A novel biomarker TLCD1 correlates with prognosis and immune infiltrates in hepatocellular carcinoma

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Abstract

Background The HCC has seen a spike in the morbidity and the mortality rate in recent times. This calls for an urgent understanding of the underlying molecular mechanisms of the HCC and to speed up the quest for the search of the target molecules to ensure quick diagnosis and prognosis subsequently. TLCD1 is a gene only reported in membrane fluidity. The variations in the TLCD1 expression levels related to the infiltration of the immune cells in HCC and the prognosis shall be examined initially.

Methods The data received from TCGA shall provide the details of the gene expression, clinicopathology analysis and immune infiltration, along with the enrichment analysis. Moreover, we also performed additional analysis of the bioinformatics available. The immune responses of TLCD1 expression in HCC were analyzed using CIBERSORT and TIMER, while the statistical analysis was handled through R.

Results Higher TLCD1 expression is strongly correlated with a poor prognostic and worse overall survival. Specifically, the increase in TLCD1 expression positively correlated with Tregs cells and T cells CD4 memory resting. The pathways strongly associated with TLCD1 was fatty acid metabolism and PPAR signaling pathway.

Conclusions From the outcome of the study, it could be surmised that TLCD1 could be considered as a potential target for future treatment of HCC, as it was observed to be associated with the tumor-infiltrating immune cells in tumor microenvironment, establishing itself as a novel potential prognostic biomarker in HCC.

Introduction

Liver cancer is one of the most common cancer with a rate of high mortality around the world. In the cancer of the liver, the HCC is the most common type, with an increasing trend of incidence presently (Fan et al., 2018; Gong et al., 2013). China accounts for about 50% of the morbidity number of liver cancer in the world (Fan et al., 2018). For the HCC patients till now, early diagnosis such as the B-mode, CT scan and the serum AFP have been the common tools (Mehta, Dodge, Grab, & Yao, 2020; Thein et al., 2017). Despite the developments in the detection and management of HCC, patients with HCC remain suffered a bad life for current therapies (D. Zhang, Ma, Sun, Cui, & Lu, 2015). Hence, it becomes necessary to understand the underlying molecular mechanisms of HCC and identify the target molecules to assist quick diagnosis and prognosis.

TLCD1 is a protein coding gene. The human and mouse TLCD1 gene expression is evident in a variety of tissues, with muscle, heart, fat and liver being the strongest for TLCD1 presence. Using a series of careful immune-detection experiments and the Myc-epitope tagging mechanisms, the TLCD1 was initially found in the plasma membrane of the mammalian cells (Papanayotou et al., 2013). Its action at the level of the plasma membrane in limiting the amounts of LCPUFA-containing phospholipids was proved in the previous study by its localization and the effect on PUFA content in membrane phospholipids (Ruiz et al., 2018). In human physiology the LCPUFA are substances with a range of important structural and

regulatory functions (Bannenberg et al., 2017). LCPUFA are involved in numerous biological processes and are major components of complex lipid molecules (Castro, Tocher, & Monroig, 2016; Zhu et al., 2019). Liver is an important place of lipid metabolism and lipid is a crucial component of membrane. Lipid destruction can reflect their important roles during tumor initiation and disease progression at different points such as disruption of normal tissue architecture, cancer cell migration, interaction of cancer cells with components of the tumor stroma and lipid metabolic reprogramming in cancer cells (Baenke, Peck, Miess, & Schulze, 2013; Beloribi-Djefalia, Vasseur, & Guillaumond, 2016; Joyce & Pollard, 2009; J. B. Park et al., 2012). Lipid alterations which might be involved in the onset of cancer and its development, such as lung cancer (Smith et al., 2008), breast cancer (Doria et al., 2012; Hilvo et al., 2011), lung cancer (Smith et al., 2008) and colon cancer (Fhaner, Liu, Ji, Simpson, & Reid, 2012). When TLCD1 was removed more unsaturated fats were incorporated, thereby leaving the membranes in a healthy restored state, albeit the excess saturated fat being the medium in which the cells were being grown (Ruiz et al., 2018).

HCC is a common type of cancer. However, the role of TLCD1 in HCC remains unknown. In the research, we explored the expression of TLCD1 in human HCC samples founded on microarray data that we downloaded from the TCGA database. Meanwhile, we used R language (Version 3.5.3) and statistical analysis to examine the correlation of TLCD1 expression with clinical parameters as well as the prognosis in patients with HCC. GEPIA, KM were used to confirm the relationship between TLCD1 and overall survival. Moreover, we performed an evaluation of tumor-infiltrating immune cells via TIMER and CIBERSORT. To delve deeper into the biological processes involved in the pathogenesis of HCC associated with the TLCD1 regulatory network, we performed the GSEA along with the enhanced GO and KEGG analyses.

Materials And Methods

Gene Expression Analysis

The TCGA official website for the liver provided the pertinent gene expression data (424 files, Workflow Type: HTSeq-FPKM) (Wang, Jensen, & Zenklusen, 2016). Certain customizable functions were made available from the GEPIA online database (<http://gepia.cancer-pku.cn/>). Adopting a standard processing pipeline the RNA sequencing expression data of 8,587 normal samples and 9,736 tumors from the GTEx and the TCGA projects were analyzed through GEPIA (Tang et al., 2017). The TCGA database provided the normal and tumor samples in the GEPIA database. To determine the differential expression of TLCD1, Boxplot, using the disease state as a variable, was graphed.

Survival analysis and prognosis analysis

The TCGA official website for the liver provided the data of systematic analysis of immune infiltrates and the clinical information (377 cases, Data Type: Clinical Supplement). The cases in the TNM stage, distant metastasis, lymph node metastasis, local invasion, overall survival time, and insufficient or missing data on age, were excluded. The clinical data was further analyzed and retained. The TCGA provided the

guidelines for the publication of this study. The GEPIA database computed the correlations of the disease-free survival rate with the TLCD1 expression in HCC. To determine the relationship between the survival days of HCC patients and the expression of high degree TLCD1, the Kaplan–Meier survival analysis () was performed(Lanczky et al., 2016).

Immune Infiltrates Analysis

The relationships between the possible tumor-infiltrating immune cells and the expression of TLCD1 was evaluated with the correlation module of TIMER, an efficient resource helping in the systematic analysis of the immune infiltrates across several types of cancer (<https://cistrome.shinyapps.io/timer/>)(T. Li et al., 2017). To determine the abundance of tumor-infiltrating immune cells from gene expression profiles, a previously published statistical deconvolution method was applied by TIMER(B. Li et al., 2016). The abundance of immune infiltrates could be estimated from the TIMER database that included 10,897 samples across 32 cancer types from TCGA. The correlation of TLCD1 expression with the abundance of immune infiltrates, including the dendritic cells, neutrophils, macrophages, CD8 + T cells, CD4 + T cells, through the gene modules, and the TLCD1 expression in liver cancer was duly analyzed. The left-most pane displays the gene expression levels against the tumor purity(Aran, Sirota, & Butte, 2015). Moreover, a deconvolution algorithm based on gene expression, CIBERSORT (<http://cibersort.stanford.edu/>), can evaluate the changes in the expression of all the sets of the other genes in the sample against one specific set of genes. In the current analysis, via CIBERSORT, the immune response of 22 immune infiltrates cells in HCC, for determining it's correlation with the molecular subpopulation and survival, was gauged. The gene expression datasets were uploaded to CIBERSORT web portal using the standard annotation files with the algorithm running at 1,000 permutations, its default signature matrix. Establishing a measure of confidence in the results a p-value for deconvolution through the Monte Carlo sampling was estimated by CIBERSORT. We used 374 tumor samples from the TCGA divided into 2 groups in order to assess the influence of TLCD1 expression in the immune microenvironment. To select the lymphocyte possibly affected by the expression of TLCD1 the p-value < 0.05 was set as the criterion.

Gene Set Enrichment Analysis

GSEA was performed using normalized RNA-Seq data by TCGA(Subramanian et al., 2005). The annotated gene sets of c5.all.v7.0.symbols.gmt and c2.cp.kegg.v7.0.symbols.gmt in the Molecular Signatures Database (MSigDB) were selected in GSEA version 3.0. The number of permutations was set at 1,000 to determine the normalized enrichment score. GO terms, KEGG pathways were performed to explore the potential biological functions of TLCD1 by using GSEA. Enrichment results satisfying a nominal P-value < 0.05 and a false discovery rate FDR q-value < 0.25 were considered statistically significant.

Statistical Analysis

The R language (Version 3.5.3) conducted the download of the statistical analyses from TCGA. To calculate the 95% CI and the HR the multivariate Cox and the Univariate proportional hazards models were utilized. The comparison of several clinical characteristics with survival was done using the

Univariate survival analysis. To evaluate the influence of TLCD1 expression and other clinical pathological factors (lymph node, distant metastasis, tumor status, grade, gender, and age) on survival, the Multivariate Cox analysis was conducted. The cut-off criterion was set with the P-value of TLCD1 expression < 0.05 . Using the logistic regression, the correlations between the TLCD1 expression and the clinical characteristics were analyzed.

Results

TLCD1 expression is significantly upregulated in HCC

The TLCD1 mRNA levels in the normal and the tumor tissues of liver cancer type were analyzed by using TCGA database, in order to determine the differences between the TLCD1 expression in the normal and tumor tissues. 50 normal files along with 374 tumor files were transformed to convert count data to values more consistent with the microarray results. The boxplot displayed the expression of TLCD1 between the HCC and the normal data (Fig. 1A). A significantly higher TLCD1 expression was revealed in the tumor tissues (p-value = 1.017×10^{-24}) by this analysis. Whereas, a significantly increased TLCD1 mRNA expression in HCC compared normal group with liver cancer group (p-value < 0.010 , $|\text{Log}_2\text{FC}| > 1$) (Fig. 1B) was found using the GEPIA database.

Relationship between TLCD1 expression and clinical characteristics

χ^2 tests revealed the relationship between the TLCD1 expression and the clinical characteristics (Table.1). To investigate the association with multivariable characteristics and tumor progression in TCGA patients, we using cox regression (Table.2). Univariate analysis of correlation revealed that some factors, including pathological stage (HR = 1.865, p-value < 0.001), tumor (HR = 1.804, p-value < 0.001) along with the expression of TLCD1 (HR = 1.036, p-value = 0.003) are significantly associated with tumor development. In multivariate analysis as a forest boxplot was observed in Fig. 1C, the TLCD1 (p-value = 0.041) expression is an independent prognostic factor for tumor progression.

Relationship between TLCD1 expression and poor overall survival

To discover the associations with TLCD1 expression and overall survival in HCC patients, we firstly validated by TCGA datasets, as shown in Fig. 2A. Patients with higher TLCD1 expression had particularly shorter OS (P = 0.019), Furthermore, we used GEPIA database find high levels of TLCD1 mRNA also strongly correlated with a worse survival (p-value = 0.0035) (Fig. 2B). KM survival plots was also used to validate the group of high TLCD1 expression will lead to poor overall survival. The result was considered statistically significant (p-value = 0.024) (Fig. 2C). Univariate analysis using logistic regression revealed that TLCD1 expression was associated with poor prognostic clinicopathologic characteristics. Increased

TLCD1 expression in HCC as significantly associated with grade (III vs I, p-value = 0.00; IV vs I, p-value = 0.01), stage (II vs I, p-value = 0.01), tumor status (III vs I, p-value = 0.00). These results suggested that liver cancer patients with high TLCD1 expression are more susceptible to a more advanced grade, stage and tumor status than those with low TLCD1 expression (Table.3).

Correlation between TLCD1 expression and tumor-infiltrating immune cells

It was aptly established that tumor-infiltrating lymphocytes were an independent predictor of survival and the sentinel lymph node status in cancers(Ohtani, 2007). Hence, whether TLCD1 expression was correlated with the immune infiltration levels in liver cancer was investigated. The correlations of TLCD1 expression with the immune infiltration levels in liver cancer was assessed from TIMER. It was observed that TLCD1 expression had positive correlations with dendritic cells (p-value = 3.67×10^{-2}), macrophages (p-value = 2.09×10^{-4}) and B cell (p-value = 4.04×10^{-5}) as indicated in Fig. 3A. A specific role in the immune infiltration in liver cancer was played by the TLCD1 as evidenced from the findings. Besides, we examined if the TLCD1 expression was associated with immune infiltration in the liver cancer cases. According to TLCD1 expression, the 374 tumor samples were divided into 2 parts. Overall, the screening criteria was met by the 187 samples of low and high expression groups. To infer the density of 22 types of immune cells and to explore the gene expression profiles of the downloaded samples, the established computational resource CIBERSORT was used. The assessment of the differing concentrations in the low and high TLCD1 expression groups of the 22 immune cell subtypes was done by applying the CIBERSORT algorithm. The results were exhibited in Fig. 3B. T cells CD4 memory resting, T cells follicular helper, T cells regulatory (Tregs), Monocytes, Macrophages M0, Macrophages M2, and Mast cells resting were affected by TLCD1 expression.

We observed considerable differences in T cells CD4 memory resting and Tregs, macrophages and mast cells between high group and low group. Afterwards, compared with low expression group, Tregs apparently increased (p-value < 0.001) in high expression group. Moreover, as shown in Fig. 3C the correlations between the 22 types of immune cells were compared as a correlation heat map. The outcome revealed that the different tumor-infiltrating immune cells subpopulations ratios were moderate to weakly correlated.

GO and KEGG pathway analysis

To explore the potential biological functions and to study the regulatory mechanism of the TLCD1, the KEGG pathways and GO terms were performed using GSEA. In the enrichment of the KEGG pathways and the GO terms the GSEA revealed significant differences (FDR < 0.25, p-value < 0.050). We selected the most significantly enriched signaling pathways based on their normalized enrichment score (NES). As shown in Table.4, the GO annotation in high TLCD1 expression resulted five negative correlated parts: protein activation cascade, vitamin B6 binding, retinoic acid metabolic process, cellular amino acid catabolic process and fatty acid catabolic process. The results revealed that the biological processes and

molecular functions strongly associated with TLCD1 was fatty acid catabolic process, as shown in Fig. 4A. The KEGG pathway analysis showed the TLCD1 was significantly enriched in five negative pathways: tryptophan metabolism, fatty acid metabolism, drug metabolism cytochrome p450, retinol metabolism and PPAR signaling pathway, as shown in Fig. 4B. It was indicated that the metabolism pathways were strongly associated with TLCD1. In HCC patients all these functions and mechanisms are critically important.

Fatty acid metabolism markers correction analysis

The relation between TLCD1 and fatty acid metabolism markers in the liver tissue was determined using the correlation module of GEPIA Pearson correlation analysis. PPARA, CPT1A, PNPLA2(ATGL), ACOX1, HNF4A, NR1H3, ACADM and ACADL are serve as potential biomarkers of fatty acid metabolism. We analyzed the relationship between these metabolic genes and TLCD1 (tumor VS normal), as shown in Table 5, The correlation between TLCD1 expression and biomarkers expression in the TCGA database in circos (Fig. 5).

Discussion

TLCD1 is a gene only reported in membrane fluidity. Firstly, the variations in TLCD1 expression level related to prognosis and infiltration of immune cells in HCC were determined. Responding to the hormonal signals, the major organ, the liver controls the glucose and the lipid metabolism (Qiu et al., 2018). The action of TLCD1 at the level of the plasma membrane by limiting the amounts of LCPUFA-containing phospholipids was suggested by the previous study (Ruiz et al., 2018). In the maintenance of the structure and function of the cell membrane and cancer metabolism the polyunsaturated fatty acids played a significant role (Mikami & Murata, 2003; F. Zhang & Du, 2012). When lipid metabolism and fatty acid catabolic are disorder, a series of pathological changes will occur in the liver. Our studies suggested that TLCD1 could be used as a promising cancer biomarker in HCC as it was found to be having a potential influence on tumor immunology.

During our current research, the expression of TLCD1 as a prognostic biomarker in HCC was first explored. All data of HCC patients downloaded from TCGA were performed to estimate the prognostic value. From the perspective of clinical pathology, tumor-infiltrating immune cells and biological functions, it was observed that the up-regulated TLCD1 was an independent prognostic factor for the overall poor survival rate. The liver cancer patients with high TLCD1 expression were found to be more susceptible to a more advanced tumor, grade, and stage status against the low expression of TLCD1. The potential influences of high TLCD1 expression levels on the mechanisms of tumor immunology and tumorigenesis in HCC progression were proposed by our results. For human HCC prognosis, the TLCD1 could serve as a predictor.

The correlation between the diverse immune infiltration levels and TLCD1 expression in liver cancer was another important aspect of our study. The study also demonstrated that the infiltration levels of immune cells in HCC could be detected with TIMER. The outcomes revealed that TLCD1 had strongest

relationships with B cells, macrophage and dendritic cells. Besides, CIBERSORT confirmed the presence of a moderate to strong positive relationships between the infiltration levels of immune cells and the TLCD1 expression, especially Tregs and dendritic cells. The results in our study could indicate correlation between possible mechanism where TLCD1 regulates Tregs functions in HCC. Regulatory T cells contributes to failure of T cell-mediated immunity(Accapezzato et al., 2004). Through cytokine secretion and via cell-to-cell contact, Tregs suppress activation and differentiation of many cell type and sustain tolerance to self-antigens and regulate the immune system(Thompson & Powrie, 2004). There are opinions that Tregs have a central role in emergence of HCC persistence.

GO term and KEGG pathway analysis in this study revealed that the up-regulated TLCD1 to be primarily linked with fatty acid catabolic process and PPAR signaling pathway. Lipid metabolism, including fatty acid catabolic, is a primary function of the liver(Yan et al., 2017). Our study here implicated that overexpression of TLCD1 in HCC patients could induce lipid accumulation and disorder of lipid metabolism. We investigated TLCD1 has a moderate to strong effect on gene (PPARA(Du et al., 2016), CPT1A(Schlaepfer & Joshi, 2020), PNPLA2(Liu et al., 2019), ACOX1(H. Park et al., 2019), SREBF1(Tian et al., 2019), HNF4A(Hayhurst, Lee, Lambert, Ward, & Gonzalez, 2001), NR1H3(Becares et al., 2019), ACADM(X. H. Li et al., 2014), ACADL(van der Meer et al., 2010)) expression related to lipid metabolism. Further studies are needed to confirm if and how TLCD1 supports HCC metastasis in vivo. Evidently, our results could establish the development in the field of TLCD1 biological function in promoting the motility of the HCC cancer cells.

To conclude, our study was first to identify TLCD1 as a new biomarker of the hepatocellular carcinoma thereby helping to determine how the immune cell infiltration and the fatty acid catabolic process could promote the development of liver cancer. In HCC studies, it could be a brand-new biomarker. The biomarker therapies could become a promising future option in the treatment of liver diseases with a better understanding of the functional diversity and heterogeneity of TLCD1. An effective design of the therapeutic strategies and diagnosis for treating human HCC could be contributed by TLCD1.

Abbreviations

HCC: hepatocellular carcinoma, TCGA: The Cancer Genome Atlas, AFP: alpha-fetoprotein, TLCD1:TLC Domain Containing 1, LCPUFA: Long-chain Polyunsaturated Fatty Acids, GEPIA: Expression Profiling Interactive Analysis, KM: Kaplan–Meier survival analysis, GSEA: Gene Set Enrichment Analysis, GO: Gene Ontology, KEGG: Kyoto Encyclopedia of Genes and Genomes

Declarations

Acknowledgements

None.

Authors' contributions

All authors contributed to the conception and design of the study. In addition, they all collected and processed the samples, acquired the data, and finally, analyzed and interpreted those data. All authors wrote and reviewed the manuscript, and finally approved the submitted manuscript

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Availability of data and materials

The data was downloaded from TCGA database.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Clinical Characteristics of the Patients at Baseline.

characteristic	n	low	high	Pearson χ^2	p
total	234	117	117		
age					
≤60	130	58	72	3.3923	0.0655
>60	104	59	45		
gender					
male	160	82	78	0.3162	0.57389
female	74	35	39		
grade					
I	29	18	11	10.2567	0.01651
II	103	59	44		
III	92	38	54		
IV	10	2	8		
stage					
I	113	66	47	7.8372	0.0494
II	49	19	30		
III	67	31	36		
IV	5	1	4		
tumor					
I	115	67	48	14.246	0.002588
II	51	20	31		
III	58	27	31		
IV	10	3	7		

Table 2. Association between TLCD1 expression and clinicopathologic characteristics using logistic regression.

Clinical characteristic	Odds ratio (OR.95L-OR.95H)	P-Value
Age	0.69(0.45-1.04)	0.08
Grade (II vs I)	1.60(0.86-3.05)	0.14
Grade (III vs I)	2.78(1.45-5.49)	0.00
Grade (IV vs I)	9.47(2.22-65.89)	0.01
Grade (I,II vs III,IV)	2.12(1.38-3.29)	0.00
Stage (II vs I)	2.00(1.18-3.43)	0.01
Stage (III vs I)	1.60(0.95-2.72)	0.08
Stage (IV vs I)	5.44(0.79-107.72)	0.13
Tumor (III vs I)	2.09(1.26-3.52)	0.00

Table 3. Correlation between overall survival and multivariable characteristics in TCGA patients via (a) Cox regression (b) Multivariate survival model.

characteristic	HR	HR.95L	HR.95H	pvalue
age	1.005	0.987	1.023	0.591
gender	1.282	0.801	2.053	0.301
grade	1.017	0.746	1.387	0.914
stage	1.865	1.456	2.388	0.000
T	1.804	1.434	2.270	0.000
M	3.850	1.207	12.281	0.023
N	2.022	0.494	8.276	0.328
TLCD1	1.036	1.012	1.060	0.003

characteristic	HR	HR.95L	HR.95H	pvalue
age	1.008	0.989	1.028	0.392
gender	1.003	0.601	1.674	0.992
grade	1.063	0.766	1.475	0.713
stage	0.895	0.333	2.407	0.826
T	1.961	0.808	4.760	0.137
M	0.975	0.256	3.710	0.971
N	2.519	0.399	15.904	0.326
TLCD1	1.026	1.001	1.051	0.041

Table 4. Signaling pathways most significantly correlated with TLCD1 expression based on their normalized enrichment score (NES) and p-value.

	NAME	NES	NOM p-val	FDR q-val
GO	tryptophan metabolism	-2.20	0.00	0.00
	fatty acid metabolism	-2.06	0.00	0.00
	drug metabolism cytochrome p450	-2.03	0.00	0.00
	retinol metabolism	-2.03	0.00	0.00
	PPAR signaling pathway	-1.87	0.00	0.01
KEGG	protein activation cascade	-2.19	0.00	0.00
	vitamin B6 binding	-2.16	0.00	0.00
	retinoic acid metabolic process	-2.11	0.00	0.01
	cellular amino acid catabolic process	-2.10	0.00	0.01
	fatty acid catabolic process	-2.01	0.00	0.01

Table 5. Correlation analysis between TLCD1 and relate genes and markers of fatty acid *metabolism*

gene markers	LIHC			
	Tumor		Normal	
	cor	p	cor	p
PPARA	-0.26	0.00	-0.11	0.44
CPT1A	-0.16	0.00	-0.02	0.92
PNPLA2	-0.14	0.01	0.28	0.05
ACOX1	-0.19	0.00	-0.16	0.26
SREBF1	0.04	0.49	-0.11	0.46
HNF4A	-0.27	0.00	0.02	0.91
NR1H3	0.33	0.00	0.06	0.71
ACADM	-0.32	0.00	-0.11	0.45
ACADL	-0.31	0.00	0.13	0.37

Figures

Figure 1

(A) The expression of TLCD1 between normal and tumor tissues in TCGA (B) TLCD1 mRNA expression levels in normal and HCC tissues, as obtained from GEPIA (C) Multivariate Cox analysis of TLCD1 expression and other clinicopathological variables

Figure 2

Kaplan-Meier analyses of RELL1 expression for patient survival. (A)TCGA database (B)GEPIA database (C)K-M database

Figure 3

(A) Correlations between TLCD1 expression and immune infiltration levels (B) The varied proportions of 22 subtypes of immune cells in high and low TLCD1 expression groups in tumor samples (C) Heatmap of 22 immune infiltration cells in tumor samples.

A

B

Figure 4

(A) GO term analysis revealed five correct groups (B) KEGG pathway showed five correlated groups.

Figure 5

Spearman's correlation between REL1 mRNA expression and of fatty acid metabolism related genes expression.