

# Prognostic Value of a Novel Multi-mRNA Signature for Predicting Disease Progression of Hepatoblastoma

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**Wang *et al.*: Novel Multi-mRNA Signature for Predicting Disease Progression of Hepatoblastoma**

Hepatoblastoma is the most common tumor of liver in childhood. The onset of the disease is insidious, and the early stage is mostly asymptomatic. About 20 % of the children had distant metastasis at diagnosis. Only 50 %-60 % of the tumors can be completely resected by surgery, and the relapse percentage of children who undergo surgical treatment alone is still high. Therefore, the aim was to conduct a multi-messenger RNA signature to improve hepatoblastoma progression prediction. Messenger RNA expression profiling of Gene Expression Omnibus dataset (GSE131329) was analyzed in a large hepatoblastoma cohort and screened the significantly differential expressed Messenger RNAs between normal tissues and tumors. Weighted correlation network analysis was used to select genes that correlated to disease progression. Data fitting analysis was performed and three samples were removed from subsequent analyses in GSE131329. A total of 1263 differentially expressed genes were screened, consisting 569 up-regulated genes and 694 down-regulated genes. Gene ontology and Kyoto encyclopedia of genes and genomes pathway enrichment analyses were performed and by using weighted correlation network analysis, 8 hub genes were selected and an 8-messenger RNA-based signature was established that was significantly related to the disease progression of hepatoblastoma. Our results might provide an efficient clinical multiple-messenger RNA signature for predicting disease progression and be potentially efficient targets for hepatoblastoma patients.

**Key words:** Hepatoblastoma, gene expression omnibus database, weighted correlation network analysis, messenger RNA signature, hub gene

Hepatoblastoma (HB) is a vulgar primary malignant pediatric hepatic tumor worldwide and over 90 % of the patients are younger than 5 y old<sup>[1,2]</sup>. Despite of the rarity, the incidence is increasing slowly<sup>[3]</sup>. Premature birth and very low birth weight are generally considered as major risk factors of HB<sup>[3-6]</sup>. There are also many factors such as oxygen therapy, medications, Total parenteral nutrition (TPN) and some toxins, but the exact mechanisms still remain unclear<sup>[3,4]</sup>.

An increasing amount of evidences have demonstrated that genetic abnormalities may play an important role and can be identified as molecular biomarkers of HB<sup>[7,8]</sup>. Numerous studies are focused on genes involved in the Wnt signaling pathway<sup>[7,9]</sup>, as the activation of this pathway could lead to the proliferation of HB and promote its development. However, functions of numerous genes and signal pathways were involved

in HB, thus targeting single gene as a prognostic or therapeutic factor for HB was lack of accuracy. Therefore, the aim of this study was discovering multi-messenger RNA signature to get a better prediction of disease progression of HB.

Gene Expression Omnibus database (GEO) is one of the public databases that offer large amounts of raw and normalized array-related and sequence-related data for study<sup>[10,11]</sup>. Large amounts of data for studies could be obtained and analyzed by the usage of bioinformatic methods<sup>[11,12]</sup>. We identified significant differential genes between HB tumors and normal liver tissues in a dataset of GEO database. Then, we conducted weighted correlation network analysis (WGCNA)<sup>[13,14]</sup> and established an 8-mRNA signature for disease progression prediction. Enrichment analysis and pathway analysis were performed. All of these

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may discover some efficient targets to individualized therapy for HB patients.

## MATERIALS AND METHODS

### Data collection of HB

The gene expression data of HBs were searched and downloaded from GEO (<https://www.ncbi.nlm.nih.gov/geo/>) database. There was one appropriate HB dataset from GEO database (GSE131329) met the following criteria: samples from tumor or normal tissues of homo sapiens, not from cell lines or other species; the number of samples larger than 10, consisting both tumor and non-neoplastic tissues; more than 90 % of the total transcriptional genes ( $n > 17000$ ) are annotated; and a total of more than 100 differentially expressed genes (DEGs).

### Data Preprocessing

The R package “arrayQualityMetrics” was used for preparing and normalizing the raw microarray data in order to give more accurate information of the original datasets<sup>[15]</sup>. By using averaging and data fitting methods, microarray quality was evaluated. First, a heatmap was obtained covered the range of distances of samples. Intended biological factors or batch effects could lead to the outlier of the arrays. Then, boxplots of the intensity distributions of the arrays was conducted. Similar positions and widths of the boxes indicate the normalized and accessible data. Furthermore, MA plots were conducted. M and A are defined as:  $M = \log_2(I1) - \log_2(I2)$ ;  $A = 1/2 (\log_2(I1) + \log_2(I2))$ . Finally, boxplots of normalized unscaled standard errors (NUSE) were conducted for data fitting. If the NUSE separated from 1, the samples were considered unqualified. After preprocessing data, qualified data of the dataset went on for further studying.

### Analysis of microarray datasets

The R packages “GEOquery” and “limma” were used to obtain data and screen DEGs between samples of HB and nonneoplastic tissues in GSE131329<sup>[16,17]</sup>. Calculated genes with adjusted p value  $< 0.05$  and  $|\text{fold change (FC)}| \geq 2$  were considered as DEGs. A total of 1263 DEGs were screened, among which 569 genes were up-regulated and 694 were down-regulated.

### GO enrichment analysis and KEGG pathway analysis

Gene Ontology (GO) analysis is an annotating method

of genes and its products consist of three aspects, such as biological processes (BP), cellular component (CC) and molecular function (MF). The Kyoto Encyclopedia of Genes and Genomes (KEGG), (<http://www.genome.ad.jp/kegg/>) database is used for systematic biological analysis and annotation of gene functions, and it also can be used to visualize these associations. In this study, we used the R package “clusterProfiler”<sup>[18]</sup> to provide functional systematization analysis and KEGG pathway analysis of the DEGs, respectively.

### Establishment of WGCNA

R package “WGCNA” was used for the establishment. First, the best thresholding power parameter ( $\beta$ ) was determined by the approximate scale-free topology criterion. Modules which involved similar genes were aggregated through the dynamic tree cut method. Module eigengenes (MEs) and module significance (MS) were used to identify the distinguished modules which are related to the different clinical traits of HB patients. The module which was highly related to given clinical characteristics was chosen. Hub genes were selected from the module, and accorded with the clinical feature relationship ( $\text{cor.geneTraitSignificance} > 0.2$  and the absolute value of the Pearson’s correlation ( $\text{cor.geneModuleMembership} > 0.8$ ).

### Statistical analysis

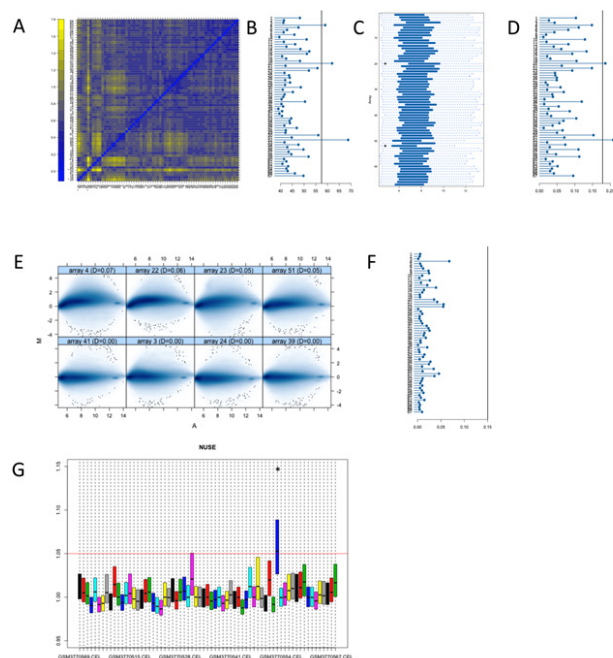
SPSS 17.0 and GraphPad Prism 6 software were used for statistical analysis. Results are exhibited as the mean  $\pm$  SEM. The results were assessed using two-tailed, unpaired or paired Student’s t-test.  $p < 0.05$  was considered statistically significant.

## RESULTS AND DISCUSSION

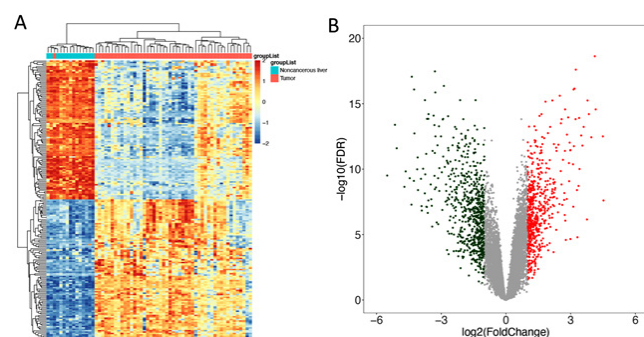
First, we performed data fitting analysis for all the original data in GSE131329 by the usage of R package “arrayQualityMetrics”. Heatmap of the distances between arrays, signal intensity distribution boxplot, MA plot, NUSE boxplot were shown (fig.1). Therefore, our results showed that GSM3770505, GSM3770521, GSM3770553 were unqualified. These three samples should be dislodged without further analysis in GSE131329.

After analyzing the HB dataset using the R packages “GEOquery” and “limma”, a total of 1263 DEGs were screened, including 569 genes up-regulated and 694 genes down-regulated in HB patients’ samples compared to normal samples. Heatmap and volcano plots (fig. 2) showed the distribution and correlation of

the total of DEGs.



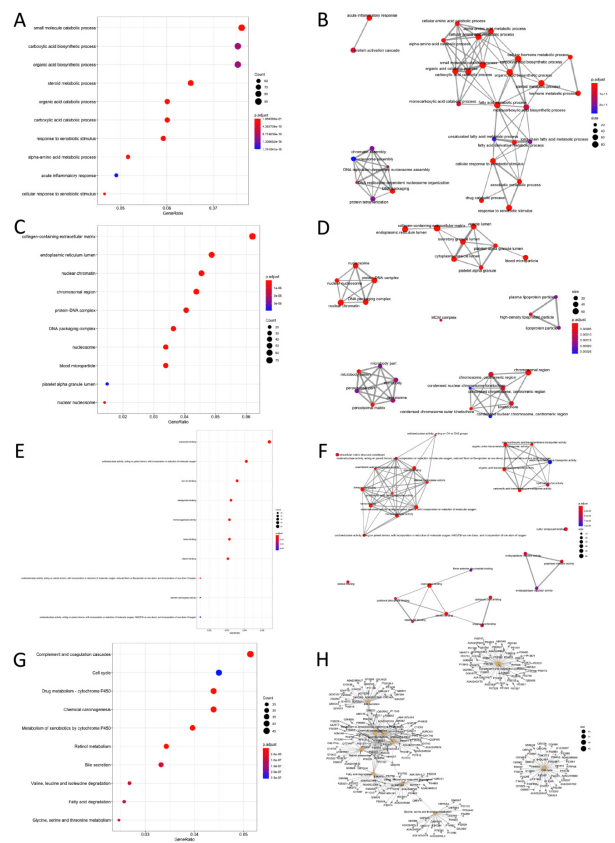
**Fig. 1:** Data preparation of a GEO dataset. (a) Heatmap of the distances between arrays. 3 arrays are detected and considered outliers. (b) A bar chart of the total of distances. 3 arrays exceed the threshold and are considered outliers. (c) Boxplots of the signal intensity distributions of the arrays. (d) A bar chart of the Kolmogorov-Smirnov statistic Ka. 2 arrays exceed the threshold and are considered outliers. (e) MA plots of the arrays. Shown are first the 4 arrays with the highest values of Hoeffding's statistic Da, then the 4 arrays with the lowest values, on the joint distribution of A and M. The value of Da is shown in the panel headings. (f) A bar chart of the Da. None of the arrays exceed the threshold and is considered an outlier. (g) The NUSE of arrays. 1 array exceeds the threshold and is considered an outlier. Arrays considered outliers are detected and marked by an asterisk, \*.



**Fig. 2:** Differentially expressed genes identification in hepatoblastoma from GSE131329. (a) A heatmap of DEGs. (b) A volcano plot of DEGs in the dataset. (X-axis:  $\log_2(\text{Fold Change})$ ; Y-axis:  $-\log_{10}(\text{FDR})$ ). Genes with  $\text{FDR} < 0.05$  and  $|\text{FC}| \geq 2$  were considered as DEGs. The color blue, grey and red represents genes down-regulated, genes non-differential and genes up-regulated, respectively).

GO and KEGG pathway enrichment analyses were conducted for these differential genes. As shown in

fig. 3A-F, differential genes were most enriched in small molecule catabolic process, collagen-containing extracellular matrix and coenzyme binding by GO-BP, GO-CC and GO-MF, respectively. Meanwhile, differential genes were most enriched in complement and coagulation cascades by KEGG pathway analyses (fig. 3G-H).

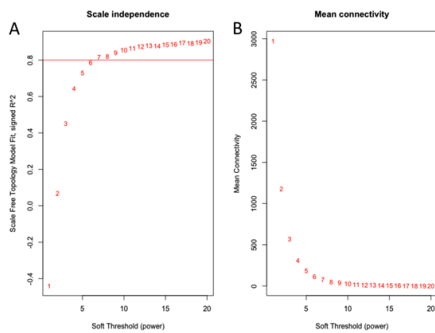


**Fig. 3:** Analysis of GO enrichment and KEGG pathway. (a-b) Dot plot and map plot of GO biological processes (BP) enrichment analysis of DEGs. (c-d) Dot plot and map plot of GO cellular component (CC) enrichment analysis of DEGs. (e-f) Dot plot and map plot of GO molecular functions (MF) enrichment analysis of DEGs. (g-h) Dot plot and net plot of KEGG pathway analysis of DEGs.

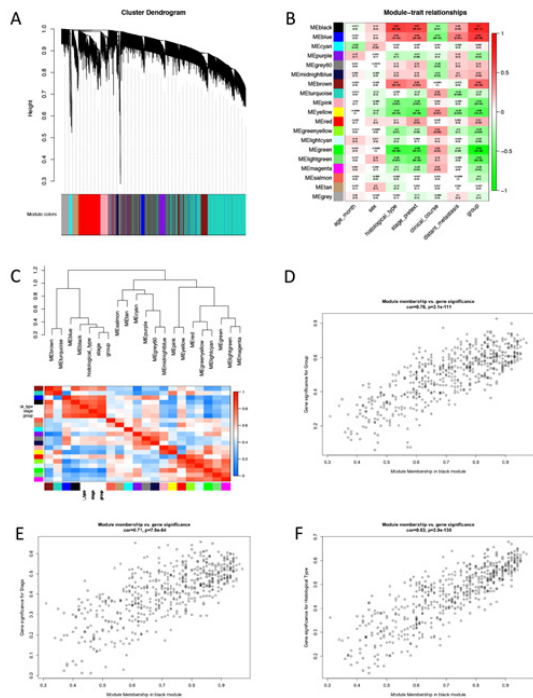
64 qualified HB samples with clinical data were all included. By the usage of the R package “WGCNA”, involved genes which have similar expression patterns were incorporating into one module. In our work, according to the calculation,  $\beta=7$  was determined as the soft-threshold (fig. 4). In this way, 19 modules were identified (fig. 5A). Relevance between key module and the disease progression of HB was tested. Our results showed that genes of the black module possessed the closely relationship with tumor progression ( $p=6 \times 10^{-9}$ ,  $R^2=0.64$  for histological type,  $p=4 \times 10^{-7}$ ,  $R^2=0.57$  for PRETEXT,  $p=4 \times 10^{-11}$ ,  $R^2=0.7$  for group, respectively), fig. 5B-C). Moreover, we also indicated the strong correlation between gene significance and the black



module (fig. 5D-F), which was considered to have more connection with tumor occurrence and development. Therefore, the black module was identified to be a clinically significant module related to HB disease progression in the dataset.



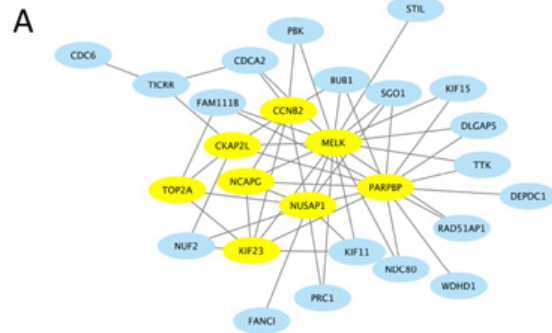
**Fig. 4: Determination of the soft-thresholding power in the WGCNA. (a) Analysis of the scale-free fit index. (b) Analysis of the mean connectivity.**



**Fig. 5: Modules identification relevant to the clinical traits of HB. (a) Clustering dendrogram of genes based on a dissimilarity measure (1-TOM). (b-c) Heatmap of the correlation between module eigengenes and clinical traits of HB. (d-f) The correlation between module membership and gene significance of clinical traits of HB.**

In this study, by the usage of R package “WGCNA”, 8 genes were identified as hub genes and an 8-mRNA signature was established, which had high connectivity in the black module and were also DEGs in GSE131329 (fig. 6). Therefore, Cyclin B2 (CCNB2), Cytoskeleton Associated Protein 2 Like (CKAP2L), Maternal Embryonic Leucine Zipper Kinase (MELK),

DNA Topoisomerase II Alpha (TOP2A), Non-SMC Condensin I Complex Subunit G (NCAPG), Poly [ADP-ribose] polymerase 1 Binding Protein (PARPBP), Nucleolar And Spindle Associated Protein 1 (NUSAP1) and Kinesin Family Member 23 (KIF23) were candidate hub genes.



**Fig. 6: Hub genes identification. (a) mRNA-mRNA interaction network constructed by WGCNA of the hub nodes represented genes.**

HB is fatal malignancy. Over the past decades, surgery alone can cure very few patients with HB, mostly ones with the pure fetal HB variant<sup>[19]</sup>. With the help of pre- and pro-operative chemotherapy, the overall survival rate has increased from 30 % to more than 70 %<sup>[20,21]</sup>. Despite of the increasing survival rate, the occurrence and the development of HB still remain unclear. Therefore, prediction of HB is still arduous and urgent.

From past to nowadays, tumor biomarkers have been used in patients with HB. An elevated serum alpha-fetoprotein (AFP) level was observed in approximately 90 % of HB patients at diagnosis<sup>[21]</sup>. Other two studies have demonstrated that HB patients with low AFP level (<100 ng/ml) at diagnosis are a risk factor, which could lead to negative response to chemotherapy and ultimate poor outcome<sup>[22,23]</sup>. However, this tumor marker is not specific to HB, since its elevation can also be observed in hepatocellular carcinoma (HCC) or hepatitis. It has also been reported in numerous studies that  $\beta$ -catenin/Wnt pathway, deletion and mutation of Catenin Beta 1 (CTNNB1) are important in the pathogenesis of HB<sup>[7,9,24]</sup>. In a recent study, a signature has been designed, and the combination of four genes (Hydroxysteroid 17-Beta Dehydrogenase 6 (HSD17B6), Integrin Subunit Alpha 6 (ITGA6), TOP2A and Vimentin (VIM)), is established and observed to distinguish each subgroup<sup>[25]</sup>. Nevertheless, the association with the genetic prediction with some clinical features, such as tumor size, stage, remains controversial.

The PRE-Treatment extent of tumor (PRETEXT) system, which was established as part of the risk

stratification and treatment planning process, has become the most widely used staging system for pediatric HB patients nowadays. This staging system has been certified useful, not only in selecting treatment plans, but also in predicting overall and disease-free survival outcomes<sup>[26]</sup>. However, this system still had some limitations. It is lack of assessment on account of dependable data<sup>[27]</sup>. It might also neglect the backgrounds of different gene and epigenetics among different tumor subtypes. In conclusion, disease progression prediction of HB still remains obstacle, thus a multi-mRNA signature was conducted to improve more accurately predicting the disease progression for HB patients.

In this study, firstly we selected a dataset from GEO database. Then, we checked data quality, eliminated outlier samples, and finally obtain 1292 DEGs conform to selection standards ( $\text{adj.pval} < 0.05$  and  $|\text{FC}| \geq 2$ ). WGCNA was utilized to identify the most relevant modules and genes associated with clinical features, such as histological type and stage of HB. Finally, a multiple-mRNA signature was established for HB patients. The black module was identified and selected, in which 8 genes were screened as hub genes.

Functions of majority of the 8 genes have been demonstrated in numerous tumor-related studies. Qian *et al.* found that overexpression of CCNB2 protein was associated with clinical progression and poor prognosis in non-small cell lung cancer<sup>[28]</sup>. Shubbar *et al.* also confirmed that cytoplasmic CCNB2 may act as an oncogene and could be a potential prognosis biomarker in breast cancer<sup>[29]</sup>. Xiong *et al.* found that CKAP2L played an important role in lung adenocarcinoma (LAD) development and could predict poor prognosis of LAD patients<sup>[30]</sup>. Chen *et al.* found that MELK enhances tumorigenesis, migration, invasion and metastasis of esophageal squamous cell carcinoma cells via activation of forkhead box protein M1 (FOXO1) signaling pathway<sup>[31]</sup>. Xu *et al.* demonstrated a similar result which suggested that the MELK axis may play a crucial role in the disease progression of endometrial carcinoma (EC)<sup>[32]</sup>. Zhang *et al.* found that the knockdown of TOP2A could increase the sensitivity to paclitaxel in EC cells<sup>[33]</sup>. Abraham *et al.* demonstrated that TOP2A was required for T-cell factor transcription as a DNA-binding factor, thus could be an epithelial-mesenchymal transition regulator and be the metastatic colorectal cancer therapeutic target<sup>[34]</sup>. Gong *et al.* found that NCAPG functioned as an oncogene in HCC and its overexpression could promote proliferation and antiapoptosis of tumor cells through PI3K/AKT/

FOXO4 pathway activation<sup>[35]</sup>. Meanwhile, Zhang *et al.* found that expression level of NCAPG in clinical HCC samples was in accordance with recurrence and poor survival<sup>[36]</sup>. Nicolae *et al.* showed that in myeloid leukemia cells, PARPBP was overexpressed, and PARPBP downregulation could reduce cell proliferation via the activation of the NF- $\kappa$ B pathway<sup>[37]</sup>. Zhang *et al.* found that NUSAP1 was overexpressed in ovarian cancer tissues and cell lines, and knocking down NUSAP1 could lead to significantly proliferation and colony forming, migration and invasion ability inhibition, meanwhile promote apoptosis and affect cell cycle distribution<sup>[38]</sup>. Also, Gao *et al.* found that NUSAP1 was upregulated in bladder cancer, and its expression was relevant to the poor prognosis of patients via the TGF- $\beta$  signaling pathway<sup>[39]</sup>. Li *et al.* found that KIF23 expression was correlated with poor prognosis of gastric cancer and associated between KIF23 and Pathological Tumor-Node-Metastasis (pTNM) stage<sup>[40]</sup>. In summary, these 8 genes had been researched in many other types of tumors, while few studies involved in HB, both in clinical and basic fields.

There were still some limitations in our study. Firstly, we only used a dataset of HBs in only one database. Because of the lack of clinical information (such as recurrence free survival time and overall survival time), the GEO dataset could not be used to construct a more precise model. Besides, the most commonly used database, The Cancer Genome Atlas (TCGA) database, does not include HB. Secondly, in addition to mRNA, the predictive value of whole transcriptome, including microRNA, lncRNA and circRNA in tumor prognosis and outcomes had been validated to some extent. Multi-dimensional data analysis could enhance the predictive accuracy and efficiency. Finally, because of the lack of clinical samples, this signature model needs to be further validated. In conclusion, our study established an 8-mRNA signature for the prediction of the occurrence and development of HB, which might serve as potential molecular targets for personalized medicine in HB.

Therefore, it is found that the chondrorepair activity of progenitor cells can be stimulated by the modification of various genes in stem cells by the independent gene transduction of slow virus vector, which provides the basis for controlling the cartilage repair process after hADSCs are implanted into cartilage defects.

The benefit of co-transduction of therapeutic lentiviral vector gene repairer hADSCs provides evidence that

enhancing the expression of Sox9 and knockdown  $\beta$ -catenin can significantly improve its cartilage repair activity *in vitro*.

### Author's contributions:

Bingrui Wang and Qiucheng Han have contributed equally to this research.

### Conflict of interests:

The authors declared no conflicts of interest.

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