# Novel Insight: CUEDC2 Expression of Potential Prognostic Value in Esophageal Squamous Cell Cancer

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**Research**

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# Abstract

**Background** Esophageal squamous cell cancer (ESCC) poses serious threats to human life. Hence, the search for effective bio-markers to predict the occurrence and development of ESCC is of emerging significance.

**Methods** We used immunohistochemistry to semi-quantitatively detect CUEDC2 expression in 50 ESCC cases and 20 adjacent tissues, analyzing the relationship with clinicopathological parameters and prognosis outcomes. Additionally, investigating the differences between CUEDC2 and CD68 in ESCC.

**Result** CUEDC2 expression was higher in 9 ESCC tissues and lower in 41 ESCC tissues. Whereas, CUEDC2 expression was higher in 11 adjacent tissues and lower of the rest 9 cases, and the differences were statistically significant (P<0.05).CUEDC2 and ESCC clinicopathological characteristics exerted no significant difference (P>0.05). Via the Kaplan-Meier method, CUEDC2 and tumor grade demonstrated an impact on ESCC prognosis ( P<0.05). By double-immunofluorescence, there was an expression difference between CD68 and CUEDC2, and the difference was statistically significant (P<0.05). There showed co-localization of CUEDC2 and CD68 fluorescence.

**Conclusion** CUEDC2 was relatively lower expressed in ESCC and higher in adjacent tissues. There was no significant difference between CUEDC2 and ESCC clinicopathological characteristics.CUEDC2 and tumor grade presented an impact on ESCC prognosis. There might be an interaction between CD68 and CUEDC2.


# Background

Esophageal cancer, originating from esophageal epithelium, is one of the most catastrophic malignancies, whose disease incidence rate ranks 13th in developed countries such as the United States and 8th in developing countries. According to a survey [1], more than 450,000 new cases of esophageal cancer were diagnosed around the world during 2012, and approximately 400,000 patients died from the disease in the same year. In perspective of histopathology, esophageal carcinoma can be classified into two categories: ECSS, taking up more than 90% in China, and esophageal adenocarcinoma, showing an increasing trend in western countries [1–4]. Owing to lacking in typical clinical manifestation during the early stage of Esophageal tumor, confirmed diagnosis inevitably often occur in the middle or late stage. Over half of the patients may manifest distant metastasis, more seriously, losing the opportunity to receive radical surgery. Despite the dramatic development of medicine, the combination of surgery and a variety of complementary and comprehensive treatments is getting advanced [5, 6]. However, the prognosis is still unfavorable, which is revealed by a five-year survival rate of no more than 18% [4]. And, its pathogenesis still remains to be further understood.

CUE domain-containing protein 2, CUEDC2, is a multifunctional protein with a molecular weight of 32 kDa, which has been reported to contain a small and somewhat conserved ubiquitin-binding motif

comprised of approximately 40 amino acids. Chinese scholar professor, Xuemin Zhang's research team was the first to discover and report this protein [9], whereas its function has not been fully clarified at present. As a protein ubiquitously expressed in heart, testicles, brain and other tissues and organs, CUEDC2 also plays a pivotal role in a variety of physiological and pathological processes and is involved in the regulation of cell cycle, inflammation, and carcinogenesis [10]. CUEDC2 expression has been reported in lung adenocarcinoma [11], colorectal cancer [12], bile duct cell carcinoma [13], breast cancer [14, 15], hepatocellular carcinoma [16, 17], ovarian serous carcinoma [18], glioma [19], etc. Intriguingly, in contrast to the high levels of expression of CUEDC2 in breast cancer and ovarian serous carcinoma [18], its expression in lung adenocarcinoma [11] and infiltrated macrophages in colon cancer [12] have shown noticeably decreased levels of expression. Specifically, this lower expression of CUEDC2 in macrophages infiltrated in colon tissues resulted in intestinal mucosa to be continuously exposed to inflammation, eventually participating in the formation of cancer.

Macrophages, a kind of widely distributed immune cells in the body, mainly function as an essential role in mediating the inflammatory processes and regulating the immune response. When infected, monocytes could rapidly be recruited to the infected area, and then differentiate and be activated into inflammatory macrophages, secreting a large amount of substances, such as pro-inflammatory cytokines, which mediate inflammation and help the body respond to infection promptly. If macrophages are continuously activated, and the inflammatory response does not get terminated in time, which eventually bring about tissue damage in the body, leading to autoimmune diseases and even inducing the formation of tumors [22, 23]. If macrophages infiltrate in tumor tissues, they may be named as tumor-associated macrophages (TAMs). As a vital inflammatory cell, it may be involved in a variety of pathophysiological processes, such as immune suppression, promotion and progression of tumorigenesis, lymphatics [23]. CD68, a cytoplasmic protein with the molecular weight of about 110 kDa, serves as an important marker of TAMs [27]. Expressions of CD68 have been detected in colorectal cancer [28], breast cancer [29], gastric cancer [30], esophageal cancer [31] and so forth, all of which correlate with the clinical and pathological characteristics of patients with different tumors. Studies have found that [21], CUEDC2 plays a significant role in regulating the differentiation of monocytes into macrophages, during which process the expression of CUEDC2 in macrophages is exceedingly up-regulated, and this mechanism is regulated by the miR-324-5p axis. By using CUEDC2 knockout mice to build inflammatory bowel disease model, it was found that the mice were more sensitive to glucansulfate induced colitis; Meanwhile, the expression of inflammatory factors such as interleukin-6 in mice was significantly increased at this time, presenting as more obvious blood stool and weight loss, which were more likely to induce colitis related malignant tumors.

When it comes to CUEDC2 expression in esophageal squamous cell carcinoma and whether there exists a correlation between CUEDC2 and CD68, there seems to be no relevant research. In this study, the expression of CUEDC2 in esophageal squamous cell carcinoma tissues and adjacent tissues was determined by immunohistochemistry. To explore the relationship between the expression of CUEDC2 and the general clinical and pathological parameters, survival and prognosis of esophageal squamous cell

carcinoma patients, so as to discover the potential role of CUEDC2 in the occurrence and development of esophageal cancer. Immunofluorescence method was used to detect the expression and locational distribution of CUEDC2 and CD68 labeled macrophages in esophageal cancer tissues, and to explore the differences in the expression of the two, so as to provide experimental basis for the early diagnosis of patients with esophageal cancer, guidance of targeted therapy and the corresponding prognosis assessment.

## Methods

# Patients' and specimen's collection

Patients were collected from the Department of Thoracic Surgery in Xiangya Hospital, Central South University, between February 2012 and June 2013. Tumor tissues were diagnosed with ESCC by two different pathologists based on the World Health Organization (WHO) criteria. Tumor differentiation and staging were classified according to the 8th edition of the TNM classification of UICC[33]. Specimens were gained, including 50 cases of ESCC tissues, 20 cases of adjacent tumor tissues (≥3 cm away from the tumor margin). Patient clinical parameters were summarized in Table 1. Patients were included if they met the following criteria: (1) younger than 75 years of age; (2) first-diagnosed cases, and they had not been exposed to chemotherapy, radiotherapy or other treatments before being sampled; (3) The pathological diagnosis was primary esophageal squamous cell carcinoma with complete clinicopathological data. Exclusion criteria: (1) patients with incomplete clinical or pathological data;(2) patients with serious systemic diseases concerning heart, liver and kidney and so on;(3) patients with other anti-tumor treatments before surgical treatment;(4) patients and their families refuse to participate due to various reasons. The subjects were followed-up for a period of 5 years after the first postoperative year for survival and recurrence inquiry until death or the end of the investigation in February 2019.

Table 1
CUEDC2 expression in esophageal squamous cell carcinoma and normal tissues

| Variables | n | CUEDC2 expression | | | χ2 | P value |
|---|---|---|---|---|---|---|
| | | high | low | Low expression ratio | | |
| ESCC tissues | 50 | 9 | 41 | 82.00% | 9.583 | 0.002 |
| ESCC adjacent tissues | 20 | 11 | 9 | 45.00% | | |

# Reagents and instruments.

Rabbit polyclonal anti-CUEDC2 antibodies were purchased from Abcam(ab109649); the PV-6001 two-step immunohistochemistry kit (cat. no. PV-6001) was from OriGene Technologies, Inc. (Beijing, China); CD68 mouse mAb was obtained from Immunoway (YM6022); CY3 goat versus rabbit /488 goat versus mouse and DAPI were both bought from Google biology, Inc. (GB21303/GB25301 G1012).

# Immunohistochemistry.

Slices with a thickness of 4 μm were grilled at 65˚C for 3 hours, followed by dewaxing in xylene I and II for 15 min each. After that, they were rehydrated in an increasing diluted ethanol series. High-temperature antigen retrieval in a microwave in 0.1 M citrate solution (pH 6.0) for 10 min. Subsequently, the slices were incubated with 3% H2O2 at room temperature for 20 min, followed by incubation with goat serum at room temperature for 20 min, blocking endogenous peroxidase activity, and subsequent incubation with the diluted CUEDC2 antibody (1:100) at 4˚C overnight. Next day, the samples were then re-warmed in room temperature and incubated with the secondary antibodies for 20 min. The slices were stained with DAB (diaminobezidine) colorimetric visualization, prior to microscopic examination.
Immunohistochemical staining was scored independently by two pathologists without knowledge of the patients' characteristics. Any discrepancy was resolved by taking the consensus view. The score of immunoreactivity was performed by calculating the extent and intensity of the staining positivity of the cells in a semi-quantitative manner. The standards for evaluation included the following: Positive stain intensity (0, negative; 1, weak positive; 2, moderate positive; 3, strongly positive) and proportion of positive areas (≤ 10%=1, 1050%=2, ≥ 50%=3). The staining score was the multiplication of the positive stain intensity and the proportion of positive areas. Five fields in each specimen were selected at random, and the final score was the average of the five scores. For every experiment of CUEDC2, a group of sections was replaced by PBS solution as the negative control.

# double immunofluorescence

Slices were grilled at 65˚C for 4–5 h, followed by dewaxing in xylene I and II for 10 min each. After that, they were rehydrated in an increasing diluted ethanol series. These slices were then placed in the repair box of EDTA antigen repair buffer with PH = 8.0 and antigen repair was conducted in the microwave oven. Medium fire for 8 min, cease-fire for 8 min and turn to medium-low fire for 7 min. (During this process, excessive evaporation of buffer fluid should be prevented, and dry slices should be avoided.) After natural cooling, the slides were placed in PBS solution, all of which were shaken and washed on the decolorizing shaker for 3 times, 5 min each time. Utilization of a histochemical pen to draw a circle around the tissue, add an appropriate amount of self-fluorescence quenching agent in the circle, cover the tissue for about 5 min, and rinse with water for 10 min. Subsequently, BSA was added to seal endogenous peroxidase and incubated for 20 min. Shake off the sealing fluid covering the tissue surface, drop a primary antibody prepared in proportion of 1:100 on the slices, lay the slices flat in a wet box at 4 °C and incubate overnight. (add a small amount of water in the wet box to prevent the antibody from evaporating). Next day, take out the wet box and restore it to room temperature; The slides were placed in PBS and washed on the decolorizing shaking table for 3 times, 5 min each. After slicing and drying, the secondary antibody covering tissues of corresponding species and species of primary antibody were added in the circle (fluorescence secondary antibody CY3 goat anti-rabbit /488 goat anti-mouse 1:30/1:400), and incubated at dark room temperature for 50 min. Slides were placed in PBS, and then washed in the decolorization shaking table for 3 times, 5 min each. After the slices were slightly dried, DAPI dye was added in the circle and incubated at room temperature under dark for 10 min. Slides were placed in PBS, and washed in the

decolorizing shaking table for 3 times, 5 min each. The slices were slightly shaken dry and sealed with anti-fluorescence quenching sealant. Finally, microscopic examination and photography sections were observed and images were collected under Nikon inverted fluorescence microscope.(the excitation wavelength is 330–380 nm, and the emission wavelength is 420 nm; The excitation wavelength of FITC green light is 465–495 nm, and the emission wavelength is 515–555 nm. The excitation wavelength of CY3 red light is 510–560 and the emission wavelength is 590 nm).

Three representative areas were selected from each section, and fluorescence photos of red, green, red/green overlay (or yellow) were collected respectively. The nucleus stained by DAPI is blue under the excitation of ultraviolet excitation wave, the positive expression of CUEDC2 protein is the red light marked by corresponding fluorescein, and the CD68 protein is green light.

Green fluorescence reaction (CD68 positive) and red fluorescence reaction (CUEDC2 positive) occurred in some parts of the cancer tissue, and the two markers overlapped as red-green or yellow fluorescence, indicating the co-localization of the two proteins.

# Statistical analysis.

The patients were categorized into two groups according to the level of CUEDC2 expression (high and low). all data were created by excel and SPSS 24.0 software was used for statistical analyses. Image Pro Plus Image software was used to analyze immunofluorescence images. T-test or u test was used to compare the data groups. Wilcoxon sign rank-sum test was used for paired sample population and difference values that did not conform to normal distribution. $\chi 2$ test or Fisher's exact test was used to analyze the relationship between the difference of CUEDC2 expression and clinicopathological parameters in the samples of the counting data groups. Spearman rank correlation was adopted to analyze the correlation between the rank data. Kaplan–Meier method was used to determine the relationship between univariate variables and prognosis in survival analysis. Log-rank was used to test whether the difference in survival was statistically significant. Survival time was calculated in months.$P < 0.05$ was considered to indicate a statistically significant difference.

# Results

Classification of the patients and their characteristics.

A total of 50 patients were included in the study. 45 cases of male and 5 cases of females were added. Patients’ mean age was 60.14 ± 6.79 years (ranging from 44–73 years old). According to the 8th edition of the International Union Against Cancer's esophageal cancer staging (2017 edition), the enrolled patients were classified as follows: 0 cases of stage IA, 13 cases of stage IB, 7 cases of stage IIA, 8 cases of stage IIB, and 6 cases of stage IIIA, 16 cases of stage IIIB.

CUEDC2 expression is mostly downregulated in ESCC tissues.

In the study, immunohistochemical staining was used to detect CUEDC2 protein. The presence of yellowish-brown or yellow substances in the cytoplasm or nucleus of esophageal squamous cell carcinoma is positive staining of CUEDC2. Compared with CUEDC2 staining in ESCC tissues, which has been shown in Fig. 1, ESCC adjacent tissues presented its CUEDC2 expression in Fig. 2. According to the standards used for evaluating the samples (as detailed in the Materials and methods section), CUEDC2 was highly expressed in 9 cases (9/50 = 18.00%) and poorly expressed in 41 cases (41/50 = 82.00%) of 50 cases of esophageal cancer. However, in 20 cases of esophageal carcinoma, the expression of CUEDC2 in para-cancer tissues was high in 11 cases (11/20 = 55.00%) and low in 9 cases (9/20 = 45.00%). The difference between the different tissue groups was statistically significant ($P < 0.05$, Table 1).

CUEDC2 Expression is not associated with ESCC clinicopathological characteristics.

The association between CUEDC2 expression and the clinicopathological characteristics of ESCC was determined by the $\chi 2$ test and Fisher exact test. As summarized in Table 3, no significant difference was identified between CUEDC2 expression and age ($P = 0.594$), gender ($P = 0.570$), smoking history ($P = 0.410$), tumor differentiation ($P = 0.925$) or tumor size ($P = 0.462$) (shown in Table 2).

Table 2
The association between CUEDC2 and the clinicopathological characteristics of ESCC

| Variables | n | | CUEDC2 expression | | | |
|---|---|---|---|---|---|---|
| | | | High | Low | χ2 | P |
| Gender | male | 45 | 9 | 36 | | 0.570 |
| | female | 5 | 0 | 5 | | |
| Age | ≤ 60 y | 28 | 6 | 22 | 0.507 | 0.477 |
| | >60y | 22 | 3 | 19 | | |
| Smoking | Yes | 33 | 7 | 26 | 0.678 | 0.410 |
| | No | 17 | 2 | 15 | | |
| Tumor size | ≥ 5 cm | 10 | 1 | 9 | 0.542 | 0.462 |
| | <5 cm | 40 | 8 | 32 | | |
| Tumor differentiation | well | 16 | 3 | 13 | 0.009 | 0.925 |
| | moderate and poor | 34 | 6 | 28 | | |
| Tumor location | Upper | 1 | 1 | 0 | 5.296 | 0.071 |
| | Middle | 24 | 5 | 19 | | |
| Invasion of the outer membrane | Lower | 25 | 3 | 11 | 0.090 | 0.764 |
| | Yes | 30 | 5 | 25 | | |
| | No | 20 | 4 | 16 | | |
| lymph node metastasis | Yes | 24 | 6 | 18 | 1.532 | 0.216 |
| | No | 26 | 3 | 23 | | |
| | I-II | 28 | 4 | 24 | 0.595 | 0.441 |
| TNM staging | III | 22 | 5 | 17 | | |

Table 3
Summary of 5 years survival time of 50 patients with ESCC

| No. | Survival time | No. | Survival time |
|---|---|---|---|
| 1 | 0 | 26 | 0 |
| 2 | 18 | 27 | 0 |
| 3 | 5 | 28 | 16 |
| 4 | 0 | 29 | 6 |
| 5 | 20 | 30 | 10 |
| 6 | 9 | 31 | 0 |
| 7 | 0 | 32 | 60 |
| 8 | 12 | 33 | 11 |
| 9 | 8 | 34 | 5 |
| 10 | 4 | 35 | 0 |
| 11 | 6 | 36 | 0 |
| 12 | 0 | 37 | 0 |
| 13 | 0 | 38 | 10 |
| 14 | 5 | 39 | 0 |
| 15 | 10 | 40 | 5 |
| 16 | 5 | 41 | 0 |
| 17 | 12 | 42 | 10 |
| 18 | 14 | 43 | 6 |
| 19 | 30 | 44 | 60 |
| 20 | 0 | 45 | 24 |
| 21 | 6 | 46 | 9 |
| 22 | 7 | 47 | 12 |
| 23 | 8 | 48 | 6 |
| 24 | 12 | 49 | 4 |
| 25 | 0 | 50 | 15 |

downregulation of CUEDC2 expression is associated with the prognosis of ESCC.

Of the 50 cases of esophageal cancer patients, 15 cases were lost to follow-up due to the wrong number, family members repeatedly failing to answer the phone or missing the number, and 35 cases were successfully followed up, among which only 2 patients were still alive. The follow-up period ended on January 30, 2019 (as seen in Table 3). The analysis is as follows:

To investigate the feasibility of using CUEDC2 expression as an ESCC prognostic factor, The results of Kaplan-Meier univariate regression survival analysis showed that CUEDC2 protein expression along with tumor grade had an effect on the prognosis of esophageal squamous cell carcinoma (all $P < 0.05$), (as seen in Table 4) which indicated that ESCC patients who had lower levels of CUEDC2 expression also manifested shorter overall survival times (median survival time: 5 months) compared with the higher CUEDC2 expression group (median survival time: 14 months) (shown in Fig. 3).

Table 4
Kaplan-Meier uni-variate regression survival rate analysis of CUEDC2 in ESCC

| Variables | $\chi 2$ | P value |
|---|---|---|
| Gender | 0.194 | 0.660 |
| Age | 25.117 | 0.242 |
| Differentiation grade | 0.150 | 0.928 |
| Smoking | 1.065 | 0.302 |
| Tumor size | 1.214 | 0.271 |
| Lymph node metastasis | 0.595 | 0.441 |
| Invasion depth | 1.533 | 0.460 |
| Tumor stage | 12.389 | 0.015* |
| Tumor location | 3.245 | 0.197 |
| Expression level | 5.188 | 0.023* |
| * $P < 0.05$ was considered to indicate a statistically significant difference. | | |

Correlation analysis of CUEDC2 and CD68 expression in ESCC

This study adopts a double immunofluorescence method labeling 12 cases of esophageal squamous cell carcinoma tissue, specifically green fluorescent markers for CUEDC2 protein expression and red fluorescent tags for CD68 protein expression. The Image Pro Plus image analysis software and SPSS 24.0 software were chosen to identify two proteins expression in 12 cases of esophageal cancer tissue, including analyzing images with a total Area of value (Area) and optical density (IOD), calculating the average optical density (MOD) = IOD/Area (as shown in Table 5 and Figs. 4 and 5). We discovered that there were significant differences between the expression and CUEDC2 CD68 protein expression. The MOD values of CD68 and CUEDC2 expression analyzed by SPSS software were not in line with normal

distribution. We chose the Wilcoxon symbol rank-sum test of paired samples, results suggesting z-value =-3.059b (b. Based on positive rank) and P-value = 0.02 < 0.05, which means the difference was statistically significant. Further, we identified that CD68 was relatively highly expressed than CUEDC2 in esophageal squamous cell carcinoma tissue in comparison with their different MOD value.

Table 5
records of immunofluorescence expression of CUEDC2 and CD68 proteins

| | **CD68(green light)** | | | **CUEDC2(red light)** | | |
|---|---|---|---|---|---|---|
| | Area | IOD | MOD | Area | IOD | MOD |
| 1 | 697570 | 1278278.9 | 1.832474017 | 19997 | 15052.644 | 0.752745112 |
| 2 | 414188 | 712745.31 | 1.720825591 | 42113 | 39623.059 | 0.940874766 |
| 3 | 20262 | 40443.223 | 1.996013375 | 61008 | 58035.688 | 0.951279963 |
| 4 | 864438 | 1527315.5 | 1.766830588 | 6321 | 6622.9917 | 1.047775937 |
| 5 | 76323 | 151514.72 | 1.985177731 | 9161 | 9824.6865 | 1.072446949 |
| 6 | 30624 | 61022.02 | 1.99262082 | 42548 | 43616.559 | 1.0251142 |
| 7 | 741613 | 1520935.4 | 2.050847814 | 657 | 711.75317 | 1.083338158 |
| 8 | 190395 | 294024.16 | 1.544285092 | 2010 | 2190.2698 | 1.089686468 |
| 9 | 3568 | 6892.0581 | 1.931630633 | 42548 | 43616.559 | 1.0251142 |
| 10 | 281264 | 517094.84 | 1.838467916 | 9507 | 8982.6348 | 0.944844304 |
| 11 | 472375 | 945674.44 | 2.001957004 | 46005 | 46852.719 | 1.018426671 |
| 12 | 42724 | 85042.68 | 1.990513061 | 31715 | 29565.219 | 0.932215639 |

At the same time, yellow fluorescence was found in the overlap of red and green light of CUEDC2 and CD68 proteins, suggesting that there may be interaction between them during the occurrence and development of esophageal cancer.

# Discussion

Esophageal cancer is one of the common malignant tumors in the digestive system. The incidence rate of esophageal cancer in China is relatively high, such as in Fu Jian and other provinces. The incidence and death rate of esophageal cancer in China accounts for more than 50% of the world's total, which brings a great burden to China's medical industry and economic development [34].

This study is the first to explore the expression of CUEDC2 in esophageal squamous cell carcinoma tissues and cancer adjacent tissues. Double immunofluorescence method was also the first used to explore the differences in the expression of CUEDC2 and CD68-labeled macrophages in esophageal cancer tissues, and it was believed that the expression of CUEDC2 protein might be involved in the

occurrence and progression of esophageal squamous cell carcinoma. Now the results are discussed as follows.

CUEDC2 is involved in various pathophysiological processes, such as the regulation of cell cycle and inflammatory response, as well as tumor occurrence and development [10, 36]. CUEDC2 can modulate the inflammatory response by inhibiting the activation of NF-κB and STAT3 signaling pathways [37]. CUEDC2 can regulate the process of mitosis as well. When exposed to ultraviolet radiation, there occurs the degradation of CUEDC2 which may increase the instability of genes in the cell cycle, thus exerting its role in the development of tumors [38]. Current research demonstrates that During the survival and proliferation of tumor cells, CUEDC2 can show its influence in the aerobic glycolysis of cells, and increase the production of lactic acid and the uptake of glucose by tumor cells during the process, so as to provide energy for tumor cells [17].

The inactivation of tumor suppressor genes and the activation of proto-oncogenes can participate in the proliferation and differentiation of tumor cells.

In this study, 9 cases (9/50 = 18%) of 50 esophageal squamous cell carcinoma tissues showed high expression of CUEDC2, while 41 cases (41/50 = 82%) showed low expression. There were 11 cases (11/20 = 55%) with high expression of CUEDC2 in the para-cancerous tissues of 20 cases of esophageal squamous cell carcinoma, and 9 cases (9/20 = 45%) with low expression, the difference was statistically significant. Imaginably,this result indicates that with the progress of the disease, the expression of CUEDC2 in ESCC is lower than that in normal tissues, that is to say, the expression of CUEDC2 protein may play a role as an oncogene in the occurrence and development of ESCC. However, how CUEDC2 acts on ESCC and the specific mechanism need to be further studied.

Till now, there appear to be no reports on the expression of CUEDC2 protein in ESCC patients and its relationship between the general clinical and pathological data. We found that CUEDC2 protein expression and esophageal squamous cell carcinoma of the patient's age and gender had no significant correlation, and may be due to women's groups were of less samples (5 cases in this group, including 0 women patients appeared CUEDC2 high expression). Taken together, in future follow-up experiments we have to further increase the sample size to enhance the experimental conclusion more persuasive. Meanwhile, the relationship between CUEDC2 and esophageal squamous cell carcinoma patients' smoking status, tumor size and location, differentiation degree, invasion depth, lymph node metastasis and tumor grade were analyzed. No significant correlation was found between CUEDC2 expression and ESCC in this study, which may be attributed to the limited sample size included in this study.

Interestingly, by analyzing the relationship between CUEDC2 and tumor size of esophageal squamous cell carcinoma, it was acknowledged that when the tumor diameter was < 5 cm, the low expression of CUEDC2 was found in 32 cases (32/50 = 64.00%); whereas, if the diameter was ≥ 5 cm, and the low expression was found in 9 cases (9/50 = 18.00%). That is to say, although there is no significant difference between the expression of CUEDC2 in this study and the tumor size, we may come to a conclusion that the smaller the tumor diameter is, the higher proportion of the low expression of CUEDC2,

which also simultaneously renders some implications for the further study on the expression of CUEDC2 in ESCC.

Therefore, it cannot be concluded that the expression of CUEDC2 protein has nothing to do with the general clinical data and pathological classification of patients with esophageal squamous cell carcinoma.

Through the postoperative follow-up survival conditions in patients with esophageal squamous cell carcinomas and usage of Kaplan Meier-method of single factor regression analysis, we found that CUEDC2 protein expression and tumor grade have an impact on the survival prognosis of esophageal squamous carcinoma, and the lower expression of CUEDC2 corresponds to lower survival rate, which provides a new basis for the prognosis prediction of patients with esophageal squamous carcinoma assessment. Besides, what cannot be ignored is that CUEDC2 may serve as a potential tumor suppressor gene in the development of esophageal cancer.

Infectious diseases and chronic inflammatory disease account for about 25% of cancer-inducing factors. As we said before, the macrophages not only play an important role in the immune system but also, they are indispensable parts of the inflammatory response. CD68, as one of the markers of tumor tissue macrophages, its expression may impact on immunosuppression, tumor invasion, inflammation and other mechanisms involved in tumor progression [25].

In this study, it was identified that CD68 was relatively highly expressed in esophageal squamous cell carcinoma tissue, which was consistent with the study of Rongling Xie[32]. Meanwhile, the infiltration density of CD68-labeled macrophages in esophageal squamous cell carcinoma tissue was relatively increased, which means that CD68-labeled macrophages may participate in the disease process of esophageal squamous cell carcinoma. In immunofluorescence, it showed that CUEDC2 was relatively low in esophageal carcinoma tissues, which was basically consistent with the results of immunohistochemistry in this study. Statistical analysis of the Wilcoxon sign rank sum test showed that there were significant differences in the expression of CD68 and CUEDC2 in esophageal cancer tissues, and the differences were statistically significant. Combined with immunofluorescence double standard atlas, we are the first to discover the phenomenon that the green light of CD68 markers and the red light of CUEDC2 markers, which resulted in yellow fluorescence, suggesting there were positioning phenomenon. Besides, there may be a potential interaction between both proteins, participating in the development of esophageal cancer, but the specific area of effect and mechanism needs further research.

Chen yuan et al. [40] found that CUEDC2 expression was increased when monocytes differentiated into macrophages. As mentioned in the introduction, when inflammatory stimulation is experienced in the mouse model of inflammatory bowel disease, the knockout of CUEDC2 will significantly increase the expression of inflammatory factors such as interleukin-6, and meanwhile cause the continuous activation of macrophages, which may induce tumor formation. However, when infiltrated into macrophages of colon cancer, the expression of CUEDC2 is significantly reduced or hardly expressed, which means that

CUEDC2 can participate in the regulation of macrophage-induced inflammatory response and tumor progression.

In conclusion, CUEDC2 has a low expression in esophageal cancer and is possibly capable of regulating inflammatory responses mediated by macrophages. Furthermore, CD68, relatively higher expression in esophageal cancer, can be involved in the inflammatory response function in tumor progression. Finally, the double immunofluorescence test method has shown that there were co-localization phenomenon, supporting the assumption that there were potential interactions between the expression of CUEDC2 and CD68 labeled macrophages in esophageal cancer which indicates their possible roles of providing new exploration evidence for the pathogenesis of esophageal cancer in "inflammation and cancer" chain [42]. However, it remains to be verified.

In this study, only semi-quantitative analysis of esophageal squamous cell carcinoma tissues was conducted from the perspectives of immunohistochemistry and immunofluorescence, and the expression of CUEDC2 was not completely quantified by PCR and other experiments, which could be improved to make the experimental conclusions more convincing. Further study on the interaction between CUEDC2 and CD68 will be helpful to elucidate the pathogenesis of esophageal cancer and is expected to be a new target for the treatment and diagnosis of esophageal cancer.

## Abbreviation

| | |
|---|---|
| CUEDC2 | CUE domain containing protein 2 |
| TAMs | tumor associated macrophages |
| NF—κB | nuclear factor –κB |
| JAK/STAT | the Janus kinase/signal transducer and activator of transcription |
| GLUT3 | glycolytic proteins glucose transporter 3 |
| LDHA | lactate dehydrogenase A |
| GR | glucocorticoid receptor |
| APC/C | The anaphase-promoting complex or cyclosome |
| SOCS-3 | suppressor of cytokine signaling-3 |
| ROS | reactive oxygen species |

## Declarations

# Ethics approval and consent to participate

Informed consent was acquired from all patients prior to surgery. This study was approved by the Xiangya Hospital, Central South University Ethics Committee, and the usage of the information and specimens collected has been handled and anonymized conformed to the ethical and legal standards.

# Consent for publication

Not applicable

# Availability of data and materials

All data generated or analysed during this study are included in this published article

# Conflict of interest

The authors declare that they have no competing interest.

# Authors' contributions

Junbo Xiao wrote the article; Rong Wu, Xue-mei Peng, Bin Chen, Yan-qiu Zhang, Zhen Wen, Jian He, Guan-nan Ye helped in the preparation of the manuscript, patients follow-up investigation, construction of tables and citation of references and the critical revision of the literature respectively.

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# Figures

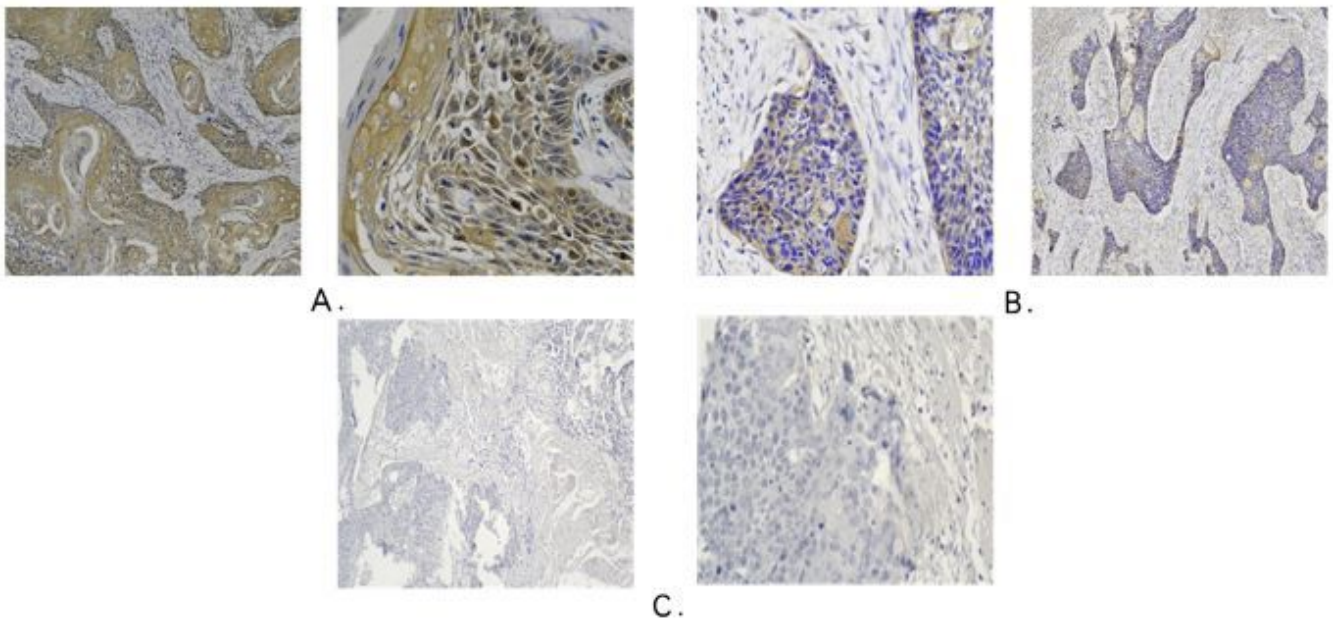


**Figure 1** Different CUEDC2 expression levels in ESCC tissues, magnification in sequence of x100 and x400 **A**. relatively higher CUEDC2 expression in ESCC tissues **B**. relatively lower expression in ESCC tissues **C**. Negative control of CUEDC2 expression by PBS resolution in ESCC tissues

**Figure 1**

Figure 1

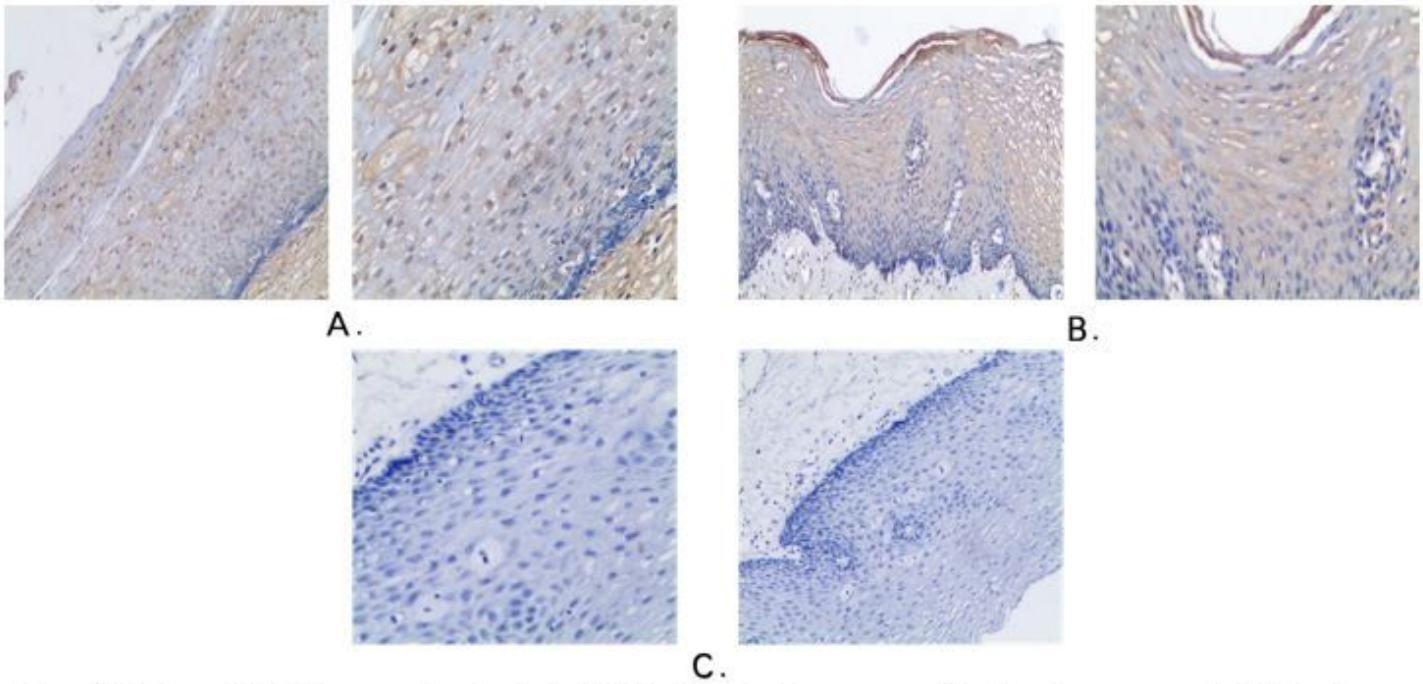


**Figure 2** Different CUEDC2 expression levels in ESCC adjacent tissues, magnification in sequence of x100 and x400 **A.** relatively higher CUEDC2 expression in ESCC adjacent tissues **B.** relatively lower expression in ESCC adjacent tissues **C.** Negative control of CUEDC2 expression by PBS resolution in ESCC adjacent tissues

## Figure 2

Figure 2

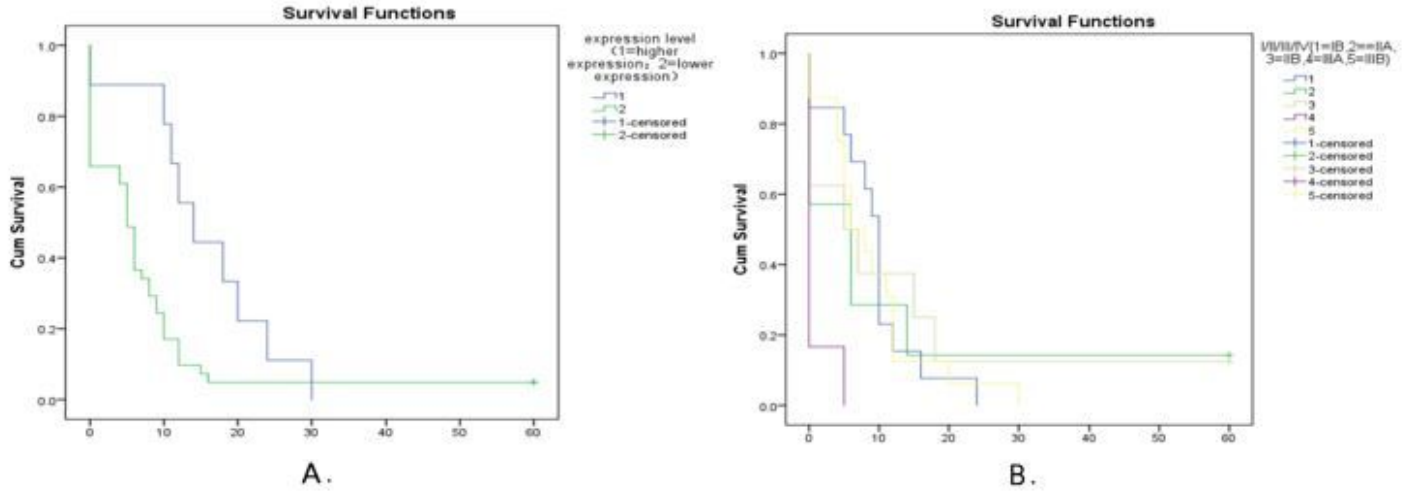


**Figure 3** postoperative survival analysis in 5 years among patients with ESCC **A.** CUEDC2 expression **B.** tumor grade

## Figure 3

Figure 3

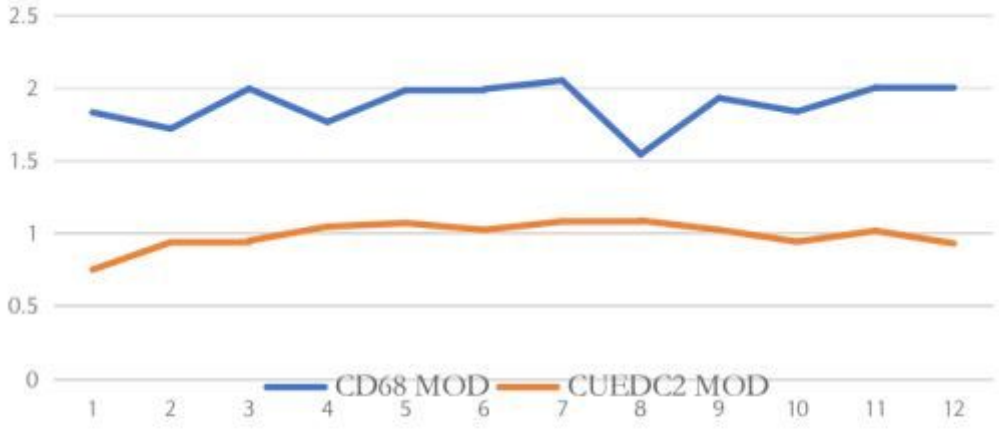


**Figure 4** The mean optical density values of CUEDC2 and CD68 protein immunofluorescence expression

## Figure 4

Figure 4

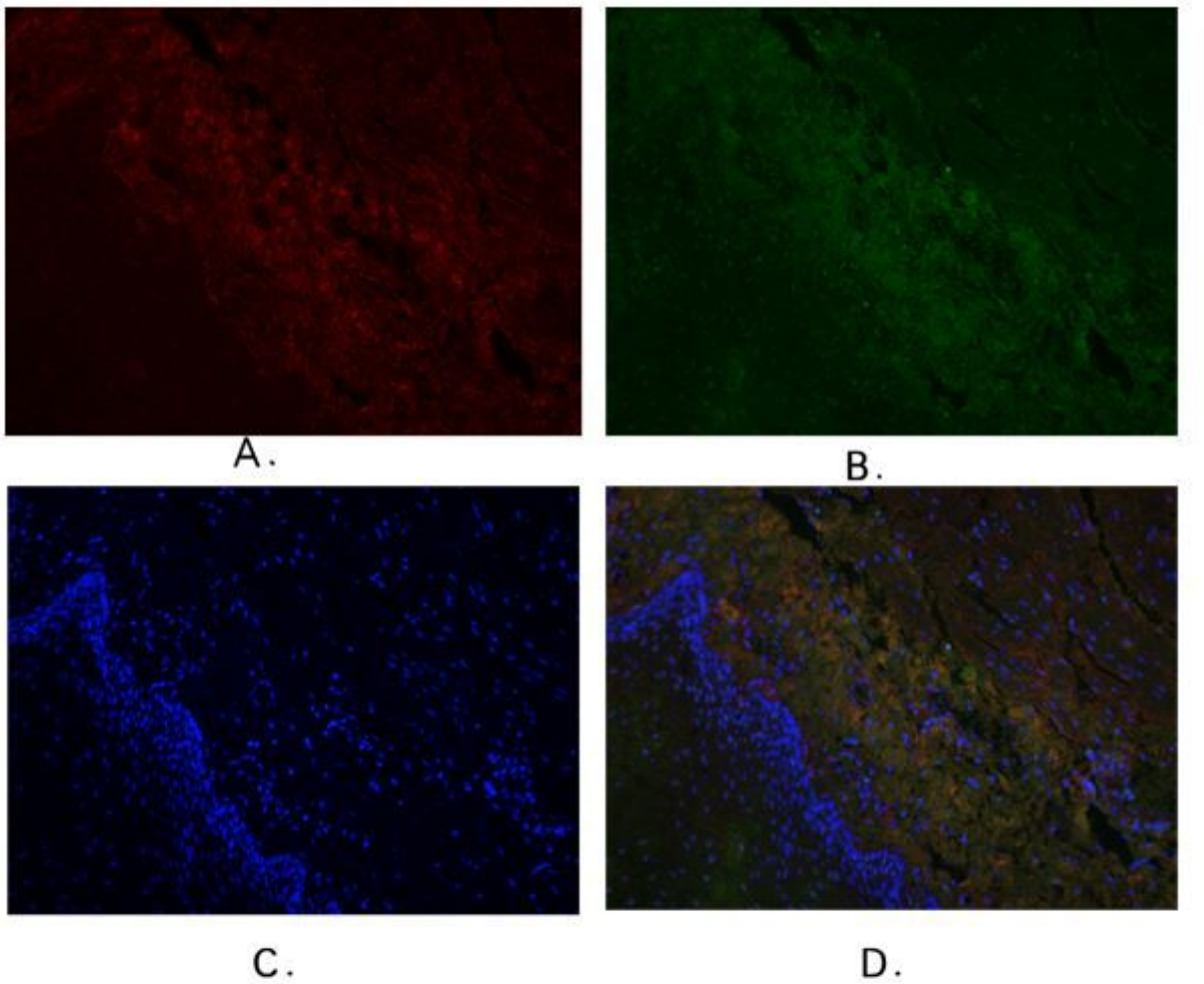


**Figure 5** CUEDC2 and CD68 expression in ESCC tissues, magnification x100 **A**. red fluorescence reaction represented expression of CUEDC2 protein **B**. green fluorescence reaction represented CD68 expression **C**. blue light showed the stain of nucleus by DAPI **D**. overlap of CEDC2 and CD 68 manifested yellow signal

## Figure 5

Figure 5

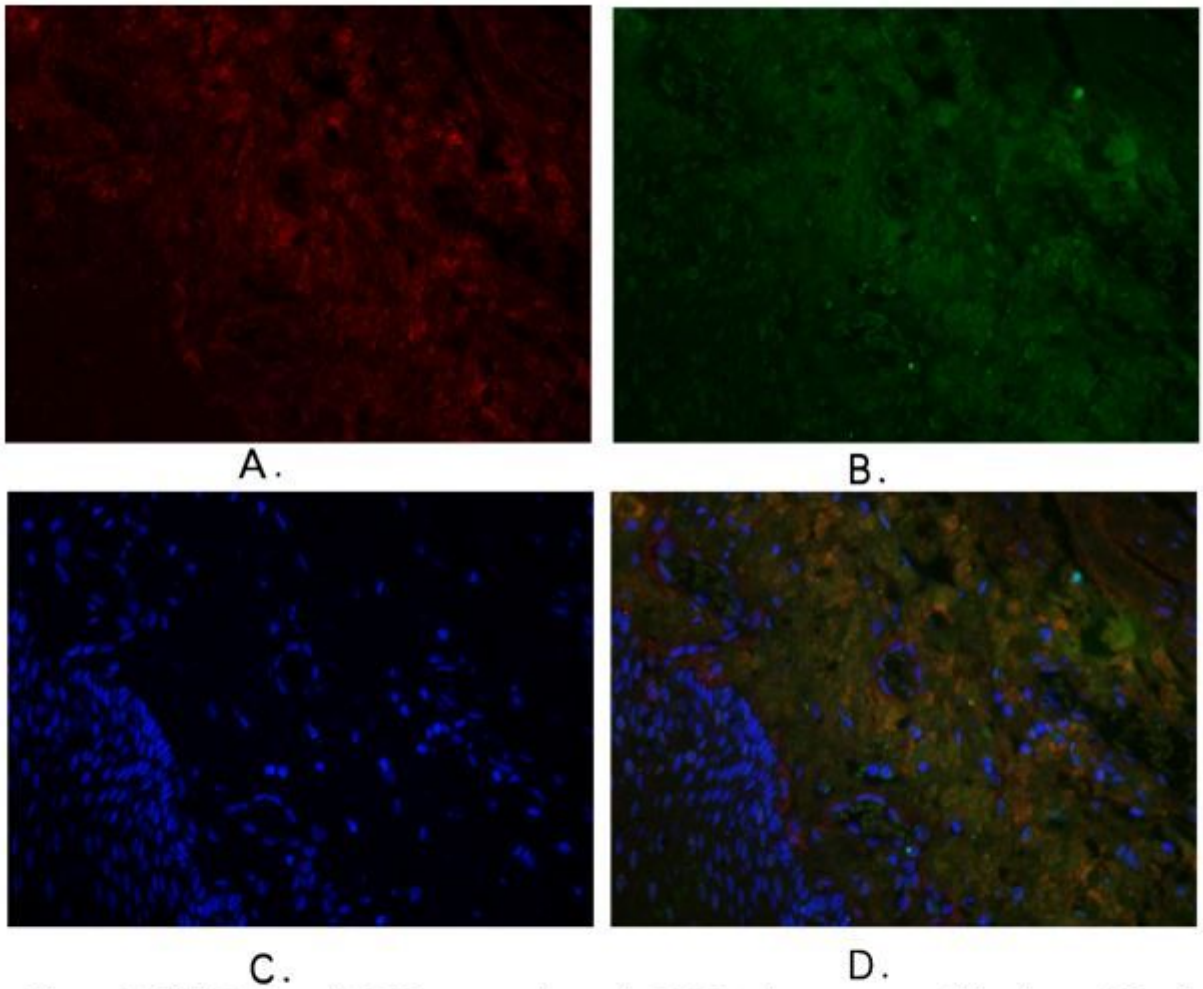


**Figure 6** CUEDC2 and CD68 expression in ESCC tissues, magnification x100 **A**. red fluorescence reaction represented expression of CUEDC2 protein **B**. green fluorescence reaction represented CD68 expression **C**. blue light showed the stain of nucleus by DAPI **D**. overlap of CEDC2 and CD 68 manifested yellow signal

## Figure 6

Figure 6