Received: 9 November 2020 | Revised: 15 December 2020 | Accepted: 30 December 2020

DOI: 10.1002/jcla.23700

RESEARCH ARTICLE

WILEY

TNK2 promoted esophageal cancer progression via activating egfr-akt signaling

Anqing Zhang | **Rongxin Zhang** | **Zhiming Yang** | **Rui Tian**

Department of Thoracic Surgery, The First Affiliated Hospital of USTC, Division of life Sciences and Medicine, University of Science and Technology of China, Hefei, Anhui, P.R. China

Correspondence
Anqing Zhang, Department of Thoracic Surgery, The First Affiliated Hospital of USTC, Anhui Provincial Cancer Hospital, No. 107 Huanhu East Road, Hefei, P.R. China.
Email: z_720Aqin@21cn.com

Abstract

Background: This study investigated the clinical implication of TNK2 expression in esophageal cancer patients' cancer tissue samples.

Methods: The expression of TNK2 in esophageal cancer tissues and para-carcinoma tissue was assessed with immunohistochemistry and Western blot analysis; besides, the proteins of CDC42, EGFR, and Akt were also analyzed. Then, Kaplan-Meier survival curves of TNK2 protein expression level were assayed with 184 esophageal cancer patients from TCGA database. Moreover, with multiple linear regression analysis, we detected the correlations of TNK2 expression associated with tumor differentiation degree and metastasis status.

Results: It revealed that TNK2 was highly expressed in the cytoplasm of esophageal cancer tissues compared with para-carcinoma tissue; besides, the proteins of CDC42, EGFR, and Akt were also up-regulated in different levels of esophageal cancer tissues. However, there was no significant difference of the overall survival time of TNK2 protein expression in 184 esophageal cancer patients from TCGA database ($p = 0.37$). But, in the included study samples of our study, there was positive coefficience between TNK2 protein expression and differentiation degree in esophageal cancer with multiple linear regression analysis [$R = 0.928$, 95% confidence interval (0.085-0.12)].

Conclusion: Our results indicated that TNK2 was a potential diagnostic marker and promoted esophageal cancer progression through activating EGFR-AKT signaling.

KEYWORDS
CDC42, EGFR-AKT signaling, esophageal cancer, TNK2

1 | INTRODUCTION

Esophageal cancer (EC) was a kind of disease with vague symptoms, such as difficult to swallow some solid foods, pain with swallowing, or unexplained weight loss.[1] It ranked 7th in incidence and 6th in mortality worldwide,[2] while in China, the incidence rate and mortality of EC were 17.9/100,000 and 13.7/100,000, respectively,[3] and its five-year survival rate was 19%,[4] all of which caused great burden to patients, families, and society. Therefore, it is of great importance to early detection and find new biomarkers to diagnose EC for timely prevention and treatment. In clinical, it was divided into two main histological subtypes: esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC),[5] while it is estimated that the people in central and southeastern Asia mostly occurred

with ESCC subtypes, which was also common in China.[5,6] Besides, the highly epidemiological characteristics were happened in Linxian of Henan Province and Cixian of Hebei Province.[7]

Nowadays, with the development of medical medicine and high-tech preventive screening methods, such as microarray, next-generation sequencing (NGS) and even single strand Adaptor Library Preparation-sequencing (SALP-seq) in combination with machine learning, the genetic alternations were more convenient to find in cancer samples, which was also accelerated the research of small-molecule anti-cancer targeted drugs.[8–10] Hence, in the study of Jing Chen et al[11] found that 766 cancer-related genes were significantly differentially expressed in esophageal normal and carcinoma tissues with BeadArray™ technology, such as tumor suppressor genes, FANCD2, CTNNB1, and APC, and the oncogenes, TNK2, TNFSF10, FGF12, DVL3, MLF1, ABCC5, BCL6, and AGTR1. The oncogene TNK2 was also called activated cell division cycle 42 (Cdc42)-associated tyrosine kinase (ACK1), and it was proved that activated ACK1 could sense extracellular signals through interacting with activated receptor-tyrosine kinases, including AKT, EGFR, HER2, and MERTK,[12,13] and it was involved in cell survival, invasion, migration, and tumorigenesis[14]; therefore, in this study, we attempted to investigate the clinical implication of TNK2 expression in esophageal cancer patients' cancer tissue samples.

2 | MATERIALS AND METHODS

2.1 | Clinical study

We collected 45 esophageal cancer tissues (15 poorly differentiated, 15 moderately differentiated, 15 well differentiated) and 15 para-carcinoma tissue (2-3 cm from the resection margin) from the patients who underwent esophagectomy at The First Affiliated Hospital of USTC, Division of life Sciences and Medicine, University of Science and Technology of China during 2017.5-2019.12. The tumor differentiation degree was determined by the pathological report of tissue samples, while the metastasis status was judged from the CT or PET-CT or ultrasonography method. Besides, all recruited patients were provided with written informed consent before surgical resection, and the study was approved by the Ethics Committee of The First Affiliated Hospital of USTC, Division of life Sciences and Medicine, University of Science and Technology of China.

2.2 | Immunohistochemistry

The formalin-fixed esophageal cancer tissues and para-carcinoma tissue were washed, dehydrated, and transparented with ethanol of different concentrations and embedded with paraformaldehyde; then, they were cut with 3-5 μm thickness and repaired in sodium citrate buffer solution for 30 min; afterward, the sections were stained with BSA staining liquids; then, they were incubated with the primary antibody TNK2 (#:ab185726, Abcam) for 4 h. Later, the samples were visualized with fluorescence microscope system (Leica). Besides, the detailed procedures were accorded with the manufacturer's instructions. The immunostaining levels and average percentage of positive cells were visualized in high magnification fields.

2.3 | Western blot analysis

The liquid nitrogen stored esophageal cancer tissues and para-carcinoma tissue were washed and lysed with RIPA Lysis Buffer containing with PMSF (catlog: 329-98-6, Sigma); then, we analyzed the protein concentration in each sample with BCA Protein Assay Kit (catlog: PA115-01, TIANGEN). Afterward, the protein were denaturated and detected with the SDS-PAGE gel; besides, the incubated primary antibody were anti-TNK2 (#:ab185726, Abcam), anti-CDC42 (#:ab187643, Abcam), anti-Akt (#:ab8805, Abcam), anti-EGFR (#:ab52894, Abcam), and anti-GAPDH (#:ab8245, Abcam). And the secondary goat anti-rabbit antibody was IgG (#:ab172730, Abcam). Afterward, the blots were conjugated with ECL and were visualized with the bandscan; then, we analyzed the proteins' gray intensity with Image J Software.

2.4 | Clinical implication of TNK2 expression in esophageal cancer

We assayed the survival curves of TNK2 protein expression with 184 esophageal cancer patients from TCGA database. Besides, with multiple linear regression analysis, we detected the correlations of TNK2 expression associated with tumor differentiation degree and metastasis status in esophageal cancer.

2.5 | Statistical analysis

Statistical analysis was analyzed with the SPSS 19.0 statistical software package (SPSS Inc., Chicago, USA). A one-way ANOVA analysis, a Kaplan-Meier plot, and a Spearman correlation coefficient analysis were used to evaluate statistical significance. Data were shown as the mean ± SEM. $p < 0.05$ was considered to be statistical significance. Besides, the graphs were depicted with GraphPad Prism 6.0 software.

3 | RESULTS

3.1 | TNK2 was a potential diagnostic marker in esophageal cancer via activating EGFR-AKT signaling

Immunohistochemistry (IHC) results revealed that TNK2 was highly expressed in the cytoplasm of esophageal cancer tissues (Figure 1A) compared with para-carcinoma tissues (Figure 1B, $p < 0.05$); besides,

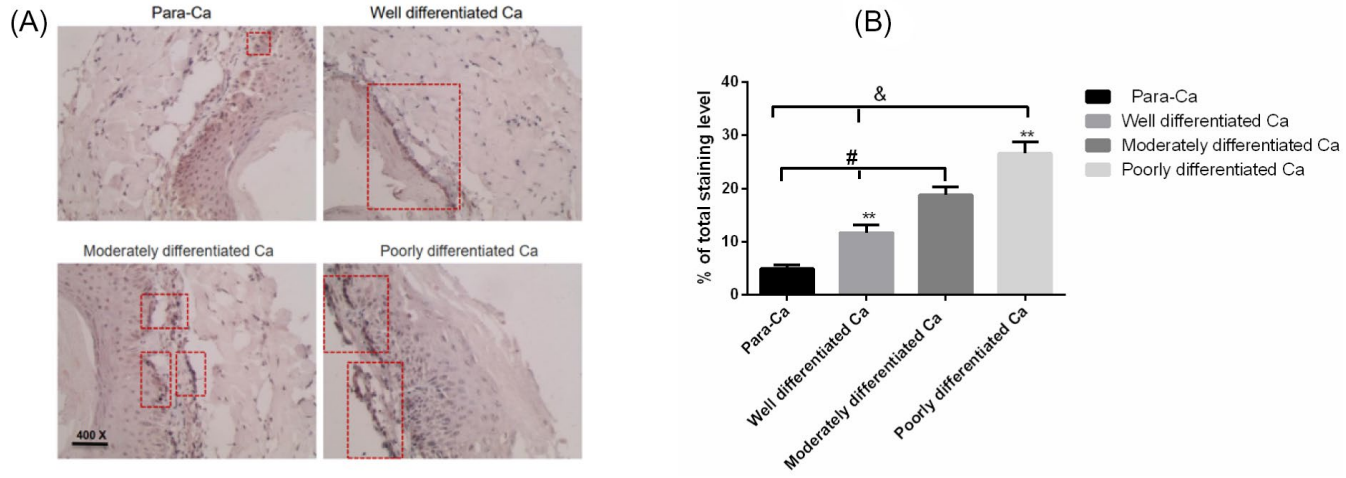

FIGURE 1 (A) TNK2 expression in esophageal cancer and adjacent tissues. Red boxes indicate representative expression. Scale bars were 200 mm. (400 × magnification.) (B) High staining level of TNK2 in esophageal cancer tissues compared with para-carcinoma tissue. &$p < 0.05$ in poorly differentiated esophageal cancer and #$p < 0.05$ in moderately differentiated esophageal cancer VS para-carcinoma tissue and well differentiated esophageal cancer, **$p < 0.05$ in poorly differentiated esophageal cancer VS well differentiated esophageal cancer (1b). Results were means ± SD for six tissue samples in each group, and $p < 0.05$ was of significance

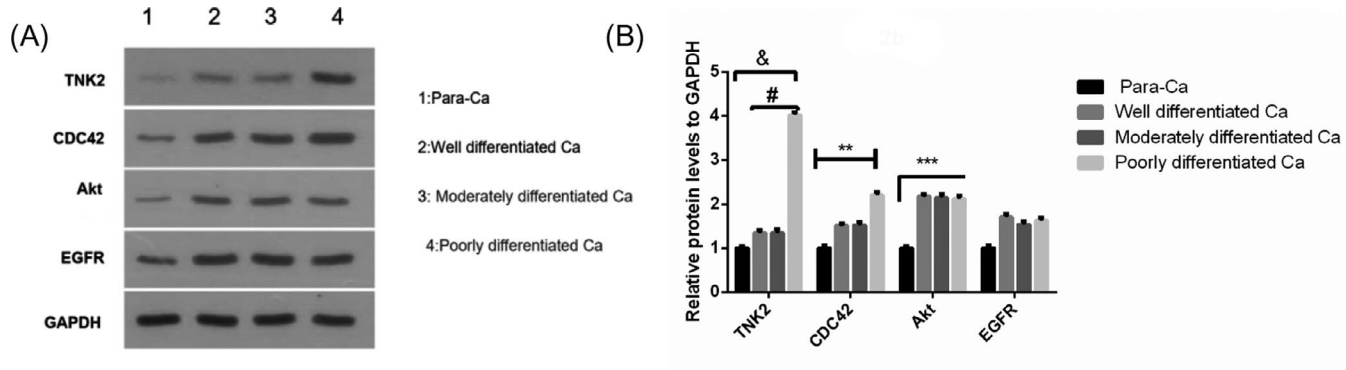

FIGURE 2 (A) Western blot analysis of proteins TNK2, CDC42, Akt, and EGFR. All proteins were increased in esophageal cancer tissues compared to adjacent tissue. (B) TNK2 in poorly differentiated esophageal cancer VS para-carcinoma tissue (&$p < 0.05$), VS well-differentiated esophageal cancer and moderately differentiated esophageal cancer (#$p < 0.05$). CDC42 in poorly differentiated esophageal cancer VS para-carcinoma tissue (**$p < 0.05$). Akt in esophageal cancer tissues VS para-carcinoma tissues (***$p < 0.05$). Results were means ± SD for six tissue samples in each group, and $p < 0.05$ was of significance

IHC and Western blot analysis all proved that TNK2 was obviously up-regulated in poorly differentiated esophageal cancer tissues (Figures 1, 2); meanwhile, the proteins of CDC42, EGFR, and Akt were also up-regulated in different levels in esophageal cancer tissues compared with para-carcinoma tissues (Figure 2).

3.2 | Overall survival time and multiple linear regression analysis of TNK2 protein expression in esophageal cancer

With data from TCGA database of 184 esophageal cancer patients (n = 46, high expression, n = 138, low/medium expression), we found that there was no significant difference of the overall survival time associated with TNK2 protein expression (Figure 3, p = 0.37). However, in the included study samples of our study, there was positive coefficience between TNK2 protein expression and differentiation degree in esophageal cancer with multiple linear regression analysis [R =0.928, 95% confidence interval (0.085-0.12)] (Figure S1).

4 | DISCUSSION

As cancer treatment had come into a new era of targeted therapy,[15] hence, in this study, we studied the molecular target of esophageal cancer with clinical tumor samples. We found that TNK2 was highly expressed in the cytoplasm of esophageal cancer tissues, especially, in the poorly differentiated tissues; besides, in the esophageal cancer tissues, the proteins of CDC42, EGFR, and Akt were also up-regulated in different levels. As TNK2 was an intracellular tyrosine kinase, which was found to be implicated in regulating multiple aspects of tumor progression.[16,17] Likely, in the study of George J N Tetley

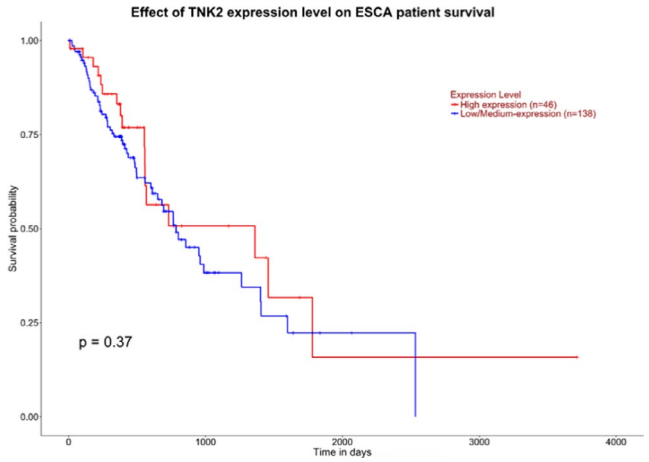

FIGURE 3 Overall survival time of TNK2 protein expression in 184 esophageal cancer samples (n = 46, high expression; n = 138, low/medium expression. Log-rank, p = 0.37 of no significance in TNK2 high expression VS TNK2 low/medium expression

et al[18] revealed that Cdc42 bound to TNK2 CRIB region via hydrophobic interactions, while CDC42 was identified to play pivotal roles in controlling the actin cytoskeleton and it was engaged in oncogenic signaling networks.[19] Besides, TNK2 (ACK1) was proved to regulate ligand-induced EGFR degradation and integrin-mediated cell adhesion and migration through the TNK2 functional domain, the EBD {EGFR [EGF (epidermal growth factor) receptor]-binding domain}.[20–22] Even more, it is showed that the activated TNK2 (ACK1) could activate AKT Tyr176 phosphorylation to promote prostate cancer progression.[23] Hence, the activated TNK2 was potential to bound and trigger CDC42 to promote esophageal cancer progression through activating EGFR-AKT signaling. Moreover, it may be the limitation of limited study samples that we found no significant difference of the overall survival time about TNK2 protein expression (p = 0.37), while there was positive coefficience between TNK2 protein expression and differentiation degree in esophageal cancer [R =0.928, 95% confidence interval (0.085-0.12)]. This finding was similar to the study of Binhui Xie et al, in hepatocellular carcinoma study.[24] Therefore, TNK2 was a potential diagnostic marker in esophageal cancer. However, due to the defects of limited study samples, there was necessity to conduct extensive and large cohort studies of esophageal cancer patients to further ensure the potential diagnostic marker, TNK2. Taken together, the oncogene TNK2 potentially promoted esophageal cancer progression through activating EGFR-AKT signaling.

5 | CONCLUSION

TNK2 was a potential diagnostic marker, and it possibly promoted esophageal cancer progression through activating EGFR-AKT signaling.

ACKNOWLEDGMENTS

We would like to acknowledge the contributions of our cooperation in this paper.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The research protocol was reviewed and approved by the Ethical Committee and Institutional Review Board of The First Affiliated Hospital of USTC, Division of life Sciences and Medicine, University of Science and Technology of China.

ORCID

Anqing Zhang https://orcid.org/0000-0003-3043-8014

REFERENCES

1. Ioana B, Leah B. Esophageal cancer surgery. *JAMA*. 2020;324(15):1580.
2. Hong Y, Ding Z-Y. PD-1 inhibitors in the advanced esophageal cancer. *Front Pharmacol*. 2019;10:1418.
3. Chen R, Zheng RS, Zhang SW, et al. Analysis of incidence and mortality of esophageal cancer in China, 2015. *Zhonghua Yu Fang Yi Xue Za Zhi*. 2019;53(11):1094-1097.
4. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA: A Cancer J Clin*. 2019;69(1):7-34.
5. Melina A, Isabelle S, Jacques F, David F. Global incidence of oesophageal cancer by histological subtype in 2012. *Gut*. 2015;64(3):381-387.
6. Zhou X, Li Y, Wang W,, et al. Regulation of Hippo/YAP signaling and esophageal squamous carcinoma progression by an E3 ubiquitin ligase PARK2. *Theranostics*. 2020;10(21):9443-9457.
7. Lin Y, Totsuka Y, Shan B, et al. Esophageal cancer in high-risk areas of China: research progress and challenges. *Ann Epidemiol*. 2017;27(3):215-221.
8. Hu N, Wang C, Hu Y, et al. Genome-wide association study in esophageal cancer using GeneChip mapping 10K array. *Cancer Res*. 2005;65(7):2542-2546.
9. Liu S, Wu J, Xia Q, et al. Finding new cancer epigenetic and genetic biomarkers from cell-free DNA by combining SALP-seq and machine learning. *Comput Struct Biotechnol J*. 2020;18:1891-1903.
10. Miller Kimberly D, Siegel Rebecca L, Chieh LC, et al. Cancer treatment and survivorship statistics, 2016. *CA Cancer J Clin*. 2016;66(4):271-289.
11. Jing C, Liping G, Peiffer Daniel A, et al. Genomic profiling of 766 cancer-related genes in archived esophageal normal and carcinoma tissues. *Int J Cancer*. 2008;122(10):2249-2254.
12. Kazuya S, Shinichiro K, Kiyoko N, et al. TNK2 gene amplification is a novel predictor of a poor prognosis in patients with gastric cancer. *J Surg Oncol*. 2014;109(3):189-197.
13. Jillian H, Jeanette R, Tommy A. TNK2 preserves epidermal growth factor receptor expression on the cell surface and enhances migration and invasion of human breast cancer cells. *Breast Cancer Res*. 2008;10(2):R36.
14. Kiran M, Mahajan NP. ACK1 tyrosine kinase: targeted inhibition to block cancer cell proliferation. *Cancer Lett*. 2013;338(2):185-192.
15. Shayan K, Craig LA, Livingstone Alan S, Wael E-R. Targeted molecular therapies in the treatment of esophageal adenocarcinoma. *Are We There Yet? Cancers (Basel)*. 2020;12(11):3077.

16. Kiran M, Domenico C, Ann CY, et al. Ack1 tyrosine kinase activation correlates with pancreatic cancer progression. *Am J Pathol*. 2012;180(4):1386-1393.
17. Xia L, Xuan W, Lifang LI, Baolin H. Research progress of the functional role of ACK1 in breast cancer. *Biomed Res Int*. 2019;2019:1018034.
18. Tetley George JN, Mott Helen R, Neil CR, Darerca O. A dock and coalesce mechanism driven by hydrophobic interactions governs Cdc42 binding with its effector protein ACK. *J Biol Chem*. 2017;292(27):11361-11373.
19. Stengel Kristy R, Yi Z. Essential role of Cdc42 in Ras-induced transformation revealed by gene targeting. *PLoS One*. 2012;7(6):e37317.
20. Feng S, Lin Qiong GU, Yan CC, Wannian Y. Activated Cdc42-associated kinase 1 is a component of EGF receptor signaling complex and regulates EGF receptor degradation. *Mol Biol Cell*. 2007;18(3):732-742.
21. Qiong L, Jian W, Chandra C, Wannian Y. The activation mechanism of ACK1 (activated Cdc42-associated tyrosine kinase 1). *Biochem J*. 2012;445(2):255-264.
22. Qiong L, Lo Charles G, Cerione Richard A, Wannian Y. The Cdc42 target ACK2 interacts with sorting nexin 9 (SH3PX1) to regulate epidermal growth factor receptor degradation. *J Biol Chem*. 2002;277(12):10134-10138.
23. Kiran M, Domenico C, Sridevi C, et al. Ack1 mediated AKT/PKB tyrosine 176 phosphorylation regulates its activation. *PLoS One*. 2010;5(3):e9646.
24. Binhui X, Qinshan Z, Xiaonong W, et al. ACK1 promotes hepatocellular carcinoma progression via downregulating WWOX and activating AKT signaling. *Int J Oncol*. 2015;46(5):2057-2066.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Zhang A, Zhang R, Yang Z, Tian R. TNK2 promoted esophageal cancer progression via activating egfr-akt signaling. *J Clin Lab Anal*. 2021;35:e23700. https://doi.org/10.1002/jcla.23700