

Original Article

Expression of COL6A1 predicts prognosis in cervical cancer patients

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Abstract: COL6A1 has been shown to play an important role in tumor initiation and progression. The present study is to investigate the clinical significance of COL6A1 in cervical cancer. In this study, the COL6A1 expression levels in 10 paired cervical cancer tissues and the adjacent non-tumor tissues were examined by real-time PCR. The expression of COL6A1 protein was examined in 162 cervical cancer samples by immunohistochemistry, and the correlation of COL6A1 expression with clinicopathologic factors was analyzed. The overall and recurrent-free survival rates were estimated using Kaplan-Meier method and compared with the log-rank test. The prognostic analysis was carried out with multivariate Cox regressions model. The result showed that COL6A1 expression was up-regulated in cervical cancer tissues in compared with that in non-tumor tissues. High expression of COL6A1 was significantly correlated with FIGO stage ($P < 0.001$), tumor size ($P = 0.025$) and lymph node metastasis ($P = 0.028$) of the disease. Moreover, survival analysis showed that high expression of COL6A1 was significantly associated with poorer overall (OS) and recurrent free (RFS) survival ($p = 0.004$ and $= 0.001$, respectively) of cervical cancer patients. Multivariate analysis suggested that COL6A1 expression was an independent prognostic marker of cervical cancer ($P = 0.029$). Thus, COL6A1 may serve as an oncogene in the initiation and progression of cervical cancer, and as a predictor of poor prognosis in cervical cancer patients.

Keywords: COL6A1, collagen IV, cervical cancer, prognosis

Introduction

The incidence and mortality of cervical cancer have declined over the past several decades, but cervical cancer remains a major public health issue, ranking as the third-most common malignancy in female reproductive system [1]. Nearly 500000 cervical cancer new cases are occurring worldwide each year, responsible for 274000 deaths [2]. According to the International Federation of Gynecology and Obstetrics (FIGO), cervical cancer is classified into 4 stages based on the level of disease severity (tumor size, tumor extension, and organ involvement). Radical surgery and radiotherapy are effective in early stage diseases, but there are limited treatment options for patients with advanced diseases [3]. Thus, understanding of the molecular mechanisms of cervical cancer development and identification

of novel biomarkers are required for the early detection and treatment of cervical cancer.

COL6A1 belongs to a family of collagens, which are important in maintaining the integrity of tissues. The COL6A1 gene is located at chromosome 21q22.3 and encodes the $\alpha 1$ chain of type VI collagen, a component of microfibrillar structures in several tissues [4]. Studies have also indicated that COL6A1 has an anchoring function in cells, and it may be involved in cell migration, differentiation and embryonic development, albeit in combination with other fusion proteins [5]. In addition, the COL6A1 gene plays an important role in tumor cell proliferation and progression. The expression of COL6A1 transcripts and the level of collagen VI protein in tumor tissues are much higher than those of the corresponding normal tissues [6]. COL6A1 has also been shown to have potent effect in

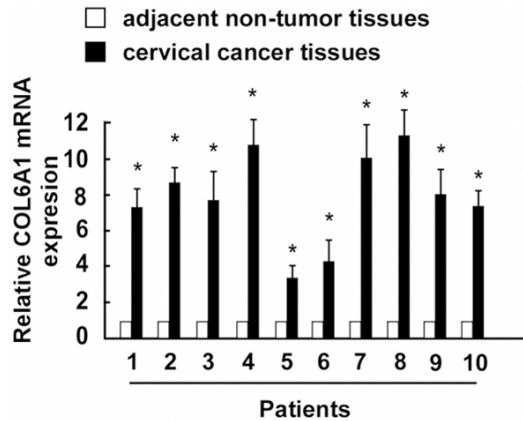


Figure 1. Expression of COL6A1 mRNA in 10 pairs of cervical cancer tissues and adjacent non-tumor tissues through real-time PCR. Bars represent the means of three independent experiments; * $P < 0.05$.

stimulating proliferation and preventing apoptosis of cancer cells [7]. In vivo studies using the MMTV-PyMT mouse model have shown that ablation of COL6A1 reduced proliferation and increased tumor cell apoptosis [8]. Wright et al. reported that collagen IV treatment increased the motility of malignant human lung epithelial carcinoma cells, suggesting that collagen VI promotes tumor metastasis [9]. In agreement with this, Zhu et al. demonstrated that COL6A1 is upregulated in castration-resistant prostate cancer, and that COL6A1 promotes tumorigenesis of prostate cancer cells in vivo [10]. These studies implied the oncogenic functions of COL6A1 in human cancers. However, the potential role of COL6A1 in cervical cancer and its biological functions in the initiation and progression of the disease has not yet been described.

In the present study, we evaluated the significance of COL6A1 expression in cervical cancer. By analyzing the correlation of COL6A1 expression and clinicopathologic features, we found that COL6A1 expression was associated with tumor stage, tumor size, lymph nodes metastasis, and the clinical prognosis of the disease. Multivariate analysis suggested that COL6A1 expression was an independent prognostic marker for overall and recurrent-free survival in patients with cervical cancer. These findings suggest that COL6A1 is related to the initiation and progression of cervical cancer, and that it could be used as a potential prognostic biomarker in patients with cervical cancer.

Materials and methods

Patients and tissue specimens

Patients: A total of 162 consecutive patients treated in our institution due to cervical carcinoma, between January 2001 and December 2008, were retrospectively reviewed in the study. The material was retrieved from archival paraffin-embedded surgical samples at Sun Yat-Sen University Cancer Center. In addition, 10 pairs of snap-frozen cervical cancer and normal cervical samples were collected for Real-time PCR. None of the patients had received chemotherapy or radiotherapy before surgery. In all cases, the diagnoses and grading were peer-reviewed according to the principles laid down in the latest International Federation of Gynecology and Obstetrics criteria [11]. Prior written consent was obtained from all patients and this study was approved by the Research Ethics Committee of Sun Yat-Sen University Cancer Center.

Real time RT-PCR

The expression status of COL6A1 in cervical cancer and paired normal cervical tissues was determined by Real time RT-PCR. Total RNA from tumor and normal tissues was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted RNA was pretreated with RNase-free DNase, and 2 μ g RNA from each sample was used for cDNA synthesis with random hexamers. Real-time PCR was performed using the Applied Biosystems 7500 Sequence Detection system. The primers used are as follows: COL6A1: forward, 5'-TCAAGAGCCTGCAGTGGATG-3', reverse, 5'-TGGACACTTCTGTCTATGC AG-3'; GAPDH, forward, 5'-AGA AGGCTGGGCTCATTG-3', reverse, 5'-AGGG GCCATCCACAGTCTTC-3'.

Immunohistochemistry (IHC) analysis

The paraffin-embedded archival specimens were cut in 4- μ m-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

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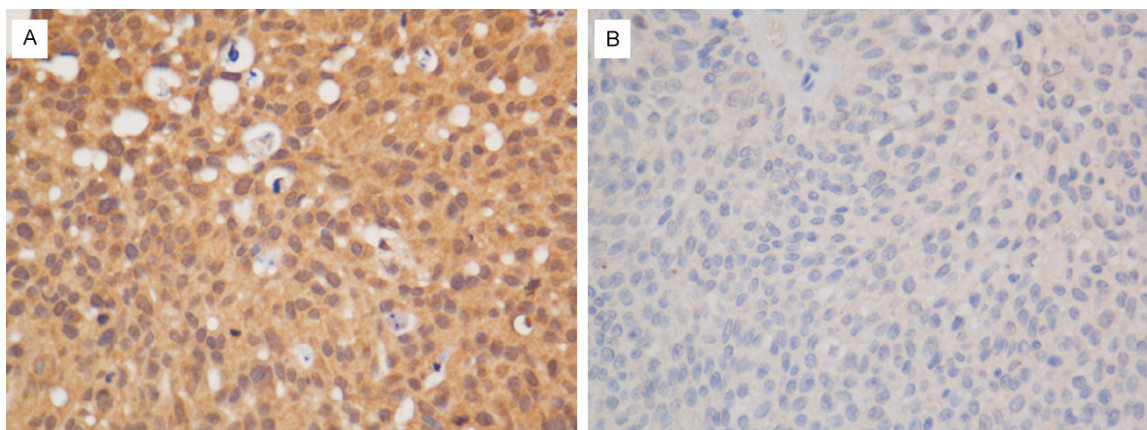


Figure 2. Representative immunohistochemical images for high (A) and low (B) COL6A1 expression in cervical cancer tissues.

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; 1:200). 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 was separately reviewed and scored by two independent pathologists who were blinded as to the patients. Expression of COL6A1 was analyzed by an individual labeling score considering the proportion of positively stained tumor cells and the intensity of staining. Intensity of stained cells was graded semi-quantitatively into four levels: 0: no staining; 1: weak staining; 2: positive staining; and 3: strong staining. The area of staining was evaluated and recorded as a percentage: 0: no staining; 1: positive staining in <10% of tumor cells; 2: positive staining in 10% to 50% of tumor cells; 3: positive staining in 51% to 80% of tumor cells; 4: positive staining in >80% of tumor cells. Intensity and fraction of positive cell scores were multiplied and thus the scoring system was defined as low expression for scores of 0-3, and as high expression for scores of 4-12.

Statistical analysis

All statistical analysis was performed using SPSS (version 16.0, SPSS Inc, Chicago, USA)

software. The overall survival (OS) and recurrence-free survival (RFS) were calculated as the time from the date of primary surgery to the date of first death or recurrence. Survival curves were plotted using the method of Kaplan-Meier, and the log-rank test was used to determine statistical differences between life tables. The correlations between clinicopathologic characteristics and recurrence were analyzed using the χ^2 test. The significance of various variables for survival was analyzed by the Cox proportional hazards model in the multivariate analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

COL6A1 expression is upregulated in cervical cancer

In this study, we evaluated the expression levels of COL6A1 in 10 pairs of frozen cervical and adjacent non-tumor tissues by real time RT-PCR. As shown in **Figure 1**, COL6A1 was dramatically upregulated in cervical cancer tissues compared with the paired adjacent normal cervical tissues. The representative immunostaining of COL6A1 in cervical cancer was shown in **Figure 2**.

COL6A1 expression is associated with clinical parameters

Expression of COL6A1 was associated with various clinicopathological parameters according to the IHC assay results. COL6A1 expression showed no significant relation with age, SCC level, histological type, or differentiation.

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Table 1. Relationship between COL6A1 expression and clinicopathological characteristics

| Parameters | Group | Total | COL6A1 | | P |
|-------------------|------------|-------|--------|------|--------|
| | | | Low | High | |
| Age | ≤50 y | 109 | 62 | 47 | 0.846 |
| | >50 y | 53 | 31 | 22 | |
| SCC level | ≤1.5 ng/ml | 40 | | | |
| | >1.5 ng/ml | 122 | | | |
| FIGO stage | IB | 117 | 77 | 40 | <0.001 |
| | >IB | 45 | 16 | 29 | |
| Tumor size | ≤4 cm | 105 | 67 | 38 | 0.025 |
| | >4 cm | 57 | 26 | 31 | |
| Differentiation | 1/2 | 128 | 76 | 52 | 0.343 |
| | 3 | 34 | 17 | 17 | |
| Histological type | SCC | 103 | 58 | 45 | 0.709 |
| | AC | 59 | 35 | 24 | |
| LN Metastasis | No | 134 | 87 | 57 | 0.028 |
| | Yes | 18 | 6 | 12 | |

SCC: squamous cell cancer; AC: Adenocarcinoma.

However, COL6A1 expression was found to be related with FIGO stage ($P < 0.001$), tumor size ($P = 0.025$) and lymph node metastasis ($P = 0.028$) (Table 1).

Expression of COL6A1 in cervical cancer is associated with clinical survival

The prognostic impact of COL6A1 in cervical cancer was evaluated using the Kaplan-Meier survival curve analysis. As shown in Figure 3, expression of COL6A1 was significantly associated with the clinical prognosis of cervical cancer patients. Patients with high expression of COL6A1 had significantly decreased overall and recurrent free survival compared to those with low expression ($P = 0.004$, and $P = 0.001$, respectively). Multivariate Cox proportional hazards model analysis showed that COL6A1 could be used as a potential prognostic marker for cervical cancer patients ($P = 0.029$) (Table 2).

Discussion

In this study, we found that elevated expression of COL6A1 was present in cervical cancer tissues in compared with normal cervical tissues. Tumors of advanced stage were associated with higher COL6A1 expression as compared to that of early stage. We also found that upregulated COL6A1 expression was associated with

poor clinical prognosis in patients with cervical cancer. Moreover, overexpression of COL6A1 was correlated with FIGO stage, tumor size and lymph nodes metastasis. The current retrospective study represents the first comprehensive survey of the clinical characteristics and outcome of cervical cancer patients in relation to COL6A1 expression features.

The extracellular matrix (ECM) provides essential signals in regulating cell growth and apoptosis. In the tumor microenvironment, it is commonly disorganized, thus contributing to cancer progression by promoting cancer cell growth and migration directly, and by educating other microenvironment components indirectly [12]. Collagen VI is one of the main ECM and contributes to the properties of the local ECM microenvironment by forming a discrete network of beaded microfilaments that interact with other ECM molecules [13]. It is composed of three major polypeptide chains ($\alpha 1$, $\alpha 2$ and $\alpha 3$), and the $\alpha 1$ chain is encoded by COL6A1, which is involved in multiple signaling pathways that regulate cell apoptosis, proliferation, angiogenesis, fibrosis, and inflammation [14]. Mutations in the COL6A1 gene affect the protein building blocks (amino acids) in the $\alpha 1$ (VI) chain, which have been found to cause some cases of collagen VI-related myopathy, including muscle weakness and joint deformities called contractures [15]. Recent studies indicate that COL6A1 is differentially expressed in tumors, and is associated with tumor progression. Fujita et al. found that COL6A1 gene was differentially expressed in normal glia and the different grades of gliomas [16]. Voiles et al. demonstrated that COL6 is overexpressed in neoplastic lung tissues and may promote tumor development [17]. Our data shown that the expression level of COL6A1 was increased in cervical cancer tissues in compared with that in normal cervical tissues, suggesting the potential role of COL6A1 in promoting cervical tumorigenesis.

It has also been demonstrated that Collagen VI is involved in tumor metastasis. Collagen VI has a potent ability to promote the adhesion and spreading of tumor cells, thus enhancing the extension, penetration, and invasion of tumor cells in an autocrine manner [18]. In a previous global secretome analysis, high expression of COL6A1 was found to promote bone metastasis in vivo [19]. Chiu et al. used quantitative

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Table 2. Multivariate Cox regression analyses of OS and RFS for cervical cancer patients

| Prognostic variables | OS | | RFS | |
|--------------------------------------|-----------------------|-------|-----------------------|-------|
| | Hazard Ratio (95% CI) | P | Hazard Ratio (95% CI) | P |
| Age (>50 y vs ≤50 y) | 1.498 (0.291-5.026) | 0.664 | 1.805 (0.971-4.046) | 0.672 |
| SCC level (>1.5 ng/ml vs ≤1.5 ng/ml) | 1.796 (0.293-4.611) | 0.527 | 1.246 (0.549-4.820) | 0.602 |
| FIGO Stage (>IB vs IB) | 4.421 (0.053-5.278) | 0.005 | 4.447 (0.046-6.600) | 0.012 |
| Tumor size (>4 cm vs ≤4 cm) | 2.769 (0.091-5.002) | 0.038 | 2.597 (0.135-5.643) | 0.063 |
| Differentiation (Grade 3 vs 1/2) | 2.206 (0.097-4.145) | 0.625 | 2.259 (0.145-4.620) | 0.438 |
| Histological type (SCC vs AC) | 2.797 (1.091-7.426) | 0.078 | 2.087 (1.348-5.410) | 0.110 |
| LN Metastasis (+ vs -) | 4.758 (1.062-10.879) | 0.036 | 4.606 (1.763-12.073) | 0.018 |
| COL6A1 expression (High vs Low) | 3.502 (1.301-7.212) | 0.029 | 3.417 (1.106-8.050) | 0.058 |

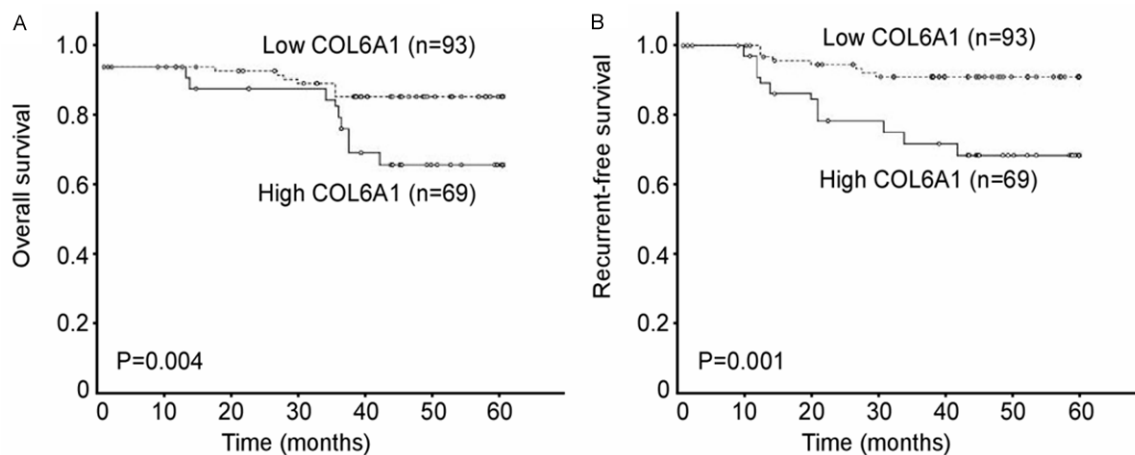


Figure 3. Kaplan-Meier curves of overall survival (A) and recurrent-free survival (B) according to COL6A1 expression in cervical cancer patients.

secretome analysis to reveal that overexpression of COL6A1 increased the metastatic ability of lung cancer cells, while knock-down of COL6A1 suppressed the metastatic ability of cancer cells [20]. In line with the studies, we found that upregulation of COL6A1 expression was associated with lymph nodes metastasis in cervical cancer patients, further indicating that COL6A1 is related to the metastatic functions of cancer cells.

Importantly, we reported that high COL6A1 expression was correlated with poor clinical prognosis in patients with cervical cancer. The prognostic role of COL6A1 has been reported in a few human cancers. Wan et al. uncovered that upregulation of COL6A1 was predictive of poor prognosis in 2 cohorts of clear cell renal cell carcinoma patients [21]. Turtoi et al. retrospectively analyzed public gene-expression data sets from over 300 glioma patients and demonstrated a significant correlation of poor patient outcome and high COL6A1 expression

[22]. Together, these studies suggested the potential useful application of COL6A1 in predicting clinical outcome of cancer patients.

In conclusion, we have revealed that COL6A1 could be used as a marker of cervical cancer progression. Although further investigations are needed to uncover the mechanisms underlying these functional differences, this study demonstrates that COL6A1 expression is related to the biological functions of cervical cancer cells and the clinical outcome of cervical cancer patients.

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Disclosure of conflict of interest

None.

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