Gastroenterology and Hepatology From Bed to Bench.

ORIGINAL ARTICLE

Identification of gene signature in RNA-Seq hepatocellular carcinoma data by Pareto-optimal cluster algorithm

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ABSTRACT

Aim: This study aimed to detect gene signatures in RNA-sequencing (RNA-seq) data using Pareto-optimal cluster size identification.
Background: RNA-seq has emerged as an important technology for transcriptome profiling in recent years. Gene expression signatures involving tens of genes have been proven to be predictive of disease type and patient response to treatment.
Methods: Data related to the liver cancer RNA-seq dataset, which included 35 paired hepatocellular carcinoma (HCC) and non-tumor tissue samples, was used in this study. The differentially expressed genes (DEGs) were identified after performing pre-filtering and normalization. After that, a multi-objective optimization technique, namely multi-objective optimization for collecting cluster alternatives (MOCCA), was used to discover the Pareto-optimal cluster size for these DEGs. Then, the k-means clustering method was performed on the RNA-seq data. The best cluster, as a signature for the disease, was found by calculating the average Spearman's correlation score of all genes in the module in a pair-wise manner. All analyses were performed in the R 4.1.1 package in virtual space with 100 Gb of RAM memory.
Results: Using MOCCA, eight Pareto-optimal clusters were obtained. Ultimately, two clusters with the greatest average Spearman's correlation coefficient scores were chosen as gene signatures. Eleven prognostic genes involved in HCC's abnormal metabolism were identified. In addition, three differentially expressed pathways were identified between tumor and non-tumor tissues.
Conclusion: These identified metabolic prognostic genes help us to provide more powerful prognostic information and enhance survival prediction for HCC patients. In addition, Pareto-optimal cluster size identification is suggested for gene signature in other RNA-Seq data.

Keywords: Hepatocellular carcinoma, Gene expression signature, RNA-Seq, Clustering.

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Introduction

In 2012, hepatocellular carcinoma (HCC), also known as hepatoma, was the world's seventh most common cancer (1), and in 2018, it was the second leading cause of cancer death (2). However, in 2020, HCC was reported as the third leading cause of cancer death, with an estimated 830,180 people having died from this disease. GLOBOCAN estimated in 2018 that approximately 72% of all liver cancer cases arise in Asian countries (3).

HCC accounts for 90% of all primary liver malignancies. Hepatitis B virus (HBV), hepatitis C virus (HCV), and cirrhosis are all risk factors for this disease (1).

HCC is treated with resection of the tumor region (resection) and liver transplantation, in addition to chemotherapy, which has not been very successful. Where there are no extrahepatic metastases, tumor resection is effective. For HCC patients who do not

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have metastatic disease, liver transplantation is the most appropriate treatment option (4). In addition to prevention, early diagnosis of the tumor, when the patient is asymptomatic and liver function is still intact, is one of the principal ways to improve the prognosis of HCC, because in this clinical setting, effective medicines with survival benefits can be implemented (5).The range of the median overall survival (OS) for HCC with a late diagnosis is 6 to 20 months (6).

Due to disease development, old age, and other factors, the majority of patients are not appropriate candidates for treatment. As there are few liver donors, most people awaiting a liver transplant die because of the tumor and disease progression (7).

Today, gene expression signatures containing tens of genes have been shown to be predictive of disease type and patient response to treatment. They have also been shown to be useful in numerous studies investigating biological mechanisms. A gene signature or gene expression signature is a single or combined group of genes in a cell with a uniquely characteristic pattern of gene expression that occurs as a result of an altered or unaltered biological process or pathogenic medical condition (8).

RNA-sequencing (RNA-seq) has emerged as an important technology for transcriptome profiling in recent years. The number of mapped reads for a given gene is determined not only by its expression level and gene length, but also by the sequencing depth. To normalize these dependencies, gene or transcript expression levels are measured using FPKM (fragments per kilobase of transcript per million fragments mapped) (9). There are many methods used to analyze such data, but one of the most important and widely used methods of data analysis, which is also one of the exploratory methods, is the use of cluster analysis (10).

In partitioning clustering, such as k-means clustering which allows the user to specify the number of clusters k to be created, determining the optimal number of clusters in a data set is a fundamental problem. This question, unfortunately, has no definitive response, and the error rate could rise as a result. Multi-objective optimization can be combined with variability analysis for cluster number estimation. Cluster number estimation is commonly thought of as a single-objective optimization problem with determining the best cluster number k as its goal. This method is highly dependent on the cluster algorithm and cluster validation measure used, as different algorithms and validity measures predict different optimal k values (11). Cluster analysis, on the other hand, is an exploratory data mining technique that has the ability to illustrate many aspects of data when used with various parameter configurations, resulting in several potentially valid solutions. Accordingly, for the set of cluster alternatives, a multi-objective optimization technique (MOCCA) was used in the current study. Cluster variability analysis was used in the first step to produce a set of k cluster results for various cluster algorithms. The collection of cluster results was then evaluated in the second stage using various cluster validation steps. The Pareto set of alternative cluster results was computed in the final step. The elements of the Pareto set were also classified according to the number of dominated objectives (12). Because of the importance and application of clustering in genetics, the Pareto optimization algorithm was used herein to find gene-signature clusters in liver cancer data. In other words, this study used a multi-objective optimization strategy for the set of cluster alternatives (MOCCA).

Methods

Data collection and gene expression analysis

In this study, mRNA expression datasets of HCC were searched using the keywords “FPKM,” “mRNA,” “Hepatocellular carcinoma,” and “Homo sapiens” [porgn: txid9606] in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo). Datasets with tumor and non-tumor tissue samples were considered. The Research Ethics Committee of the University of Social Welfare and Rehabilitation Sciences approved the current study (Code: IR.USWR.REC.1398.158).

After a systematic review, a GSE profile (GSE124535 based on GPL20795, HiSeq X Ten [Homo sapiens]) was selected and analyzed. Gene expression analysis was performed by the limma package in Bioconductor that was utilized to mine statistically significant differentially expressed genes (DEGs) based on the difference in their expression values between non-tumor and tumor samples with log2 fold change ≥ 1, and an adjusted p-value threshold of 0.05 were considered as significantly differentially

expressed genes (13). After that, an expression matrix from up- and down-regulated genes (mural DEGs) was created for analysis. The volcano plot was also applied using bi-filtering approaches (p-value filtering and fold change filtering) consecutively.

Identification of gene signatures

The R package MOCCA was used on the data of the mural DEGs to determine the optimal (robust) number of clusters after identifying the collection of differentially expressed genes (up-regulated and down-regulated genes).

The stable cluster numbers are determined using a bootstrapping method based on multiple cluster validity indices. It is worth noting that these estimates can vary depending on the clustering technique used and the cluster validation index.

It is worth noting that these estimates can vary depending on the clustering strategy and cluster validation index used. The MOCCA method is based on aggregating the best cluster numbers from various clustering algorithms and various cluster validation indices in a multi-objective setting to determine the robust (Pareto-optimal) cluster numbers. To begin, MOCAA performs a multi-objective optimization to collect cluster alternatives. The data-matrix is then used to extract R number of bootstrap samples. It uses three clustering techniques to compute clustering for all unique cluster numbers K (kmeans, single-linkage, and neuralgas clustering).

Following that, it clusters using a variety of cluster validation indices (MCA, Jaccard, FM, and CQS). To achieve the Pareto ideal (robust) number of clusters, a total of twelve objective functions (kmeans.MCA, kmeans.Jaccard, kmeans.FM, kmeans.CQS, neuralgas.MCA, neuralgas.Jaccard, neuralgas.FM, neuralgas.CQS, single.MCA, single.Jaccard, single.FM, single.CQS). These results (cluster validity indices) were then compared by determining the Pareto-optimal cluster sizes and ranking them by dominance.

The Pareto-optimal cluster sizes were classified based on the lowest number of goals for which they outperformed the remaining cluster sizes. Then, k-means clustering with the optimal cluster size was used to identify the cluster information of each participating gene after determining the optimal number of clusters.

Next, the average Spearman's correlation coefficient score of each resultant cluster was calculated using the Spearman's correlation coefficient score computed among the participating pairwise genes. The best cluster was chosen as the one with the highest average Spearman's correlation coefficient score. The best cluster's combined gene set was used as a gene signature in this study. Our strategy is presented in Figure 1.

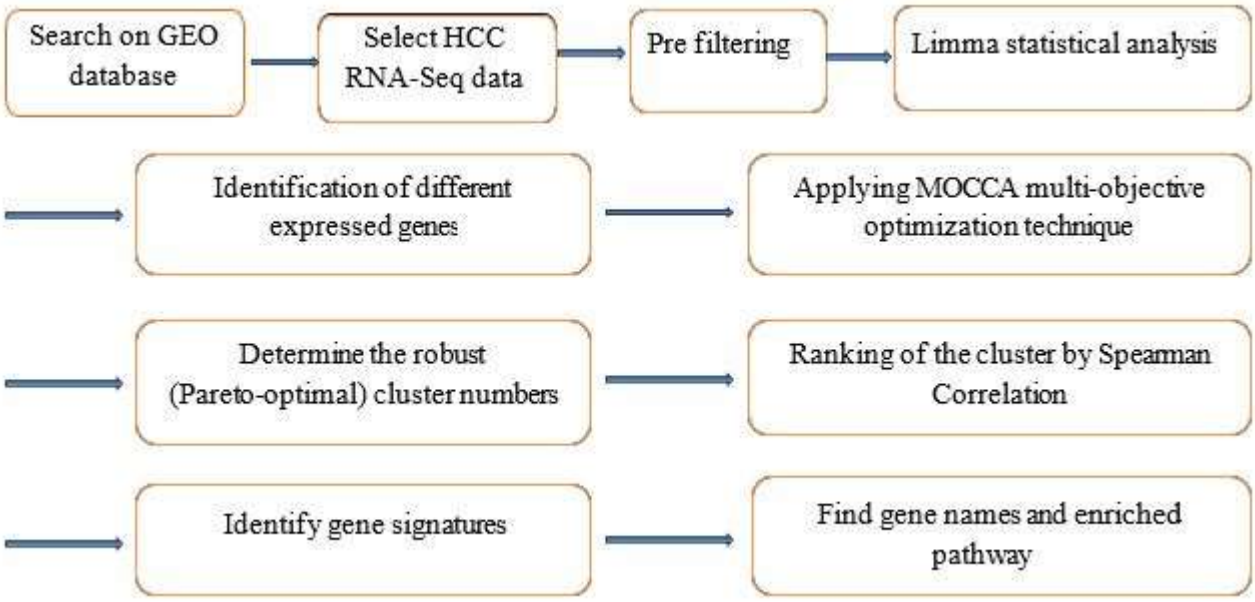

Figure 1. Flowchart of the suggested framework to identify gene signature

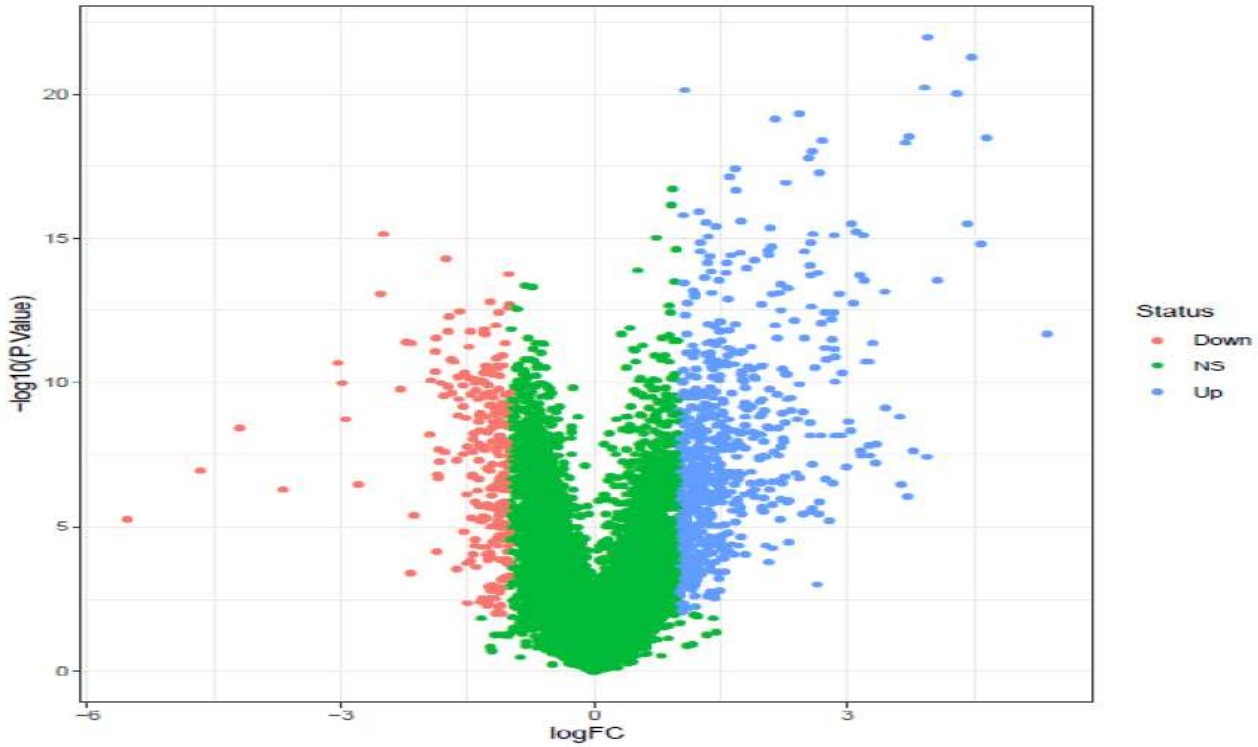

Figure 2. Volcano plot for identifying up-regulated and down-regulated genes for liver cancer dataset

Gene Set Enrichment Analysis

The DAVID database was used to conduct KEGG pathway analyses for the signature's participating genes. Only KEGG pathways or Gene-Ontology words with an enriched p-value of less than 0.05 were included in this study.

Results

Identification of DEGs

In this study, GSE124535 (GPL20795) was used and the RNA-seq dataset contained 70 samples (35 non-tumors and 35 tumors). Using the limma package in R language programming, differential expression analysis was carried out between non-tumor and tumor tissues. A total of 1157 DEGs (410 up-regulated and 747 down-regulated genes) were identified (supplementary file 1). Moreover, the volcano plot (Figure 2) was utilized using bi-filtering approaches (p-value filtering and fold change filtering) consecutively.

Determination of gene signatures

The R package MOCCA was used on the data of these genes to evaluate the Pareto-optimal (robust) number of clusters after the collection of up-regulated and down-regulated genes was identified. As a result, the Pareto-optimal cluster size was found to be 8. Table 1 shows the objective values for each of the twelve objective functions.

Table 1. Twelve objectives in MOCCA and their values from the hepatocellular carcinoma (HCC) cancer RNA-seq dataset.

Objective	Objective value
kmeans.MCA	0.551903
kmeans.Jaccard	0.389215
kmeans.FM	0.511502
kmeans.CQS	0.993375
neuralgas.MCA	0.589965
neuralgas.Jaccard	0.376686
neuralgas.FM	0.505094
neuralgas.CQS	0.993018
single.MCA	0.480104
single.Jaccard	0.299592
single.FM	0.464825
single.CQS	0.993618

Ultimately, k-means clustering with the optimal cluster size was used to collect cluster information for each participating gene after determining the optimal number of clusters (n=8).

The average Spearman's correlation coefficient scores of the eight clusters were 0.0706, 0.7070, 0.4973, 0.5110, 0.5530, 0.4195, 0.5923, and 0.1975, respectively. “Gene-signature” was chosen as the second and seventh cluster with the highest average

Spearman's correlation coefficient scores (0.7070, 0.5923, respectively). It is worth noting that the gene signature included 11 differentially expressed genes (Table 2).

Table 2. Names of genes and gene identities related to the HCC gene signature.

Gene Symbol	Full Name
TF	Transferrin
APOC3	Apolipoprotein C3
APOA1	Apolipoprotein A1
RBP4	Retinol Binding Protein 4
APOH	Apolipoprotein H
AMBP	Alpha-1-Microglobulin/Bikunin Precurso
TTR	Transthyretin
FGG	Fibrinogen Gamma Chain
FGA	Fibrinogen Alpha Chain
FGB	Fibrinogen beta chain
ORMI	Orosomucoid 1

Pathway enrichment analysis

The DAVID online tool was utilized to find the enriched pathways with a p-value of ≤0.05. In GSEA analysis, three biological pathways were significantly enriched. KEGG pathway analysis indicated that complement and coagulation cascades, platelet activation, and PPAR signaling pathways were most prevalent in the final list (Table 3).

Discussion

In this study, a Pareto optimum based clustering framework method was utilized to identify gene signature in RNA-Seq liver cancer data. This method uses a single genomic or epigenetic data set. There are many other gene signature identification methodologies based on co-expression in the literature; however, the vast majority of existing approaches use either the WGCNA module detection method or something similar, in which the generalized modules are not optimized. The number of modules is likely to change if the input criterion for the minimum number of modules is altered. A typical clustering methodology was used to locate gene modules after optimizing the number of clusters in our method. Finally, the average Spearman's correlation coefficient for each module was computed, and the top-ranked module was employed as a gene signature. It should be noted that, although there are several bioinformatic methods for detecting gene signatures, no one has ever tried gene signature detection using Parto's ideal method for liver cancer.

Using the mRNA expression dataset, the current study created an 11-gene signature for HCC prognosis assessment. Previous bioinformatic studies have investigated the AMBP gene in liver cancer. AMBP is differently expressed in seven liver cancer cell lines and 17 HCC tissues, according to research. Since hepatitis B is linked to HCC, AMBP might be considered new hepatitis B virus-related HCC hallmark gene (14). The role of *ApoA1* in many cancers has been established (15). Because HCC patients who receive curative therapy are in the very early or early stage of the tumor, *ApoA1* might be a potential biomarker for HCC early detection, prognosis, and surveillance (16). The mechanism of *ApoA1* is still unexplored in HCC, but based on microarray data analysis, decreased *ApoA1* levels have been observed in both the cancerous liver tissue and the serum of HCC patients (15, 16). The role of *ApoA-I* in the synthesis of high-density lipoproteins (HDL), the decrease in ApoA1 transcription, intracellular and secreted ApoA-I, and systemic HDL levels in HCC suggest that this pathway may play a tumor-suppressive function (15). The role of *APOC3* in HCC has been confirmed by bioinformatic studies. *APOC3* is part of the APOC family which consists of components of chylomicrons (CM), very low density lipoprotein (VLDL), and HDL. In individuals with chronic hepatitis B, the *APOC3* polymorphism is thought to represent an independent risk factor for hepatocarcinogenesis and HCC development (17, 18). One of the roles of *APOH*, a complex efficiency and financial produced by the human *APOH* gene, is to bind cardiolipin. In hepatitis B-related HCC tissue, *APOH* was significantly overexpressed. Cardiolipin is a phospholipid found in the inner membrane of mitochondria. Cardiolipin oxidation causes apoptosis, which has implications for the hepatocellular cancer etiology (HCC) (17, 19). Fibrinogen alpha (FGA), beta (FGB), and gamma

Table 3. Differentially expressed pathways between tumor and non-tumor tissues based on KEGG results.

Pathway name	p-value	Genes
Complement and coagulation cascades	0.000038	FGA, FGB, FGG
Platelet activation	0.000144	FGA, FGB, FGG
PPAR signaling pathway	0.001737	APOC3, APOA1

(FGG) are three polypeptide chains that make up the extracellular matrix protein fibrinogen (20). Several studies have found that hyperfibrinogenemia is a common prognostic marker in individuals with malignancies, and that is strongly linked to tumor invasion, metastasis, angiogenesis, and tumor development. However, the mechanism through which fibrinogen promotes cancer development is unknown (21). FGG is one of three peptide chains found in fibrinogen, and it is often seen in various malignant cancers, including HCC. Furthermore, it has been established that FGG is involved in the regulation of fibrinogen synthesis and function. For example, it reduces platelet adherence to fibrinogen by binding with hepatitis B spliced protein (22). *Orm1* is a member of the lipocalin protein family which functions as a transporter of basic and neutrally charged lipophilic substances. Hepatocytes (HPCs) are the primary cells that express Orm1 when they are stressed. Its precise function, on the other hand, remains unknown. Qin, Xian-Yang et al. evidenced that *Orm1* plays a role in HPC growth. Serum levels of Orm1 were shown to be higher in individuals who had their liver cancer surgically removed and in mice who had a partial hepatectomy (PH) (23). The liver and adipose tissues produce retinol binding protein 4 (RBP4) (24), an adipokine that causes obese individuals to develop hyperinsulinemia and type 2 diabetes (25). Furthermore, obesity is a well-known cancer risk factor, and it is closely linked to the development of numerous cancers, including those of the liver (26). TTR has been shown to be a useful biomarker for a variety of cancers, including lung, ovarian, advanced cervical, and endometrial carcinomas (27, 28). It is also involved in the metabolism of retinol. TTR is generated in part by the liver, and its levels may be reduced in severe liver illness, starvation, and inflammation (29, 30). We did not find an effective role for gene TC in HCC or other liver diseases.

The predicted genes were predominantly implicated in several pathways, according to KEGG pathway analysis, including complement and coagulation cascades, platelet activation and PPAR signaling pathway. Zhang et al. (2021) proved that associations exist between enriched differentially expressed genes (DEGs) in complement and coagulation cascades and hepatitis B virus (HBV)-related HCC patients (31). Complement and coagulation pathway dysregulation is generally caused by innate immune system malfunctions (32). Complement, contact/coagulation, and fibrinolytic mechanisms make up the majority of this system. Activation of these systems causes endothelial cells, leukocytes, and platelets to become activated, resulting in thrombosis and inflammatory responses (33). New data suggests that thrombo-inflammation has a role in cancer development (34). Increased platelet activation can induce venous thromboembolism, which is a risk factor for malignant prognoses (35). Abnormal blood emboli, especially in advanced stages of cancer, have a significant impact on tumor recurrence and metastasis as well as the disease's prognosis (36). Furthermore, liver tumor tissue produces more prothrombin than normal liver tissue, resulting in platelet activation. As a result, platelet function is likely to represent the health of HCC patients (35, 37). PPAR signaling has manifested its role in the field of carcinogenesis, type 2 diabetes, and other metabolic disorders such as obesity; it could be an interesting field of research for the development of new strategies useful in preventing and treating cancer that understand the potential molecular mechanisms which underlie the interplay between metabolism, PPAR signaling, and cancer (38). Mello et al. (2016) discussed the roles of PPAR in modulating liver mitochondrial metabolism from HCC to nonalcoholic fatty liver disease, hypothesizing new treatment methods to preferentially cope with HCC fuel requirements in the future (39).

Conclusion

The current study identified eleven prognostic genes that participate in aberrant metabolism in HCC and developed a metabolic eleven-gene signature that provides more powerful prognostic information and improves the survival prediction for HCC. Moreover, several significant pathways have been identified to provide new insights into the development of HCC.

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Conflict of interests

The authors declare that they have no competing interests.

Restrictions

We need a computer with 100 GB of RAM to run programs. RNA-Seq data is not available in Iran and we used data from the GEO database.

Suggestion for future investigations

Researchers can use this algorithm for other types of cancer. This algorithm can be applied to other diseases such as Alzheimer's.

Researchers can merge RNA-seq and microarray data and then apply the algorithm.

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