ZC3H13 Can Serve as Potential Prognostic Biomarker and Therapeutic Target for Hepatocellular Carcinoma

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Research

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Abstract

Background: Recently, an increasing number of studies have revealed that N6-methyladenosine (m6A) functions as a significant post-transcriptional modification which plays a critical role in the occurrence and progression of enriched tumors by regulating coding and non-coding RNA biogenesis. However, the roles of m6A RNA modification in hepatocellular carcinoma (HCC) remain largely unknown.

Results: In this study, the expression levels, genetic variations, prognostic values and gene–gene interaction network of m6A-related genes in hepatocellular carcinoma were analyzed using the Oncomine, Gene Expression Profiling Interactive Analysis (GEPIA), cBioPortal, Kaplan–Meier plotter and GeneMANIA database. The results show that zinc finger CCCH-type containing 13 (ZC3H13) was significantly lowly expressed in Hepatocellular Carcinoma. Survival outcome analysis suggested that significant correlations occurred between ZC3H13 downregulation and poor overall survival (OS) and poor recurrence free survival (RFS) in patients with liver cancer. Whereupon, ZC3H13 was selected for analyzing the biological functions and pathways of its co-expressed genes via Metascape, and its correlation with immune system responses via the TISIDB database, respectively. Further analyses indicated that ZC3H13 may be involved in transcriptional misregulation in cancer. Additionally, ZC3H13 expression was significantly correlated with lymphocytes and immunomodulators.

Conclusions: Therefore, ZC3H13 is a high candidate as a novel biomarker and therapeutic target for hepatocellular carcinoma.

1. Background

Primary liver cancer is the sixth most commonly diagnosed cancer and the third leading cause of cancer death worldwide in 2020, with approximately 906,000 new cases and 830,000 deaths. Primary liver cancer includes hepatocellular carcinoma (HCC)(comprising 75%-85% of cases) and intrahepatic cholangiocarcinoma (10%-15%), as well as other rare types [1]. HCC usually develops from chronic hepatitis and cirrhosis. The main risk factors for HCC are hepatitis B virus (HBV) infection in the eastern region and hepatitis C virus (HCV) infection and alcohol consumption in the western region. Early diagnosis and multidisciplinary treatment are critical to improve HCC prognosis [2]. Early diagnosis largely depends on monitoring high-risk patients as well as advances in diagnostic techniques. The rapid progression of imaging techniques (such as diffusion-weighted magnetic resonance imaging) has provided us powerful tools for monitoring and diagnosing chronic hepatitis and HCC [3]. Multidisciplinary treatments offer the best treatment efficacy based on currently available therapies [2–4]. However, treatment outcomes for HCC are far from satisfactory, mainly due to two major barriers: low rates of receiving potentially curable treatments and high rates of recurrence or metastasis after treatment [5]. In order to further improve the prognosis of HCC, we need to explore the detailed molecular mechanisms of oncogenesis and cancer progression.

N6-methyladenosine (m6A), the methylation modification at the sixth N atom of adenine, is the most common post-transcriptional modification on mRNA, mediating > 60% RNA methylation [6]. Abnormal m6A methylation levels are closely related to stem cell differentiation, immune response, embryonic development, and microRNA (miRNA) editing; they also play an essential role in the progression of various cancers [7]. The epigenetic modification of m6A methylation affects tumor development by modulating the mRNA expression levels of related oncogenes or suppressor genes. The m6A methylation levels in tumors mainly depend on the expression of m6A methylation regulators. The m6A methylation is highly correlated with the expression of intracellular methyltransferases (“writers”) and demethylases (“erasers”), whereas binding proteins (“readers”) bind to m6A methylation sites in performing a series of biological functions [8, 9]. The “Writers,” which included methyltransferase like 16 (METTL16), Wilms tumor 1 associated protein (WTAP), zinc finger CCCH-type containing 13 (ZC3H13), KIAA1429 and RNA-binding motif protein 15 (RBM15), promote m6A RNA methylation [10, 11]. The “Erasers,” comprising fat mass- and obesity-associated protein (FTO), alkyl homolog 5 (ALKBH5), remove m6A methylation groups from RNA [12]. The “Readers,” which include YTH domain-containing 1 (YTHDC1), YTH N6-methyladenosine RNA-binding protein 1 (YTHDF1), YTHDF2, YTHDF3, and heterogeneous nuclear ribonucleoprotein C (HNRNPC), play a specific role by binding to the m6A methylation site [13]. The aberrant expression of m6A regulators plays a vital regulatory role in tumor progression, prognosis, and radio-resistance [12]. However, despite recent advances in the role of m6A, the role of m6A RNA modification in hepatocellular carcinoma (HCC) is still worthy of further investigation.

Here, we searched for mRNA expression levels of m6A-related genes between hepatocellular carcinoma and normal liver tissues using the Oncomine and Gene Expression Profiling Interactive Analysis (GEPIA) databases, analyzed the prognostic value of each m6A family member in liver cancer using the Kaplan-Meier plotter database, and constructed a gene–gene interaction network for Annexins in order to explore their mechanisms of function. We further analyzed ZC3H13, which was significantly correlated with the prognosis of patients with hepatocellular carcinoma. ZC3H13 was subjected to gene set enrichment analysis using The Cancer Genome Atlas (TCGA) database to explore its biological functions and relevant pathways. Correlation between ZC3H13 and the immune system were analyzed using the TISIDB database. Our study was aimed at exploring the clinical significance of ZC3H13 and providing a theoretical basis for the early diagnosis, prognostic judgments, and targeted therapy of hepatocellular carcinoma.

2. Results

2.1 Bioinformatics analyses revealed differential mRNA expression levels of m6A-related genes in hepatocellular carcinoma

The mRNA expression levels of m6A-related genes in hepatocellular carcinoma and normal liver tissues were analyzed using the Oncomine database (Figure.1). Roessler et al compared the mRNA expression

levels between 22 cases of hepatocellular carcinoma and 21 cases of normal liver tissues [Table I], and found that ZC3H13 expression was significantly downregulated in malignant tumor tissues ($P = 1.54E-11$, fold change = -3.105). However, the study carried out by Wurmbach et al showed that KIAA1429 was upregulated based on 35 cases of hepatocellular carcinoma ($P = 1.17E-09$, fold change = 2.117). The results are generally consistent with those obtained using the GEPIA database. In detail, we analyzed the differential mRNA expression levels of m6A-related genes between hepatocellular carcinoma and normal liver tissues using the GEPIA database (Figure 2), which indicated that KIAA1429 expression levels were significantly upregulated in hepatocellular carcinoma (Figure 2D). In contrast, ZC3H13 expression was significantly downregulated (Figure 2L). Both databases showed that KIAA1429 mRNA expression levels were upregulated in liver cancer compared with normal tissues, while ZC3H13 mRNA expression levels were downregulated.

Table 1						
Datasets of m6A-related genes in hepatocellular carcinoma (Oncomine)						
gene	tumor(cases)	normal(cases)	fold change	t-test	p-value	dataset
KIAA1429	Hepatocellular Carcinoma (35)	liver(10)	2.117	7.522	1.17E-09	Wurmbach et al.
RBM15	Hepatocellular Carcinoma (225)	liver(220)	1.324	8.681	4.53E-17	Roessler 2 et al.
WTAP	Hepatocellular Carcinoma (225)	liver(220)	1.42	8.763	2.81E-17	Roessler 2 et al.
ZC3H13	Hepatocellular Carcinoma(22)	liver(21)	-3.105	-9.418	1.54E-11	Roessler et al.
HNRNPC	Hepatocellular Carcinoma(22)	liver(21)	1.551	7.65	5.44E-09	Roessler et al.
YTHDF1	Hepatocellular Carcinoma(26)	liver(26)	1.044	4.5	3.63E-05	Guichard 2 et al.
YTHDC1	Hepatocellular Carcinoma(104)	liver(76)	1.888	10.315	5.48E-20	Chen et al.
YTHDF2	Hepatocellular Carcinoma(35)	liver(10)	1.44	3.639	2.00E-03	Wurmbach et al.
YTHDF3	Hepatocellular Carcinoma(99)	liver(86)	1.106	7.092	9.24E-11	Guichard et al.
ALKBH5	Hepatocellular Carcinoma(104)	liver(76)	1.171	2.41	9.00E-03	Chen et al.
FTO	Hepatocellular Carcinoma(26)	liver(26)	-1.056	-3.225	2.00E-03	Guichard 2 et al.

Further analysis using the TISIDB database to study the relationship between m6A-related genes expression levels and the tumor pathological grade of hepatocellular carcinoma (Figure 3) showed that the expression levels of ZC3H13 (Figure 3L, Spearman: $\rho = -0.186$, $P = 0.000347$), HNRNPC (Figure 3C, Spearman: $\rho = 0.207$, $P = 6.58e-05$), KIAA1429 (Figure 3D, Spearman: $\rho = 0.181$, $P = 0.000507$), YTHDF1 (Figure 3I, Spearman: $\rho = 0.175$, $P = 0.000795$), FTO (Figure 3B, Spearman: $\rho = -0.15$, $P = 0.00397$) and ALKBH5 (Figure 3A, Spearman: $\rho = -0.129$, $P = 0.0135$) increased significantly with advanced grade. However, the expression levels of METTL16, RBM15, WTAP, YTHDC1, YTHDF2, and YTHDF3 did not change significantly with grade.

Combining the results of three databases, ZC3H13 mRNA expression levels were downregulated in liver cancer and the expression levels decreased significantly with the tumor pathological grade of hepatocellular carcinoma. In addition, KIAA1429 mRNA expression levels was upregulated in liver cancer and the expression levels increased significantly with tumor pathological grade of hepatocellular carcinoma.

2.2 Amplification, deletion, mutation, and fusion of m6A-related genes in hepatocellular carcinoma

Genetic variations of m6A in 372 cases retrieved from this studies (TCGA, Pancancer Atlas) was analyzed using the cBioPortal database. Amplification and deep deletion mutations were the most common genetic alteration (Figure 4). In liver hepatocellular carcinoma (LIHC), 9% of patients presented KIAA1429 alterations, with the most common being copy number alteration (CNA), followed by missense mutation and deep deletion. The distribution of CNA and deletion in ALKBH5 alterations was similar with that of KIAA1429. However, only 2.8% of LIHC patients showed genetic ZC3H13 alterations, deep deletion and missense mutation were the two most common alteration types. We also showed mutations in each m6A-related genes in detail (Figure S1).

2.3 ZC3H13 showed the greatest prognostic value in patients with hepatocellular carcinoma

To validate the prognosis of the m6A family members in LIHC, the Kaplan-Meier Plotter platform (<http://kmplot.com>) was further used to determine the association between the survival of LIHC patients and the expression of m6A-related genes. The analysis revealed that the mRNA expression levels of ALKBH5, WTAP, YTHDF1, YTHDF2, YTHDF3 and ZC3H13 were correlated with patient prognosis. We observed that high ZC3H13 mRNA expression was associated with better RFS (Figure 5K, HR=0.67, 95% CI: 0.47-0.95, $P=0.026$) and OS (Figure 6K, HR =0.45, 95% CI: 0.3-0.68, $P=7.9E-05$). The expression level of KIAA1429 was correlated with OS (Figure 6D, HR=1.58, 95% CI: 1.07-2.34, $P=0.0266$) in HCC patients, but RFS (Figure 5D, HR=1.43, 95% CI: 0.98-2.07, $P=0.059$) was not.

ZC3H13, which showed the greatest prognostic significance for OS and PFS in patients with liver cancer, was selected to study correlations between its mRNA expression level and PFS during different degrees of differentiation (Grade 1 is highly differentiated, grade 2 is moderately differentiated, grade 3 is poorly differentiated and grade 4 is undifferentiated.), FIGO stages, alcohol consumption and hepatitis virus (Figure 7). ZC3H13 mRNA downregulation was correlated with poor PFS with moderate and high differentiation, and the correlation was even more pronounced with poor differentiation (Figures 7A, B). ZC3H13 mRNA upregulation indicated poor PFS in patients with FIGO stage III–IV (Figure 7I). A significant correlation was observed between of ZC3H13 mRNA downregulation and poor PFS in patients with alcohol consumption and hepatitis virus, when compared to patients without alcohol consumption and hepatitis virus (Figures 7D, F). Therefore, ZC3H13 can better reflect the prognosis in patients with liver cancer, and its correlation with prognosis was even more pronounced in patients with poor differentiation, advanced FIGO stages, alcohol consumption and hepatitis virus.

2.4 Correlations between m6A-related genes and construction of a gene–gene interaction network

A gene–gene interaction network for the 11 m6A-related genes were constructed, and their functions were analyzed using the GeneMANIA database. The 11 central nodes representing m6A family members were surrounded by 20 nodes representing genes that greatly correlated with the family in terms of physical interactions, co-expression, predictions, co-localization, and genetic interactions. A series of genes associated with the m6A family are shown in figure 8 according to their association. The top five genes displaying the greatest correlations with the m6A family included ALKBH7 (alkB homolog 7), YTHDC2 (YTH domain containing 2), CBLL1 (Cbl proto-oncogene like 1), ALKBH3 (alkB homolog 3, alpha-ketoglutarate dependent dioxygenase), ALKBH6 (alkB homolog 6), among which CBLL1 was correlated with WTAP and KIAA1429 in terms of physical interactions, and co-localized with HEATR6. ALKBH7 was correlated with ALKBH5 in terms of Shared protein domains. ZC3H13 was correlated with WTAP and KIAA1429 in terms of physical interactions and predicted. ZC3H13 was connected with YTHDC1 and YTHDF3 in terms of co-expression. The remaining genes interaction networks are not enumerated in detail, as detailed in the following document (Figure S2).

2.5 Functional and pathway enrichment analyses of ZC3H13

A total of 357 genes co-expressed with ZC3H13 having an average Spearman's correlation coefficient of 0.5 were obtained from the cBioPortal database based on the selection criteria (Table S1). In this study, the genes co-expressed with ZC3H13 were subjected to functional and pathway enrichment analyses using the xiantao academic website (www.xiantao.love). Bubble plots represented the top 20 enriched biological functions and pathways (based on P-value). The functional analysis results showed that the following biological processes were enriched for the genes co-expressed with ZC3H13: transcriptional

misregulation in cancer (Figure 9A). We also used Metascape to perform functional annotation and pathway enrichment analysis of ZC3H13 and their adjacent genes. The first 20 items of GO enrichment are mainly distributed in the biological process (11 items), Reactome Gene Sets (7 items), CORUM (1 items) and Canonical Pathways (1 items) (Figures 9B, C). Five of the first seven projects are in the biological process, which are cellular protein catabolic process; translational elongation; mRNA processing; protein modification by small protein removal; viral transcription, and the other two are macroautophagy; negative regulation of cell cycle. Canonical Pathways Refers to ST JNK MAPK pathway. Signaling by TGFB family members included the Reactome Gene Sets. And, the first 100 items of GO enrichment are mainly distributed in the biological process, Reactome Gene Sets, CORUM and Canonical Pathways (Figure S3). Among signaling pathways showing the greatest correlation for genes co-expressed with ZC3H13, hsa05202 (transcriptional misregulation in cancer), the result was generally consistent with those obtained using the xiantao.academic website.

2.6 Regulation of immune molecules by ZC3H13

The Spearman's correlations between ZC3H13 expression and lymphocytes and immunomodulators were analyzed using the TISIDB database (Figure 10). Figure 10A shows the correlation between ZC3H13 expression and tumor-infiltrating lymphocytes (TILs), and the lymphocytes displaying the greatest correlations included CD56dim (Spearman: $\rho = -0.417$, $P < 2.2 \times 10^{-16}$), Tgd (Spearman: $\rho = -0.286$, $P = 2.18 \times 10^{-8}$), Eosinophil (Spearman: $\rho = 0.272$, $P = 1.03 \times 10^{-7}$), and Act-CD8 (Spearman: $\rho = -0.269$, $P = 1.52 \times 10^{-7}$) (Figure 10B). Immunomodulators can be further classified into immunoinhibitors, immunostimulators, and major histocompatibility complex (MHC) molecules. Figure 10C shows correlations between ZC3H13 expression levels and immunoinhibitors. The immunoinhibitors displaying the greatest correlations included KDR (Spearman: $\rho = 0.468$, $P < 2.2 \times 10^{-16}$), PVRL2 (Spearman: $\rho = -0.261$, $P = 3.42 \times 10^{-7}$), LAG3 (Spearman: $\rho = -0.261$, $P = 3.38 \times 10^{-7}$), and LGALS9 (Spearman: $\rho = -0.255$, $P = 6.8 \times 10^{-7}$) (Figure 10D). Figure 10E shows correlations between ZC3H13 expression and immunostimulators, and the immunostimulators displaying the greatest correlations included IL6R (Spearman: $\rho = 0.424$, $P < 2.2 \times 10^{-16}$), TNFRSF18 (Spearman: $\rho = -0.407$, $P = 2.2 \times 10^{-16}$), TNFRSF4 (Spearman: $\rho = -0.369$, $P = 2.45 \times 10^{-13}$), and TNFRSF14 (Spearman: $\rho = -0.287$, $P = 1.93 \times 10^{-8}$) (Figure 10F). Figure 10G shows correlations between ZC3H13 expression and MHC molecules, and the MHC molecules displaying the greatest correlations included TAPBP (Spearman: $\rho = -0.309$, $P = 1.33 \times 10^{-9}$), HLA-DMA (Spearman: $\rho = -0.226$, $P = 1.08 \times 10^{-5}$), HLA-DOB (Spearman: $\rho = -0.214$, $P = 3.1 \times 10^{-5}$), and TAP1 (Spearman: $\rho = -0.211$, $P = 4.04 \times 10^{-5}$) (Figure 10H). Therefore, ZC3H13 may be involved regulating the above immune molecules.

2.7 Comparison of expression levels of ZC3H13 in different tissue with IHC

We used the human protein atlas database find that, taking all the tissues of the body as observation points, the RNA expression level of ZC3H13 was in the first place (Figure S4).

In addition, we compared the expression level of ZC3H13 protein in normal tissues and tumor tissues (Figure 11). It is interesting to note that IHC assays demonstrated that ZC3H13 was found mainly in the membranous nuclear or cytoplasmic. We used the human protein atlas database to detect the protein expression of ZC3H13 in LIHC and its paired normal tissue. We found that the protein levels of ZC3H13 is higher in tumor tissue than in the adjacent tissue.

3. Discussion

m6A mRNA modifications are evolving to be a new layer of posttranscriptional gene regulation. The dynamic and delicate interplay between m6A-methyltransferases and demethylases plays crucial roles in many fundamental biological processes, such as RNA splicing, protein translation, and cell fate transition. However, the roles and clinical significance of m6A modification in human diseases, especially cancer, are just beginning to be explored [14].

Sustaining mitogenic signaling and evading growth suppressors in tumor cells are feasibly the most fundamental characteristics of tumor cells, unlike in normal cells that regulate cell homeostasis, especially pertaining to releasing growth-promoting signals [15]. For instance, HCC occurs largely as a result of uninhibited cellular proliferation resulting from a series of dysregulations in normal cell cycle regulators such as cyclin-dependent kinases (CDKs). Given the unique regenerative aptitude of hepatocytes, any reprobate cell proliferation, upregulation of CDKs, or alterations in CDK-related downstream signaling pathways and CDK inhibitors could potentiate the onset of hepatocarcinogenesis [16]. In our study, the enrichment analysis showed that the biological process results of candidate gene included transcriptional misregulation in cancer and negative regulation of cell cycle. JNK MAPK pathway and signaling by TGFB family members have been found to be involved.

In this study, we also investigated the expression, prognostic value, genetic variations, and potential roles of m6A family members in hepatocellular carcinoma. ZC3H13, which displayed the greatest correlation with the prognosis of patients with liver cancer, as well as the closest association with its tumorigenesis and progression, was selected for subsequent in-depth investigations of its biological processes, as well as its correlations with immunity. In addition, its expression level was correlated with tumor grades. Patients with low ZC3H13 expression had a relatively poor prognosis. The results of enrichment pathway showed negative regulation of cell cycle, which further suggested the antitumor effect of ZC3H13 (Figure S3). We also validated the expression of ZC3H13 in liver cancer by performing the human protein atlas with liver cancer.

m6A-related genes were frequently dysregulated in cancers but with a cancer-specific pattern, which is consistent with the findings of previous studies [17]. For the m6A “writers”, several previous studies have demonstrated that dysregulation of METTL3 and METTL14 could contribute to human carcinogenesis. METTL3 was up-regulated in acute myeloid leukemia cells, enhanced translation of c-MYC, BCL2, and PTEN mRNAs, blocks cell differentiation and apoptosis, and promotes leukemia progression [18, 19]. METTL3 was also over-expressed in pancreatic cancer, bladder cancer, glioma and gastric cancers, and

promotes proliferation, invasion, and drug resistance of cancer cells [20–23]. By contrast, METTL14 was down-regulated in colorectal cancer and hepatocellular carcinoma and could inhibit cancer cells' growth and metastasis by regulating m6A-dependent primary microRNA processing [24, 25]. Previous studies had shown that ZC3H13 enhance m6A by bridging Wilms Tumor 1 associated protein (WTAP) to the mRNA binding factor Nito [26]. We used GeneMANIA database to know that ZC3H13 was connected with YTHDC1 and YTHDF3 in terms of co-expression, the results were in line with previous studies. WTAP can ensure the localization of the METTL3-METTL14 heterodimer to the nuclear speckle and promotes catalytic activity [27, 28]. At the same time, METTL14 function was to help METTL3 to recognize the substrate, and METTL3 can catalyze m6A modification [29]. In HCC, Jin-zhao Ma and colleagues have found that METTL14 positively manipulates primary microRNA126 processing via an m6A-dependent manner, which impairs the metastatic potential of HCC. Overexpression of METTL14 inhibits the migration and invasiveness of HepG2 cells in vitro and restrains cell proliferation and metastasis in vivo. METTL14 not only enhances the recognition and binding of microprocessor protein DGCR8 to pri-mi126, but also promotes the subsequent processing to mature miRNA [30]. On the other hand, further analysis suggested that ZC3H13 may be involved in the transcriptional misregulation of cancer. Thus, it can be inferred that ZC3H13 affects the migration and invasion of HCC cells through the indirect effect of WTAP and METTL14, thus inhibiting tumor metastasis.

Cancer cells carry different types of mutations that can lead to uncontrolled cell replication. Copy number alterations or deletion are ubiquitous in cancer, and are associated with cancer outcomes such as recurrence and death [31]. Although some studies have suggested that copy number or deletion variations correlate with mRNA expression [32], we found that gene amplification of m6A family and mRNA expression were not significantly associated. However, we found gene alteration in 33.5% of m6A family in the TCGA database. The incidence rate of genetic variations main in the KIAA1429, YTHDF3, ALKBH5 and ZC3H13 genes was up to 21.6%. Therefore, genetic variations in KIAA1429, YTHDF3, ALKBH5 and ZC3H13 genes may be correlated with liver cancer tumorigenesis and progression. Therefore, increased amplifications of m6A family may be associated with poor prognosis in liver cancer.

In recent years, increasing attention has been paid to the treatment of tumors via immunotherapy, which targets cancer by activating the body's own immune system. Recent findings have suggested that liver cancer is an immunogenic tumor and, thus, developing vaccines, adoptive T cell therapy, and immunomodulators has broad prospects for reducing mortality rates in patients with liver cancer. In our study, we assessed the correlation between ZC3H13 and the immune system via the TISIDB database, and the results showed that ZC3H13 had the greatest correlation with lymphocytes (such as CD56dim, Tgd, Eosinophil and Act-CD8), immunoinhibitors (such as KDR, LAG3, PVRL2 and LGALS9), immunostimulators (such as TNFSF4, TNFSF18, TNFSF14, and IL6R), and MHC molecules (such as HLA-DMA, HLA-DOB, DPA1, and TAPBP). LIGHT, also known as Tumor Necrosis Factor Superfamily member 14 (TNFSF14), is one of the costimulatory molecules that can regulate T-cell activation [33]. TNFSF14 in particular has shown promise as an immunomodulator that can enhance T-cell and natural killer (NK)-cell proliferation, function, and antitumor responses [34]. Therefore, it appears that TNFSF14 (LIGHT), in the tumor microenvironment may reflect or contribute to an antitumor immune response. In addition, cellular

metabolic switches in the liver can regulate the pre-tumor function of monocytes in a specific human tumor microenvironment [35]. Although the underlying mechanism of hepatocellular carcinoma induced immunosuppression has not been determined, there is growing evidence that immunotherapy of anti-tumor immunity can be accomplished by changing the function or number of immune cells, the expression of immune receptors or ligands, and the level of cytokines.

ZC3H13, which is associated with these immune molecules, may provide a new target for studying the immune evasion of hepatocellular carcinoma cells and can potentially serve as a immunotherapeutic target for hepatocellular carcinoma. ZC3H13 is a high candidate as a biomarker for early diagnosis, immunotherapy, and prognostic judgments of hepatocellular carcinoma. However, functional mechanisms of m6A still require further experimental validations, owing to differences found among databases, limited sample sizes, and few relevant experimental studies.

4. Conclusions

In summary, m6A family members display varying degrees of abnormal expressions, and play important roles in the progression of hepatocellular carcinoma. Our data revealed that ZC3H13 was significantly lowly expressed in malignant liver tumor tissues and that low ZC3H13 expression was significantly correlated with poor prognosis in patients with hepatocellular carcinoma and showed that ZC3H13 had the greatest correlation with lymphocytes. Therefore, ZC3H13 may prove to be a novel biomarker for the early diagnosis, immunotherapy, and prognostic judgment of patients with hepatocellular carcinoma.

5. Materials And Methods

5.1 Oncomine analysis

The Oncomine database (<http://www.oncomine.org>) is an online microarray database that includes 715 datasets, as well as 86,733 cancer and normal tissue samples [36]. In this study, the Oncomine database was employed to analyze the mRNA expression levels of m6A-related genes in hepatocellular carcinoma and liver tissue. The search was carried out based on the following criteria: (a) type of analysis: cancer versus normal tissues; (b) type of data: mRNA; (c) thresholds: fold change = 2 and P value = 0.01.

5.2 GEPIA dataset analysis

GEPIA (<http://gepia.cancer-pku.cn/>) is a database of data retrieved from the UCSC Xena server, which includes 9736 tumor samples and 8587 normal samples. The database can be used to analyze differential gene expression levels in tumor tissues and paracancerous tissues, as well as patient survival and prognosis [37]. In this study, we validated the differential mRNA expression levels of m6A-related genes in cancerous and paracancerous tissues of hepatocellular carcinoma using the database. $P < 0.05$ indicated statistically significant differences.

5.3 TCGA and cBioPortal analyses

The open-source cBioPortal database (<http://www.cbioportal.org>) contains data retrieved from the TCGA database for interactively exploring cancer genomic datasets [38]. It contains a wide variety of data, including DNA Keywords: ZC3H13, hepatocellular carcinoma, prognostic value, m6A-related genes. Bioinformatics analyses copy numbers, DNA methylation, mRNA and micro-RNA expression levels, and nonsynonymous mutations. In this study, the cBioPortal database was used to analyze genetic variations in m6A-related genes (such as amplifications, deep deletions, and mutations). In addition, the database was used to evaluate correlations between m6A-related genes.

5.4 Kaplan–Meier plotter analysis

As a tool for assessing biomarkers, the Kaplan–Meier plotter (<http://kmplot.com>) can be used to assess the functions of 54,675 genes and 10,188 tumor tissue samples, including breast cancer, liver cancer, lung cancer and gastric cancer [39]. In this study, we analyzed the prognostic value of m6A-related genes in hepatocellular carcinoma using the Kaplan-Meier plotter. The prognostic values of high- and low-expression groups were evaluated according to the hazard ratio (HR), 95% confidence interval (CI), and log-rank P-values. $P < 0.05$ indicated statistically significant differences.

5.5 GeneMANIA analysis

GeneMANIA (<http://www.genemania.org>) provides a flexible web interface for deriving hypotheses based on gene functions. GeneMANIA generates a list of genes with similar functions to the query gene and constructs an interactive functional-association network to illustrate relationships between genes and datasets [40]. In this study, the database was adopted to construct a gene-gene interaction network for 6A-related genes in terms of physical interactions, co-expression, predictions, colocalization, and genetic interaction, as well as to evaluate their functions.

5.6 Functional and pathway enrichment analysis

We identified genes co-expressed with ZC3H13 using the cBioPortal database. A Spearman's correlation coefficient exceeding 0.50 indicated a good correlation between ZC3H13 and co-expressed genes. Metascape (<https://metascape.org/>) is a free and well-maintained online bioinformatics database for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis [41]. In this study, we used Metascape for functional annotation and pathway enrichment analysis of ZC3H13 and co-expressed genes significantly associated with ZC3H13. On the other hand, data from cBioPortal database were normalized and then differential expression analysis was performed for ZC3H13 using R package ggplot2 and clusterProfiler. Only terms with P-value < 0.05 , minimum count > 3 , and enrichment factor > 1.5 were considered as significant.

5.7 TISIDB analysis

The TISIDB database (<http://cis.hku.hk/TISIDB>) integrates 988 reported immune-related anti-tumor genes, high-throughput screening techniques, molecular profiling, and paracancerous multi-omics data, as well as various resources for immunological data retrieved from seven public databases. The database enables analyses of correlations for selected genes with lymphocytes, immunomodulators, and chemokines [42]. In this study, we employed the TISIDB database to analyze the relationship between the expression levels of m6A-related genes and tumor grades in hepatocellular carcinoma, as well as to investigate correlations between ZC3H13 expression and lymphocytes and immunomodulators.

5.8 The human protein atlas (HPA)

M6A-related genes expression was analyzed by IHC using the available scanned tumor macro array staining from the human protein atlas database (www.proteinatlas.org/). We used this database to study RNA expression levels of ZC3H13 in different tissues. At the same time, we compared the expression of ZC3H13 in liver tissues and tumors based on protein levels.

5.9 Statistical analysis

The statistical analysis in this study was automatically calculated by the online database mentioned above. p value <0.05 or log rank p value <0.05 was considered as statistically significant.

Abbreviations

m6A N6-methyladenosine

HCC hepatocellular carcinoma

HBV hepatitis B virus

HCV hepatitis C virus

TCGA The Cancer Genome Atlas

GO Gene Ontology

KEGG Kyoto Encyclopedia of Genes and Genomes

METTL16 methyltransferase like 16,

WTAP Wilms tumor 1 associated protein,

ZC3H13 zinc finger CCCH-type containing 13,

RBM15 RNA-binding motif protein 15,

FTO fat mass- and obesity-associated protein,

ALKBH5 alkb homolog 5

YTHDC1 YTH domain-containing 1

YTHDF1 YTH N6-methyl-adenosine RNA-binding protein 1

HNRNPC heterogeneous nuclear ribonucleoprotein C

Declarations

Author contributions

SW, GFH and SHL conceived the project and wrote the manuscript. SW, SHL and YXC participated in data analysis. SW, GFH, SHL and YXC participated in discussion and language editing. HZP reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Data Availability

The datasets presented in this study can be found in online repositories. The names of the repository can be found in the article.

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Figures

Figure 1

mRNA expression levels of m6A-related genes in various types of cancer (Oncomine). The threshold was designed with following parameters: fold change = 2 and P-value = 0.01. The cell number represents the number of dataset that meets the thresholds. The color intensity (red or blue) is directly proportional to the significance level of upregulation or downregulation, respectively.

Figure 2

mRNA expression levels of m6A-related genes in liver cancer and normal liver tissues(GEPIA). A-L mRNA expression levels of each member of the m6A-related genes in liver cancer and normal liver tissues. Box plots show mRNA expression of in liver cancer(red plot) and the corresponding normal tissues (gray plot). Axis units are Log2 (TPM + 1). *P < 0.01.

Figure 3

Correlation analysis of the m6A-related genes expression and tumor grades in LIHC(TISIDB) . A–L Correlation between the expression of each m6A-related genes and tumor grades in hepatocellular carcinoma. Spearman's correlation analysis between biomarkers and tumor grades is based on whole tumor grades . Grade 1 is highly differentiated, grade 2 is moderately differentiated, grade 3 is poorly differentiated and grade 4 is undifferentiated.

Figure 4

Analyses of genetic variations in m6A-related genes in Hepatocellular Carcinoma((cBioPortal)). OncoPrint visual summary of variations on a query of m6A-related genes and overview of the analyses of genetic variations in genes. The variations frequency included amplification (red), deep deletions (blue), mutation (green), fusion (purple) and multiple alterations (grey).

Figure 5

Prognostic values of m6A-related genes in hepatocellular carcinoma (RFS in Kaplan–Meier plotter). A-K Prognostic significance of individual of m6A-related genes in hepatocellular carcinoma. The P-values were calculated using the log-rank test. RFS Recurrence free survival.

Figure 6

Prognostic values of m6A-related genes in hepatocellular carcinoma (OS in Kaplan–Meier plotter). A-K Prognostic significance of individual m6A family members in hepatocellular carcinoma. The P-values were calculated using the log-rank test. OS Overall survival.

Figure 7

Survival analyses of ZC3H13 in liver cancer(PFS in Kaplan–Meier plotter). A-C Prognostic significance of ZC3H13 expression in liver cancer with different grades. D-E Prognostic significance of ZC3H13 in liver cancer patients with or without alocahal consumption. F-G Prognostic significance of ZC3H13 in liver cancer patients with or without hepatitis virus .H-I Prognostic significance of ZC3H13 in liver cancer with different FIGO stages. The P-values were calculated using the log-rank test. PFS progression-free survival, FIGO International Federation of Gynecology and Obstetrics.

Figure 8

Gene–gene interaction network among m6A family members (GeneMANIA). Each node represents a gene. The node size represents the strength of interactions. The inter-node connection lines represent the types of gene–gene interactions, and the line color represents the types of interactions.

Figure 10

Spearman's correlation of ZC3H13 with lymphocytes and immunomodulators (TISIDB) in LIHC. a Relations between the abundance of TILs and ZC3H13 expression. b Top 4 TILs displaying the greatest Spearman's correlation with ZC3H13 expression. c Relations between the abundances of immunoinhibitors and ZC3H13 expression. d Top 4 immunoinhibitors displaying the greatest Spearman's correlation with ZC3H13 expression. e Relations between abundances of immunostimulators and ZC3H13 expression. f Top 4 immunostimulators displaying the greatest Spearman's correlation with ZC3H13 expression. g Relations between abundance of MHC molecules and ZC3H13 expression. h Top 4 MHC molecules displaying the greatest Spearman's correlation with ZC3H13 expression. Red and blue cells indicate positive and negative correlations, respectively. The color intensity is directly proportional to the strength of the correlations. TILs tumor-infiltrating lymphocytes, MHC major histocompatibility complex.

Figure 11

The expression level of ZC3H13 (The human protein atlas). (A) Representative images of immunohistochemical staining in liver cancer ; (B) Representative images of immunohistochemical staining in liver tissue ;Scale bars: upper, 100 μ m; lower, 50 μ m.

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