Identification of potential biomarkers of peripheral blood mononuclear cell in hepatocellular carcinoma using bioinformatic analysis
A protocol for systematic review and meta-analysis

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Abstract
Background: Hepatocellular carcinoma (HCC) is the cause of an overwhelming number of cancer-related deaths across the world. Developing precise and noninvasive biomarkers is critical for diagnosing HCC. Our research was designed to explore potentially useful biomarkers of host peripheral blood mononuclear cell (PBMC) in HCC by integrating comprehensive bioinformatic analysis.

Methods: Gene expression data of PBMC in both healthy individuals and patients with HCC were extracted from the Gene Expression Omnibus (GEO) to identify differentially expressed genes (DEGs). The gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were applied to annotate the function of DEGs. Protein-protein interaction analysis was performed to screen the hub genes from DEGs. CBioportal database analysis was performed to assess the prognostic significance of hub genes. The Cancer Cell Line Encyclopedia (CCLE) and The Human Protein Atlas (HPA) database analyses were performed to confirm the expression levels of the hub genes in HCC cells and tissue.

Results: A total of 95 DEGs were screened. Results of the GO analysis revealed that DEGs were primarily involved in platelet degranulation, cytoplasm, and protein binding. Results of the KEGG analysis indicated that DEGs were primarily enriched in focal adhesion. Five genes, namely, myosin light chain kinase (MYLK), interleukin 1 beta (IL1B), phospholipase D1 (PLD1), cortactin (CTTN), and moesin (MSN), were identified as hub genes. A search in the CCLE and HPA database showed that the expression levels of these hub genes were remarkably increased in the HCC samples. Survival analysis revealed that the overexpression of MYLK, IL1B, and PLD1 may have a significant effect on HCC survival. The aberrant high expression levels of MYLK, IL1B, and PLD1 strongly indicated worse prognosis in patients with HCC.

Conclusions: The identified hub genes may be closely linked with HCC tumorigenicity and may act as potentially useful biomarkers for the prognostic prediction of HCC in PBMC samples.

Abbreviations: AFP = α-fetoprotein, BP = biological processes, CC = cellular components, CCLE = the Cancer Cell Line Encyclopedia, CTTN = cortactin, DAVID = the Database for Annotation, Visualization and Integrated Discovery, DEG = differentially expressed gene, GEO = Gene Expression Omnibus, GO = gene ontology, HBV = hepatitis B virus, HCC = hepatocellular carcinoma, HCV = hepatitis C virus, HPA = The Human Protein Atlas, IL1B = interleukin 1 beta, KEGG = Kyoto Encyclopedia of Genes and Genomes, MCC = maximal clique centrality, MF = molecular functions, MSN = moesin, MYLK = myosin light chain kinase, PLD1 = phospholipase D1, PPI = protein-protein interaction, STRING = Search Tool for the Retrival of Interacting Genes/Proteins.

Keywords: bioinformatics analysis, differentially expressed genes, hepatocellular carcinoma, peripheral blood mononuclear cell
1. Introduction

Human hepatocellular carcinoma (HCC) is among the highly prevalent types of malignancy all over the world and is a principal cause of tumor-associated mortality; the mortality and occurrence rate of HCC reportedly increased in recent years.1,2 Its incidence is recorded to be approximately 2 to 7/100,000 populations per year across the whole earth, most of HCC patients comes from South Africa and East Asia,3 especially in China. Nearly half of the new patients with HCC in the world are in China. The survival of patients with advanced HCC stages still remains unsatisfactory across the world. Effective diagnosis of HCC at the early stages is challenging and urgent. However, the conventional methods for HCC detection, which frequently hinge on liver biopsy, α-feto-protein (AFP), and ultrasound scanning, have disadvantages in terms of accuracy and sensitivity.4 A detection method with high efficacy for the early discovery of HCC is urgently needed.

A novel biomarker for the early diagnosis of HCC needs to be found; thus, numerous researchers have shifted their research focus to molecular biomarkers.5 Experimental analysis with the assistance of bioinformatics analysis is a novel useful tool for thoroughly analyzing data. Numerous researchers have explored the mechanism underlying all kinds of cancers by mining the gene expression data using bioinformatics,6 peripheral blood mononuclear cell (PBMC) are inflammatory cells that ultimately lead to disease progression through different ways. Studies on altered genes have been extensively performed to investigate tumor carcinogenesis. In the past few decades, altered genes in PBMCs were found to be closely linked to multiple malignancies.7 Accumulated evidence indicated that multiple genes in PBMCs participate in the initiation of HCC.8 Identification of hub genes and the molecular mechanism involved in PBMCs are crucial for developing potential diagnostic and prognostic strategies. In the present study, we identified the overlapped differentially expressed genes (DEGs) of PBMCs by analyzing the gene profile data from the Gene Expression Omnibus (GEO) database between healthy individuals and patients with HCC. The Database for Annotation, Visualization and Integrated Discovery (DAVID) platform was utilized to understand the function of the selected DEGs. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) website was performed to construct the protein-protein interaction (PPI) network. Cytoscape was utilized to screen the potential hub genes. The Cancer Cell Line Encyclopedia (CCLE) and The Human Protein Atlas (HPA) databases were subsequently combined to verify the expression levels of the screened hub genes in HCC cells and tissues. cBioportal was used to precisely evaluate the prognostic significance of hub genes. In conclusion, 5 key genes that may be developed as candidate biomarkers for the occurrence of HCC were defined in the present analysis.

2. Materials and methods

Institutional Review Board in the First Affiliated Hospital of Guangxi Medical University approved this study [(approval nos. 2020 (KY-E-133)]. This study was conducted based on available data in previously published literatures; hence, patient consent for participation and ethical approval was not necessary in the study.

2.1. Data resources

Gene expression data in PBMC samples from patients with HCC and healthy individuals were extracted from the GEO database (http://www.ncbi.nlm.nih.gov/geo/).9 The 2 gene expression profile datasets (GSE36076 and GSE58208) were based on the GPL570 platform (Human Genome U133 Plus 2.0). The GSE36076 dataset consisted of 10 healthy individuals and 10 patients with HCC. The GSE58208 dataset contained the gene data of 5 normal samples and 10 HCC samples.

2.2. Identification of DEGs

The DEGs between healthy individuals and patients with HCC in PBMC samples were identified by using the online web tool GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/).10 Genes that met the filter criteria of log2FC (fold-change) > 1 and adjusted P < .05 were considered to be differentially expressed. The overlapped genes of 2 datasets was screened out by drawing Venn diagrams with an online webtool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

2.3. Functional annotation of DEGs

To further elucidate the function and signaling pathways involved in the enrichment of the identified DEGs, we inputted the DEGs into the Database for DAVID (http://david.abcc.nicifcrf.gov/) for GO function analysis and KEGG pathway analysis.11 GO analysis was composed of 3 categories, namely, biological processes (BPs), cellular components (CCs), and molecular functions (MFs).12 Results that met the threshold value with P < .05 were regarded as significant.

2.4. Selection of hub genes

To reveal the functional interactions among the proteins encoded by the identified DEGs, the DEGs were uploaded into STRING tools (http://string-db.org) to map the PPI network.13 The results with a combined interaction score of >0.4 were considered to be significant. Cytoscape (https://cytoscape.org/download.html) was utilized to visualize the constructed PPI networks.14 The CytoHubba plugin was applied to explore the potential hub genes of PPI networks.

2.5. Verification of hub gene expression analysis

Data obtained from CCLE database platform (http://portals.broadinstitute.org/ccle/home) were utilized to validate the expression levels of the selected hub genes in HCC cells.15 The threshold value with P < .05 and fold changes of >2 were deemed significant. HPA database (https://www.proteinatlas.org/) was used to map the protein expression levels of the hub genes in the tumor tissues of HCC.16

2.6. Survivals analysis of hub genes

The overall survival and disease-free survival of the selected hub genes in HCC were estimated by using the cBioportal online database (http://www.cbioportal.org/).17 A log-rank of P ≤ .05 was used as the cutoff value.

3. Results

3.1. Identification of DEGs in PBMCs from healthy individuals and HCC samples

A total of 1721 and 1014 genes could be considered as the DEGs in the GSE36076 and GSE58208 datasets, respectively. The overlap across the 2 datasets finally contained 95 genes (Fig. 1A),

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1. Peng et al. Medicine (2021) 100:2

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as determined by drawing a Venn diagram. The overlapped DEGs consisted of 71 upregulated and 24 downregulated genes in the HCC samples.

3.2. Enrichment analysis of KEGG and GO of the overlapped DEGs

Seven remarkably enriched BP terms, 13 notably involved CC terms, and 3 remarkably involved MF terms were screened. The platelet degranulation was the most significant enrichment in the biological processes category, followed by the negative regulation of the extrinsic apoptotic signaling pathway. The most significantly enriched gene sets were aligned to the cytoplasm and the focal adhesion in the cell component analysis. The molecular function analysis indicated that these changed genes were primarily enriched in the structural constituent of muscle and protein binding. KEGG analysis demonstrated that the selected DEGs were predominantly involved in “focal adhesion.”
“regulation of actin cytoskeleton,” “gap junction,” and “platelet activation” (Table 1).

### 3.3. Identification of key genes

A total of 39 DEGs, which comprised 33 upregulated and 6 downregulated DEGs, were filtered into the PPI network by using the STRING database to assess the interaction relationships among the DEGs. The network contained 88 nodes and 36 edges and was visualized by using the Cytoscape software (Fig. 1B). The top 5 genes with the most interaction lines according to the MCC method were sequentially considered as hub genes, including myosin light chain kinase (MYLK), interleukin 1 beta (IL1B), phospholipase D1 (PLD1), cortactin (CTTN), and moesin (MSN) (Fig. 1C). MYLK had the highest scores of nodes (Table 2). All these identified hub genes were upregulated in patients with HCC.

### 3.4. Expression validation of hub genes in HCC

The results obtained from CCLE revealed that the MYLK expression was elevated in HCC cells. Data obtained from HPA database revealed that MYLK expression is enhanced in HCC tissue compared with liver tissue. Data obtained from CCLE demonstrated that the expression levels of IL1B were significantly elevated in HCC cells. Data available from CCLE revealed that PLD1 was overexpressed in HCC cells. Data provided by HPA demonstrated that IL1B’s expression was remarkably enhanced in HCC tissue. We observed that the CTTN expression was remarkably upregulated in HCC cells and tissues. The expression levels of MSN were found to be upregulated in HCC cells and tissues (Figs. 2 and 3).

### 3.5. Survival analysis of hub genes expression in HCC

Results revealed that patients with HCC with upregulated MYLK expression showed reduced overall survival, whereas MYLK alterations in patients with HCC did not show any impact on disease-free survival prognosis. A high expression of IL1B was drastically correlated with reduced disease-free survival in patients with HCC, whereas its correlation with overall survival was not significant. Patients with HCC who overexpress PLD1 showed worse overall survival, whereas no changes were observed in patients with HCC with PLD1 alterations in terms of disease-free survival. CTTN or MSN alterations were not closely related to the reduced disease-free and overall survival (Figs. 4 and 5).

### 4. Discussion

Human HCC is a widespread malignant tumor with high mortality and poor prognosis, which are due to the absence of appropriate early biomarkers for its diagnosis. The effective diagnosis of HCC is crucial for intervening in the progression of...
HCC and improving the prognosis. Altered genes in PBMC have a remarkably clinical value in the diagnosis and prognosis of multiple human malignancies. Accumulating evidence noted that gene expression profiles in PBMC samples were altered in many types of malignancies, thereby contributing to the initiation and progression of HCC. Hence, the identification of biomarkers based on PBMCs to determine the underlying HCC tumorigenesis and prognosis is urgent. Microarray technology combined
with bioinformatics analysis has rapidly developed and is widely
used for exploring genetic alterations in multiple types of
malignancies, thereby enabling researchers to identify novel
molecular biomarkers in malignant tumors.\textsuperscript{19} With the help of
systematic bioinformatics analysis, we attempted to discover
the promising biomarkers of PBMCs that can be used for
noninvasively diagnosing and preventing HCC in its early
stages.

The differences among the gene expression profiles of PBMC
samples from healthy individuals and patients with HCC chosen
from GEO online database were the focus of the analysis. Ninety-
five common DEGs were determined for further analysis. These

Figure 3. Expression of the MYLK, IL1B, PLD1, CTTN, and MSN in HCC according to CCLE. (A) MYLK was overexpressed in HCC cells according to CCLE. (B) IL1B was overexpressed in HCC cells according to CCLE. (C) PLD1 was overexpressed in HCC cells according to CCLE. (D) CTTN was overexpressed in HCC cells according to CCLE. (E) MSN was highly expressed in HCC according to CCLE. CTN = cortactin, HCC = hepatocellular carcinoma, HPA = Human Protein Atlas database, IL1B = interleukin 1 beta, MSN = moesin, MYLK = myosin light chain kinase, PLD1 = phospholipase D1.
comprised 71 upregulated and 24 downregulated genes. The functional analysis revealed the identified DEGs as participants in platelet degranulation among the biological processes. GO analysis implied that these identified DEGs were predominantly involved in cytoplasm and focal adhesion with regard to the cell component category. GO analysis also illustrated that these identified DEGs were markedly enriched in the structural constituents of muscle and protein binding in the molecular function category. KEGG analysis suggested that these DEGs were closely associated with focal adhesion and gap junction. The following top 5 genes (ordered by connectivity) were finally set as the hub genes in the PBMCs of HCC: MYLK, IL1B, PLD1, CTTN, and MSN.

Figure 4. Overall survival analysis of hub genes. (A) Overexpression of MYLK presented with a reduced overall survival prognosis. (B) No changes were observed in patients with HCC with IL1B alterations for overall survival prognosis. (C) Overexpression of PLD1 presented with a reduced overall survival prognosis. (D) No changes were observed in patients with HCC with CTTN alterations for overall survival prognosis. (E) No changes were observed in patients with HCC with MSN alterations for overall survival prognosis. CTTN = cortactin, HCC = hepatocellular carcinoma, IL1B = interleukin 1 beta, MSN = moesin, MYLK = myosin light chain kinase, PLD1 = phospholipase D1.
CTTN, and MSN, and accumulating evidence recorded that those hub genes were tightly involved in the progression and deterioration of HCC through various ways.

MYLK belongs to the immunoglobulin gene super family. MYLK predominantly contributes to the modulation of a wide variety of biological processes that are primarily linked to regulation of cell adhesion, cell migration, division, tumor invasion, and tumor metastasis.[20,21] Previous studies have reported that abnormal MYLK expression was frequently related to carcinogenesis, invasion, and tumor metastasis in multiple types of malignancies, such as prostate cancer,[22] non-small cell lung cancer,[23] colorectal cancer,[24] and HCC.[25] Lin et al

Figure 5. Disease free survival analysis of hub genes. (A) No changes were observed in patients with HCC with MYLK alterations for disease-free survival prognosis. (B) Overexpression of IL1B presented with a reduced disease-free survival prognosis. (C) No changes were observed in patients with HCC with PLD1 alterations for disease-free survival prognosis. (D) No changes were observed in patients with HCC with CTTN alterations for disease-free survival prognosis. (E) No changes were observed in patients with HCC with MSN alterations for disease-free survival prognosis. CTTN = cortactin, HCC = hepatocellular carcinoma, IL1B = interleukin 1 beta, MSN = moesin, MYLK = myosin light chain kinase, PLD1 = phospholipase D1.
reported that higher expression levels of MYLK were observed in HCC specimens through comparison with the matched adjacent liver specimens. MYLK overexpression was closely related to the clinicopathological characteristic features of aggressive behavior. Upregulated MYLK served a function in the promotion of HCC microvascular invasion and metastasis. Results from in vitro functional experiments established that MYLK knockdown can repress the migration and invasion of HCC cells. Epithelial-mesenchymal transition (EMT) of carcinoma cells usually induces the increased mobility of the tumor cells and a high risk of lymph node or distant metastases. EMT of HCC cells was reported to accelerate HCC progression and deterioration.

Previous research recorded MYLK could promote HCC progression and development by means of altering cytoskeleton to enhance EMT. In vitro assays revealed the expression of MYLK in PBMC samples from patients with HCC was higher than in samples from healthy individuals, according to GEO data analysis. Results from the HPA showed that MYLK had higher protein expression in human HCC tissues than in normal liver tissues. MYLK expression levels were upregulated in HCC cells. Patients with increased expression of MYLK could have significantly poor overall survival. Nevertheless, the associations of these genes with the disease-free survival of patients with HCC were not significant according to the cBioportal platform. These findings demonstrated that the increase in MYLK expression in HCC might predict unfavorable prognosis.

IL1B is a member of the interleukin 1 gene family. It participates in the induction process of the malignant transformation of human normal cells and plays an essential role in the control of migratory and invasive properties by promoting tumor angiogenesis and cell adhesion and by increasing cancer metastasis in a number of cancer types. IL1B was reportedly upregulated in multiple types of human tumors, such as lung adenocarcinoma, pancreatic ductal adenocarcinoma, and HCC and served as a useful prognostic factor in different human tumors. Previous studies have shown that some polymorphisms of IL1B were associated with HCC, and some SNPs of IL1B gene increased the risk of HCC; IL1B in the IL1 family contributes to HCC susceptibility. Researchers in Thailand found that the IL1B gene polymorphism was remarkably associated with HCC in patients with hepatitis B virus (HBV) infection. A previous study found that the genotypes of IL1B were most likely associated with the prognosis of hepatitis C virus (HCV)-related HCC, and these might function as potential factors for predicting the clinical outcome of patients with HCC. A previous research revealed that upregulated IL1B promoted tumor metastasis in HCC mouse models and was a promising prognostic factor in predicting worse clinical outcome in patients with HCC, the enhancement of HIF1α/IL1B signaling in a hypoxic microenvironment, finally contributed to EMT and metastasis process of HCC cells. IL1B was overexpressed in PBMC samples from patients with HCC, according to GEO dataset analysis; and results from the CCLE database analysis demonstrated that increased expression levels of IL1B were observed in HCC cell. These findings suggested that the overexpression of IL1B participated in the promotion of HCC onset and development. The highly upregulated expression level of IL1B led to a low disease-free survival in patients with HCC, whereas IL1B was not significantly associated with worse overall survival.

PLD1 is a member of the PLD superfamily and has been demonstrated to play pivotal roles in a series of cell biological processes. For example, PLD1 functions in the induction of cell growth, cell proliferation, cell migration, mediation of differentiation, motility, cytoskeletal reorganization, and intracellular protein trafficking. The expression levels of PLD1 were remarkably elevated in various human tumors, for example, osteosarcomas, pancreatic ductal adenocarcinoma, breast cancer, and endometrial carcinoma. Previous studies reported that the inhibition of PLD1 exerted a suppressive effect on tumor growth and on the biological process of EMT in mice with HCC. And PLD1 activation resulted in HCC progression by means of regulating the proliferation, migration and invasion, and promoting the EMT process of HCC cells. PLD1 was also revealed to be remarkably overexpressed in tissue samples from patients with HCC. PLD1 was significantly overexpressed in PBMC samples from patients with HCC according to GEO dataset analysis. The expression levels of PLD1 were upregulated in HCC cells. The protein expression of PLD1 was highly upregulated according to HPA data analysis. PLD1 expression was considered to be remarkably linked to decreased overall survival. PLD1 expression was not significantly related to unsatisfactory disease-free survival prognosis.

CTTN overexpression was reportedly robustly correlated with tumor aggressiveness, cell adhesion, and cell motility. CTTN was tightly associated with the process of human tumor pathogenesis and progression in various types of cancers. High CTTN expression level was observed in human colorectal cancer, gastric cancer, osteosarcoma, and esophageal cancer. A previous study illustrated that CTTN led to the promotion of tumor cell proliferation, invasion, and migration of HCC. Previous studies also implied that dysregulated expression levels of CTTN affected the modulation of the migration and invasion of HCC. CTTN was overexpressed in PBMC samples of patients with HCC, according to GEO dataset analysis. The protein expression of CTTN was revealed to be predominantly overexpressed in human HCC tissues according to HPA data analysis. Increased expression level of CTTN was observed in HCC cells according to CCLE data analysis. No changes were observed in the disease-free and overall survival prognosis of patients with HCC with CTTN alterations.

MSN is a member of the ezrin-radixin-moesin family, which serves an critical function in the regulation of cellular morphology, adhesion, and motility; the dysregulated expression levels of MSN were reportedly extensively implicated in the processes of cancer proliferation, tumor metastasis, and colonization of tumor cells. Moesin was reportedly remarkably overexpressed in a wide variety of human cancer types, such as breast cancer, oral cancer, pancreatic cancer, and gastric adenocarcinoma. Previous findings implied that the expression levels of MSN were remarkably upregulated in human HCC tissues samples, and MSN serves a function in the promotion of tumor metastasis of HCC cells. Our research found that MSN was remarkably overexpressed in PBMC samples of HCC patients, according to GEO dataset analysis. The expression level of MSN was remarkably upregulated in HCC cells according to CCLE data analysis. The protein expression of MSN in human HCC tissues was notably upregulated according to HPA data analysis. MSN was not closely related to disease-free survival and overall survival.

Few limitations existed in this review. Such as the subject selection bias was unavoidable due to the restrictions of English or Chinese literature. In-depth experiments with large populations is recommended to verify a compelling conclusion.
These key and noninvasive biomarkers in HCC were determined through bioinformatics analysis. Two publicly available databases were combined to screen 5 hub genes, namely, MYLK, IL1B, PLD1, MSN, and CTTN, which may be regarded as powerful and promising noninvasive biomarkers for predicting tumorigenesis and progression of HCC. The gene upregulation of MYLK, IL1B, and PLD1 was closely associated with worse survival outcome. Thus, these genes have potential as HCC survival predictors. However, further experimental verification in vitro and in vivo is warranted to confirm the biological processes and molecular pathogenesis underlying the possible effects of these eligible hub genes on HCC carcinogenesis.

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