Overexpression of Coiled-Coil Domain-Containing Protein 34 (CCDC34) and its Correlation with Angiogenesis in Esophageal Squamous Cell Carcinoma

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Background: The coiled-coil domain-containing proteins have been shown to have a series of functions in biological synthesis. Recent studies have found that CCDC34 is highly expressed in bladder cancer, but the underlying molecular mechanisms still remain unclear. Therefore, we performed the present study to assess the expression of the coiled-coil domain-containing protein 34 (CCDC34) in esophageal squamous cell carcinoma (ESCC) patients. We also explored the relationships between CCDC34 expression and clinicopathologic characteristics, tumor angiogenesis, and prognosis.

Material/Methods: We detected the expressions of CCDC34, VEGF, and MVD by immunohistochemical technique in 100 cases of ESCC and 80 cases of corresponding paracarcinomatous normal tissues. The relationship between CCDC34 expression and clinicopathologic characteristics, tumor angiogenesis, and prognosis were also explored.

Results: The expression of CCDC34 protein was obviously increased in ESCC tissues, which was significantly correlated with sex ($p=0.038$), TNM stage ($p=0.003$), and lymphatic metastasis ($p=0.024$). In addition, we found that the expression of CCDC34 was an independent prognostic factor for ESCC patients. The overexpression of CCDC34 protein in ESCC was associated with tumor progression, angiogenesis, and poor survival.

Conclusions: Our results demonstrate that CCDC34 is overexpressed in ESCC and can be used as an independent parameter for indicating the poor prognosis of ESCC patients, suggesting that CCDC34 might be a new potential therapeutic target for ESCC patients in the future.

MeSH Keywords: Esophageal Neoplasms • Microvessels • Prognosis • rho-Associated Kinases • Vascular Endothelial Growth Factor A

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Background

Esophageal squamous cell carcinoma (ESCC), which ranked as the sixth leading cause of cancer-associated mortality worldwide, is a common cancer with poor prognosis [1]. Although great progress has been made in treatments for ESCC, including surgical operation, chemoradiotherapy, and neoadjuvant chemotherapy, the diagnosis and prognosis of patients with ESCC remains unsatisfactory. Thus, better therapies may be developed by exploring molecular biomarkers that can predict recrudescence, progression, and prognosis of ESCC.

Coiled-coil domain (CCDC) is a particular structural motif that had been identified in proteins. Studies have shown that the structural motif is involved in a series of biological functions, such as cell division, drug extrusion, regulation of gene expression, drug delivery, and membrane fusion [2,3]. In many types of tumors, coiled-coil domain-containing proteins have been found to be abnormally expressed, including gastric cancer [4,5], breast cancer [6,7], nasopharyngeal carcinoma [8], prostate tumor [9], pancreatic cancer [10], colorectal carcinoma [11,12], and bladder cancer [13]. Studies have shown that the expression of CCDC proteins is closely associated with tumor cell migration, invasion, and metastasis [14–16]. Coiled-coil domain-containing 34 (CCDC34), also named renal carcinoma antigen 41, is a protein-coding gene that is related to disease. It is made up of 373 amino acid and is located on chromosome 11. The clinical significance of CCDC34 was first developed by exploring molecular biomarkers that can predict recrudescence, progression, and prognosis of ESCC.

Therefore, in the present study, we detected the expression levels of CCDC34, VEGF, and MVD by immunohistochemical staining in ESCC tissues and corresponding paracarcinomatous normal tissues. We analyzed the relationship between CCDC34 expression level and clinicopathological parameters using the chi-square test. Kaplan-Meier univariate and Cox multivariate survival analyses were used to explore the prognosis of CCDC34 in patients with ESCC.

Material and Methods

Patients and specimens

We acquired 100 ESCC tissues from Anhui Provincial Hospital from January 2006 to December 2009. In the pathological tissues of cases numbers 1–80, ESCC tissues and paracarcinomatous tissues were paired. In the pathological tissues of cases numbers 81–100, there were only the ESCC tissues. These tissues all came from patients who underwent esophagectomy. No patient had a history of radiotherapy, chemotherapy, or immunotherapy. The specimens were taken from 74 male and 26 female patients, aged 48–82 years (average 65.3 years) who were all clearly diagnosed as having ESCC basing on pathological confirmation. Clinicopathologic data of all patients were collected from the case management system in the medical records room; these patients were followed up with a deadline of July 2015. The data mentioned above covered sex, age, tumor size, lymphatic metastasis, TNM stages, and degree of tumor differentiation. This study was approved by the Ethics Committee of Anhui Provincial Hospital and all patients signed the written informed consent form.

Immunohistochemical staining

The CCDC34 and VEGF levels and the presence of CD34 were assessed by immunohistochemistry. The experimental method is a 2-step method. First, sections were first dewaxed in phosphate-buffered saline (PBS), then incubated in 10 mmol/l pH 6.0 citrate buffer, heated in a microwave oven, and incubated in 3% hydrogen peroxide for 15 min at room temperature. Second, sections were incubated with primary antibodies (CCDC34 antibody [ab122396; Abcam Inc, Shanghai, People’s Republic of China], VEGF antibody [ab2349; Abcam Inc, Shanghai, People’s Republic of China], and CD34 antibody [ab81289; Abcam Inc, Shanghai, People’s Republic of China]), all at a dilution of 1: 100 at 4°C overnight. We removed the humidified chamber with sides from the freezer, equilibrated it to room temperature for 45 min, and then absorbed the surplus primary antibody. The sides were incubated at room temperature for 30 min with the appropriate secondary antibody (Envision+HRP, Rabbit, DAKO). Finally, sections were colored through reacting with 3,3-diaminobenzidine. Hematoxylin was applied as counterstain, and negative-control sections were processed with PBS.

Determination of immunohistochemical results

CCDC34: There were brown-yellow or light-yellow granules in the cytoplasm or nuclei of cells with positive staining. The results were overall evaluated on 2 aspects: one is the average percentage of positive cells per 100 cells in 10 high-power fields, and the other is the power of staining. Staining was scored as follows: (1) staining intensity score: 0 points (negative), 1 points (1+), 2 points (2+), and 3 points (3+); and (2) staining power score: 0 points (negative), 1 points (1–25%), 2 points (26–50%), 3 points (51–75%), and 4 points (76–100%). The total score was calculated by multiplying both. The low-expression group was defined as less than or equal to 4 points, and the high-expression group was the opposite. The principal of the VEGF evaluation was the same as that of CCDC34. MVD evaluation was conducted as follows: (1) Quantifying 5 different horizons with high expression in a high-power lens
field (400×); (2) The averages values were taken as final count measurements; and (3) Two pathologists who were unaware to diagnosis assessed the immunohistochemical results of all slices separately.

**Western blot analysis**

The fresh-frozen tumor and corresponding normal para-cancerous tissues of 2 ESCC patients were cracked on ice for 30 min with lysis solution (RIPA). The protein lysate was collected into 1.5-ml EP tubes and further centrifuged at a speed of 12 000 rpm at 4°C for 10 min. The supernatant was absorbed to a new 1.5-ml EP tube. BCA method was used to measure protein concentration in a standard curve. About 30 ug of extracted protein was electrophoresed in 10% SDS-PAGE for 5 h. The PAGE-separated proteins were transferred to a PVDF membrane in ice water. We used 5% skimmed milk solution to block the membranes for 2 h at room temperature. The blocked membrane was incubated with the primary antibodies of rabbit polyclonal anti-CCDC34 (Abcam, Inc, Shanghai, People’s Republic of China) at a dilution of 1: 200 at 4°C overnight. The membrane was further incubated with the second antibodies combined with horseradish peroxidase (HRP) for 2 h at 37°C after washing with TBST. The band intensities were quantified using a computerized densimeter.

**Statistical analysis**

Data were analyzed using SPSS 13.0 version software (SPSS, Inc., Chicago, IL). Differences were considered to be statistically significant at $p<0.05$. The Spearman’s rank correlation test and $\chi^2$ tests were applied to compare the variables. Univariate and multivariate survival analyses were conducted by the log-rank test and Cox regression model, respectively. Furthermore, survival curves were drawn by the Kaplan-Meier method.

**Results**

**Expression of CCDC34 in ESCC tissues and its correlation with clinicopathological characteristics of patients**

CCDC34 showed strong positive staining in ESCC tissues, mainly locating in cytoplasm (Figure 1A, 1B, 1D). As shown in

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**Figure 1.** Immunohistochemical staining of CCDC34, VEGF, and CD34 in esophageal squamous cell carcinoma (ESCC) tissues. CCDC34 was mainly expressed in the cytoplasm of ESCC tissues. (A) High CCDC34 staining; (B) low CCDC34 staining; (C) negative for CCDC34 staining in para-carcinoma tissues; (D) high CCDC34 staining; (E) positive for VEGF; (F) positive for CD34. Bar=100 μm.
Table 1. Differential expression of CCDC34 between ESCC and corresponding paracarcinomatous normal tissues (cases).

| Tissues                  | Case number | CCDC34 expression | High rate% |
|--------------------------|-------------|-------------------|------------|
| Paracarcinomatous tissues| 80          | 33                | 41%        |
| ESCC tissues             | 100         | 74                | 74%        |

Table 2. The relationship between CCDC34 expression and the selected clinicopathologic features in 100 ESCC patients (cases).

| Clinicopathologic parameters | Case number | CCDC34 | χ² | P value |
|------------------------------|-------------|--------|----|---------|
|                              |             | High   | Low |         |
| Gender                       |             |        |     |         |
| Male                         | 74          | 59     | 15  | 4.856   | 0.038   |
| Female                       | 26          | 15     | 11  |         |         |
| Age (years)                  |             |        |     |         |
| £65                          | 51          | 37     | 14  | 0.114   | 0.821   |
| >65                          | 49          | 37     | 12  |         |         |
| Lymphatic metastasis         |             |        |     |         |
| No                           | 46          | 29     | 17  | 5.315   | 0.024   |
| Yes                          | 54          | 45     | 9   |         |         |
| TNM stages                   |             |        |     |         |
| I/II                         | 48          | 29     | 19  | 8.852   | 0.003   |
| III/IV                       | 52          | 45     | 7   |         |         |
| Pathological grading         |             |        |     |         |
| I/II                         | 73          | 54     | 19  | 0.000   | 1.000   |
| III                          | 27          | 20     | 7   |         |         |
| Tumor size(cm)               |             |        |     |         |
| £5                           | 63          | 45     | 18  | 0.585   | 0.488   |
| >5                           | 37          | 29     | 8   |         |         |

Table 1, CCDC34 positive immunostaining rate was observed in 74/100 (74%) of ESCC tissues. The expression of CCDC34 in corresponding paracarcinomatous normal tissues was markedly reduced compared with ESCC tissues (33/80, 41%, χ²=19.773, p<0.001, Figure 1C). We further explored the correlation between CCDC34 expression and clinicopathological features according to the immunostaining evaluation (Table 2). We found no relationship between CCDC34 expression and sex, age, tumor size, or pathological grade.

We used Western blot analysis to further detect the expression levels of CCDC34 proteins in 2 fresh-frozen ESCC and corresponding normal para-cancerous tissues. As shown in Figure 2, the expression level of CCDC34 in tumor tissues was higher than in normal para-cancerous tissues. The results are consistent with the immunohistochemical analysis.

Prognostic value of CCDC34 expression in ESCC patients

To explore the prognostic significance of CCDC34 expression in ESCC patients, log-rank test and Cox regression model were conducted. As shown in Table 3, ESCC patients with high CCDC34 expression had dramatically worse overall survival (OS) and disease-free survival (DFS) times than those with CCDC34 low expression, as shown by the log-rank test analysis (OS: 95%CI, 15.872–24.723 vs. 58.738–90.569, p<0.001; DFS:
95% CI, 8.403–13.522 vs. 27.315–43.257, p < 0.001; Figure 3A, 3C). The patients at TNM stage I/II with high CCDC34 expression had worse overall survival (OS) and disease-free survival (DFS) times than those with CCDC34 low expression (Figure 3B, 3D). Moreover, Cox multivariate survival analysis showed that CCDC34 different expression level (low vs. high) was an independent unfavorable prognostic factor for both OS and DFS of ESCC patients (Table 4).

**High expression of CCDC34 was positively associated with both VEGF expression and MVD counts in ESCC**

As shown in Table 5, VEGF was mostly located in cytoplasm (Figure 1E) in all 100 ESCC tumor samples, and the high expression rate was 64% (64/100). The results revealed that the high expression of CCDC34 was positively associated with VEGF expression (r = 0.743, p < 0.001) in ESCC tissues. Besides, tumors with CCDC34-high expression had significantly higher MVD counts than those with CCDC34-low expression (48.38 ± 9.83 vs. 31.92 ± 8.95, p < 0.001; Figure 4).

Table 3. Univariate analysis of factors correlated with OS and DFS.

| Variable                  | OS      | DFS      |
|---------------------------|---------|----------|
| 95% CI                    | P value | 95% CI   | P value  |
| CCDC34 High               | 15.872–24.723 | <0.001 | 8.403–13.522 | <0.001 |
| CCDC34 Low                | 58.738–92.569 |       | 27.315–43.257 |       |
| Gender Male               | 22.299–36.972 | 0.024  | 11.92–18.459  | 0.019  |
| Gender Female             | 33.270–67.422 |       | 16.446–33.587 |       |
| Age (years) ≤65           | 23.294–26.047 | 0.723  | 11.355–19.619 | 0.197  |
| Age (years) >65           | 26.047–48.361 |       | 14.390–26.866 |       |
| Lymphatic metastasis No   | 35.138–59.253 | 0.002  | 18.427–30.835 | <0.001 |
| Lymphatic metastasis Yes  | 16.659–29.489 |       | 8.367–14.391  |       |
| TNM stages I/II           | 38.807–63.360 | <0.001 | 19.650–31.879 | <0.001 |
| TNM stages III/IV         | 14.606–24.394 |       | 7.503–12.052  |       |
| Pathological grading I/II | 26.451–43.631 | 0.926  | 13.283–21.603 | 0.677  |
| Pathological grading III  | 21.351–46.723 |       | 10.885–25.683 |       |
| Tumor size(cm) ≤5         | 31.716–50.728 | 0.008  | 15.368–24.923 | 0.062  |
| Tumor size(cm) >5         | 14.336–34.313 |       | 8.080–17.717  |       |
Coiled-coil domain-containing (CCDC) has many important biological functions and are thought to be involved in the invasion and metastasis of malignant tumor cells and other biological behaviors. CCDC34 is a new member of this family and its biological and clinical significance in cancer has received scant research attention. Recently, Gong et al. [13] were the first to report the enhanced expression of CCDC34 in bladder cancer tissues and cell strain, revealing that knocking down CCDC34 by lentivirus-mediated siRNA could restrain bladder cancer cells from proliferation and migration, and promote the cell cycle to remain at G2/M phase. In addition, the knockdown of CCDC34 significantly inhibited the growth of bladder tumor cells in nude mice. However, until now, there has been no detailed report focusing on the expression level of CCDC34 and its prognostic value in ESCC.

Therefore, in the present study, we are the first to demonstrate that CCDC34 is highly expressed in ESCC patients compared with matched normal tissues. Then, chi-square test results showed that CCDC34 differential expression level was significantly correlated with lymphatic metastasis and TNM stages in ESCC tissues. Moreover, survival analysis demonstrated

**Figure 3.** Kaplan-Meier analysis of overall survival (OS) and disease-free survival (DFS) curves of patients with esophageal squamous cell carcinoma (ESCC) based on CCDC34 expression as high or low. (A) OS curve of patients with ESCC based on CCDC34 expression; (B) OS curve of patients with ESCC based on CCDC34 expression in TNM stage I/II; (C) DFS curve of patients with ESCC based on CCDC34 expression; (D) DFS curve of patients with ESCC based on CCDC34 expression in TNM stage I/II. The ESCC patients with CCDC34 high expression showed significantly poorer OS and DFS rates than those with CCDC34 low expression.

**Discussion**

Coiled-coil domain-containing (CCDC) has many important biological functions and are thought to be involved in the invasion and metastasis of malignant tumor cells and other biological behaviors. CCDC34 is a new member of this family and its biological and clinical significance in cancer has received scant research attention. Recently, Gong et al. [13] were the first to report the enhanced expression of CCDC34 in bladder cancer tissues and cell strain, revealing that knocking down CCDC34 by lentivirus-mediated siRNA could restrain bladder cancer cells from proliferation and migration, and promote the

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that patients with CCDC34 high expression had remarkably lower OS and DFS than those with CCDC34 low expression. According to the NCCN guidelines, surgery alone is the treatment of choice for early stages of ESCC. TNM stages I/II are correlated with early stages. We further compared the OS and DFS of CCDC34 low expression with CCDC34 high expression in TNM stage I/II. The results showed that patients with CCDC34 high expression had lower OS and DFS than those with CCDC34 low expression in TNM stage I/II. This demonstrates that the patients with higher expression of CCDC34 in TNM stage I/II had a lower OS and DFS. Further, Cox multivariate analysis revealed that CCDC34 expression level could be used as an independent prognostic factor for OS and DFS in patients with ESCC. All these findings demonstrate that CCDC34 may predict ESCC patient prognosis as a novel biomarker.

Angiogenesis is crucial for tumor initiation, progression, and metastasis. It was found that the esophageal cancer cell lines express high levels of VEGF and MVD [17], as also supported by Oshima’s study [18]. Thus, we jointly quantified the levels of VEGF and MVD, which are widely acknowledged biomarkers for tumor angiogenesis to confirm whether there is a connection between CCDC34 protein and tumor angiogenesis. Results showed that the ESCC tissues with CCDC34-high expression expressed higher VEGF, while the values of MVD were accordingly higher compared with CCDC34-low expression group. The Spearman’s rank correlation test was used to analyze the relationship between VEGF and CCDC34. We found there was a clear correlation between CCDC34 and VEGF. All the findings mentioned above provide sufficient evidence that CCDC34 is critical to induce and promote tumor angiogenesis in ESCC. Gong’s study [13] revealed that the signal transduction factor AKT, which plays a key role in tumorigenesis and angiogenesis [19,20], was down-regulated with CCDC34 knockdown. It may be the potential mechanism by which CCDC34 promotes tumor angiogenesis.
There are some weaknesses in the present study. It was a retrospective study with small a sample size, and the conclusions obtained were based only on preliminary results. The mechanisms for CCDC34 function need to be further explored, and further research is needed on the details of mechanisms by which CCDC34 regulates ESCC angiogenesis.

Conclusions

We found that CCDC34 was overexpressed in ESCC and served as an independent poor parameter for predicting the prognosis of ESCC patients, which suggests that CCDC34 might be a new potential therapeutic target for ESCC patients in the future.

Conflicts of interest

None.

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