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*Original Article*

# Clinicopathological significance and prognostic value of CK1α expression in esophageal squamous cell carcinoma

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**Abstract:** Casein kinase 1α (CK1α) is a member of serine/threonine protein kinases, which expressed in all kinds of eukaryotes, and the sequence structure is highly conserved. In mammals, CK1α is involved in a variety of cellular physiological processes and partial signaling pathways. It has been shown that CK1α acts as a tumor suppressor and it is closely related to the occurrence and development of tumor. However, little is known about its role in esophageal squamous cell carcinoma (ESCC). Purpose: The aim of present study was to explore the role and the prognostic significance of CK1α expression in ESCC. Methods: The expression of CK1α was detected by immunohistochemistry (IHC) technique in 100 cases of ESCC and their corresponding adjacent nonneoplastic esophageal tissues (n=80), and analyzed the relationship between CK1α expression and clinicopathological parameters, survival rate and prognosis of ESCC patients. Results: The positive percentage of CK1α protein in ESCC was 56% (56/100), which was significantly lower than that in nonneoplastic esophageal tissues was 90% (72/80), ($\chi^2$=25.010, *P*=0.000). The expression of CK1α was significantly related with differentiation degree, but not with the sex, age, tumor size, depth of invasion, lymphatic invasion and AJCC clinical stage. On the basis of univariate and multivariate Cox regression analysis, we found that tumor size and depth of invasion were independent risk factors for worse prognosis in ESCC patients. Furthermore, survival analysis date revealed that the positive expression of CK1α had significantly better survival than the negative expression of CK1α in ESCC patients after curative surgery. Conclusion: CK1α may play an important role in the carcinogenesis and development of ESCC and might provide clinically useful prognostic information in cases of ESCC.

**Keywords:** Casein kinase 1α (CK1α), esophageal squamous cell carcinoma (ESCC), immunohistochemistry (IHC), tissue microarray (TMA), prognosis

## Introduction

Esophageal cancer (EC) is the eighth most common cancer and the sixth leading causes of cancer-related mortality in the world, with an estimated 456,000 new cases per year worldwide [1, 2]. Esophageal squamous cell carcinoma (ESCC) is the predominant histologic subtype of EC and China is one of the highest incidence areas, especially in Taihang Mountain [3]. Though there have been great efforts to develop methods for a better prognosis, there is still far from optimism. Therefore, it is very urgent to further study the pathogenesis of esophageal carcinoma and develop new therapeutic strategies.

The casein kinase 1 (CK1) family is a conserved serine/threonine protein kinases, ubiquitously existing in all eukaryotes, phosphorylates a large number of cellular proteins [4, 5]. Seven family members were identified, which are encoded by different genes, including CK1α, CK1β, CK1γ1, CK1γ2, CK1γ3, CK1δ, and CK1ε [6]. They are highly homologous within their kinase domains. The CK1 protein kinases are involved in multiple cellular processes including gene transcription, DNA repair, cell division, nuclear localization, membrane transport and so on. In addition, it has also been shown to be involved in other signaling pathways, such as Wnt/β-Catenin, Hedgehog and NF-κB. It was described that CK1α was identified as negative regulators of the Wnt/β-Catenin signaling pathway. And CK1α is able to phosphorylate Ser45 of β-catenin which is the priming reaction for the proteasomal degradation of β-catenin [7].

Some evidences support that CK1α plays positive roles in tumor pathogenesis, interestingly, very little is known about the expression and functional role of CK1α in tumor cells thus far. In this study, we investigated the expression of CK1α protein in ESCC and the relationship between CK1α expression and patients' clinicopathological parameters, survival rate and prognostic information.

## Materials and methods

### *Tissues and patients history*

To be eligible for participation in this study, the patients were selected at their first diagnosis and underwent esophagectomy alone (none had performed radiotherapy, chemotherapy and/or immunotherapy). The tissues evaluated in our study were obtained from 100 patients (74 female, 26 male; median age 59 years; range 36-81 years) who had undergone radical esophagectomy in the Department of Surgery in Heping Hospital affiliated Changzhi Medical College (Changzhi, Shanxi, China) from January 2006 to October 2008. The tissue samples were collected immediately during surgical procedures, which composed of liquid nitrogen snap-frozen specimens and paraffin blocks. 80 samples were contrasted with the adjacent nonneoplastic mucosa removed during the same resection, measuring 3-10 cm away from the edge of the main tumor lesion. All tissues, with a histopathologic diagnosis of ESCC and the adjacent nonneoplastic mucosa, were confirmed by two independent pathologists who were blinded to the original diagnosis. To ascertain no carcinoma, metaplasia, dysplasia, and atypical hyperplasia in the nonneoplastic mucosa tissue, we use the strict evaluation criteria to diagnose, and the chronic inflammation was allowed. In addition, clinicopathological parameters were collected including gender, age, tumor size, depth of invasion, cell differentiation, lymphovascular invasion and AJCC clinical stage. Tumors were staged according to the seventh edition of the AJCC staging system for ESCC [8].

The regular assessment of survival status was continuous after surgery until September, 2014. The mean follow-up period was 80±12 months (range, 3-99 months). We considered as uncensored only the records of patients who had died of ESCC, we considered as censored record of all patients who were alive at the end of follow-up interval or patients who died of a cause not related to ESCC. Follow-up records were made every month during the first year after surgery, then trimonthly during the second year, and once half a year, thereafter. The study was approved by the Medical Ethics and Human Clinical Trial Committee of Changzhi Medical College.

### *Tissue microarray (TMA)*

The tissue microarray was produced as described previously by Kononen et al. (in collaboration with Shanghai Biochip Company, Shanghai, China) [9]. By careful selection of the morphologically representative region with their hematoxylin-eosin-stained slides on the chosen individual paraffin-embedded blocks (donor blocks), a core tissue biopsy of 2 mm was punched and transferred to the recipient paraffin-embedded block (receiver block). Then the recipient blocks were incubated twice for 5 min at 56°C to improve adhesion between cores and paraffin. In view of tumor heterogeneity and the tissue losing, the nonneoplastic and cancer tissues were repeated thrice for each case. Then, we prepared 180 TMA blocks tissues with formalin-fixed, paraffin-embedded (100 case of ESCC and their corresponding adjacent nonneoplastic esophageal tissues (n=80)).

### *Immunohistochemistry*

The immunohistochemical reactions were performed on 4 μm thick sections obtained from the TMA blocks. For immunohistochemical analysis, the slides were deparaffinized, rehydrated, then immersed in 3% hydrogen peroxide solution for 10 min; hyperbaric heated in Ethylene diaminetetra acetic acid (EDTA) buffer (pH 9.0) at 100°C for 10 min; and cooled at room temperature for 30 min. Then we blocked the slides with 10% normal goat serum at 37°C for 30 min and then incubated with CK1α rabbit polyclonal antibody (1:300 dilution, Abcam, Cambridge, UK) overnight at 4°C. After washed with phosphate buffer solution (PBS), the sections were treated with corresponding streptavidin-peroxidase conjugated second antibody (Zhongshan Golden Bridge Corporation, Beijing, China) then color reaction was detected by diaminobenzidine (DAB) reagent. Negative and positive controls were included for experime-

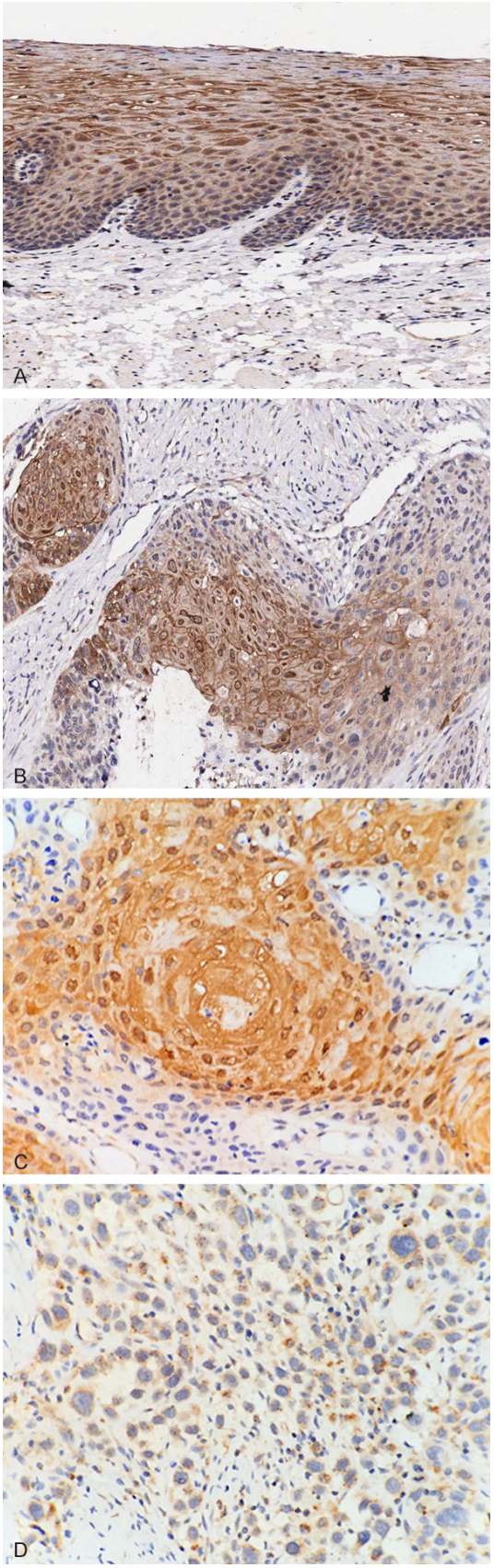


**Figure 1.** A. Expression of CK1α in nonneoplastic esophageal tissue (SP×200); B. Expression of CK1α in ESCC (SP×200); C. Expression of CK1α in high grade ESCC (SP×200); D. Expression of CK1α in low grade ESCC (SP×200).

nts. The gastric carcinoma specimen was utilized as positive control for CK1α. Both stains were performed with the same procedures; only the primary antibodies were replaced by PBS as negative controls.

*Slide evaluation of immunohistochemical staining*

Immunostaining for CK1α was graded by a semiquantitative method based on a scale; both the distribution and intensity of the staining were considered. Evaluation of the IHC was performed by two pathologists, who were blinded to the original histological diagnosis. The result of the tissues was determined from at least 1,000 cells that were counted systematically at ×400 magnification in five visual fields. In the IHC test for CK1α, the presence of diffuse cytoplasm staining was considered to be significant. The intensity was scored as follows: 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). The percentage of positive cells was scored as follows: 1 (0-25%), 2 (26-50%), 3 (51-75%), or 4 (76-100%). The two scores were combined to obtain the final one: negative (0-3), weakly positive (4-5), or strongly positive (6-7) [10].

*Statistical analysis*

All analysis was performed with SPSS 18.0 for Windows (SPSS Inc., Chicago, USA). The Chi-square test was used to analyze the correlation between CK1α expression and clinicopathological parameters. The data are expressed as mean ± standard deviation (SD). Kaplan-Meier method was used to draw survival curves; Comparative analysis of subgroups was determined with the log-rank test. Univariate proportional hazards regression was used to estimate the dependence of survival on each variable. Multivariate survival analysis was based on Cox proportional hazard model to test the variables selected by univariate analysis as having prognostic value. All statistical significance was considered for $P<0.05$.

**Table 1.** Correlation of CK1α expression with clinicapathological parameters

| Variable | | n | CK1α expression (%) | | $\chi^2$ | *P*-value |
|---|---|---|---|---|---|---|
| | | | + | - | | |
| Overall frequency | Nonneoplastic | 80 | 72 (90.0) | 8 (10.0) | 25.010 | 0.000* |
| | ESCC | 100 | 56 (56.0) | 44 (44.0) | | |
| Gender | Male | 74 | 40 (54.1) | 34 (45.9) | 0.437 | 0.508 |
| | Female | 26 | 16 (61.5) | 10 (38.5) | | |
| Age (yr) at surgery | ≥60 | 68 | 39 (57.4) | 29 (42.6) | 0.158 | 0.691 |
| | <60 | 32 | 17 (53.1) | 15 (46.9) | | |
| Tumor size (cm) | <4 | 24 | 12 (50.0) | 12 (50.0) | 0.471 | 0.790 |
| | 4-7 | 71 | 41 (57.7) | 30 (42.3) | | |
| | ≥8 | 5 | 3 (60.0) | 2 (40.0) | | |
| Cell differentiation | High-grade | 6 | 4 (66.7) | 2 (33.3) | 7.811 | 0.020* |
| | Middle-grade | 69 | 44 (57.9) | 25 (42.1) | | |
| | Low-grade | 25 | 8 (48.0) | 17 (52.0) | | |
| Depth of invasion | T1 | 5 | 3 (60.0) | 2 (40.0) | 0.198 | 0.978 |
| | T2 | 13 | 7 (53.8) | 6 (46.2) | | |
| | T3 | 79 | 44 (55.6) | 35 (44.4) | | |
| | T4 | 3 | 2 (66.7) | 1 (33.3) | | |
| Lymphatic invasion | (-) | 46 | 25 (54.3) | 21 (45.7) | 0.094 | 0.759 |
| | (+) | 54 | 31 (57.4) | 23 (42.6) | | |
| AJCC clinical stage | I+II | 46 | 27 (50.0) | 19 (50.0) | 0.251 | 0.616 |
| | III+IV | 54 | 29 (53.7) | 25 (46.3) | | |

*$P$<0.05.

## Results

*Expression of CK1α protein in ESCC and the relationship between CK1α expression and patients' clinicopathological parameters*

As performed by IHC, CK1α expression was found in cell cytoplasm, few in nuclei as well. The CK1α expression in ESCC was significantly lower than in nonneoplastic esophageal tissues (**Figure 1A**, **1B**). CK1α expression was noted in 56 out of 100 (56%) cases of ESCC and 72 out of 80 (90%) cases of nonneoplastic esophageal tissues. A significant down-regulation of CK1α immunoreactivity was found in ESCC compared to the nonneoplastic esophageal tissue ($\chi^2$=25.010, $P$=0.000). The statistical analyses also show the relationship between CK1α expression and clinicopathological parameters. The percentage of CK1α positive expression showed an decreasing trend from high grade to low grade, a significant difference was found among them ($P$=0.020) (**Figure 1C**, **1D**). However, there were no statistically significant correlations between CK1α expression and Sex ($P$=0.508), age ($P$=0.691), tumor size ($P$=0.790), depth of invasion ($P$=0.978), lymphatic invasion ($P$=0.759) and AJCC clinical stage ($P$=0.616) (**Table 1**).

Univariate and multivariate Cox-regression analyses have performed to identify independent predictors for survival. All the clinicopathological parameters were entered into the analysis. Both univariate and multivariate survival analysis proved tumor size and depth of invasion were poor prognostic factors (P<0.05, **Tables 2**, **3**).

*Survival analysis*

In order to determine whether CK1α expression is a prognostic factor, Kaplan-Meier survival curves for all 100 patients have demonstrated. The median survival time for patients with positive CK1α expression was 23 (95% CI: 22.2-33.6) months compared to 16 (95% CI: 18.2-29.9) months for patients with negative CK1α expression. The 1-year, 3-year and 5-year survival rate were 73.6%, 32.1% and 21.4% in the CK1α positive group (n=56) compared with 60.5%, 22.7% and 15.9% in the negative expression group (n=44), respectively. The CK1α positive expression group had significantly a better survival rate than the negative group ($\chi^2$=4.367, $P$=0.037) (**Figure 2**).

## Discussion

CK1α is a multifunctional Ser/Thr kinase that phosphorylates several substrates. Recent investigations have demonstrated that CK1α plays a role in tumorigenesis and changes in the CK1α expression level and/or activity could promote tumorigenesis and tumor progression through a multitude of cellular signal transduction pathways.

At present, the research on CK1α is mostly focused on the mechanism of phosphorylation. Surprisingly, very little is known about the

**Table 2.** Univariate analysis of predictive factors for survival

| Prognostic factors | Relative risk (95% CI) | *P*-value |
|---|---|---|
| Univariate | | |
| CK1α (+) (-) | 0.520 (0.252-1.071) | 0.076 |
| Gender (Male) (Female) | 0.762 (0.483-1.202) | 0.242 |
| Age (≥60 years) (<60 years) | 0.796 (0.421-1.505) | 0.482 |
| Tumor size (<4 cm) (4-7 cm) (≥8 cm) | 1.315 (1.028-1.647) | 0.042 |
| Cell differentiation (High-grade) (Middle-grade) (Low-grade) | 1.302 (0.769-2.204) | 0.326 |
| Depth of invasion (T1) (T2) (T3) (T4) | 1.742 (1.204-2.119) | 0.025 |
| Lymphatic invasion (-) (+) | 0.800 (0.335-1.913) | 0.616 |
| AJCC clinical stage (I+II) (III+IV) | 1.457 (0.834-2.545) | 0.186 |

**Table 3.** Multivariate analysis of predictive factors for survival

| Prognostic factors | Relative risk (95% CI) | *P*-value |
|---|---|---|
| Tumor size (<4 cm) (4-7 cm) (≥8 cm) | 1.899 (1.256-2.468) | 0.036 |
| Depth of invasion (T1) (T2) (T3) (T4) | 1.654 (1.116-2.085) | 0.024 |

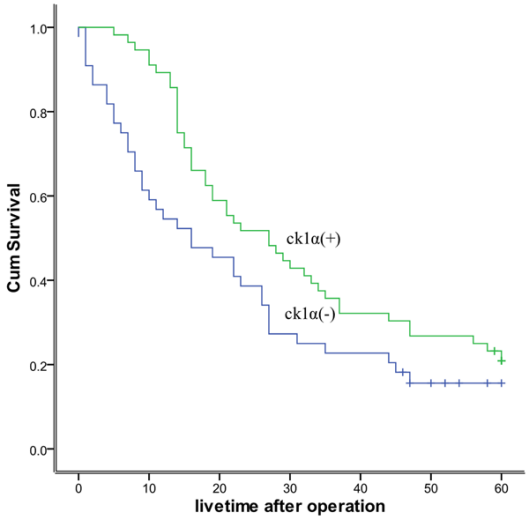


**Figure 2.** Overall survival curves of patients with ESCC according to the status of CK1α expression. ($\chi^2$=4.367, *P*=0.037).

expression and functional role of CK1α in tumor tissues or cells and there are only few reports on CK1α and tumor. Zou et al. [11] found that CK1α as a gene that is downregulated in 46.7% lung cancer tissues correlated with histological type and differentiation degree, the expression of CK1α in different differentiation degree of lung squamous cell carcinoma and adenocarcinoma of the lung tissue were different and a significant increase was detected in higher grade lung cancer tissues. Sinnberg et al. [12] detected that CK1α re-expression in metastatic melanoma cells reduces growth in vitro and metastasis formation in vivo, and induces cell cycle arrest and apoptosis, whereas suppression of CK1α in melanoma cells induces a switch in β-Catenin signaling to promote tumor progression. Vaid M proved that silymarin as an inhibitor of Wnt/β-catenin pathway significantly inhibited cell migration of Mel 1241 cells, which was associated with the elevated levels of casein kinase 1α and glycogen synthase kinase-3β, and decreased accumulation of nuclear β-catenin and inhibition of MMP-2 and MMP-9 levels [13]. Thorne CA's result suggested that pyrvinium as a potent inhibitor of Wnt signaling selectively potentiates CK1α kinase activity which inhibits the proliferation of colon cancer cells with mutation of the gene for adenomatous polyposis coli (APC) or β-catenin [14]. Li et al. [15] had showed that glioma pathogenesis-related protein 1 (GLIPR1) promoted c-Myc protein ubiquitination and degradation by glycogen synthase kinase-3α- and/or CK1α-mediated c-Myc phosphorylation in prostate cancer. Above research evidences indicated that CK1α acted as a tumor suppressor and CK1α was involved in tumorigenesis by regulating Wnt/β-Catenin signaling pathway. However, different to above, CK1α also plays opposing effect in partial tumor cells. The researchers' study indicated that the inhibition of CK1α expression can significantly inhibit the proliferation of A549 cells in lung cancer cells [16]. Bowman's study show that phosphorylation of FADD by the kinase CK1α promotes the formation of lung cancer in mice [17]. Bidère N's studies have indicated that CK1α as a bifunctional regulator of NF-κB and CK1α has thus a dual 'gating' function which first promotes and then terminates receptor-induced NF-κB in ABC DLBCL cells [18]. Hu et al. [19] detected that CK1α is

expressed in all the tested multiple myeloma (MM) cell lines and patient MM cells, inhibition of CK1α kinase activity in MM cells with targeted therapy D4476 or small hairpin RNAs triggers cell G0/G1-phase arrest, prolonged G2/M phase and apoptosis. These findings suggest that CK1α functions as a conditionally essential malignancy gene.

In this study, we analyzed expression of CK1α in ESCC and in nonneoplastic esophageal tissues, and identified the deregulation of CK1α expression in ESCC than the nonneoplastic esophageal tissues. Our study also found that the expression of CK1α was significantly correlated with the differentiation degree of ESCC and showed a decreasing trend from high grade to low grade, but not with the sex, age, tumor size, depth of invasion, lymphatic invasion and AJCC clinical stage. So, we show that the expression the CK1α might play an important role in the tumorigenesis and tumor progression of ESCC.

Analysis of survival data revealed that positive expression of CK1α was significantly associated with a better prognosis of ESCC. Furthermore, by using univariate and multivariate Cox-regression analysis proved that tumor size and depth of invasion were poor prognostic factors in ESCC patients. These results suggested that CK1α protein could be a useful indicator for prognosis of ESCC.

In conclusion, our preliminary study demonstrates that CK1α could be a helpful indicator that provides clinically useful prognostic information in cases of ESCC. Current evidence indicates that the role of CK1α expression in different tumors is still controversial, a novel example of the dual functions of CK1α activity to either oppose or promote the occurrence and development of tumor. Therefore, further investigation is still required to explore the molecular mechanisms of CK1α in ESCC.

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## Disclosure of conflict of interest

None.

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