BMC Cancer

RESEARCH Open Access

ADH4—a potential prognostic marker for hepatocellular carcinoma with possible immune-related implications

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Abstract

Objective This study aims to explore ADH4 expression in hepatocellular carcinoma (HCC), its prognostic impact, and its immune correlation to provide novel insights into HCC prognostication and treatment.

Methods HCC prognostic marker genes were rigorously selected using GEO database, Lasso regression, GEPIA, Kaplan-Meier and pROC analyses. The expression of interested markers (ADH4, DNASE1L3, RDH16, LCAT, HGFAC) in HCC and adjacent tissues was assessed by Immunohistochemistry (IHC). We observed that ADH4 exhibited low expression levels in liver cancer tissues and high expression levels in normal liver tissues. However, the remaining four genes did not manifest any statistically significant differences between hepatocellular carcinoma (HCC) tissue and adjacent non-cancerous tissue. Consequently, ADH4 became the primary focus of our research. ADH4 expression was validated by signed-rank tests and unpaired Wilcoxon rank sum tests across pan-cancer and HCC datasets. Clinical significance and associations with clinicopathological variables were determined using Kaplan-Meier, logistic regression and Cox analyses on TCGA data. The ADH4-related immune responses were explored by Spearman correlation analysis using TIMER2 data. CD68, CD4, and CD19 protein levels were confirmed by IHC in HCC and non-cancerous tissues.

Results ADH4 showed significant downregulation in various cancers, particularly in HCC. Moreover, low ADH4 expression was associated with clinicopathological variables and served as an independent prognostic marker for HCC patients. Additionally, ADH4 affects a variety of biochemical functions and may influence cancer development, prognosis, and treatment by binding to immune cells. Furthermore, at the immune level, the low expression pattern of ADH4 is TME-specific, indicating that ADH4 has the potential to be used as a target for cancer immunotherapy.

Conclusion This study highlights the diagnostic, prognostic and immunomodulatory roles of ADH4 in HCC. ADH4 could serve as a valuable biomarker for HCC diagnosis and prognosis, as well as a potential target for immunotherapeutic interventions.

Keywords Alcohol dehydrogenase 4 (ADH4), Hepatocellular carcinoma, Immune-related

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Introduction

Primary liver cancer is a common type of cancer, with increasing prevalence and mortality rates globally [1, 2]. In fact, from 1972 to 2017, 30.53% of all cancer-related fatalities were attributable to liver cancer [3]. Although clinical interventions like surgical resection [4], radiofrequency ablation [5], and liver transplantation have been beneficial in treating hepatocellular carcinoma (HCC) [6, 7], the prognosis and therapeutic effect remain sub-optimal. Thus, the pathogenesis, recurrence, and metastatic mechanisms of HCC must be studied, and it is imperative to find possible efficient molecular biomarkers with diagnostic significance in the clinical setting. One vital member of the ADH family implicated in alcohol metabolism is alcohol dehydrogenase 4 (ADH4) [8, 9]. ADH4 variants perform a significant function in alcohol dependence susceptibility and have been linked to alcohol and drug dependence [10–12]. Recent research has also shed light on the relationship between ADH4 and cancer. Specifically, ADH4 variants could be linked to an elevated risk of Esophageal squamous cell carcinoma (ESCC) [13], a decreased risk of ovarian cancer, and an increased upper aerodigestive tract (UAT) cancer risk [14, 15]. Additionally, ADH4 may be a putative therapeutic target and prognostic biological marker for gastric cancer (GC) [16]. Studies demonstrate the potential significance of ADH4 in cancer. With ADH4 playing a critical role in liver metabolism, several investigations have linked ADH4 to liver disease. For instance, ADH4 is a putative biomarker for nonalcoholic steatohepatitis (NASH) while being a candidate prognostic marker for HCC patients [17–19]. Other bioinformatics studies illustrate that ADH4 is implicated in the development and progression of HCC [20, 21]. Despite these findings, most of these studies were partial and lacked a systematic validation of ADH4 expression in HCC. As such, this study establishes a standard process for finding and validating cancer markers in HCC with a focus on the role of ADH4 in immunity. Our study contributes to the study of ADH4 in other cancers and their markers.

Herein, we perform a comprehensive analysis of ADH4 in HCC using several available databases. Firstly, we identify the prognostic key gene, ADH4, in HCC and analyze its pan-cancer expression alongside its expression level in the clinical specimens of HCC tissue and adjacent tissue. Further, we explore the clinical prognostic value of ADH4 through the use of Kaplan-Meier survival curves, among other techniques. Through gene enrichment analysis and immune infiltration analysis, we investigate the critical involvement of ADH4 in the cell cycle and immune microenvironment while exploring its potential application in immunotherapy and chemoresistant treatment. Based on our findings, ADH4 may be considered an immune-related prognostic biological marker and a candidate treatment target in HCC patients.

Materials and methods

Microarray data

We filtered the NCBI-Gene Expression Omnibus database for the GSE39791, GSE54236, GSE76427, and GSE101685 datasets associated with liver cancer. Using bioinformatics and R analysis, we screened for differentially expressed genes (DEGs) with Adj. |logFC| > 2 and p-value < 0.05 representing the cut-off standards. Employing the Draw Venn Diagram online tool, we calculated the intersection of the three DEGs sets, which produced the most common DEGs.

ROC curves and survival curves analysis

We utilized the "survminer" and "pROC" packages to analyze the ROC curves and survival curves of Hub Genes. An AUC cutoff value (>95%) was set to determine the diagnostic relevance of the Hub Genes, where a P-value < 0.05 indicates statistical significance.

GEPIA database

Using GEPIA, which integrates big data of TCGA cancer and GTEx normal tissues, we generated box diagrams to observe Hub Genes distribution in normal liver and liver cancer tissues. This bioinformatics technology helps to deal with significant problems in cancer biology, reveal alleles, driver genes, cancer subtypes, oncogenic factors, or differential expression, and explore new cancer markers and targets. In this study, GEPIA was applied in the interactive analysis of Hub Genes expression in HCC [22].

Ualcan database

We examined the mRNA and protein expression of ADH4 in HCC utilizing the Ualcan online tool. Ualcan is an interactive, user-friendly, and comprehensive web resource to analyze data on cancer omics [23].

Kaplan-meier plotter survival analysis

We conducted a survival analysis of ADH4 utilizing the Kaplan-Meier (KM) Plotter to evaluate its prognostic significance in HCC. The database has 54,000 genes (protein, miRNA, mRNA) and 21 tumors, and it employs the prognostic analysis Meier algorithm to analyze survival curves. The statistical significance level for the log-rank test was established at $P < 0.05$ [24].

TIMER2.0 (tumor immune estimation resource) database extraction data

We utilized TIMER (https://cistrome.shinyapps.io/timer/) to explore ADH4 immune infiltration in immune cells [25]. The database offers a comprehensive and

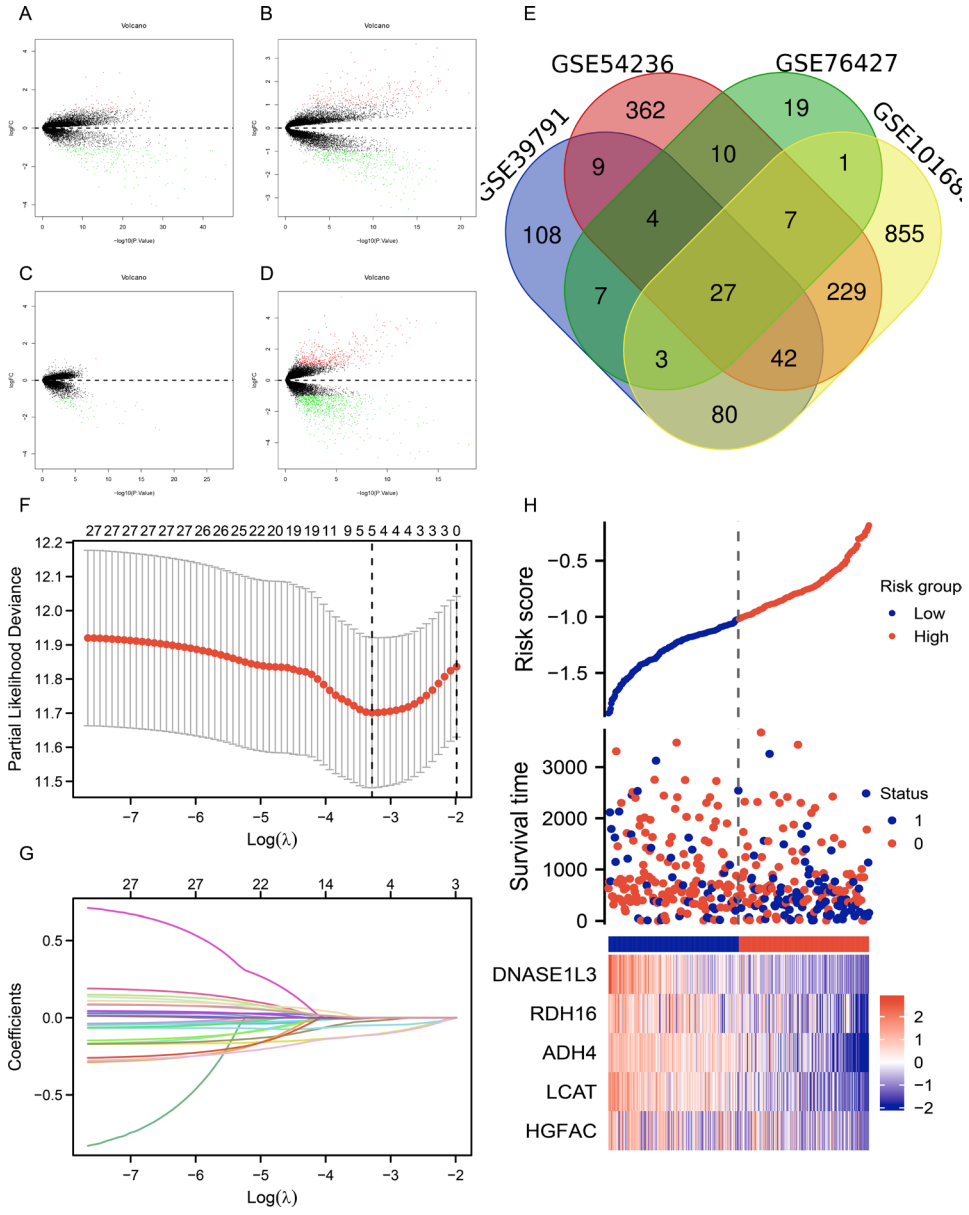

Fig. 1 The differential gene analysis and screening of prognostic marker genes in HCC. (**A-D**) The volcano plot of the four data sets. (**E**) The DEG intersection in a Venn diagram. (**F**) The LASSO model-related ten-time cross-validation for tuning parameter selection. (**G**) The profiles of LASSO coefficients. (**H**) The risk scores, survival statuses, and heat map of the five genes in patients with HCC

flexible assessment and visualization of tumor-infiltrating immune cells (TIICs). In the current research, the database of TIMER was utilized to explore ADH4 immune infiltration in the immune cells [26, 27].

Immunohistochemistry (IHC)

In this study, ADH4 expression was examined by IHC on liver cancer tissues and adjacent normal tissues from 30 patients, and CD68, CD4, and CD19 expression(from 20 HCC patients) at Guangxi Zhuang Autonomous Region's People's Hospital. The experiment was conducted following standard procedures with a primary antibody (ADH4, Proteintech, 16474-1-AP; CD68(PG-M1)ZSGB-BIO, ZM-0464;CD4(SP35),MXB, RMA-0620;CD19(LE-CD19),MXB, MAB-0646) on paraffin-fixed materials that were cut into serial sections. After HE staining, an IHC staining was conducted following the guidelines of the SP kit. The antigen content, distribution density, tag technique, and susceptibility all influenced determining the count of positive cells. Positive results were indicated by a darker color, ranging from light yellow for slightly positive markers to dark brown for highly positive markers. Representative images were captured using an Olympus X21 microscope, and each image underwent a generic

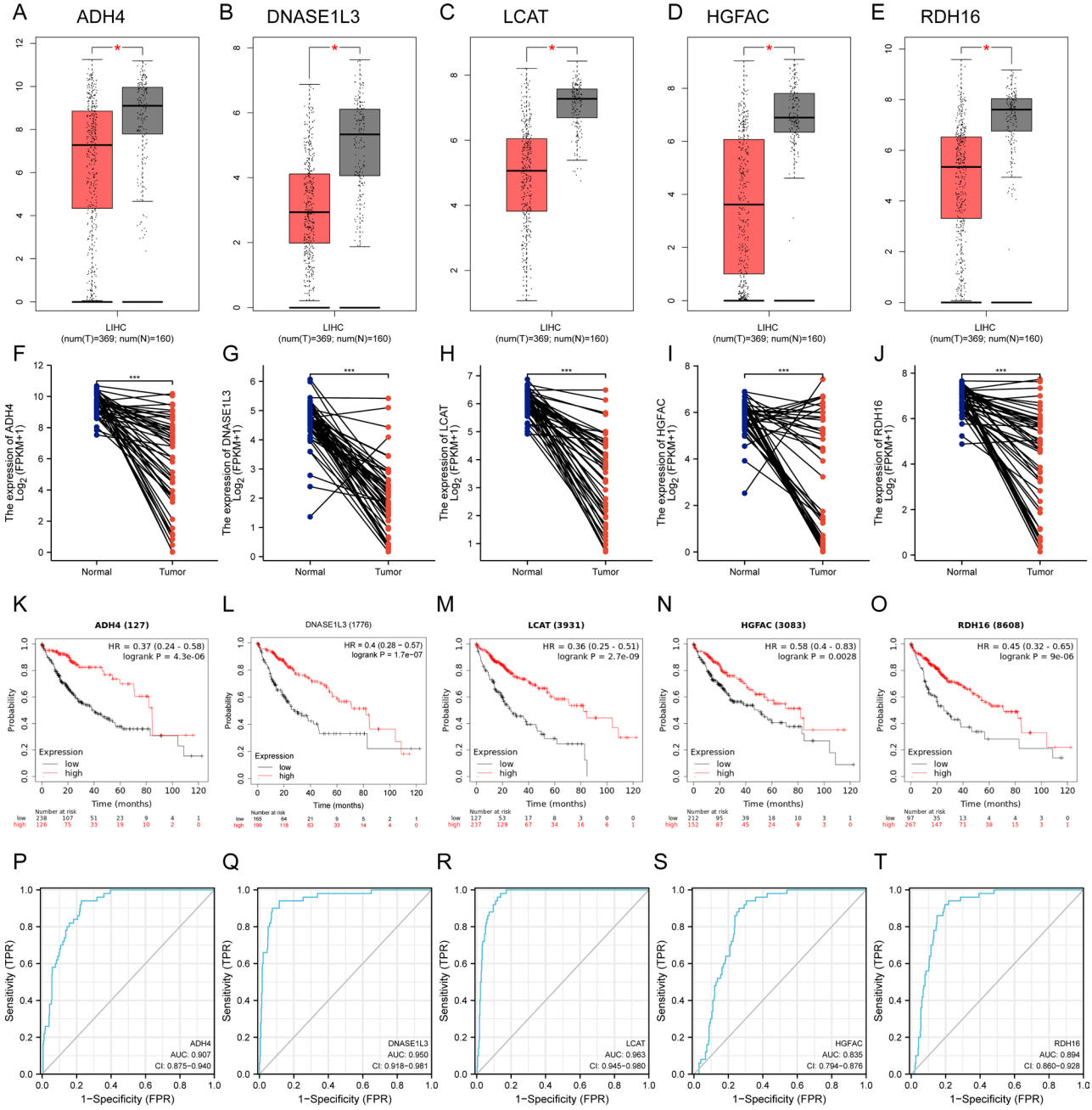

Fig. 2 The expression, survival, and ROC curve analysis of the hub genes. (**A-E**) The levels of the 5 hub genes in LIHC and normal samples (Tumor Color: red; Normal Color: gray). (**F-J**) The levels of the five hub genes in paired LIHC samples. (**K-O**) Survival analyses for the five hub genes. (**P-T**) ROC curve analyses for the five hub genes

morphometric analysis using Image J. The optical density and positive staining area data were measured based on Image J parameters, and the average level of ADH4 expression was determined for the normal and cancer groups. Statistical methods were used to analyze the data and determine if there was a variation in the expression of ADH4 between cancer and normal groups.

Statistical analysis

We used Pearson or Spearman's coefficients to explore correlations between variables. We utilized the t-test to compare continuous variables that were normally distributed between groups. Otherwise, we employed the Mann-Whitney U test. The chi-square test or Fisher's exact test was applied to compare categorical variables. Based on the KM approach, we produced survival curves for prognostic analyses of categorical variables, with the

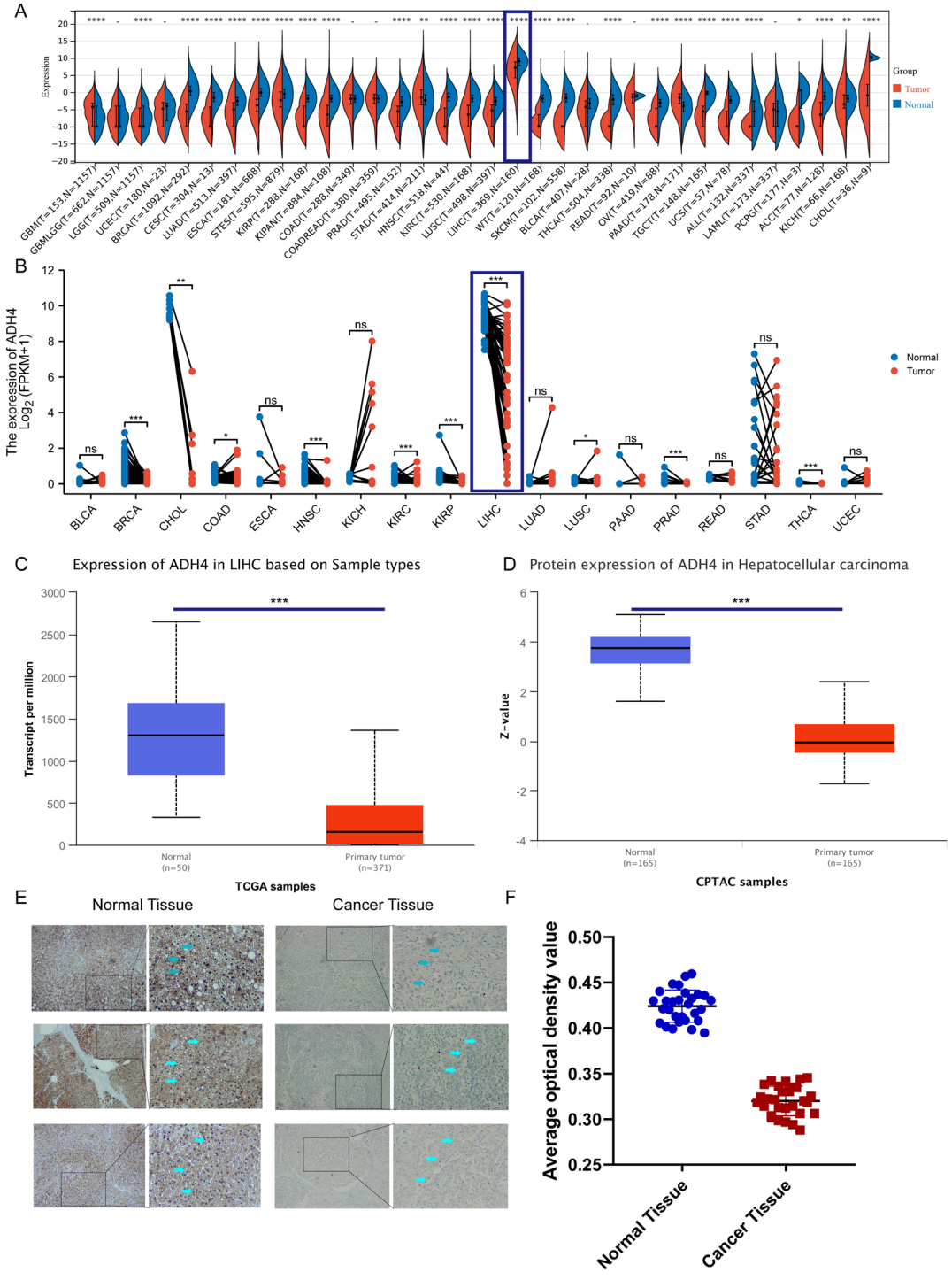

Fig. 3 Analysis of ADH4 Expression in Pan-Cancer and Hepatocellular Carcinoma. (**A**) Expression levels of ADH4 across different cancer types. (**B**) Analysis of ADH4 expression in matched pairs of tumor and normal tissues. (**C-D**) ADH4 mRNA and protein expression in hepatocellular carcinoma according to the UALCAN database. (**E**) Representative immunohistochemical images of ADH4 expression in liver cancer tissues and normal controls. (**F**) Statistical analysis of mean staining intensity of immunohistochemical images from 30 patients with liver cancer and their adjacent tissues. *$p < 0.05$, * *$p < 0.01$, * **$p < 0.001$, ** **$p < 0.0001$, ns: not statistically significant

log-rank test used to determine statistical significance. We used two-sided statistical tests for all analyses with $P<0.05$ as the significance level. R software v.4.2.0 was utilized to conduct all analyses of statistical data.

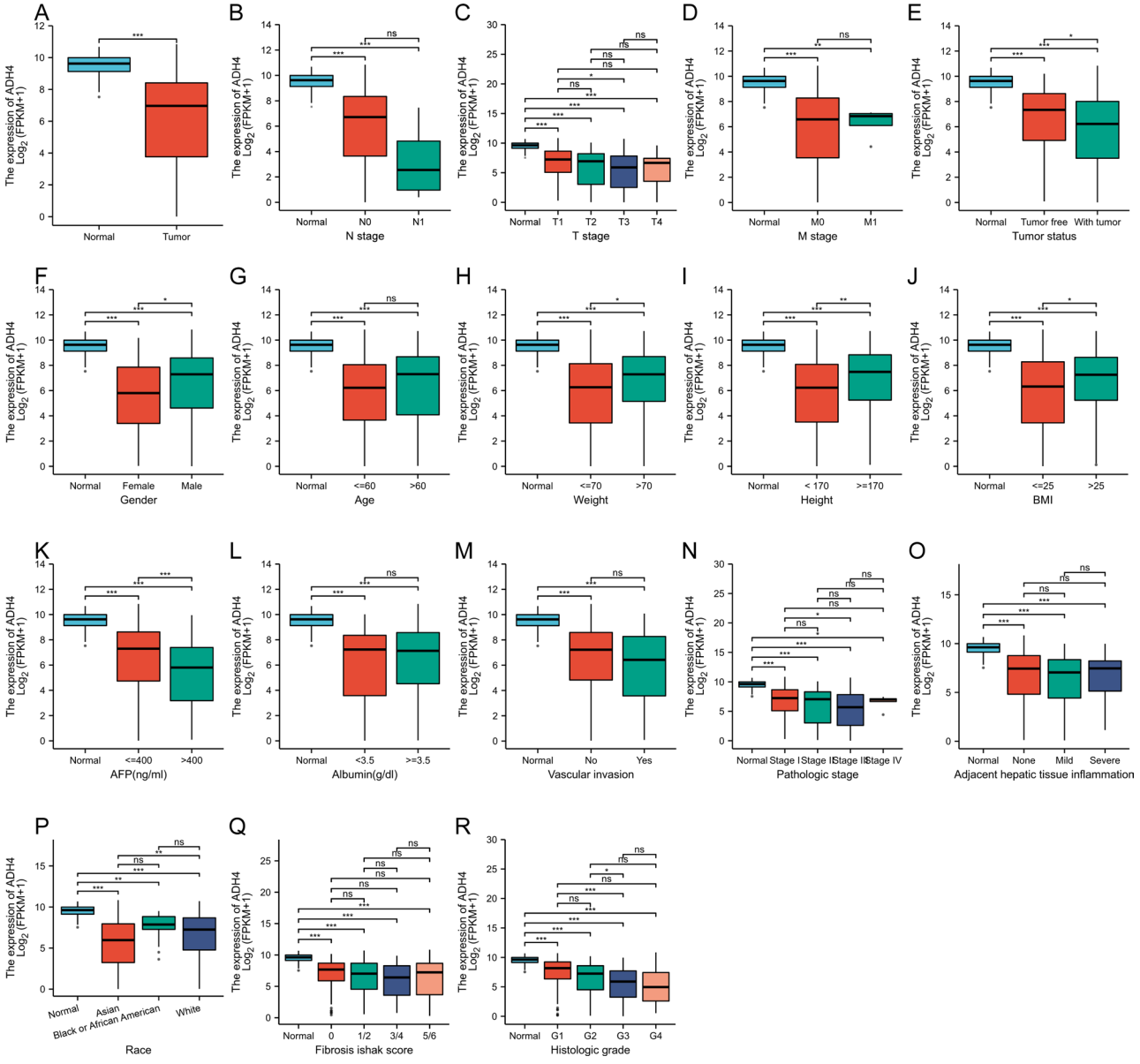

Fig. 4 Association between ADH4 mRNA Expression and Clinical Parameters in HCC. (**A**) Comparison of ADH4 mRNA expression between HCC tissues and normal controls. (**B-R**) The ADH4 mRNA expression in HCC based on analysis of the Cancer Genome Atlas (TCGA) using R. Parameters investigated include N stage, T stage, M stage, tumor status, gender, age, weight, height, BMI, AFP (ng/ml), albumin (g/dl), vascular invasion, pathologic stage, adjacent hepatic tissue inflammation, race, Fibrosis Ishak score, and histologic grade. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Results

Differential gene analysis and screening of prognostic marker genes in hepatocellular carcinoma

Through gene analysis, we distinguished differentially expressed genes (DEGs) between cancerous and normal liver tissues. For this purpose, we screened the GEO database and selected four datasets associated with liver cancer (GSE101685, GSE76427, GSE54236, and GSE39791). The volcano diagrams of DEGs in these datasets are presented in Fig. 1 (A-D). By examining these datasets, we obtained a total of 27 different genes, as shown in Fig. 1(E). We further used Lasso regression to analyze the 27 common differential genes and identified five marker genes related to the prognosis of liver cancer (Fig. 1F, G), namely DNASE1L3, RDH16, HSPA6, ADH4, LCAT, and HGFAC (Fig. 1H). The LASSO (Least Absolute Shrinkage and Selection Operator) regression is a statistical method that performs both variable selection and regularization to enhance the prediction accuracy and interpretability of the resulting statistical model.The model fit was the best with the personality coefficient set at 5.

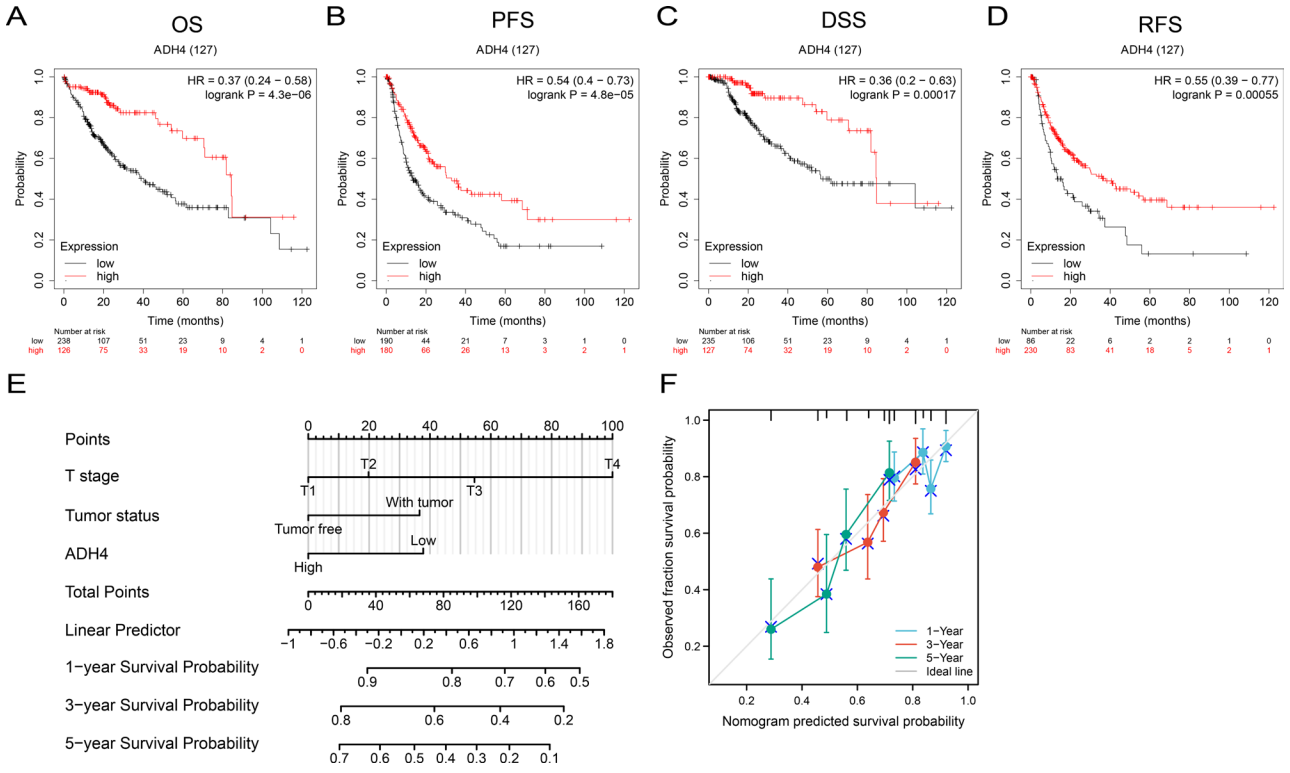

Fig. 5 The prognostic analysis of ADH4 in HCC. (**A-D**) Analysis results for Overall Survival (OS), Recurrence-Free Survival (RFS), Disease-Specific Survival (DSS), and Progression-Free Survival (PFS), respectively. (**E**) The Nomogram for predicting 1-year, 3-year, and 5-year OS probabilities for patients with HCC. (**F**) The Nomogram Calibration Plots for predicting 1, 3, and 5-year OS probabilities. *(*Note* OS = overall survival, RFS = recurrence-free survival, PFS = progression-free survival, DSS = disease-specific survival, HCC = hepatocellular carcinoma, ADH4 = alcohol dehydrogenase 4)*

Expression, survival and ROC curve analysis of hub genes

We detected five identified hub genes's expression levels in cancerous and normal samples using GEPIA. The differential analysis was based on the selected datasets, with one-way ANOVA used to calculate differential expression. As opposed to normal liver samples, the retrieved genes were expressed at low levels in HCC samples (Fig. 2A-E). The findings of paired gene expression study in liver cancer were in line with those of the unpaired expression analysis, indicating low expression in HCC and high expression in normal tissue (Fig. 2F-J).

We leveraged the KM plotter database to examine the hub gene-related overall survival (OS) rate. We discovered that the high-expression group of the five genes had a better prognostic outcome than the low-expression group, with statistical significance ($P<0.05$), indicating that all five genes are good prognostic indicators of hepatocellular carcinoma (Fig. 2K-O).

We further investigated the ROC curves of the five hub genes through the pROC software package and identified three genes with AUC > 90%, namely ADH4, DNASE1L3, and LCAT (Fig. 2P-T). These genes exhibited high accuracy in distinguishing liver cancer from normal tissues and thus can serve as an underlying tumor biomarker with great importance in accurately diagnosing liver cancer. Based on previous research, we selected ADH4 as the research gene for further in-depth analysis.

Analysis of ADH4 expression in pan-cancer and hepatocellular carcinoma and the IHC results

Firstly, we ascertain the variation in ADH4 expression between cancerous and normal samples in each cancer type. The results show that ADH4 was remarkably upregulated in 10 cancer types, whereas it was remarkably downregulated in 22 cancer types, such as BRCA and LIHC. The expression differences were more pronounced in LIHC (Fig. 3A).

Next, we observed that ADH4 was significantly downregulated in multiple cancer groups through the Wilcoxon signed-rank test for the pan-cancer paired difference significance analysis.In the LIHC group, the Tumor group showed a median difference of -3.513 (-4.595 - -2.589) compared to the Normal group ($P<0.001$). Likewise, ADH4 expression was significantly lower in LIHC (Fig. 3B).

Furthermore, we separately analyzed ADH4 expression level in HCC and discovered that it was substantially reduced in the tumor tissue relative to the normal liver tissue, both at the mRNA level (Fig. 3C) and protein level (Fig. 3D) ($P<0.001$). To validate this observation,

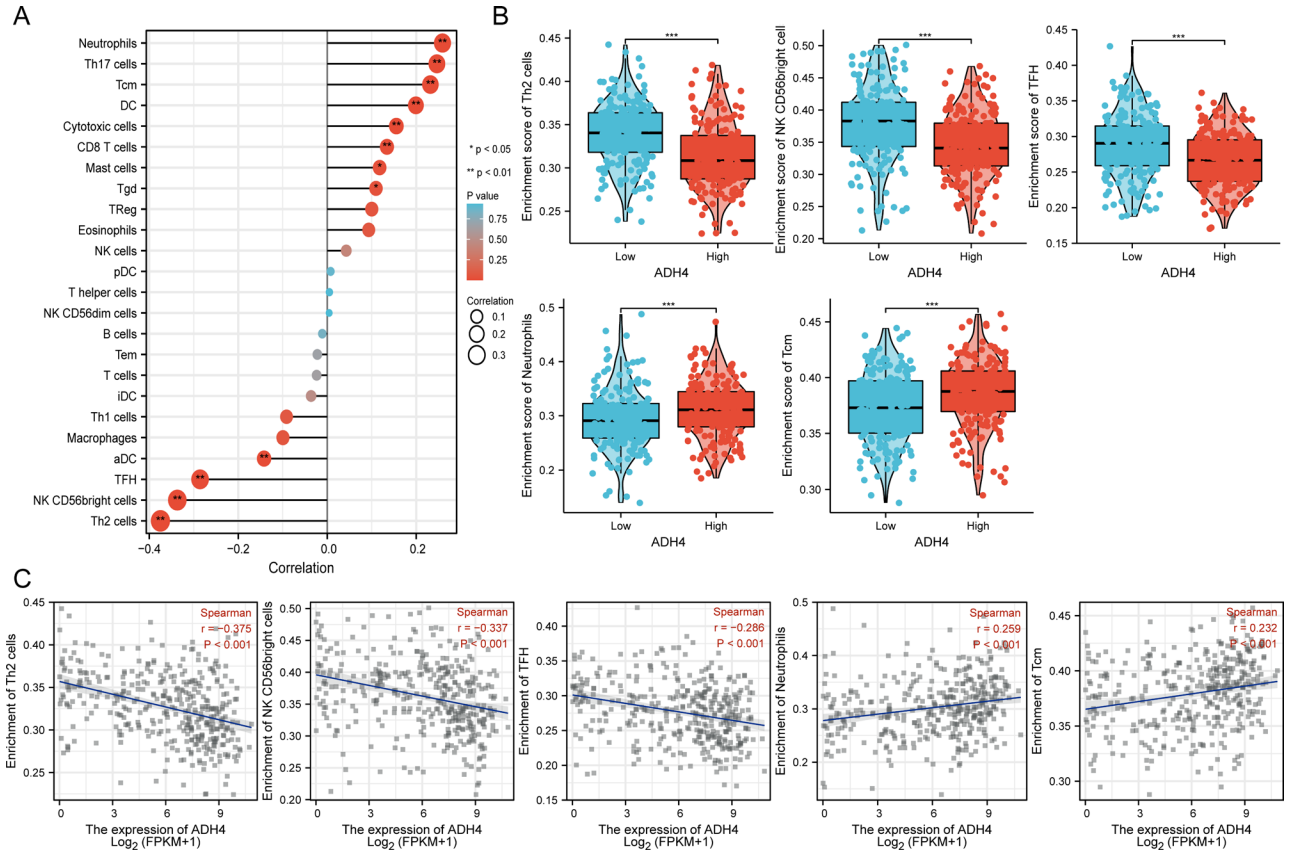

Fig. 6 The analysis of immune cell infiltration in relation to ADH4 expression is presented in this figure. (**A**) Forest plot that depicts the correlation between the level of immune cells and ADH4 mRNA expression. (**B**) The contrast in the abundance levels of neutrophils, Tcm, Th2, NK CD56bright, and TFH cells between high and low ADH4 expression groups. (**C**) The correlation between the expression level of ADH4 and immune cell infiltration in hepatocellular carcinoma, focusing on neutrophils, Tcm, Th2, NK CD56bright, and TFH cells

we conducted immunohistochemistry analysis on 30 pairs of samples and confirmed that the ADH4 expression level was remarkably reduced in hepatocellular carcinoma in contrast with normal liver tissues(Fig. 3E and F) ($P<0.001$). These data illustrate that ADH4 could be a promising tumor marker for liver cancer in the future.

Association with ADH4 expression and clinicopathological variables

The study examined the link between ADH4 levels and clinicopathological variables in HCC patients from the TCGA cohort. The study utilized the R tool to investigate the link between ADH4 expression and clinical characteristics of patients with liver cancer based on multiple indicators. Tumor patients exhibited lower expression levels of ADH4 compared to those with different clinical parameters (Fig. 4A-R, $p<0.001$). Univariate logistic regression was implemented to examine the link between ADH4 levels and clinical features as per the substantial variations in ADH4 levels across groups with various clinical characteristics.The results indicated a significant association between ADH4 downregulation and clinicopathological variables such as tumor status, histologic grade, sex, race, height, residual tumor, AFP, and OS event (all $p<0.05$). This suggests that ADH4's deficiency may have a remarkable role in the metastasis, proliferation, and prognosis of HCC.

Clinical prognostic value of ADH4 in HCC

ADH4's clinical predictive significance in HCC was the focus of this investigation. We first explored the clinical prognostic value of ADH4 through employing the Kaplan-Meier survival curves.The outcomes reveal that HCC patients containing low expression of ADH4 revealed poorer OS (Fig. 5A), RFS (Fig. 5B), PFS (Fig. 5C) and DSS (Fig. 5D) (all $P<0.05$).

We generated nomograms based on T stage, tumor status, and ADH4 mRNA expression to further elucidate the clinical prognostic significance of ADH4 in HCC. These nomograms were included in multivariate studies to determine whether they were independent risk factors for OS (Fig. 5E). The calibration chart showed excellent agreement between actual survival probability and that predicted by the nomogram (1-year, 3-year, and 5-year OS) (Fig. 5F).

Immune infiltration analysis of ADH4 in LIHC

In the LIHC microenvironment, samples with high ADH4 expression had a higher proportion of neutrophils and Tcm cells. Meanwhile, TFH cells, NK CD56bright cells, and Th2 cells had higher levels ($p<0.001$; Fig. 6A-C) in samples with low ADH4 expression. This highlights the potential of ADH4 as a significant immunoregulatory factor in LIHC.

We leveraged the GEPIA database to correlate the expression of ADH4 with immune cell indicators in HCC to learn more about the function of ADH4 in tumor immunity. The results in Table 1 demonstrate that ADH4 expression is significantly and negatively associated with the biomarkers for B cells (CD19),Macrophages (CD68), M1 macrophage biomarkers (IRF5),neutrophils (ITGAM), and DCs in liver cancer (HLA-DQB1, NRP1, and ITGAX). These results point to a potential negative link between immune cell infiltration and ADH4 expression.

Expression correlation of ADH4 and biomarkers of immune cells in HCC by IHC

In order to further elucidate the relationship between ADH4 and immune cell indicators, we conducted an investigation based on the GEPIA analysis results presented in Table 1. Three specific immunological markers(CD68 marks macrophages, CD4 labels CD4+T cells, and CD19 identifies B cells) were selected for in-depth examination. We employed immunohistochemical(IHC) techniques to validate the protein expression levels of CD68, CD4, and CD19 in both liver cancer tissues and adjacent non-cancerous tissues, while concurrently comparing these expressions with ADH4. The immunohistochemical findings were rigorously evaluated by two senior pathologists, following a standardized protocol which involved the random counting of positively stained macrophage cells in ten high-power fields. Notably, Kupffer cells in normal liver tissues were excluded from the count. The positively stained macrophage cells in each high-power field were stratified into four categories: (-) 0–5 cells, (+) 5–10 cells, (++) 10–15 cells, and (+++)≥15 cells.

The findings unveiled that among the 20 liver cancer tissues and their corresponding adjacent tissues, CD68 protein exhibited expressions of 9 cases (+), 3 cases (++), and 8 cases (+++), while being undetectable (-) in all adjacent tissues. Conversely, ADH4 protein showed no detectable expression (-) in all 20 liver cancer tissues, yet demonstrated robust expression in the 20 adjacent tissues (+++). These outcomes were consistent with the negative correlation observed at the mRNA level between CD68 and ADH4, as illustrated in Table 1.(Representative staining results are presented in Fig. 7.)

However, we did not detect any difference in the expression of CD19 and CD4 proteins in cancer and adjacent tissues. This disparity may be attributed to differential regulation of CD19 and CD4 at the transcription and translation stages, resulting in disparities between their protein and mRNA expression in liver cancer tissues and adjacent non-cancerous tissues. We intend to explore this issue further in forthcoming experiments.

Table 1 Correlation analysis between ADH4 and HCC immune cell biomarkers determined by GEPIA database (correlation coefficient: Spearman)

Immune cell	Biomarker	*R* value	*P* value
B cell	CD19	-0.14[a]	0.0049**[a]
	CD79A	0.023	0.64
$CD8^+$T cell	CD8A	0.027	0.58
	CD8B	-0.054	0.27
$CD4^+$ T cell	CD4	0.19[a]	6.6E-05***[a]
Macrophage	CD68	-0.18[a]	2.7E-05***[a]
	MARCO	0.29[a]	8.2E-10***[a]
M1 macrophage	NOS2	-0.021	0.67
	IRF5	-0.35[a]	1E-13***[a]
	PTGS2	0.16[a]	0.00074***[a]
M2 macrophage	CD163	0.18[a]	0.00028***[a]
	VSIG4	0.17[a]	0.00069***[a]
	MS4A4A	0.079	0.11
Neutrophil	CEACAM8	0.057	0.25
	ITGAM	-0.17[a]	0.00052***[a]
	CCR7	0.023	0.64
Dendritic cell	HLA-DPB1	-0.073	0.14
	HLA-DQB1	-0.18[a]	0.00029***[a]
	HLA-DRA	-0.05	0.31
	HLA-DPA1	-0.027	0.58
	CD1C	0.019	0.71
	NRP1	-0.12[a]	0.015*[a]
	ITGAX	-0.21[a]	1.2E-05***[a]

[a]These results are statistically significant

*p value < 0.05; **p value < 0.01; ***p value < 0.001

Association of ADH4 with immune checkpoints

Since immune checkpoint blockade (ICB) therapy represents the most remarkable progress in tumor immunotherapy in recent years, achieving considerable efficacy in the treatment of various cancers, we studied the link between ADH4 expression and immune checkpoints. Finding the cancer types that might respond to ADH4 immunotherapy requires conducting a pan-cancer analysis focused on determining the immune function of ADH4. Our analyses illustrated that most immunomodulators in LIHC had a negative correlation with ADH4 (Fig. 8A). Among these immunomodulators, VEGFB, CD276, TGFB1, TNFRSF4, TNFRSF18, TNFSF9, PDCD1, and CTLA4 were remarkably and inversely linked to ADH4 expression (Fig. 8B-C).Additionally, the levels of these immune checkpoints (VEGFB, CD276, TGFB1, TNFRSF4) were considerably greater in

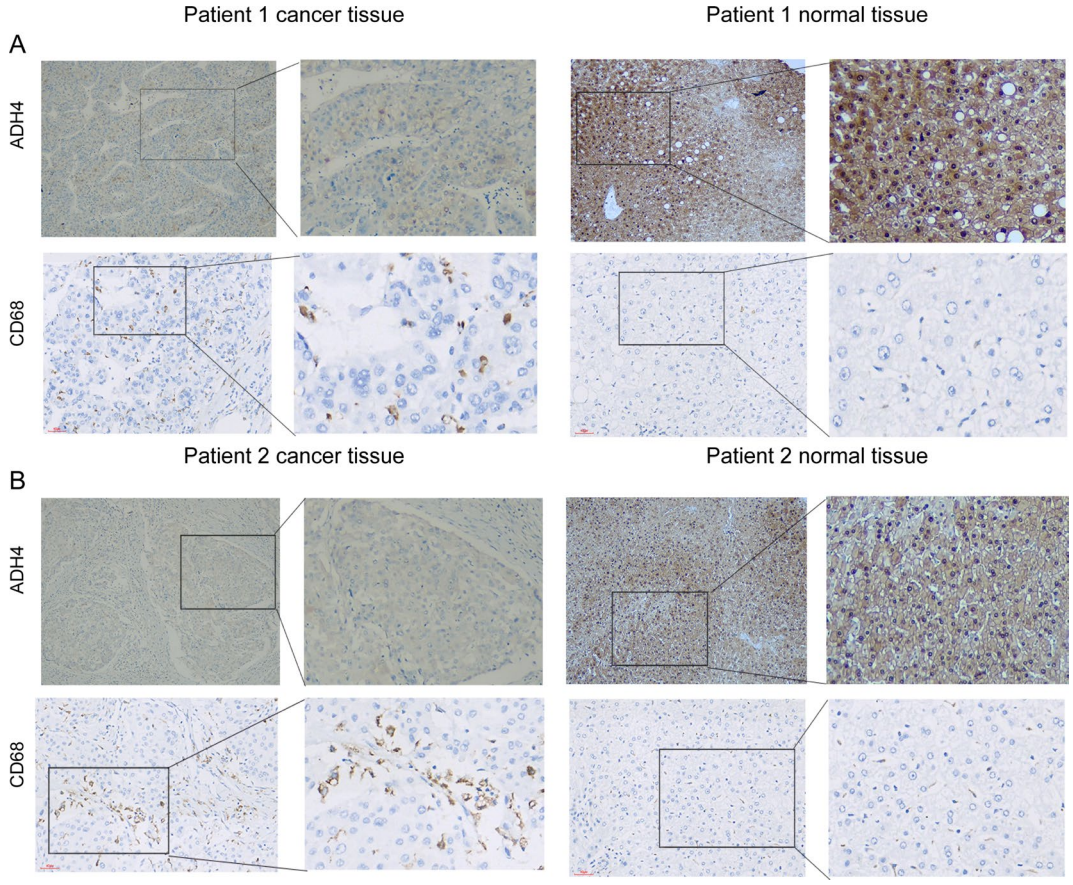

Fig. 7 ADH4 negatively correlated with tumor-associated macrophage (TAM) infiltration in hepatocellular carcinoma (HCC) tissues. (**A** and **B**) Representative immunohistochemical (IHC) staining images of ADH4 and CD68 in HCC tissues and in adjacent normal tissues. Scale bar: 40 μm

the groups with low levels of ADH4 expression than in the groups with high levels of ADH4 (Fig. 8D).

Discussion

There is accumulating evidence that patients with HCC can live longer when receiving a combination of several immunotherapies and targeted treatments [28, 29]. The ability to intervene with genes with varying expression levels and have a therapeutic effect is made possible by the identification of the difference in gene expression between malignant and healthy liver tissues.

The GEO database was screened using the Lasso regression method to analyze the differential genes present in multiple data sets. Five genes, DNASE1L3, RDH16, ADH4, LCAT, and HGFAC, were found to be linked to the prognosis of HCC individuals. Expression analysis was conducted using the GEPIA database, and prognostic and ROC curve analyses were carried out using the Kaplan-Meier plotter database. From the screening process, five genes, namely (ADH4,DNASE1L3,RDH16,LCAT and HGFAC)were identified to have significant clinical prognostic value. And the differential expression of ADH4 was verified by immunohistochemistry.ADH4 exhibited low expression levels in liver cancer tissues and high expression levels in normal liver tissues.Although Wei et al. reported in their 2012 study that immunohistochemical results showed a significant reduction in ADH4 protein expression in 59.3% of hepatocellular carcinoma tissues [30], our immunohistochemical results indicate a significant reduction in ADH4 protein expression in over 95% of HCC tissues. Our study employed a standardized immunohistochemical protocol used in clinical settings, as opposed to the research-oriented laboratory protocols. Despite these differences, the results are complementary and provide a broader understanding of ADH4 expression in HCC.

ADH4, being a crucial member of the ADH family, is capable of metabolizing a wide range of substrates, including retinol and ethanol [18]. ADH4 is a key enzyme involved in alcohol metabolism. Variations in ADH4 contribute to susceptibility to alcohol dependence and are associated with both alcohol and drug dependence [31–33]. In liver samples from patients with alcoholic hepatitis (AH), the expression of alcohol metabolism genes, including ADH4, is significantly downregulated [34]. Similarly, protein levels of ADH4 are reduced in

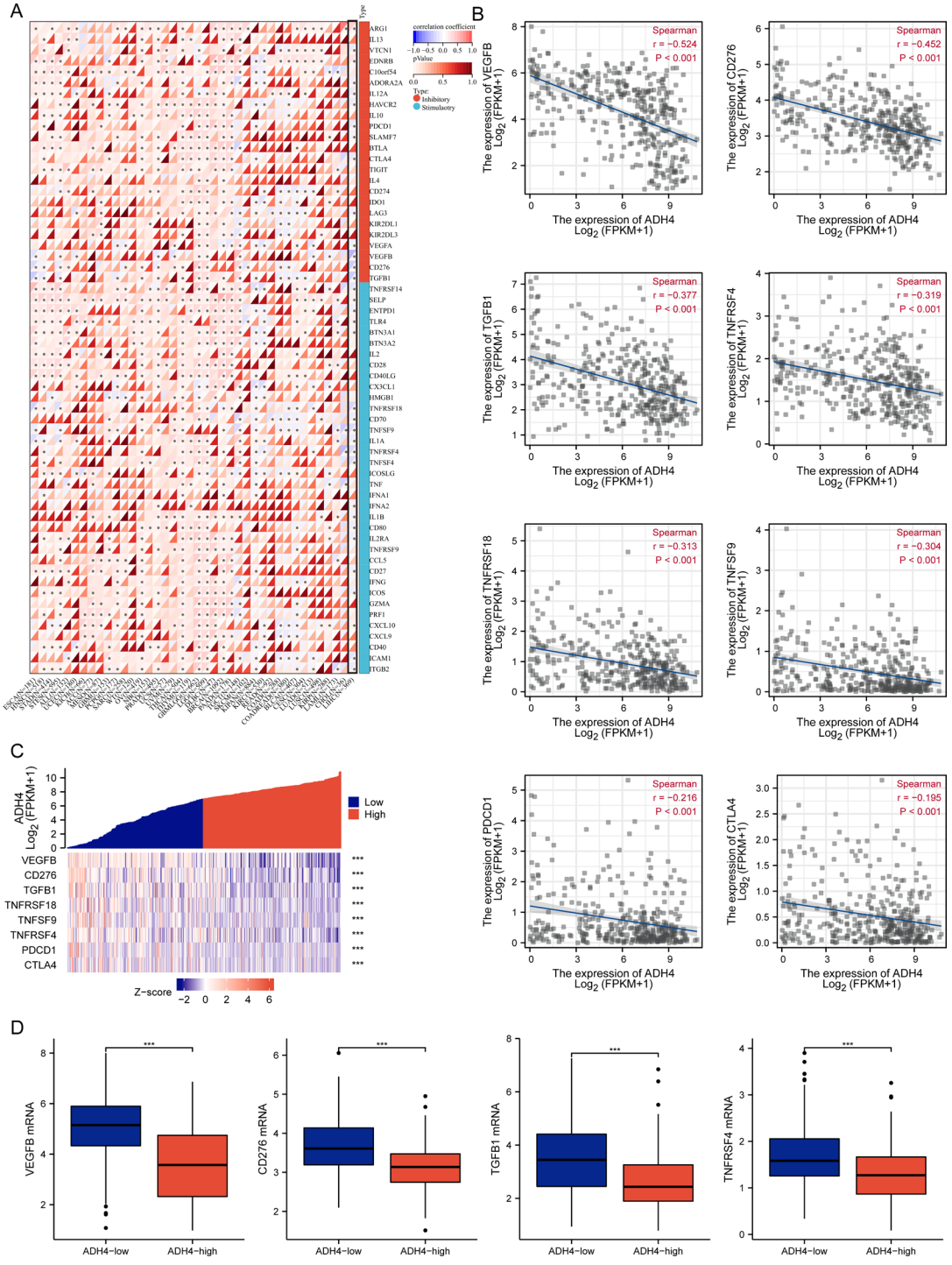

Fig. 8 The correlation between ADH4 expression and immune checkpoint proteins was investigated in liver hepatocellular carcinoma (LIHC). (**A**) Correlation between ADH4 and immune checkpoints molecules in pan-cancers. (**B**) Scatter diagrams demonstrated a negative correlation between ADH4 mRNA expression and the expression of these proteins. (**C**) A heatmap of co-expression between ADH4 and the proteins VEGFB, CD276, TGFB1, TNFRSF4, TNFRSF18, TNFSF9, PDCD1, and CTLA4. (**D**) In the group with low ADH4 expression, VEGFB, CD276, TGFB1, and TNFRSF4 proteins were significantly up-regulated compared to the group with high ADH4 expression. (***$P < 0.001$)

nonalcoholic steatohepatitis (NASH). Both Wei et al.'s study and our research indicate that ADH4 expression is significantly decreased in hepatocellular carcinoma (HCC). These findings suggest that ADH4, as a critical enzyme in alcohol metabolism, plays an important role in the pathogenesis and progression of HCC.Additionally,

ADH4 performs a protective function in immune metabolism, and its low levels can be associated with a high-risk prognosis for HCC [35]. The functions of ADH4 make it a promising ideal choice for further investigation into its involvement in liver cancer.

The Wilcoxon signed rank test was conducted in the pan-carcinoma and LIHC to assess the importance of paired differences. Data confirmed that the expression of ADH4 was down-regulated in tumor tissues, and this pattern was particularly significant in liver cancer. To subsequently analyze the expression of ADH4 in liver cancer, mRNA and protein expression which revealed that ADH4 levels were substantially reduced in cancerous tissues relative to normal liver tissues.

Univariate logistic regression analysis revealed that decreased ADH4 expression in HCC was significantly related to a high T stage, tumor status, residual tumor, AFP, sex, age, histological grade, height, weight, and fibrosis IShak score. Collectively, these outcomes suggest that patients with low ADH4 expression exhibited a higher likelihood of experiencing a relapse, having a more aggressive tumor, poorer prognosis, and likely advancing to later stages.

The study investigated the clinical prognostic value of ADH4 in hepatocellular carcinoma (HCC). The data confirm that patients' OS, RFS, PFS, and DSS were all lower in those with low expression of ADH4. The study also conducted Cox regression analysis, which found that tumor stage, tumor status, and ADH4 expression were correlated with OS. Tumor status and ADH4 expression were shown to independently function as prognostic markers for HCC patients.

We investigated the link between ADH4 expression and TIICs in LIHC. We discovered by ssGSEA correlation analysis that ADH4 expression was substantially and positively linked to several TIICs, such as neutrophils and Tcm cells, but inversely linked to Macrophage, Th2 cells, NK CD56bright cells, and TFH cells.These findings suggest that ADH4 might act as a crucial immunoregulatory factor in LIHC.

We additionally compared the expression of ADH4 with immune cell indicators in HCC using the GEPIA database. The findings demonstrated a significant negative link between ADH4 and immune cell biomarkers, including B cells (CD19), Macrophages (CD68),CD4+Tcells(CD4), neutrophils (ITGAM), and dendritic cells (HLA-DQB1, NRP1, and ITGAX) (Table 1). These findings imply an inverse link between ADH4 expression and infiltrating immune cells.In addition, we employed immunohistochemical techniques to confirm a strong negative correlation at the protein level between CD68 and ADH4, consistent with the negative correlation observed between CD68 and ADH4 mRNA levels in Table 1. This suggests the negative correlation between ADH4 and immune responses.

Immune checkpoint expression and immune cell infiltration into the tumor microenvironment (TME) both affect immunotherapy efficacy [36]. Some of the proteins implicated in this process include CTLA4 [37], PDCD1 [38], TIGIT [39, 40], LAG3 [41, 42], VEGFB [43], CD276 [44], TGFB1 [45], and TNFRSF4 [46]. The relationship between ADH4 and immune checkpoints was also investigated. A significant negative link between ADH4 level and the expression of CTLA4, PDCD1, TIGIT, LAG3,VEGFB, CD276, TGFB1, and TNFRSF4 was observed.Regarding the relationship between ADH4 levels and immune checkpoints, we currently only know that there is a correlation, but the regulatory relationship requires further investigation. Tumor research is a very complex field, and only through step-by-step in-depth studies can we uncover the ultimate truth.

Therefore, this paper recommends exploring gene therapy targeting the ADH4 gene. In the future, gene therapy has the potential to become more widely used in medical practices. Liver-specific gene therapy can be achieved through molecular techniques. Consequently, this paper sought to comprehensively analyze the impact of the ADH4 gene on liver cancer, investigate the correlation between the two, and explore the possibility of gene therapy as a treatment for liver cancer.

Conclusion

ADH4 emerges as a pivotal diagnostic and prognostic marker in HCC, showcased through comprehensive analyses of gene differentials and survival evaluation. Its distinct expression patterns in HCC and diverse cancers underscore its promise as a potent biomarker. ADH4's deletion and robust correlation with clinicopathological factors emphasize its remarkable role in metastasis and proliferation, while its immunoregulatory role in the HCC microenvironment and association with immune checkpoints suggest potential values for low-ADH4 in HCC management and treatment. However, the exploration of its potential molecular mechanisms and its involvement in immune responses requires much more basic experimentation and large clinical trials in the future.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-024-12675-y.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Acknowledgements
Not applicable.

Author contributions
Mr. Xiaolong Li designed and conducted the whole research, Ling Li, Yongta Huang, Liting Wang, Zhenyu Chen, Xiaoling Wang, Shaolan Jiang and Qiuling Zeng participated in the data analysis and interpretation, meanwhile drafted the manuscript. Ling Li revised the manuscript. Ling Li and Hui-Pin Huang did this immunohistochemical test, Yongta Huang reviewed the immunohistochemical section result. All authors contributed to the article and approved the submitted version.

Funding
This research was funded by: Natural Science Foundation of Guangxi Province [grant number:2017GXNSFAA198063]. Natural Science Foundation of Guangxi Province [grant number:2023GXNSFBA026107]. Science Foundation for Young Scholars, the People's Hospital of GuangXi Autonomous Region of China [grant number: QN2021-43].

Data availability
These data can be found here: National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO), www.ncbi.nlm.nih.gov/geo/, GSE39791, GSE54236, GSE76427, and GSE101685.

Declarations

Ethics approval and consent to participate
The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of the People's Hospital of Guangxi Zhuang Autonomous Region. Written informed consent was obtained from individual or guardian participants.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 8 April 2024 / Accepted: 23 July 2024
Published online: 01 August 2024

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