients with breast cancer.

With the new data being accrued, the interactions between cancer and its microenvironment is of great importance in the development and progression of cancer. The stroma can be described as an active microenvironment that modulates the biology of the carcinoma, including cancer stem cells [10]. Cancer cells are supported by the network of beaded microfila-

Table 1. Clinicopathologic characteristics and COL6A1 expression of patients with breast cancer of the study cohort (n = 232)

Characteristics	Number of cases (%)
Gender	
Female	232 (100)
Age (years)	
≤ the median (47.5)	116 (50.0)
> the median (47.5)	116 (50.0)
T stage	
T_{1-2}	184 (79.0)
T_{3-4}	48 (21.0)
N stage	
N_0	136 (58.6)
N_{1-3}	96 (41.4)
Recurrence	
No	152 (65.5)
Yes	80 (34.5)
Vital status (at follow-up)	
Live	163 (70.3)
Death	69 (29.7)
COL6A1 expression	
Low	89 (38.4)
High	143 (61.6)

ments formed by extracellular matrix protein, the major element of which is collagen VI, composed of three major polypeptide chains (α1, α2, and α3) [11]. The COL6A1 gene is located at chromosome 21q22.3 and encodes the α1 chain, the family of collagen VI [12]. COL6A1 is involved in multiple signaling pathways that regulate apoptosis [13], proliferation, angiogenesis, fibrosis, and inflammation [14, 15]. Recent studies indicate that COL6A1 is differentially expressed in tumors and adjacent normal tissue [16, 17]. Chiu reported that COL6A1 knockdown suppresses the metastatic ability of lung cancer cells, whereas overexpression of COL6A1 has the opposite effect [18]. In a previous global secretome analysis, high expression of COL6A1 was found to promote in vivo bone metastasis [19]. The same function was also confirmed in renal clear cell carcinoma [20] and prostate cancer [21].

To date, the studies relating to the oncogenic functions of COL6A1 have been exposed, whereas the potential role and biological functions of COL6A1 in breast cancer remain unknown. In this study, we investigated COL6A1 expression in breast cancer to determine the clinical significance of COL6A1 overexpression in the development and progression of breast cancer.

Materials and methods

Patients and sample

The patients with breast cancer were diagnosed and underwent surgery at our hospital without any presurgical chemotherapy or radiotherapy between 2003 and 2007. All diagnoses were confirmed by clinical diagnosis and pathological assays. Patients or family members provided informed consent for the use of patient data and tissue samples. The exclusion criteria were as follows: (1) history of another tumor, (2) patient had presurgical chemotherapy or radiotherapy, (3) patient or family members refused the use of patient data and tissue samples. The present study was approved by the Ethics Committee of the Fifth Affiliated Hospital, Sun Yat-sen University. Initially, eight matched pairs of breast cancer and adjacent normal tissue were obtained for reverse-transcription polymerase chain reaction (RT-PCR) and western blot analysis. Paraffin-embedded breast cancer sections were obtained from 232 female patients treated in our hospital between 2003 and 2007. Patient clinical-pathological characteristics and tissue COL6A1 immunohistochemical details are summarized in **Table 1**.

Immunohistochemistry

The paraffin-embedded archival specimens were cut into 4-μm-thick sections and mounted on glass slides. Each slide was baked at 65°C for 30 minutes, then dewaxed in xylene and rehydrated in grade alcohol, followed by boiling in 10 mmol/L of citrate buffer (pH 6.0) for antigen retrieval. After inhibition of endogenous peroxidase activities by 3% hydrogen peroxide in methanol, slides were treated with 1% bovine serum albumin to block non-specific binding. The sections were then incubated overnight at 4°C with monoclonal rabbit antibody against COL6A1 (Abcam, Cambridge, USA; 1:100). After washing, the tissue sections were incubated with the prediluted secondary antibody, followed by further incubation with streptavidin-horseradish peroxidase complex. Finally, the sections were counterstained with hematoxylin and mounted in an aqueous mounting medium. For negative controls, primary antibodies were replaced with normal serum. Immunostaining

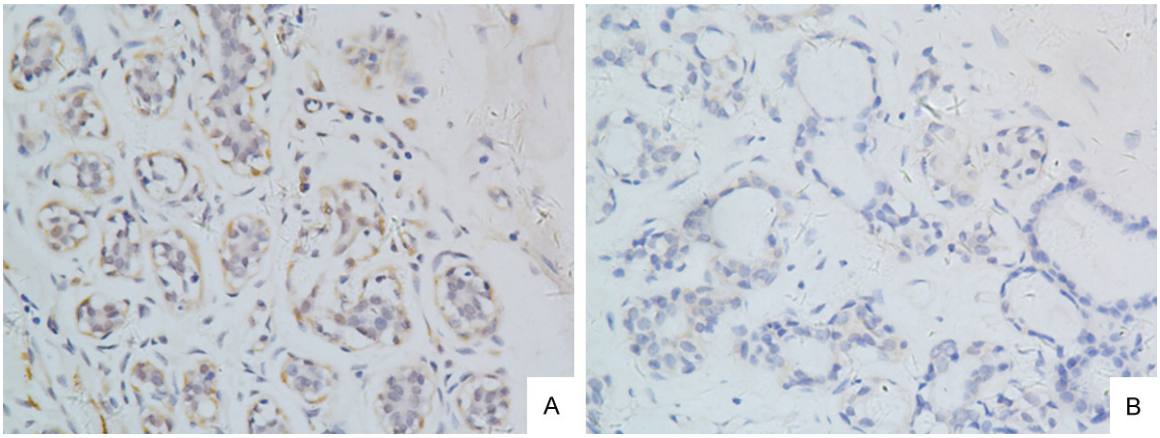

Figure 1. COL6A1 expression level in pathological tissue from the breast cancer patients. Different COL6A1 expression in breast cancer tissues (400×): (A) high for scores ≥4, (B) low for scores <4.

Table 2. Correlation between COL6A1 expression and clinicopathologic characteristics of patients with breast cancer

Characteristics	COL6A1 expression		χ^2 test *P* (Fisher's exact test *P*)
	Low or none, no. (%)	High, no. (%)	
Age (years)			0.138
≤ the median (47.5)	77 (53.8)	39 (43.8)	
> the median (47.5)	66 (46.2)	50 (56.2)	
T stage			0.001
T_{1-2}	123 (86.0)	61 (68.5)	
T_{3-4}	20 (14.0)	28 (31.5)	
N stage			0.001
N_0	82 (57.3)	14 (15.7)	
N_{1-3}	61 (42.7)	75 (84.3)	
Recurrence			0.001
No	120 (83.9)	32 (36.0)	
Yes	23 (16.1)	57 (64.0)	
Vital status			0.001
No	123 (86.0)	40 (45.0)	
Yes	20 (14.0)	49 (55.0)	

was separately reviewed and scored by two independent pathologists who were blinded to the patient information. Expression of COL6A1 was analyzed by an individual labeling score considering the proportion of positively stained tumor cells and the intensity of staining. The intensity of stained cells was graded semi-quantitatively into four levels: 0: no staining; 1: weak staining; and 2: strong staining. The area of staining was evaluated and recorded as a percentage, as follows: 0: no staining; 1: positive staining in less than 10% of tumor cells; 2: positive staining in 10% to 50% of tumor cells; 3: positive staining in 51% to 80% of tumor cells; and 4: positive staining in greater than 80% of tumor cells. The intensity and fraction of positive cell scores were multiplied and, thus, the scoring system was defined as low expression for scores of 0 to 3, and as high expression for scores of 4 to 8.

Reverse transcriptase-polymerase chain reaction

RNA was extracted from 8 matched PTC and nodular goiter tissues using TRIzol reagent, according to the manufacturer's instructions (Invitrogen), and treated with RQ1 RNase-free DNase (Pro-mega) before complementary DNA (cDNA) synthesis using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories). A semi-quantitative PCR assay was performed for COL6A1 using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The CCOL6A1 and GAPDH primers were designed using Primer Ex-press Software version 2.0 (Applied Biosystems). The PCR reaction steps were as follows: 95°C preheating 2 minutes, 40 cycles of 95°C denaturing for 15 seconds, 60°C annealing for 45 seconds, and 72°C extension for 1 minute. All experiments were performed in triplicate.

Western blot assay

Protein was extracted from eight matched pairs of breast cancer and adjacent normal tissues. Protein concentration was determined using a BCA assay. Protein was loaded on polyacryl-

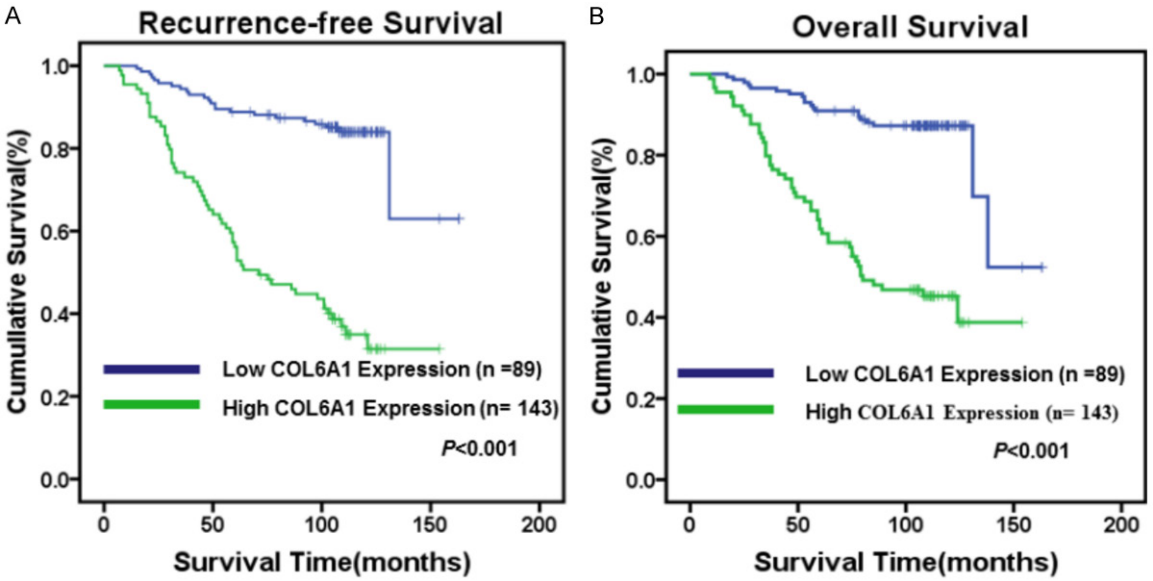

Figure 2. The level of COL6A1 protein expression affects overall survival and disease-free survival. Kaplan-Meier curves with univariate analysis (log-rank) for breast cancer patients with high COL6A1 expression (n = 143) versus low or no COL6A1 expression (n = 89) for overall survival (A) and disease-free survival (B).

Table 3. Cox proportional regression multivariate analysis of correlative factors

	B	SE	Wald	Df	Sig	Exp (B)	95.0% CI Exp (B)	
							Lower	Upper
Age	0.419	0.251	2.780	1	0.095	1.520	0.929	2.486
T stage	0.570	0.265	4.630	1	0.031	1.768	1.052	2.972
N stage	0.724	0.354	4.187	1	0.041	2.063	1.031	4.129
M stage	0.431	0.444	0.942	1	0.332	1.539	0.644	3.673
COL6A1 expression	1.205	0.300	6.117	1	0.000	3.338	1.853	6.013

amide gels for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and gels were run in buffer. Blots were transferred to polyvinylidine difluoride (PVDF) membranes using the Bio-Rad mini transfer system at 100 mA for 3 hours at 4°C. Membranes were blocked with 5% non-fat milk TBST solution and incubated for 1 to 1.5 hours at room temperature. Membranes were subsequently incubated in COL6A1 antibody at 4°C overnight (rabbit anti-human COL6A1, Abcam, 1:1000 dilution) or at room temperature for 1 hour in GAPDH mouse monoclonal antibody (Sigma, 1:1000 dilution). After washing, the membranes were incubated with a secondary anti-mouse or anti-rabbit horseradish peroxidase antibody. Protein signals were detected by membrane incubation in chemiluminescent reagent for 1 minute, followed by radiographic film exposure and scanning. Imag J was used to quantitatively analyze the expression difference by comparing the concentration of COL6A1 to GAPDH.

Statistical analyses

All statistical analyses were conducted using SPSS version 18.0. Data are expressed as mean ± standard deviation (SD). The Pearson test, chi-square test, and Fisher's exact test were used for correlation analysis between COL6A1 expression and clinicopathological phenotypes. Kaplan-Meier survival curves were produced for survival analysis. The Cox regression model was used for univariate and multivariate factor analysis. A p value of <0.05 was considered statistically significant.

Results

The relationship between COL6A1 expression and the clinicopathologic characteristic

A comparison of COL6A1 immunohistochemical staining with clinicopathological features of breast cancer is shown in **Figure 1** and **Table 2**. Of 232 cases, 224 (96.6%) were positive for COL6A1; expression was high in 143 cases

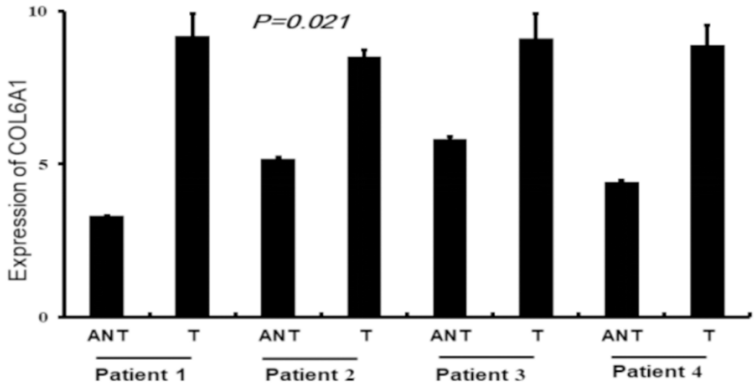

Figure 3. High COL6A1 mRNA and protein level in breast cancer tissue. RT-PCR detection 4 pairs of breast cancer tissues and adjacent normal tissues.

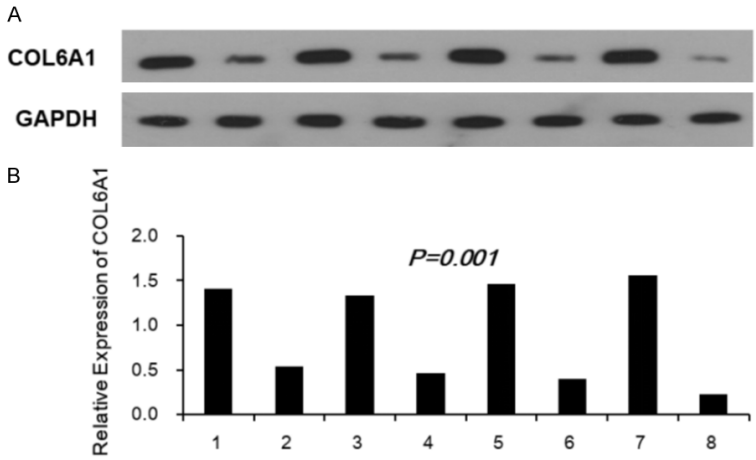

Figure 4. Western blot detection 4 pairs of breast cancer tissues and adjacent normal tissues, with GAPDH as internal control (A). Imag J was used to quantitatively analyze the expression difference by comparing the concentration of COL6A1 to GAPDH (B).

(61.6%) and low in 89 cases (38.4%). COL6A1 expression level was significantly associated with postsurgical conditions including T phase (P = 0.001), N phase (P = 0.001), and recurrence (P<0.001).

Association between FLOT1 expression and patient survival

Survival analysis indicated that COL6A1 expression in 232 immunohistochemical analyses was negatively correlated with recurrence-free survival. In breast cancer patients, the 5-year recurrence-free survival was 90.6% and 70.9% (P<0.001) for breast cancer patients with low and high COL6A1 expression, respectively (**Figure 2**).

A Cox regression analysis showed that only T phase [relative risk, 1.768, 95% confidence interval (CI): 1.052-2.972, P<0.031], N phase (relative risk, 2.063, 95% CI: 1.081-4.129, P<0.001), and COL6A1 expression (relative risk: 3.338, 95% CI: 1.853-6.013, P = 0.001) were independent prognostic factors for poor overall survival (**Table 3**).

The high expression of COL6A1 in breast cancer tissues

According to RT-PCR, COL6A1 mRNA expression was greater in the breast cancer tissues compared with the adjacent normal tissue samples (P = 0.021) (**Figure 3**). In addition, in western blot analysis, COL6A1 protein expression was greater in the breast cancer samples compared with the adjacent normal tissue samples (**Figure 4A**). The relative expression of COL6A1 to GAPDH is shown in **Figure 4B** (P = 0.001).

Discussion

Breast cancer remains a major public health problem. Although many efforts are made to prevent the disease, epidemiologic studies predict that the incidence may further increase over the next 20 years [22-25]. Lower age of menarche, late age of first pregnancy, fewer pregnancies, shorter or no periods of breast feeding, a later menopause, obesity, alcohol consumption, inactivity, and hormone replacement therapy are the proved risk factors for increasing incidence of breast cancer. But, the genetic mutation, influenced by aforementioned risk factors, is the key step for tumorigenesis, and the target of adjuvant therapy, especially targeted drugs.

Substantial evidence indicates that the interactions between tumor cells and their microenvironment are important in the development and

progression of cancer. Cancer cells may alter the surrounding extracellular stroma; in turn, cancer stromal cells and cytokines may promote cancer progression and the acquisition of invasive properties [26, 27]. Collagen VI is a major extracellular matrix (ECM) protein, which forms a network of beaded microfilaments that interact with other ECM molecules and provide structural support for cells [11]. Studies have also indicated that collagen VI triggers signaling pathways that regulate cell apoptosis [13], inflammation [15], and even tumor progression [14]. COL6A1, which is a conservative gene/protein in vertebrates and is present in all connective tissues [28], was recently found to be differentially expressed in bone cancer [19], astrocytomas [16], lung cancer [18], renal clear cell carcinoma [20], and prostate cancer [21].

In the current study, RT-PCR and western blot analysis found that COL6A1 was highly expressed in breast cancer samples compared with adjacent normal tissue, which is in accordance with previous reports mentioned here. Immunohistochemistry, based on 232 tissues, pathologically proved COL6A1 protein overexpression correlated with the clinical features of breast cancer, including T classification, N classification, recurrence, and vital status. Furthermore, the cumulative 5-year overall and recurrence-free survival analysis showed patients with high COL6A1 have a poorer prognosis than those with low COL6A1 expression, making COL6A1 a potential independent prognostic factor for breast cancer. COL6A1 has been confirmed as a tumor growth factor (TGF)-β/Smad target in human dermal fibroblasts [29]. The high expression of COL6A1, as the result of TGF-β activity, may lead to a poor prognosis. Recent studies have suggested that sorafenib inhibits TGF-β activity [30, 31]; thus, It was hypothesized that COL6A1 expression level may also reflect patient response to sorafenib treatment, although this requires further investigation. Collagen VI can also influence responses to anticancer agents by regulating access to chemotherapy and potentially forming a physical barrier to promote resistance [32].

Breast carcinomas are extraordinarily heterogeneous with different clinicopathologic features and outcomes. The statuses of ER, PR, and HER2 are important for treatment and prognosis [33-35].

The prognosis of breast cancer could be influenced by many complicated factors such as staging, Fuhrman grade, surgical performance, and response to adjuvant therapy; therefore, a single marker used in isolation will provide limited information. The current retrospective study represents the comprehensive survey of the clinical characteristics and outcomes of patients with different cancers in relation to COL6A1 expression features.

Based on the evidence presented above, we can hypothesize that COL6A1 plays a role as an oncogene in breast cancer, and regulates anti-apoptosis, proliferation, angiogenesis, and even metastasis involved in multiple signaling pathways. The underlying mechanism requires further study.

Disclosure of conflict of interest

None.

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