ADH4 Is A TP53-Associated Immune-Metabolic Signature For The Prediction Of Prognosis In Hepatocellular Carcinoma

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Research Article

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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 are 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 signatures 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 a differential immune microenvironment. Especially, ADH4 expression and the prognostic prediction values were further validated in clinical samples.

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

Background

HCC is one of the most aggressive malignancies around the world. In the past several decades, the incidence of HCC has increased and the prognosis is still poor as a result of high recurrence and metastasis rates(1). The clinical outcomes of patients with HCC remain unsatisfactory, although great progress has been made toward its prevention. Plenty of evidence indicates that the phenotypes of tumors are remarkably 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, the tumor suppressor gene TP53 is always mutated in human cancers and is thus known as a potential prognostic and predictive marker, as well as
a 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 an immune-metabolic gene signature to predict the prognosis of patients with HCC in the clinic.

**Material And Methods**

**Collection of genome-wide mutation data**

HCC genetic mutation data, transcriptome data, and clinical data were downloaded from The Cancer Genome Atlas (TCGA) database. Mutation data were visualized by using the “maftools” package in R software.

**Screening and gene enrichment analysis of DEGs**

Limma package of R software was used to screen the DEGs and ClusterProfiler package was performed to analyze the KEGG pathway and the GO function. “P<0.05 and Log (Fold Change) >1 or Log (Fold Change) <-1” were considered as the threshold.

**Selection of specific signature**

The univariate and multivariate cox regression analyses was performed and the “forestplot” package was used to show the p-value, hazard ratio (HR), and 95% confidence interval (CI) of each variable. “P<0.05” was defined as the threshold. The nomogram based on the results of multivariate cox analysis exhibited a list of the risk factors by “rms” package and predicted the 1,3,5-year OS. Analysis of risk score, survival status and heatmap were implemented by “ggrisk” package of R software. All analytical methods were performed by R software (version 4.0.3) with “ggplot2” package. “p < 0.05” was considered as statistically significant.

**Estimation of immune cell score and immune infiltration**

We utilized the “immunedown” package integrating EPIC algorithm to estimate the immune cell score and immune infiltration. CD274, CTLA4, HAVCR2, LAG3, PDCD1, PDCD1LG2, TIGIT, and SIGLEC15 were selected to be immune-checkpoint–relevant transcripts, and the expression values of these genes were extracted. Analysis was implemented by R software (version 4.0.3) with “ggplot2” and “pheatmap” packages.
Collection of HCC specimens

Surgically resected specimens were obtained in October 2021 from five patients with HCC at the Renji Hospital Affiliated with Shanghai Jiao Tong University School of Medicine and none of them had received any prior treatment. All the fresh specimens were conserved in the liquid nitrogen. This research was approved by the ethics committee of Renji Hospital and informed consent was provided by enrolled patients.

Immunohistochemistry

All HCC tissues were paraffin-embedded and cut into 4 µm thick sections. Then, sections were dewaxed with xylene and hydrated with alcohol. After blocking non-specific sites with serum albumin, sections were incubated with primary antibody (cat. no. Ab137077; Abcam; 1:500 dilution) overnight at 4°C and secondary antibody (cat. no. Ab6721; Abcam; 1:1,000 dilution) for 1 h at room temperature.

Western blot assay

The tissues were treated with lysis solution, supplemented with 1% protease inhibitors (P8340, Sigma-Aldrich), to extract interest protein. After homogenizing, the proteins extracted from HCC samples were quantified using the Bradford method. Western blot analysis was then performed as previously described(14). Primary antibody (cat. no. Ab137077; Abcam; 1:1000 dilution), and second antibody (cat. no. Ab6721; Abcam; 1:1,000 dilution) were used.

Immune checkpoint blockage (ICB) response prediction

Potential ICB response was predicted with TIDE algorithm(15) and software packages “ggplot2” and “ggspr”。 All analytical methods were performed using R software version v4.0.3. “P < 0.05” was considered as statistically significant.

Statistical analysis

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

Results

TP53 is the most frequent mutation in HCC

The flowchart of our research was shown in Figure 1. A total of 270 mutant genes in HCC were screened from the TCGA cohort. The screened genes like TP53 (28%), TTN (25%), CTNNB11(21%), MUC16 (16%), and PCLO (10%) had higher mutation frequency and were exhibited directly by the horizontal histogram
Missense mutation was the most common type of TP53 mutation (Fig. 2Ba). Single nucleotide polymorphism showed a predominant position compared with deletion or insertion (Fig. 2Bb). C>T was the most distinct mutation type (Fig. 2Bc). The number of mutations per sample was shown in Fig. 2Bd. Each color in the box diagram represented one kind of mutation (Fig. 2Be). The stacked barplot in Fig. 1Bf showed the top ten mutant genes. Figure 2C showed a lollipop plot for TP53, a highly mutated gene, in HCC.

DEGs in HCC by TP53 status was 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, mutant-type TP53 was 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). All these results showed that TP53-related mutation genes may probably play a significant role in the 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 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, indicates 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 the previous hypothesis.

Differential immune analysis was remarkable

To explore the distribution of immune scores (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 a differential immune microenvironment.
For the expression distribution of immune checkpoints 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 successfully 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-off 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 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 the 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 the low-ADH4 group showed a worse response than the high-ADH4 group (Fig.7G).

Discussion

HCC is one of the main cancer-related causes of death worldwide with a poor prognosis(16). Importantly, HCC always developed fast as a result of immunosuppression and reprogramming of metabolism(17). An increasing number of studies indicated that the combination of different immunotherapies and targeted therapies could prolong the survival of patients with HCC(18, 19). Besides, changes in metabolic reprogramming were significant for hepatocarcinogenesis and prognosis but how this reprogramming occurs is unknown(20). 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(21). Therefore, we developed a TP53-associated immune-metabolic signature for the 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(22). 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 subverts the immune function, which is associated with immune dysfunction, thereby promoting tumorigenesis, invasion, and metastasis(23). In order to make a deeper exploration on the changes in immunometabolism of TME, we constructed a nomogram and discover a distinct prognostic
signature ADH4 (Fig. 4), which was an effective independent prognostic model for HCC. Besides, we found that the predictors including the 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 a huge family of dehydrogenase enzymes and are associated with the positive prognosis of various cancers(24).

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 the previous hypothesis. In conclusion, ADH4 is an immune-metabolic protective factor and low-ADH4 can be regarded as a high-risk factor for the prognosis of HCC.

As we know, immune-metabolic relationships existed in various diseases like cancer, metabolic syndrome, and immune-mediated diseases(25). The changes in the TME were closely associated with the phenotype and function of immune cells (26). In fact, immune cells played a variety of critical roles in the development of malignant tumors, especially in HCC. For example, the tumor-infiltrating leucocyte was found to significantly impact the development progress of HCC(27). Researchers had discovered that the functional interaction between tumor-infiltrating T cells and B cells is contributed to local immune activation and a better prognosis for HCC. Accordingly, our results indicated that there was significant immune infiltration with higher expression of B cell groups and lower expression of T cell groups showing a differential immune microenvironment (Fig. 6A-B). The results may suggest that the different prognosis is related to the 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 (28).

The distribution of ADH4 expression and the prognostic prediction value were further explored in clinical specimens. We found that ADH4 protein was remarkably overexpressed in normal tissues compared with those in tumor tissues and the 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 information in the database, further we need more clinical trials to verify the research results.

**Conclusions**

Our results identified ADH4, as an immune-metabolic 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; Kyoto Encyclopedia of Genes and Genomes, KEGG; Gene Ontology, GO; The Cancer Genome Atlas, TCGA; Hazard ratio, HR; Confidence interval, CI; Immune checkpoint blockage, ICB.

Declarations

Ethics approval and consent to participate

This investigation was approved by the ethics committee of Renji Hospital and followed 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.

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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 is 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
364 HCC samples in **TCGA cohort**

- Genome-wide **mutation profiling** in HCC
  - Altered in 270 (74.18%) samples
    - 105 TP53-MT and 254 TP53-WT
    - 81 up-regulated and 27 down-regulated

- Identification of **differential expression genes**
  - KEGG pathway analysis
    - Cell cycle
    - Metabolism of cytochrome P450
  - GO analysis
    - Nuclear division
    - Regulation of humoral immune response
    - Fatty acid metabolic process

- **Univariate** Cox regression
- **Multivariate** Cox regression

1-y, 3-y and 5-y overall survival

- **Nomogram**
- **Optimal prognostic gene ADH4**

- **Validation of** ADH4 in HCC
  - **Kaplan-Meier analysis** of overall survival
    - With and without TP53 mutation
    - High- and low-ADH4 with TP53 mutant type
    - High- and low-ADH4 with TP53 wild type
    - The expression type of ADH4 in HCC
  - **Immune analysis**
    - Immune cell score
    - Immune infiltration
    - Immune checkpoint
  - IHC staining
  - Western blot
  - Risk score
  - Kaplan-Meier survival
  - ROC analysis
  - Sankey diagram
  - Potential ICB response

**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. (SNP: single nucleotide polymorphism; DEL: deletion; Insertion: INS)

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 proteins.

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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 the 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. The upper statistical table indicates the amounts of positive immune response of samples.