Exploration of key genes combining with immune infiltration level and tumor mutational burden in hepatocellular carcinoma

Haozhen Ren  
Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School

Lu Zhang (✉ zhangludoc@126.com)  
Nanjing Drum Tower Hospital Clinical College of Xuzhou Medical University

Xiaolei Shi  
Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School

Chen-Zhuo Xu  
The First Hospital of Jiaxing, Affiliated Hospital of Jiaxing University

Research Article

Keywords: Hepatocellular carcinoma, keratin 17, immune infiltration biomarker

Posted Date: October 17th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2096302/v1

License: ☑️ This work is licensed under a Creative Commons Attribution 4.0 International License.  
Read Full License
Abstract

**Background:** Hepatocellular carcinoma (HCC) is the second most common cancer-associated cause of death globally. It is thus vital that the novel diagnostic and prognostic biomarkers associated with early-stage HCC be identified. Keratin 17 (KRT17) has previously been reported to be associated with certain cancer types. However, its relationship with HCC remains to be defined.

**Methods:** The expression of KRT17 in the TCGA LIHC database and in 44 pairs of samples collected from patients with HCC was assessed using qRT-PCR, WB, and IHC. The prognostic relevance of KRT17 was assessed using Kaplan–Meir curves. The important cancer- and KRT17-related biological processes were defined through gene set enrichment analysis (GSEA). The functional link between KRT17 expression and tumor cell proliferation/survival was assessed using flow cytometry, colony formation assay, CCK-8 assay, and subcutaneous tumor model approaches. Protein–protein interaction (PPI) networks and analyses of immune cell infiltration were also employed to define the signaling pathways associated with KRT17 expression in HCC.

**Results:** HCC tissue samples exhibited increased KRT17 mRNA and protein expression that was predictive of poorer patient survival \((P<0.001)\). GSEA and functional experiments revealed that KRT17 functioned as a regulator of HCC. PPI network analyses also revealed that KRT17 expression was linked to immune cell infiltration and activation in patients with HCC.

**Conclusions:** We found that increased KRT17 levels were associated with poorer survival, more aggressive disease, and altered immune cell infiltration in patients suffering from HCC. KRT17 may function as an oncogene and a prognostic biomarker in this cancer type.

Introduction

Hepatocellular carcinoma (HCC) is a malignancy responsible for approximately 780,000 deaths each year, ranking as the second leading cancer-associated cause of death globally (Bray et al. [1]). Hepatectomy can be used to remove tumors from patients with HCC. However, the overall prognosis remains poor with approximately 65% of patients ultimately succumbing to disease even after undergoing this form of treatment (Hanouneh et al. [2]). It is thus vital that the molecular mechanisms governing HCC progression be better understood to guide targeted treatment efforts for this deadly disease.

Keratin is an intermediate protein family member that plays vital structural and protective roles (Herrmann et al. [3], Jacob et al. [4]). Cysteine found in keratin can protect the liver against cirrhosis, fatty liver disease, and other related conditions (Jang et al. [5]). Nam-On Ku et al. further determined that keratin could prevent the necrotic and apoptotic death of liver cells (Ku et al. [6]). During apoptosis, keratin 18 (KRT18) and KRT19 undergo caspase-mediated cleavage at conserved aspartate residues. Keratins are also important in oncogenesis, with elevated expression of KRT17 associated with poor prognosis observed in the context of colorectal cancer, pancreatic cancer, and nonsmall-cell lung cancer (Wang et al.
Prior evidence suggests that KRT17 can influence tumor proliferation and metastasis (Li et al. [9], Li et al. [10], Chivu-Economescu et al. [11], Sarlos et al. [12], Yan et al. [13]), but its function in HCC remains to be defined.

In this study, KRT17 expression levels and prognostic relevance were assessed using extant transcriptomic data and samples collected from 44 patients with HCC at the Affiliated Hospital of Nantong University. Through functional analyses, KRT17 was found to be associated with poor prognosis of patients with HCC, indicating that it might represent a viable target for the treatment of this form of cancer.

**Materials**

**Patients and HCC tissues**

A total of 44 pairs of HCC tumor and paracancerous normal tissue samples were collected from patients at the Affiliated Hospital of Nantong University from 2012 to 2016. The patients included in this study were those who met the following criteria: (1) patients with HCC diagnosed through alpha-fetoprotein serology, imaging studies, and pathological findings as per the guidelines of the American Association for the Study of Liver Diseases (Trieweiler et al. [14]), (2) patients positive for chronic viral hepatitis B infection as defined by hepatitis B surface antigen positivity (+), (3) patients who had not undergone any prior liver resection of cancer treatment; (4) patients without any tumor invasion of the trunk or branches of the hepatic or portal veins as established via magnetic resonance imaging, and (5) patients free of other diffuse liver diseases such as primary biliary cirrhosis or primary sclerosing cholangitis. The Human Research Ethics Committee of Nantong University Affiliated Hospital approved this study, with all patients having provided written informed consent to participate.

**qRT-PCR**

RNA was isolated from tumor tissue and paracancerous tissue samples and reversed transcribed to yield cDNA, which was used for qRT-PCR analyses. KRT17 primers used in this study were as follows: forward: 5' -GGTGTCGATGATATCACGGA-3' and reverse: 5' -CAGGTTCGCTTCTCTGTC-3'; GAPDH forward: 5' -GGACCTGACCTGCCGTCTAG-3' and reverse: 5' -GTAGCCAGGATGCCCTTGA-3'.

**Western blotting**

Western blotting was performed as in prior studies (Xiong et al. [15]), with anti-GAPDH (Santa Cruz Biotechnology) used as a loading control. Rabbit polyclonal anti-KRT17 (1:1000, # 12509S, Cell Signaling Technology, MA, USA) was used to stain tumor and paracancerous tissue sections from patients with HCC.

**Immunohistochemical staining**

Rabbit polyclonal anti-KRT17 (1:100, # 12509S, Cell Signaling Technology) was used to stain tumor and paracancerous tissue sections from patients with HCC. Two experienced pathologists then independently
scored KRT17 staining intensity (0: no staining; 1: weak positivity; 2: moderate positivity; 3: strong positivity) and the percentage of positively stained tumor cells (0: 0%; 1: 1–25%; 2: 26–50%; 3: 51–75%, 4: 76–100%). These two scores were then multiplied to yield a final immunohistochemical (IHC) score ranging from 0 to 12.

**TCGA LIHC database analyses**

TCGA LIHC RNA-Seq expression matrix and clinical data, including T stage and grade, were downloaded from the XENA tool (https://xenabrowser.net/heatmap/). The expression of KRT17 in these samples was then determined based upon log2(x + 1) values and transformed RSEM normalized counts.

**Gene set enrichment analysis**

Gene set enrichment between samples with high and low levels of KRT17 expression was defined using gene set enrichment analysis (GSEA) v.2.10.1, including both gene ontology (GO) (Ashburner et al. [16]) and KEGG (Kanehisa [17]) enrichment analyses. A total of 1000 gene set permutations were conducted per analysis.

**Protein–protein interaction network construction**

A protein–protein interaction (PPI) network was constructed using the STRING (v10.0; http://string-db.org) database based upon key KRT17 co-regulated genes to assess potential interactions between proteins of interest (Franceschini et al. [18]). The resultant network ultimately incorporated all putative interactions with an interaction score > 0.4 and was visualized using Cytoscape (v.3.5) (Smoot et al. [19]). The ClueGO (v.2.5.3) and CluePedia (v.1.5.3) Cytoscape plugins, which enabled the visualization of nonredundant biological terms associated with large gene clusters in functional networks (Bindea et al. [20], Bindea et al. [21]), were used for the GO and KEGG pathway–related analyses of co-expressed genes.

**Tumor immune estimation resource analyses**

The tumor immune estimation resource (TIMER) tool was used to analyze tumor-infiltrating immune cells (https://cistrome.shinyapps.io/timer/) (Li et al. [22]), estimating B cell, CD4+ T cell, CD8+ T cell, macrophage, dendritic cell, and neutrophil intratumoral infiltration based on the patterns of gene expression. This tool was used to compare the patterns of immune cell infiltration as a function of KRT17 expression levels for samples in the LIHC database.

**Single-sample GSEA analyses**

The gsva R package was used to quantify immune cell infiltration via a single-sample GSEA (ssGSEA) approach (Hänzelmann et al. [23]), wherein the gene signatures expressed by immune cell populations were applied to individual cancer samples.

**Cell lines and culture**

SMMC-7721, HepG2, Sk-Hep-G1, Huh7, HCC-LM3 cell lines, and THLE-2 cell lines were ordered from GeneChem (Shanghai, China). ALL liver cell lines were cultured in DMEM (Gibco, MD, USA) with 10% fetal
bovine serum (Clark, Shanghai, China). The cell lines were authenticated using STR.

**Cell transfection**

KRT17 ectopic-expressing and empty vectors were obtained from GeneChem (Shanghai, China). Short-hairpin RNA (shRNA) sequences targeting KRT17 were procured from GenePharma (Soochow, China).

**CCK-8, colony formation, cell cycle analysis, and apoptosis assays**

These experiments were conducted as previously described (Lu et al. [24]).

**Mouse experiments**

Nude mice were subcutaneously implanted with $5 \times 10^6$ SMMC-7721/shKRT17 or control cells in the right axillary region. The mice were sacrificed, and tumors were collected and imaged 4 weeks after tumor implantation.

**Statistical analysis**

The expression profiles and clinical information of KRT17 and related genes in 374 patients with HCC from TCGA and 44 patients with HCC from Affiliated Hospital of Nantong University were analyzed and displayed. Statistical analyses were performed and visualized using R version 4.0.2 and GraphPad Prism version 8.0 software. The quantitative values of all experiments were expressed as the mean ± standard deviation. The differences among/between sample groups were analyzed using the independent-samples $t$ test or one-way analysis of variance (ANOVA). Pearson's correlation coefficient was employed to measure the linear correlation between KRT17 and immune infiltrated cells. $P < 0.05$ indicated a statistically significant difference.

**Results**

**Assessment of KRT17 expression and prognostic relevance in HCC**

First, KRT17 expression patterns in tissues from patients with HCC were compared using the TCGA LIHC database, revealing that KRT17 expression was elevated in HCC tissues ($n = 372$) relative to paracancerous normal tissues ($n = 50$, $P < 0.001$, Fig. 1a). This finding was confirmed using qRT-PCR, IHC, and Western blotting using 44 pairs of HCC and paracancerous tissue samples collected from the Affiliated Hospital of Nantong University (Fig. 1b–1d). Kaplan–Meier survival curves revealed that the overall survival of patients with high levels of KRT17 expression was significantly reduced relative to that of patients with lower KRT17 expression levels ($P = 0.048$); the TCGA LIHC dataset yielded comparable results ($P = 0.016$, Fig. 1e and 1f). One-way ANOVA of the TCGA LIHC dataset further indicated that KRT17 expression levels were significantly associated with increased tumor T stage ($P = 0.003$) and
grade ($P = 0.037$, Fig. 1g and 1h). Together, these data revealed that KRT17 might play an oncogenic role and have value as a prognostic biomarker in patients with HCC.

**Relationship between KRT17 expression levels and GSEA results**

Next, patients with HCC in the TCGA LIHC database were stratified into KRT17-high and KRT17-low groups to explore the mechanistic basis for the putative oncogenic role of this keratin gene. GO and KEGG pathway functional enrichment analyses were conducted using a GSEA approach (Fig. 2a–2d), revealing that KRT17 was associated with important functional pathways, including the positive regulation of apoptotic signaling pathway, Notch signaling pathway, methyl CpG binding, intestinal immune network for IGA production, and cell cycle signaling pathway (Fig. 2e–2j).

**KRT17 knockdown modulated HCC cell proliferation and apoptosis, and promoted G1-phase arrest**

We next explored the functional role of KRT17 in HCC cell lines. Of seven tested HCC cell lines, the expression of KRT17 was confirmed to be the highest in SMMC-7721 cells and the lowest in HCC-LM3 cells (Supplementary Fig. 1a). The apoptotic death and cell cycle progression of these cells was then assessed using flow cytometry, revealing higher numbers of cells in the G1 phase in the SMMC-7721-shKRT17 group relative to the control group, whereas KRT17 expression was associated with a reduced number of cells in this growth phase (Fig. 3a). The rates of KRT17-knockdown SMMC-7721 cell apoptosis were also lower than the rates in control cells, whereas KRT17 upregulation was associated with increased apoptotic death (Fig. 3b). KRT17 expression was higher, on average, for patients with a higher T stage in the TCGA LIHC database. The colony formation and CCK-8 assays were used to examine the relationship between KRT17 and tumor cell proliferation, revealing that KRT17 knockdown markedly impaired SMC-7721 cell proliferation, whereas KRT17 overexpression enhanced the proliferation of HCC-7721 (Fig. 3e–3g). Consistent with these findings, subcutaneous tumors generated using SMMC-7721/shKRT17 cells grew more slowly in nude mice than did control cells (Fig. 3h).

**Assessment of the biological function of KRT17 co-regulated genes in HCC**

For a better understanding of the potential regulatory roles of KRT17 in HCC, the genes with which KRT17 was co-expressed were identified using the STRING database (Fig. 4a). The functional enrichment analyses of 11 co-regulated genes were then conducted with ClueGO, revealing that these KRT17 co-regulated genes were associated with keratin type I bound to keratin type II regulation of water loss via skin, and keratin filament and intermediate filament cytoskeleton organization (Fig. 4b).
**Relationship between KRT17 expression and HCC tumor immune cell infiltration**

First, the immune cell levels in HCC and paracancerous tissue samples were compared to assess the relationship between KRT17 and the infiltration of immune cells into HCC tumors (Fig. 5a). TIMER and ssGSEA analyses revealed that higher KRT17 expression was linked to B cell, CD8⁺ T cell, CD4⁺ T cell, macrophage, neutrophil, dendritic cell, central memory CD4 T cell, plasmacytoid dendritic cell, natural killer T cell, myeloid-derived suppressor cell, CD56 natural killer cell, regulatory T cell, and T follicular helper cell infiltration ($P < 0.05$), whereas reduced KRT17 expression was associated with memory B cell and eosinophil infiltration (Fig. 5b–5i, $P < 0.05$). These relationships suggested that KRT17 might serve as a key regulator of T cell functionality in HCC, underscoring its importance as a regulator of immune infiltration in this oncogenic context.

**Discussion**

Keratin proteins, including KRT8 and KRT18, serve as important regulators of liver injury, fibrosis, and cancer (Strnad et al. [25], Thulin et al. [26], Govaere et al. [27], Toivola et al. [28]). The prognostic and functional relevance of KRT17 in HCC, however, has not been defined to date. In this study, both the TCGA LIHC database and a separate cohort of 44 patients with HCC were analyzed. KRT17 expression levels were found to be significantly higher in HCC tumors compared with normal paracancerous tissues. Elevated KRT17 levels were also identified as a risk factor associated with the decreased survival of patients with HCC.

GSEA and functional enrichment analyses revealed that KRT17 was closely linked to the regulation of many key biological processes, including apoptotic signaling, methyl CpG binding, development of the embryonic digestive tract, Notch signaling, and cell cycle pathways. These pathways might be linked to the mechanisms whereby KRT17 governed HCC development and progression.

Dysregulation of the cell cycle and suppression of apoptotic cell death are key hallmarks of oncogenesis, making them prime targets for treating all cancer types (Evan et al. [29]). A majority of extant antitumor drugs are anti-mitotic agents, interfering with DNA synthesis and cellular division in a nontargeted manner (Evan et al. [30], Schmitt et al. [31], Evan et al. [32]). The present study found that the knockdown of KRT17 in HCC cells was associated with G1-phase arrest and a decrease in the frequency of cells in the S phase, while the overexpression of KRT17 led to increased rates of apoptotic death of HCC cells. Despite no effective means to inhibit the growth of liver cancer cells, the development of a large number of drugs is aimed at molecular targets for cell growth (Pelengaris et al. [33]). Some recent studies proved that the growth-inhibiting pathologies (such as E2F and Myc) were effective differentiation inhibitors (Felsher et al. [34]). The present study found that the knockdown of KRT17 was sufficient to suppress the proliferation of HCC cells *in vitro* and *in vivo*. 
Notch genes encode cell surface receptors that control differentiation and development in myriad species, including humans (Terauchi et al. [35], Aster et al. [36]). Notch signaling can influence apoptosis, proliferation, pluripotent progenitor cell differentiation, and formation of cell boundaries. Notch gene mutations can result in profound signaling changes and consequent phenotypic effects (Terauchi et al. [35]). Keratin family genes are also linked to many key Notch-induced signaling activities (Arunugam et al. [37], Chen et al. [38]). The GSEA results revealed KRT17 enrichment in the Notch pathway, suggesting that KRT17 might drive dysfunctional HCC cell proliferation, cell cycle progression, and apoptosis at least in part via the Notch signaling pathway.

Tumor immunotherapeutic strategies have revolutionized the standards of care for certain cancer types, and the role of the immune system in the context of cancer progression is increasingly well understood (Camidge et al. [39], Carbone et al. [40]). The composition of the tumor microenvironment has also been studied as a prognostic biomarker (Altorki et al. [41]). Prior evidence has shown that immune cell infiltration is linked to liver cancer patient survival (Kurebayashi et al. [42]), which is consistent with the findings of the present study. This study also found that KRT17 expression levels were associated with the degree of immune cell infiltration in patients with HCC such that elevated KRT17 expression positively correlated with macrophage and activated CD4 + T cell infiltration. Together, these data offered detailed insights into the relationship between KRT17 expression and immune markers in patients with LIHC. However, further studies should be performed to understand whether KRT17 is a key factor in CD4 + T lymphocyte therapy outcomes.

In summary, the results of this study provided multiple lines of evidence confirming that KRT17 was a vital regulator of HCC and a potential prognostic biomarker that could be used to evaluate patients affected by this disease. Specifically, the study showed that KRT17 upregulation in HCC tumors was likely to impact apoptosis, proliferation, and cell cycle progression dramatically. In addition, KRT17 might function as a novel immunoregulatory gene oncogenic, underscoring the value of future genomics studies on samples from patients with HCC.

Declarations

Competing interests

No competing financial interests exist.

Funding Information

This work was funded by the National Natural Science Foundation of China (81872359).

Authors’ contributions

Hao - Zhen Ren and Xiao - Lei Shi contributed to the conception of the study; Lu Zhang performed the experiment; Lu Zhang and Chen - Zhou Xu contributed significantly to analysis and manuscript
preparation; Lu Zhang performed the data analyses and wrote the manuscript; Hao - Zhen Ren and Xiao - Lei Shi helped perform the analysis with constructive discussions.

Acknowledgments

Thanks for the technical support of the hepatobiliary surgery staff of the Department of Hepatobiliary Surgery, The Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing, China.

References


Figures
Figure 1

KRT17 upregulation is evident in HCC tumors and associated with poor prognosis. (a and b) KRT17 expression levels were assessed in HCC tumors and matched paracancerous tissue samples from 44 patients with HCC and in samples from the TCGA LIHC database. (c) KRT17 levels in tumor and paracancerous tissues from patients with HCC were assessed via Western blotting. (d) Representative images of KRT17 staining in HCC and paracancerous tissues are shown. (e and f) Overall survival with
low and high KRT17 expression levels was compared using the Kaplan–Meier approach for patients with HCC from the TCGA LIHC database and for the 44 patients with HCC analyzed in the present study. (g and h) Violin plots were used to assess T and N stages for patients from the TCGA LIHC database. P <0.05 indicated a statistically significant difference.
Gene set enrichment analysis–mediated identification of genes co-regulated with KRT17. (a–j) GO and KEGG enrichment analyses were used to identify key GSEA-related signaling pathways.

Figure 3

KRT17 downregulation was linked with HCC cell G1-phase arrest, decreased proliferation, and apoptotic cell death. (a and b) HCC cell cycle progression and apoptosis were analyzed using flow cytometry. (c–e)
CCK-8, colony formation, and in vivo analyses were used to assess the relationship between KRT17 and tumor proliferation. *P < 0.05, **P < 0.05, ***P < 0.001.

**Figure 4**

Analysis of KRT17-associated proteins. (a) A total of 11 KRT17-related proteins were detected using STRING. (b) GO and KEGG analyses were used to identify the key signaling pathways associated with these co-regulated proteins and visualized using Cytoscape.
Figure 5

KRT17 expression correlated with the levels of immune infiltration in patients with HCC. (a) (b–i) KRT17 expression levels were compared between patients with HCC having high and low immune scores as defined through ssGSEA and TIMER analyses. *$P < 0.05$, **$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.
• supfig1.1.tif