ADH4 is a TP53-associated immunometabolic signature for prediction of prognosis in hepatocellular carcinoma

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Abstract

Background: Tumor protein p53 (TP53) is one of the most frequent mutated genes in hepatocellular carcinoma (HCC), whose mutations influenced tumor microenvironment (TME) and associated with a worse prognosis. Thus, finding out an accurate prognostic signature could be beneficial for improving the prognosis of HCC.

Methods: HCC genetic mutation data, transcriptome data and clinical data were downloaded from the TCGA database to clarify specific TP53-associated signature based on differential expression genes (DEGs). We investigated the predictive value of this signature on the overall survival (OS), immune analysis and validation in clinical specimens.

Results: In total, our research exhibited 270 mutant genes and TP53 mutation occupied 28%. Besides, 81 upregulated genes and 27 downregulated genes were identified. Enrichment analysis showed that mutant-type TP53 were enriched for pathways related to cell cycle and cell metabolism, while clustered most enriched for terms related to metabolic process and immune response. The gene alcohol dehydrogenase4 (ADH4) was identified by univariate and multivariate Cox regression analysis and a nomogram was also established to validate this prognostic signature. Moreover, the low-ADH4 group in both TP53 mutant type and wild type displayed significantly worse OS than the high-ADH4 group. In addition, immune infiltration with higher expression of B cell groups showed an differential immune microenvironment. Specially, ADH4 expression and the prognostic prediction values were further validated in clinical samples.

Conclusions: The TP53-associated immunometabolic signature is a specific and independent prognostic biomarker for HCC patients, and could provide potential prognostic biomarker for the development of novel immunotherapies.

Background

Primary liver cancer is one of the most aggressive human malignancies around the world, while HCC represents 85–90% of primary liver cancers. For the last decades, the incidence of HCC has doubled and the OS of patients with HCC is still poor as a result of high recurrence and metastasis rates(1). Though great progress has been made toward its prevention, diagnosis and targeted treatment, the clinical outcome of HCC remains unsatisfactory. Growing evidence indicates that the malignant phenotypes of cancers are influenced by TME(2-4). As an immune-sensitive organ, the metabolic function of liver is powerful at the same time. In the past ten years, plenty of studies found that our immune system was strongly related to metabolic functions in a manner that was not previously recognized and it has been described as a new field, called immunometabolism(5-7). However, few studies have systematically explored the relationship between the immunometabolism of TME and its prognosis.

As a transcription factor, TP53 inhibits cell division and survival, therefore acting as a key failsafe mechanism of cellular anti-cancer defenses(8). However, tumor suppressor gene TP53 is always mutated

in human cancers and thus known as a potential prognostic and predictive marker, as well as target for pharmacological intervention(9-11). An increasing number of studies revealed that TP53 mutations in several cancers are associated with increased resistance to cancer therapies and poorer survival prognosis(12, 13). Therefore, finding out the association between immunometabolism of TP53 mutation cancer-related microenvironment and its prognosis is significant and meaningful, especially of HCC.

In this research, we suggested that the OS of patients with HCC harboring TP53 mutations might be significantly influenced by the immunometabolism of TME. Thus, we identified genes affected by TP53 mutation status, and established a immunometabolic gene signature to predict the prognosis of patients with HCC in the clinic.

Material And Methods

Data sources of genome-wide mutation profiling

We downloaded the HCC genetic mutation data, transcriptome data, and clinical data from the TCGA database (<https://tcga-data.nci.nih.gov/tcga/>). To identify the somatic mutations of the patients with HCC in the TCGA database, mutation data were downloaded and visualized using the “maftools” package in R software.

Screening of differentially expression genes

Limma package (version: 3.40.2) of R software was used to study the differential expression of mRNAs. The adjusted P-value was analyzed to correct for false positive results in TCGA or GTEX. “Adjusted $P < 0.05$ and Log (Fold Change) > 1 or Log (Fold Change) < -1 ” were defined as the thresholds for the screening of differential expression of mRNAs.

To further confirm the underlying function of potential targets, the data were analyzed by functional enrichment. Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analysis is a practical resource for analytical study of gene functions and associated high-level genome functional information. Gene Ontology (GO) is a widely-used tool for annotating genes with functions, especially molecular function (MF), biological pathways (BP), and cellular components (CC). To better understand the carcinogenesis of mRNA, ClusterProfiler package (version: 3.18.0) in R was employed to analyze the GO function of potential targets and enrich the KEGG pathway.

Selection of TP53-related prognostic signature

Univariate and multivariate cox regression analysis was performed to build the nomogram. The forest was used to show the P value, HR and 95% CI of each variable through ‘forestplot’ R package. A nomogram was developed based on the results of multivariate Cox proportional hazards analysis to

predict the 1,3,5-year overall survival. The nomogram provided a graphical representation of the factors, which can be used to calculate the risk for an individual patient by the points associated with each risk factor through 'rms' R package.

Kaplan-Meier analysis of overall survival

Raw counts of RNA-sequencing data (level 3), corresponding clinical information and tumor gene mutation MAF data were downloaded from The Cancer Genome Atlas (TCGA) dataset (<https://portal.gdc.cancer.gov/>). For Kaplan–Meier curves, p-values and hazard ratio (HR) with 95% confidence interval (CI) were generated by log-rank tests and univariate Cox proportional hazards regression. Analysis of risk score, survival status and heatmap were implemented by R foundation and software packages ggrisk, survival and survminer, timeROC. All analytical methods above and R packages were performed by R foundation for statistical computing (2020) version 4.0.3 and software packages ggplot2. $p < 0.05$ was considered as statistically significant.

Estimation of immune cell score and percentage abundance of tumor infiltrating immune cells

To make reliable immune infiltration estimations, we utilizes the immunedeconv, an R package which integrates EPIC algorithms. CD274, CTLA4, HAVCR2, LAG3, PDCD1, PDCD1LG2, TIGIT and SIGLEC15 were selected to be immune-checkpoint–relevant transcripts and the expression values of these eight genes were extracted. Analysis methods and R package were implemented by R foundation for statistical computing (2020) version 4.0.3 and software packages ggplot2 and pheatmap.

Collection of HCC samples

Surgically resected specimens were obtained in October, 2021 from 5 cases of HCC patients at the Renji Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (Shanghai, China). This investigation was approved by the ethics committee of Renji Hospital and follow the guidelines of the declaration of Helsinki. All patients provided written informed consent. The inclusion criteria: a. pathological diagnosis was HCC; b. none had received any prior treatment; c. the maximum diameter of single tumor was more than 2 cm; d. Child-Pugh score A or B. The exclusion criteria: a. Child-Pugh score C; b. exceptional circumstances, such as syphilis and Acquired Immune Deficiency Syndrome.

Immunohistochemistry

HCC tissues were paraffin-embedded and paraffin sections (4 μ m) were prepared. Before incubating with antibodies, samples were blocked with goat serum (cat. no. Ab7481; Abcam) for 10 min at room

temperature. For Ki67 staining, sections were incubated with the primary antibody (cat. no. Ab137077; Abcam; 1:500 dilution) and secondary antibody (cat. no. Ab6721; Abcam; 1:1,000 dilution). Sections were dewaxed in xylene and rehydrated in an ethanol gradient of 100, 95 and 80%, and heat-mediated antigen retrieval of the tissue sections was carried out at a temperature of 96-98°C before they were allowed to cool. Paraffin-embedded sections (4 µm) were incubated with primary antibodies overnight at 4°C. The secondary antibody was used to detect the primary for 1 h at room temperature. Confocal laser scanning microscopy was performed using an Olympus Corporation BX51 instrument.

Western blot assay

The liver tissue samples were treated with lysis solution. After homogenizing, the proteins extracted from liver tissues samples were quantified using the Bradford method. These protein samples were then separated using SDS-PAGE under a 100v voltage for 1hrs and transferred onto a PVDF membrane. After the transferred membranes were blocked in 5% skim milk for 1hrs, blots were incubated with primary antibodies (cat. no. Ab137077; Abcam; 1:1000 dilution), diluted with 5% skim milk overnight. After the transferred membranes were washed with TBST for three times, blots were re-incubated for 1hrs with second antibodies (cat. no. Ab6721; Abcam; 1:1,000 dilution) diluted with 5% skim milk. Then, sections were washed with TBST for three times. At last, transferred membranes were scanned and analyzed using the image system.

ICB (Immune checkpoint blockage) response prediction

Potential ICB response was predicted with TIDE algorithm([14](#)) and software packages ggplot2(v3.3.3) and ggpubr(0.4.0). All analytical methods above and R packages were performed using R software version v4.0.3 (The R Foundation for Statistical Computing, 2020). $p < 0.05$ was considered as statistically significant.

Statistical analysis

All statistical analyses were performed using R version 4.0.3. Two-tailed Student's t test was used for significance of differences between subgroups. One-way ANOVA test or Student's t test were applied to analyse the correlation between risk score and clinicopathological parameters. The data from two groups were compared by Wilcoxon Test and more than three groups by Kruskal-Wallis test. $P < 0.05$ was considered statistically significant.

Results

TP53 is the most frequent mutations in HCC

The flowchart of our research is shown in Figure 1. A total of 270 mutant genes in HCC were screened from the raw counts of data from TCGA cohort. To identify the somatic mutations of the patients with HCC in the TCGA database, mutation data were down loaded and visualized using the “maftools” package in R software. Horizontal histogram showed the genes have the higher mutation frequency in patients with HCC, such as TP53 (28%), TTN (25%), CTNNB11(21%), MUC16 (16%) and PCLO (10%, Fig.2A). Missense mutations were the most common type of mutation in patients with HCC (Fig.2Ba). Single nucleotide polymorphism (SNP) occupied an absolute position compared with deletion (DEL) or insertion (INS) (Fig.2Bb). C>T was the predominant mutation type detected (Fig. 2Bc). The number of mutations per sample was shown in Fig.2Bd. The box diagram of each color represents a kind of mutation in Fig.2Be. The stacked barplot in Fig.1Bf shows top ten mutated genes. Figure 2C shows a lollipop plot for a highly mutated gene, TP53, in HCC.

DEGs in HCC by TP53 status were detected

We explored DEGs by TP53 mutant-type and wild-type groups. In total, 81 upregulated genes and 27 downregulated genes were identified (Fig.3A-B). In addition, KEGG pathway analysis and GO analysis were performed. As a result, mutant-type TP53 were enriched for pathways related to cell cycle and cell metabolism (Fig.3Ca-b). Besides, TP53 mutation genes clustered most enriched for terms related to metabolic process and immune response (Fig.3Cc-d). These results showed that TP53 mutation related genes may probably play a significant role in immunometabolism of microenvironment in HCC.

ADH4 is a distinct prognostic signature for HCC

Seventeen up-regulated genes and seventeen down-regulated genes were identified through univariate (Fig.4A) and multivariate (Fig.4B) cox regression analysis to build the a predictive nomogram, respectively. The predictors included ADH4 and HPD, age of patients and pTNM_stage (Figure 4C, Left), satisfying the criteria of $P < 0.05$ in risk assessment. Significantly, the P-value of ADH4 < 0.001 , indicating that ADH4 is a distinct prognostic signature for HCC. The calibration plots for the 1, 3, 5-year OS rates were predicted well compared with an ideal model in the entire cohort (Figure 4C, Right).

TP53 and ADH4 status were correlated with the prognosis of HCC

The TP53 mutant type and wild type groups included 105 and 253 patients, respectively. Patients in the mutant-type group displayed significantly worse OS than the wild-type group ($P < 0.05$) (Fig.5A). To investigate whether ADH4 was independent of TP53 mutation status, patients with HCC were divided into high- and low-ADH4 groups based on TP53 mutation status. The high- and low-ADH4 groups in TP53 mutant type included 52 and 53 patients, while the high- and low-ADH4 groups in TP53 wild type included 126 and 127 patients, respectively. The results showed that the low-ADH4 group in both TP53 mutant

type and wild type displayed significantly worse OS than the high-ADH4 group ($P < 0.05$) (Fig.5B-C). Besides, we observed that ADH4 wild type occupied a markedly high proportion in HCC patients (chi-square test, Figure 5D), which is consistent with previous hypothesis.

Differential immune analysis were remarkable

To explore the distribution of immune score (Fig.6A) in different groups and the percentage abundance of immune infiltration (Fig.6B), 38 types of immune cells were compared. Among them, 23 types were statistically different. It was noted that there was significant immune infiltration with higher expression of B cell groups and lower expression of T cell groups, showing an differential immune microenvironment. For the expression distribution of immune checkpoint in tissues, CD274, CTLA4, HAVCR2, LAG3, PDCD1, PDCD1LG2 were discovered to be immune-checkpoint-relevant transcripts with a statistical difference (Fig.6C).

ADH4 was validated sucessfully in clinical HCC tissues

To confirm the reliability of the identified gene signature, we examined ADH4 expression levels by immunohistochemistry and western blot assay using 3 pairs of HCC tissues and adjacent normal tissues. The results showed that ADH4 proteins were significantly overexpressed in normal tissues when compared with those in tumor tissues (Fig.7A-B). The risk score of every patient was calculated and obtain the median cut-of point to divide the patients into the high-ADH4 group ($n=185$) and low-ADH4 group ($n=185$) (Fig. 7C-up). Figure 7C-middle showed the survival status of all patients with HCC and the prognostic gene ADH4 expression profile was illustrated by the heatmap (Fig.7C-down). The Kaplan–Meier survival curves showed that the low-ADH4 group had worse OS compared with the high-ADH4 group (Fig.7D). Besides, prognostic signature ADH4 showed a significant AUC values in a time-dependent ROC analysis (Fig. 7E), which meant that ADH4 had effective prediction ability in 1-year, 3-year and 5-year OS. Sankey diagram of ADH4 expression indicated the distribution of same sample in different characteristic variables and different types or stages(Fig.7F). Potential ICB responses indicated that the distribution of immune response scores in low-ADH4 group showed a worse response than high-ADH4 group (Fig.7G).

Discussion

HCC is one of the main cancer-related causes of death worldwide with a poor prognosis⁽¹⁵⁾. Importantly, HCC always developed fast as a result of immunosuppression and reprogramming of metabolism⁽¹⁶⁾. Increasing number of studies indicated that the combination of different immunotherapies and targeted therapies could prolong the survival of patients with HCC^(17, 18). Besides, changes in metabolic reprogramming were significant for hepatocarcinogenesis and prognosis but how this reprogramming occurs is unknown⁽¹⁹⁾. These conclusions indicated that immunometabolism of TME is critical to the prognosis of HCC. As we all know, TP53 mutation displays an increased mutational burden in cancers,

which may influence the immunometabolism of TME(20). Therefore, we developed a TP53-associated immunometabolic signature for prediction of prognosis in HCC, which may have an increased clinical role in the future.

Previous studies have shown that TP53 mutations may play a different role in antitumor immunity(21). In our research, we found that TP53 had a high mutation frequency in patients with HCC and genes modulated according to TP53 status were specifically enriched for GO terms related to immune and metabolic response (Fig. 3C). Wild-type TP53 plays fundamental roles in cancer immunity while mutant-type TP53 subvert the immune function, which are associated with immune dysfunction, thereby promoting tumorigenesis, invasion and metastasis(22). In order to make a deeper exploration on the changes in immunometabolism of TME, we constructed a nomogram and discovered a distinct prognostic signature ADH4 (Fig. 4), which was an effective independent prognostic model for HCC. Besides, we found that the predictors including age of patients and pTNM_stage significantly affected the overall survival of patients with HCC, which was consistent with previous assumptions. What's more, the low-ADH4 group in both TP53 mutant type and wild type displayed significantly worse OS than the high-ADH4 group (Fig.5B-C), indicating that ADH4 is a positive signature for the prognosis of HCC and the type of ADH4 suggested to be wild type. According to the literature research, we realized that ADHs are huge family of dehydrogenase enzymes and associated with the positive prognosis of various cancers(23). To investigate the accurate expression type of ADH4 in HCC, distributions of ADH4 mutant type and ADH4 wild type were compared in TP53 mutant group, TP53 wild group and total HCC group. Surprisingly, our results showed that ADH4 wild type occupied a markedly high proportion in HCC patients, which is consistent with previous hypothesis. In conclusion, ADH4 is an immunometabolic protective factor and low-ADH4 can be regarded as a high-risk factor for the prognosis of HCC.

As we know, immunometabolic relationships form the basis of life and are at the center of many diseases including cancer, immune-mediated diseases, and metabolic syndrome(24). The changes in the TME were closely associated with immune cell phenotype and function(25). According to the interactions between the types and functions of immune cells, immune cells may play a variety of roles in the development of tumors. The tumour-infiltrating leucocytes is known to impact clinical outcome in HCC. In addition, the close relationship between tumor-infiltrating T cells and B cells indicates a functional interaction which is related to local immune activation and better prognosis for HCC(26). Our results indicated that there was significant immune infiltration with higher expression of B cell groups and lower expression of T cell groups, showing an differential immune microenvironment (Fig. 6A-B). The results may suggest that the different prognosis is related to interaction between different percentage abundance of immune infiltration. Besides, CD274, CTLA4, HAVCR2, LAG3, PDCD1, PDCD1LG2 were discovered to be immune-checkpoint-relevant transcripts (Fig.6C), which may provide patients with more chances and greater benefits from immunotherapy and chemotherapy (27).

The distribution of ADH4 expression and the prognostic prediction value was further explored in clinical specimens. We found that ADH4 protein was remarkably overexpressed in normal tissues comparing with those in tumor tissues and low-ADH4 group had worse OS compared with the high-ADH4 group (Fig. 7),

which indirectly confirms the previous analysis. Consistently, our results indicated that high-risk HCC patients were more likely to respond to ICB response, which might further highlight a potential strategy for clinical guidance. Properly, limited in the amounts of clinical informations in database, further we need more clinical trials to verify the research results.

Conclusion

Our results identified ADH4, as an immunometabolic signature associated with TP53 mutation, that can independently predict the prognosis of patients with HCC and may serve as an accurate biomarker to the novel immunotherapies.

Abbreviations

Tumor protein p53, TP53; Hepatocellular carcinoma, HCC; Tumor microenvironment, TME; Differential expression genes, DEGs; Overall survival, OS; Alcohol dehydrogenase ADH.

Declarations

• Ethics approval and consent to participate

This investigation was approved by the ethics committee of Renji Hospital and follow the guidelines of the declaration of Helsinki. All patients provided written informed consent.

• Consent for publication

Not applicable.

• Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

• Competing interests

The authors declare that they have no competing interests.

• Funding

Not applicable.

• Authors' contributions

YZ has made substantial contributions to the conception and the work of manuscript editing; HHJ has made substantial contributions to the design of the work; ZYW has made substantial contributions to the acquisition and analysis of data; BZ has approved the modified version and accountable for several aspects of the work; MBL has agreed to ensure that questions related to the accuracy or integrity of all parts of the work. All authors read and approved the final manuscript.

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Figures

Figure 1

Flowchart of the whole study.

Figure 2

Genome-wide mutation profiling in HCC. (A) Oncoplot displaying the somatic landscape of HCC cohort. Genes are ordered by their mutation frequency and samples are ordered according to disease histology as indicated by the annotation bar (bottom). Side bar plot shows log₁₀ transformed Q-values estimated by MutSigCV. (B) Cohort summary plot displaying distribution of variants according to variant classification, type and SNV class. A stacked barplot shows top ten mutant genes. (C) Lollipop plot displaying mutation distribution and protein domains for TP53 in HCC with the labeled recurrent hotspots.

Figure 3

Identification of DEGs in patients with and without TP53 mutations in HCC. (A) Heatmap of DEGs. (B) Volcano plots of DEGs. (C) The enriched KEGG signaling pathways of primary biological actions of target mRNAs and GO analysis of potential targets of mRNAs. $P < 0.05$ or FDR < 0.05 is considered to be enriched to a meaningful pathway.

Figure 4

Construction of prognostic signature based on DEGs. (A-B) Prognostic values of DEGs by univariate and multivariate Cox regression analysis. (C) Nomogram to predict the 1-y, 3-y and 5-y overall survival of HCC patients. A dashed diagonal line represents the ideal nomogram, and the blue line, red line and orange line represent the 1-y, 3-y and 5-y observed nomograms.

Figure 5

Kaplan-Meier analysis of overall survival according to TP53 mutation and ADH4 status. (A) Kaplan-Meier survival by TP53 status. (B) Kaplan-Meier survival in TP53 mutant group. (C) Kaplan-Meier survival in TP53 wild group. (D) The expression type of ADH4 mRNA in different groups of HCC (chi-square test).

Figure 6

Immune analysis of high- and low-risk patients with HCC. (A) The heat map of immune cell score. (B) The percentage abundance of tumor infiltrating immune cells. The abscissa represents the sample, and the ordinate represents the percentage of immune cell content in a single sample. (C) The expression distribution of immune checkpoint in tissues. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, the significance passed the Kruskal-Wallis test).

Figure 7

Validation of the gene signature in clinical HCC tissue samples. (A) Representative images of IHC staining for ADH4 expression in adjacent non-tumor tissues (up) and HCC tissues (down). (B) Western blot assay in adjacent non-tumor tissues and HCC tissues. (C) The curve of risk score (up). Survival status of the patients (middle). The heatmap of the prognostic gene ADH4 expression profile (down). (D) Kaplan-Meier survival analysis of patients with HCC by ADH4 status. (E) Time-dependent ROC analysis of ADH4 signature. ROC receiver operating characteristic. (F) Sankey diagram of ADH4 expression, which indicates the distribution of same sample in different characteristic variables and different types or stages. (G) Potential ICB responses of HCC groups by ADH4 status, which indicates the distribution of immune response scores in different groups in the prediction results. Upper statistical table indicates the amounts of positive immune response of samples.