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Research Article

ASF1B Serves as a Potential Prognostic Biomarker and Correlates with Immune Infiltration in Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death, and anti-silencing function 1B histone chaperone (ASF1B) has been implicated in several cancers. This study aimed to investigate the role and molecular mechanism of ASF1B in HCC. ASF1B expression was analyzed using the TIMER2, GEO, Oncomine, and GEPIA2 databases, as well as western blotting. Cell viability, cell cycle distribution, and apoptosis were assessed via CCK-8 assay and flow cytometry, respectively. Survival analysis and immune infiltration analysis were performed using the TIMER2 database, and enrichment analysis was conducted via Metascape. Results showed that ASF1B expression was significantly higher in HCC tissues than in normal tissues, and high ASF1B expression predicted poor prognosis and was associated with higher tumor stage. Knockdown of ASF1B by siRNA significantly reduced cell viability, promoted apoptosis, and induced G1 phase cell cycle arrest in SNU-423 cells. Moreover, ASF1B expression was positively correlated with the infiltration levels of immune cells and tumor microenvironment signature cells, particularly functional T cells. Enrichment analysis further indicated that ASF1B may contribute to HCC progression through mechanisms involving cell cycle, cell division and differentiation, and DNA replication and repair. Collectively, these findings suggest that ASF1B overexpression predicts poor prognosis and increased immune infiltration in HCC, highlighting ASF1B as a potential therapeutic target for this malignancy.

Keywords

ASF1B, Prognosis, Immune Infiltration, Cell Cycle Arrest, Apoptosis, Hepatocellular Carcinoma

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1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third leading cause of cancer-related death, accounting for 7% of all cancers, which is a growing incidence of HCC worldwide. Unfortunately, treatment for HCC is largely palliative, with low long-term survival rates and poor quality of life for advanced HCC patients [1]. Over the past decades, immune checkpoint inhibitors, specifically inhibitors of the programmed cell death 1 (PD‐1) and the programmed cell death 1 ligand 1 (PD‐L1) axis, have shown remarkable therapeutic efficacy in a variety of cancers, including hepatocellular carcinoma [2, 3]. In addition, several biomarkers, including PD‐L1 expression, Tumor Mutational Burden (TMB), and VEGF have been reported to have predictive value for clinical responses to immune checkpoint inhibitors therapy [4-6]. Therefore, it will be of great importance to find a safe and effective strategy for treating to improve the prognosis and survival of HCC patients.

ASF1B (Anti-Silencing Function 1B Histone Chaperone), is one of two paralogs (ASF1A, ASF1B) in ASF1, which is an important histone chaperone protein that plays a role in the chromatin-based progression of cellular DNA replication, DNA damage repair, and transcription regulation. Interestingly, ASF1B has been proven to be a proliferative marker in the diagnosis and prognosis of breast cancer and cervical cancer [7, 8]. What's more, ASF1A has been shown to enhance the antiviral immune response in Vesicular Stomatitis Virus (VSV)-infected macrophages [9]. However, the relationship between ASF1B expression and HCC proliferation, immunity, and prognosis remains unknown.

In this study, we comprehensively analyzed the relationship between ASF1B expression and its prognostic value in HCC using multiple databases, including Gene Expression Omnibus (GEO), Oncomine database, Gene Expression Profile Interaction Analysis 2 (GEPIA2), and tumor immune estimation resource (TIMER2.0). Anti-silencing function 1B histone chaperone (ASF1B) was selected as a potential molecular involved in HCC and role was investigated. Moreover, according to research, tumor-infiltrating lymphocytes can serve as a predictor of survival in cancers [10]. In addition, cancer associated fibroblast, hematopoietic stem cell, common lymphoid progenitor, myeloid derived suppressor cells (MDSC), granulocyte-monocyte progenitor, T cell follicular helper, endothelial cell, and mast cell can affect tumor progression, invasion, metastasis, and prognosis by affecting tumor microenvironment [11-13], the relationships between ASF1B expression and immune infiltration as well as tumor microenvironment in HCC were investigated via TIMER. We found that ASF1B is a potential prognosis‐related biomarker in HCC and provided novel direction to understand the interactions between ASF1B expression, tumor infiltration, and T cells exhaustion.

2. Methods

2.1. Open Database Analysis

HCC and adjacent normal liver tissues gene expression profiles of GSE14520 [14], GSE19665 [15], GSE25097 [16], GSE45267 [17], GSE45436 [17], GSE55092 [18], GSE60502 [19], GSE62232 [20], GSE65372, GSE76297 [21], GSE76311 [21], GSE77314 [22], GSE84402 [23], GSE84598 [24], GSE87630 [25], GSE112790 [26], and GSE121248 [27] were downloaded from GEO (http://www.ncbi.nlm.nih.gov/geo/) database.

Oncomine database (https://www.oncomine.org/resource/login.html) can be used to analyze the expression level of the ASF1B gene in HCC, and the threshold was determined according to the following values: p-value≤1E-4, fold change ≥ 2 and top 10% gene rank.

TIMER2.0 (Tumor Immune Estimation Resource2, http://timer.cistrome.org/), is a comprehensive resource for systematical analysis of immune infiltrates, as well as tumor immunological, clinical and genomic features [28].

GEPIA2 (Gene Expression Profiling Interactive Analysis 2) [29] database (http://gepia2.cancer‐pku.cn/) can performed differential expression analysis, profiling plotting, correlation analysis, patient survival analysis, similar gene detection and dimensionality reduction analysis based on TCGA and GTEx (Genotype‐Tissue Expression) data.

STRING database [30] (https://string-db.org/) is a database of known and predicted protein-protein interactions.

Metascape [31] (http://metascape.org) was used to analysis of enrichment pathways and the construction of protein–protein interaction networks. Gene Ontology (GO) terms for the biological process (BP), cellular component (CC) and molecular function (MF) categories as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were enriched.

2.2. Tissue Source

From May 2015 to February 2021, 42 HCC tissue samples and healthy adjacent tissue samples (mean age 65.5±7 years) were collected from HCC patients hospitalized in Lishui Central Hospital (Lishui, Zhejiang, China). Hepatocellular carcinoma was diagnosed by biopsy or pathology. Patients who also had other malignancies, coronary heart disease or diabetes were excluded. In this study, all patients signed informed consent to use their tissues. This study was approved by the Ethics Committee of Lishui People's Hospital.

2.3. Cell Lines and Transfections

The HCC cell lines SNU-423 was purchased from the American Type Culture Collection (ATCC; USA). They were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 μg/mL streptomycin (Hy-

Clone, Logan, UT, USA), 100 U/mL penicillin (HyClone, Logan) and 10% fetal bovine serum (FBS; Gibco, Life Technologies, USA) in a humidified incubator at 37 °C with 5% carbon dioxide (CO2).

ASF1B was knockdown by the small interfering RNA (siRNA) (Genepharma, ShangHai, China). The target sequence for ASF1B is: siASF1B a 5'- CTG GAG TGG AAG ATC ATT TAT -3', siASF1B b 5'- CCC ACT CAA CTG CAC TCC TAT-3' and siASF1B c 5'- TTA GTT AGT AGG TAG ACT TAG -3'. A random sequence was used as a negative control (NC) with sequence: 5'-UUC UCC GAA CGU GUC ACG UTT-3'. SNU-423 cell line was selected for transfection and Lipofectamine™ 3000 Transfection Reagent (Thermo, South Logan, UT, USA) was used according to the manufacturer in all transfections.

2.4. Western Blotting Analysis

The Cell Lysis Buffer supplemented with 1% PMSF (Cell Signaling Technology, Boston, MA, USA) was used for protein extraction, 10% SDS-PAGE gels electrophoresis for separation. After being transferred by the proteins, polyvinylidene fluoride (PVDF) membranes (PALL Life Science, USA) were blocked with 5% nonfat milk for 1 h at room temperature. Then the membranes were incubated with anti- ASF1B (rabbit anti-human, 1: 500, SAB4502347, Sigma-Aldrich) over night at 4°C. The β-Action was used as an internal control. And then incubation with secondary antibody (1: 1000; Sigma-Aldrich). At last, we detected the immunolabeled protein with the electrochemiluminescence (ECL) system (Thermo). All experiments were repeated independently for three times.

2.5. Cell Proliferation Assay

Cell viability was examined using a CCK-8 kit (Beijing Solarbio Science & Technology Co., Ltd.) following the manufacturer's protocol. In brief, SNU-423 were plated in 96-well plates in an incubator for 24 h. Cells were exposed to PBS (control), unspecific scrambled and ASF1B-target siRNA (siRNA-ASF1B) for 12, 24 and 48 h. Then, CCK-8 solution was added to the cells and incubated for 4 h. Absorbance at 450 nm was recorded and evaluated using a microplate reader.

2.6. Cell Cycle/Apoptosis Assay

SNU-423 cells were seeded in 6-well plates and then transfected with NC (Normal control), siASF1B b or siASF1B c for 48 h. According to the manufacture we collected the cells and treated them with cell cycle staining solution (Multi Sciences, CCS012; China) and cell apoptosis staining solution (Multi Sciences, AP107-60; China). The cell suspension was detected by the flow cytometer (ACEA NovoCyte, NovoExpress; China). Experiments were performed in triplicate.

2.7. Statistical Analysis

Distributions of ASF1B expression levels were displayed using box plots across 32 cancer types in TIMER, with statistical significance of differential expression evaluated using Wilcoxon test. In GEPIA2 database, Wilcoxon test was used to analysis ASF1B expression levels between HCC and normal liver tissue. All continuous variables were evaluated with the Student's t test or Wilcoxon rank-sum test and the qualitative variables were carried out by Pearson Chi-square test or Fisher's exact test. Only $P<0.05$ was considered statistically significant. $^{***}\ p < 0.001$, $^{**}\ p < 0.01$, $^{*}\ p< 0.05$. The data was downloaded from open database analysis, and see the corresponding database.

3. Results

3.1. ASF1B Overexpressed in Hepatocellular Carcinoma

TIMER database was used to analyze the expression of ASF1B in human 32 tumors. The result revealed that the ASF1B expression was significantly higher compared to the normal tissues (Figure 1). To further explore ASF1B expression in HCC, HCC datasets were downloaded from GEO database to explore the expression of ASF1B in HCC compared with normal liver tissue (2A-Q), and details of these datasets are shown in Table 1. The results show that ASF1B was significantly overexpressed in HCC compared with matched nontumor tissues in GSE14520(A), GSE19665(B), GSE45267(C), GSE45436(D), GSE55092(E), GSE87630(F), GSE25097(G), GSE60502(H), GSE62232(I), GSE65372(J), GSE76297(K), GSE112790(L), GSE76311(M), GSE77314(N), GSE84402(O), GSE84598(P), and GSE121248(Q). Then, the significantly high expression of ASF1B in HCC was verified again using TCGA data from GEPIA2 database (Figure 2R). In addition, Meta-analysis of ASF1B mRNA expression levels in 4 analyses using Oncomine database was performed to once again verify expression of ASF1B in HCC (Figure 2S). What's more, six patients were randomly selected from the cohort to detect ASF1B protein expression by Western blotting to investigate the expression of ASF1B in tumor tissues and healthy adjacent liver tissues of 8 HCC patients. It was demonstrated that at least a two-fold increase in ASF1B protein expression in cancer tissues compared to healthy adjacent tissue (Figure 2T). The above results indicate that ASF1B was significantly highly expressed in HCC compared with that in normal liver tissues.

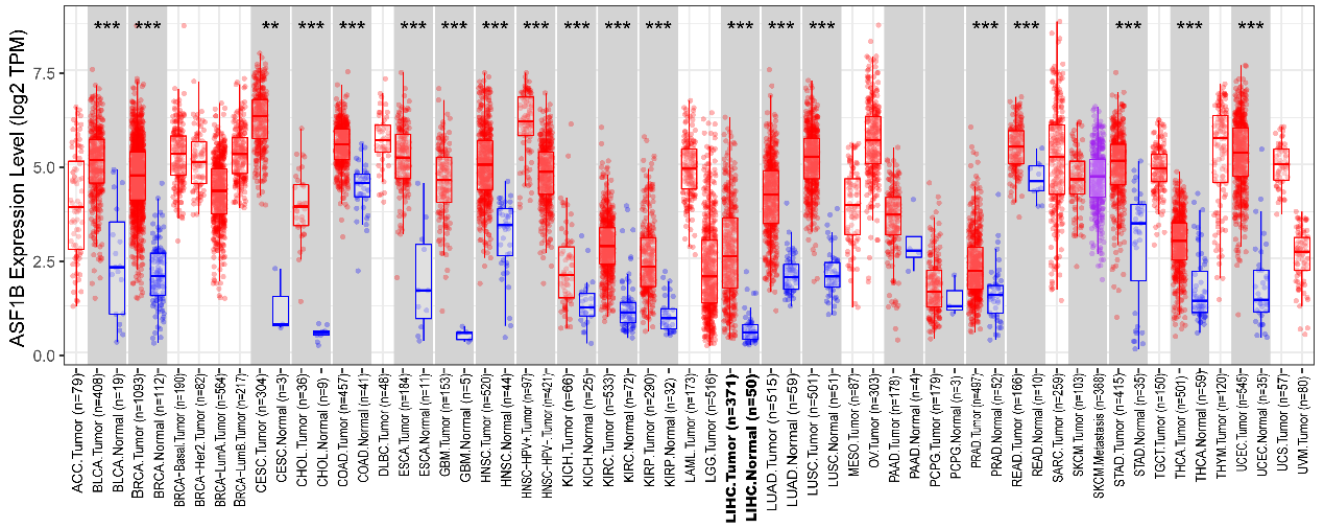

Figure 1. *The expression levels of ASF1B in different cancer types were determined by TIMER2.0. BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, Cholangiocarcinoma; COAD, colon adenocarcinoma; ESCA, Esophageal carcinoma; GBM, Glioblastoma multiforme; HNSC, Head and Neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; PRAD, Prostate adenocarcinoma; READ, Rectum adenocarcinoma; STAD, Stomach adenocarcinoma; THCA, thyroid carcinoma; UCEC, uterine corpus endometrial carcinoma.* *** *p <.001,* ** *p <.01,* * *p<.05.*

Table 1. *Details of GEO series included in this analysis.*

GSE Series	Tumor	Nontumor	Platform
GSE14520	225	220	GPL3921 [HT_HG-U133A] Affymetrix HT Human Genome U133A Array
GSE19665	10	10	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array
GSE25097	268	243	GPL10687Rosetta/Merck Human RSTA Affymetrix 1.0 microarray, Custom CDF
GSE45267	46	41	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array
GSE45436	93	41	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array
GSE55092	49	91	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array
GSE60502	18	18	GPL96 [HG-U133A] Affymetrix Human Genome U133A Array
GSE62232	81	10	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array
GSE65372	39	15	GPL14951Illumina HumanHT-12 WG-DASL V4.0 R2 expression beadchip
GSE76297	62	59	GPL17586 [HTA-2_0] Affymetrix Human Transcriptome Array 2.0 [transcript (gene) version]
GSE76311	62	59	GPL17586 [HTA-2_0] Affymetrix Human Transcriptome Array 2.0 [transcript (gene) version]
GSE77314	50	50	GPL9052Illumina Genome Analyzer (Homo sapiens)
GSE84402	14	14	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array
GSE84598	22	22	GPL10558 Illumina HumanHT-12 V4.0 expression beadchip
GSE87630	64	30	GPL6947 Illumina HumanHT-12 V3.0 expression beadchip
GSE112790	183	15	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array
GSE121248	70	37	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array

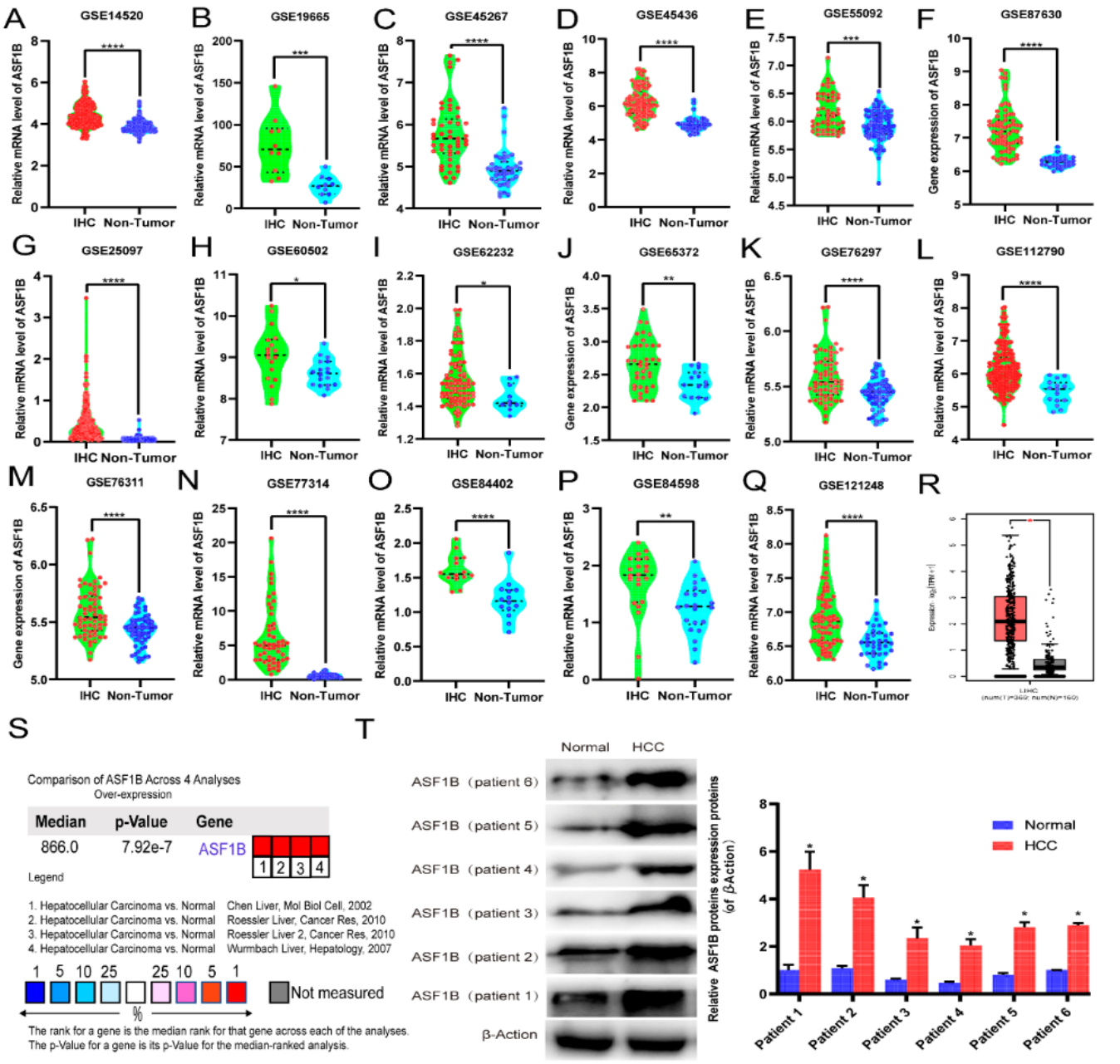

Figure 2. *ASF1B is highly expressed in hepatocellular carcinoma (HCC). (A-R) ASF1B mRNA expression levels between tumor and nontumor tissues in HCC patients in GEO database series including GSE14520(A), GSE19665(B), GSE45267(C), GSE45436(D), GSE55092(E), GSE87630(F), GSE25097(G), GSE60502(H), GSE62232(I), GSE65372(J), GSE76297(K), GSE112790(L), GSE76311(M), GSE77314(N), GSE84402(O), GSE84598(P), GSE121248(Q) and GEPIA2 database (R). (S) Meta-analysis of ASF1B mRNA expression levels in 4 analyses using Oncomine database. (T) Western blotting was performed to evaluate the protein expression of ASF1B in HCC tissues (relative to the internal control, β-Action) vs. normal (healthy adjacent liver tissues). *** p < 0.001, ** p < 0.01, * p< 0.05.*

Downregulation of ASF1B expression inhibited proliferation and induced G1/S phase arrest and cell apoptosis in SNU-423 cells

ASF1B gene expression profile data were downloaded to investigate the differential expression of ASF1B in human HCC cell lines from the CCLE database. We found that the expression of ASF1B in SNU-423 was much higher than that in other cell lines (Figure 3A).

After SNU-423 cells transfected with siASF1B a, siASF1B b and siASF1B c were cultured for 24 h, western blotting showed that siASF1B a and siASF1B c were more down-regulated than siASF1B b (Figure 3B). After that, we examined the effects of ASF1B on growth by CCK-8. CCK-8 showed the viability of SNU-423 cells transfected with siASF1B a and siASF1B c significantly decreased from 24h to 48h (Figure 3C).

To investigate the potential reason of the inhibition of SNU-

423 cells proliferation by siASF1B a and siASF1B c, flow cytometry was used to detect cell cycle and apoptosis (Figure 3D and E). After transfection with siRNA a, the percentage of cells in the G1 phase increased from 33.78% to 63.72%, and the percentage of cells in the S phase concomitantly decreased from 63.45% to 35.92% (Figure 3D). And after transfection with siRNA c, the percentage of cells in the G1 phase increased from 33.78% to 66.28%, and the percentage of cells in the S phase concomitantly decreased from 63.45% to or 32.86% (Figure 3D). As suggested in Figure 3D, ASF1B may promote HCC development through affecting the transition from the G1 stage to the S stage. Additionally, when SNU-423 cells were treated with siRNAs-ASF1B, the percentage of early and late apoptotic cells were all significantly increased (Figure 3E). The above results indicated that compared with the control, siRNA-ASF1B significantly enhanced the apoptotic and promoted the cell cycle arrest of SNU-423 cells (Figure 3D and E).

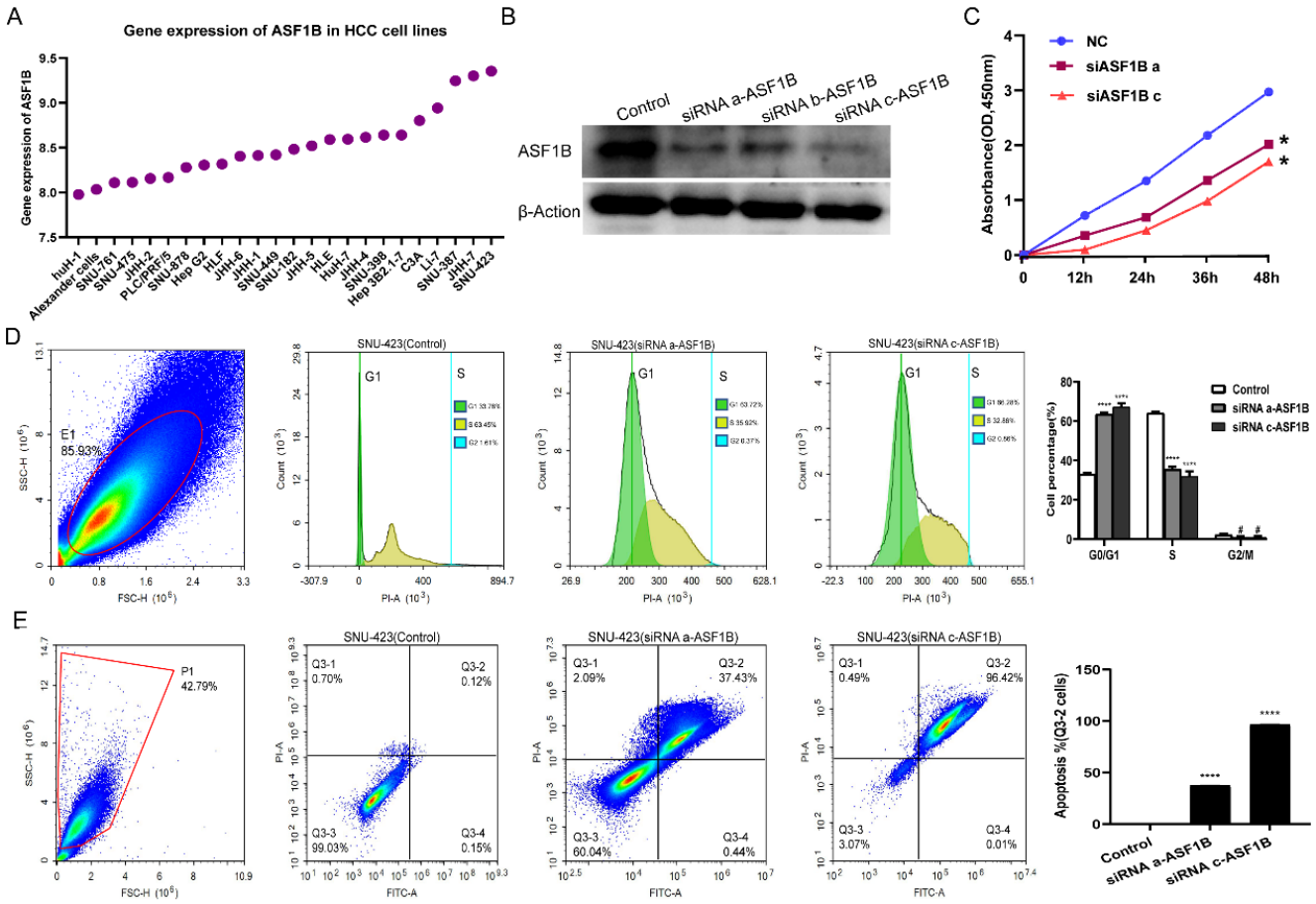

Figure 3. *Expression of ASF1B in HCC cell lines, and effects of downregulation of ASF1B on the proliferation, cell cycle and apoptosis of SNU-423 cells. (A) Expression of ASF1B in HCC cell lines. (B) Protein expression of ASF1B protein in SNU-423 cells after treatment with siRNAs-ASF1B. (C) The effect of ASF1B downregulation on proliferation. (D) Downregulation of ASF1B leads to a marked increase in G1-phase cells and a marked decrease in cells in the S phase. (E) The effect of ASF1B downregulation on cell apoptosis of SNU-423 cells. *** p < 0.001, ** p < 0.01, * p< 0.05, #P >0.05 vs. control. ASF1B, anti-silencing function 1B histone chaperone; siRNA, small inteferring RNA.*

3.2. Relationship Between High Expression of ASF1B and Prognosis of HCC Patients

Through GEPIA2 database, we investigated the relationship between the expression of ASF1B and overall survival (Figure 4A) and disease-free survival (Figure 4B) in HCC patients, and found that overexpression of ASF1B was associated with worse overall survival and disease-free survival in HCC patients (P < 0.05). Then, the relationship between the ASF1B expression and clinical characteristics of HCC patients was investigated in TIMER database (Figure 4C-G) to better understand the relevance and underlying mechanisms of ASF1B expression, the results show that high ASF1B mRNA expression was correlated with worse prognosis in HCC patients over 69 years of age and under 60 years of age (Figure 4C) as well as male and female HCC patients (Figure 4D), four types of stages of HCC patients (Figure 4E), three types of races of HCC patients in the world (Figure 4F) and purity of tumor of HCC patients (Figure 4G) (P < 0.05). In addition,

overexpression of ASF1B was correlated with worse prognosis stages of HCC patients and age of HCC (Figure 4H).

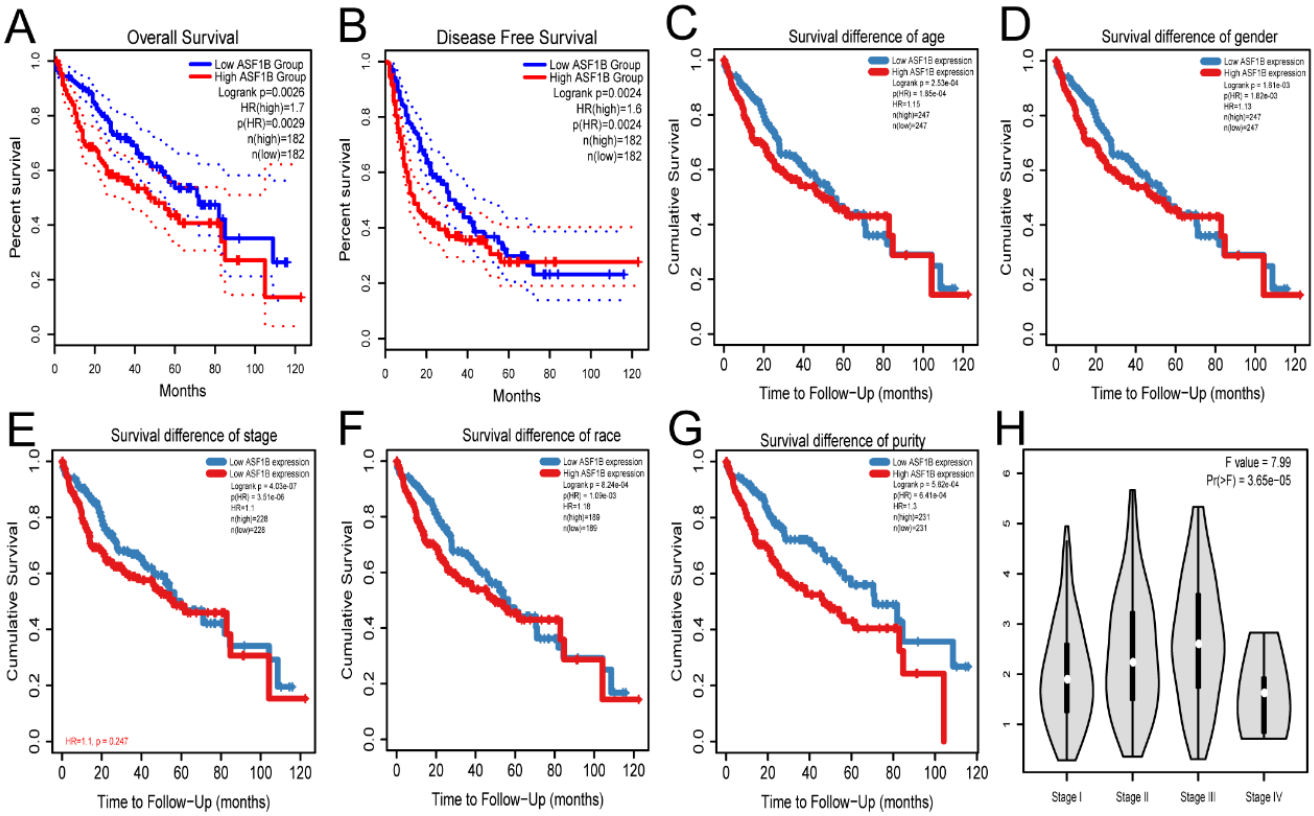

Figure 4. *Relationship between high expression of ASF1B and prognosis of HCC patients with different clinical characteristics (A-G) and clinical staging (H) of HCC patients. Overall survival (A) and disease-free survival (B) of HCC patients in GEPIA2 database. Subgroup analyses of overall survival comparison in different population [age(C), gender (D), stage (E), race (F) and purity (G)] with ASF1B expressed in HCC patients through TIMER2.0 database.*

3.3. Correlation ASF1B Expression with Immune Infiltration Level in HCC

To investigate the correlation ASF1B expression with immune infiltration level in HCC, we performed the relationships between ASF1B expression and immune infiltration cells in HCC through TIMER2.0database (Figure 5). The results showed that after purity adjusted, ASF1B expression had significant positively correlations with infiltrating levels of CD4+ T cells (Figure 5A), CD8+ T cells (Figure 5B), NK cells (natural killer cells) (Figure 5C), Tregs (regulatory T cells) (Figure 5D), B cells (Figure 5E), myeloid dendritic cells (Figure 5F), MDSC (myeloid derived suppressor cells) (Figure 5G), monocyte (Figure 5H), macrophages (Figure 5I), neutrophils (Figure 5J) in HCC. In addition, the effect of ASF1B expression on tumor microenvironment was also analyzed via TIMER2.0 database (Figure 5K-U), we found that ASF1B expression was significantly positively correlated with T cell follicular helper (Figure 5K), T cell gamma delta (Figure 5L), T cells NK (Figure 5M), cancer associated fibroblast (Figure 5O), common lymphoid progenitor (Figure 5P), mast cell (Figure 5S), and eosinophil (Figure 5U). While, ASF1B expression had a strong negative correlation with hematopoietic stem cell (Figure 5N), common myeloid progenitor (Figure 5Q), granulocyte-monocyte progenitor (Figure 5R), and endothelial cell (Figure 5T). All of the above results indicated that ASF1B plays a specific role in immune infiltration in HCC.

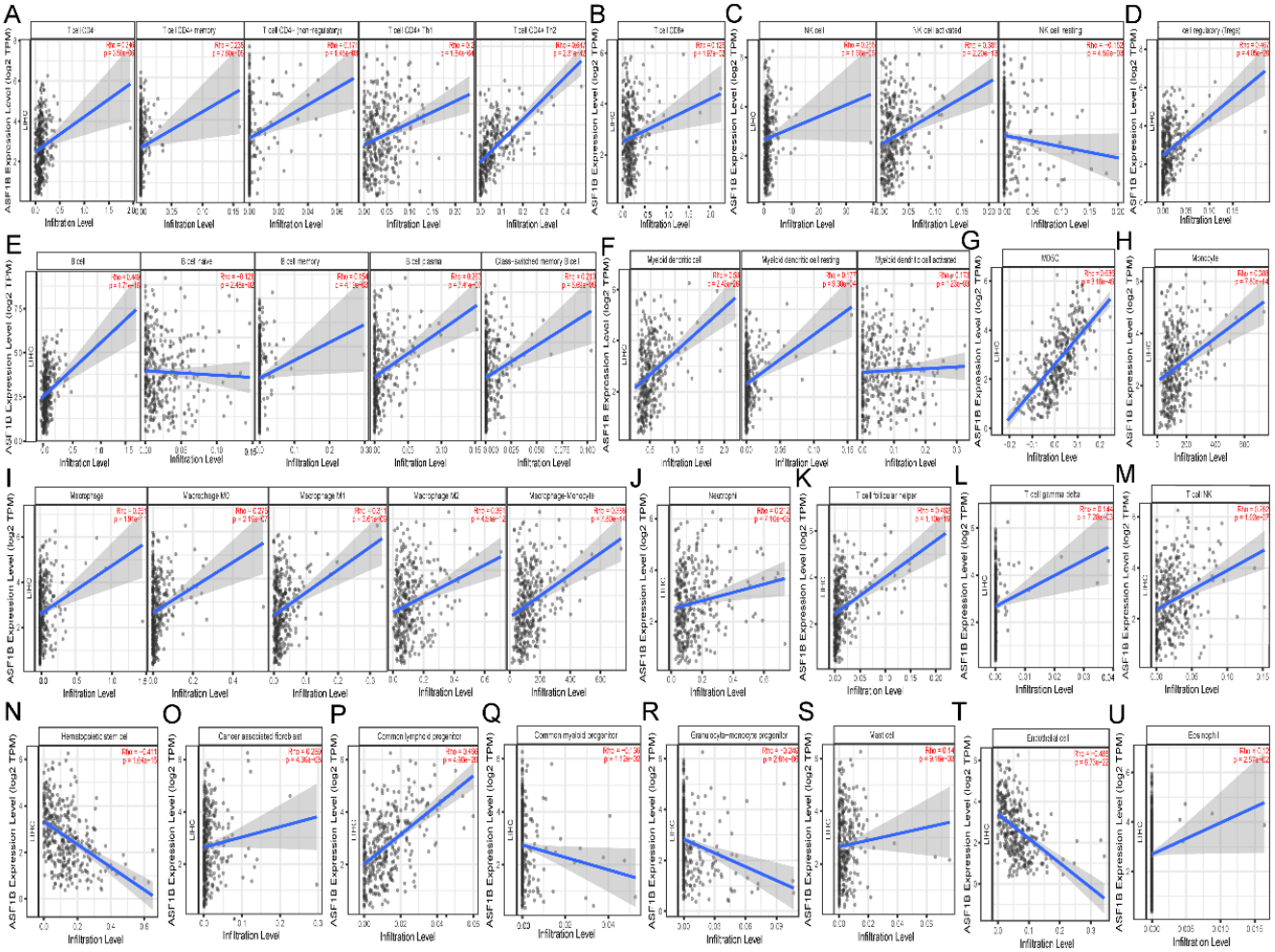

Figure 5. *Correlation of ASF1B expression with immune infiltration level in HCC. (A) ASF1B expression is significantly positive correlations with CD4+ T cell, Memory CD4+ T cell, non-regulatory CD4+ T cell, Th1 CD4+ T cell and Th2 CD4+ T cell. (B) ASF1B expression is significantly positively related to the infiltrating levels of CD8+ T cell. (C) ASF1B expression is significantly positively correlations with infiltrating levels of NK cell, activated NK cell, and significantly negatively correlations with resting NK cell. (D) ASF1B expression is significantly positively related to the infiltrating levels of Tregs. (E) ASF1B expression is significantly positively related to the infiltrating levels of B cell, memory B cell, plasma B cell, class-switched memory B cell and has significant negative correlations with infiltrating levels of native B cell in HCC. (F) ASF1B expression is significantly positively related to myeloid dendritic cell, resting myeloid dendritic cell, and activated myeloid dendritic cell. (G-H) ASF1B expression is significantly positively related to MDSC and Monocyte. (I) ASF1B expression is significantly positively related to macrophage, macrophage M0, macrophage M1, macrophage M2, and macrophage-monocyte. (J-M) ASF1B expression is significantly positively related to Neutrophil, T cell follicular helper, T cell gamma delta, and T cell NK. (N) (N-U) ASF1B expression is significantly negatively related to hematopoietic stem cell(N), common myeloid progenitor(Q), granulocyte-monocyte progenitor(R), and endothelial cell(T), but positively related to cancer associated fibroblast(O), common lymphoid progenitor(P), mast cell(S) and eosinophil(U). HCC, hepatocellular carcinoma; MDSC, myeloid derived suppressor cells; Th, T helper cell; Tfh, Follicular helper T cell; Treg, regulatory T cell.*

3.4. Correlation Analysis Between ASF1B Expression and Immune Markers

To performed the correlation analysis between ASF1B expression and immune markers, we analyzed the correlations between ASF1B expression and immune marker genes of different immune cells (Figure 6) (Table 2). After the correlation adjustment by purity, the results revealed the ASF1B expression level was significantly positive correlated with most immune marker sets of various immune cells in HCC, such as CD8A and CD8B of CD8+ T cell (Figure 6A), CD2, CD3D, and CD3E of T cell (general) (Figure 6B), CD19, CD79A, FCRL2, KIAA0125, MS4A1, PNOC, SPIB, TCL1A, and TNFRSF17 of B cell (Figure 6C), CCR7, IL7R and SELL of central memory T cell (Figure 6D), CD69, CXCR6, ITGAE, and MYADM of resident memory T cell (Figure 6E), CCR8, CTLA4, FOXP3 and TNFRSF9 of effector Treg T cell (Figure 6F), CCL2, CD68, and IL10 of TAM (Figure 6G), CX3CR1 and FCGR3A of effector T cell (Figure 6H), CD86 and CD115 (CSF1R) of monocyte (Figure 6I), IRF5 and COX2(PTGS2) of M1 macrophage (Figure 6J), CD163,

VSIG4 and MS4A4A of M2 macrophage (Figure 6K), KIR2DL3, KIR2DL4, KIR3DL2, XCL1 and XCL2 of natural killer cell (Figure 6L), CCR7, CD11b (ITGAM), FPR1, SIGLEC5, CSF3R, FCAR, FCGR3B and CEACAM3 of neutrophils (Figure 6M), BCL6 and IL21 of Tfh (Follicular helper T cells) (Figure 6N), CCR8, FOXP3, STAT5B, TGFβ (TGFB1) and STAT3 of Treg (Figure 6O), PDL1(CD274), CTLA4, GZMB, TIM-3 (HAVCR2), LAG3, PD-1 (PDCD1) and TIGIT of T cell exhaustion(Figure 6P), HDC of mast cell (Figure 6Q), IFN-γ (IFNG), STAT1, STAT4, TNF-α (TNF) and T-bet (TBX21) of Th1 (Figure 6R), GATA3, STAT6, STAT5A and IL13 of Th2 (Figure 6S), CCL13, BDCA-1(CD1C), CD209, HLA-DPA1, HLA-DPB1, HLA-DQB1, HLA-DRA, CD11c (ITGAX), BDCA-4(NRP1) and HSD11B1 of dendritic cell(Figure 6T).

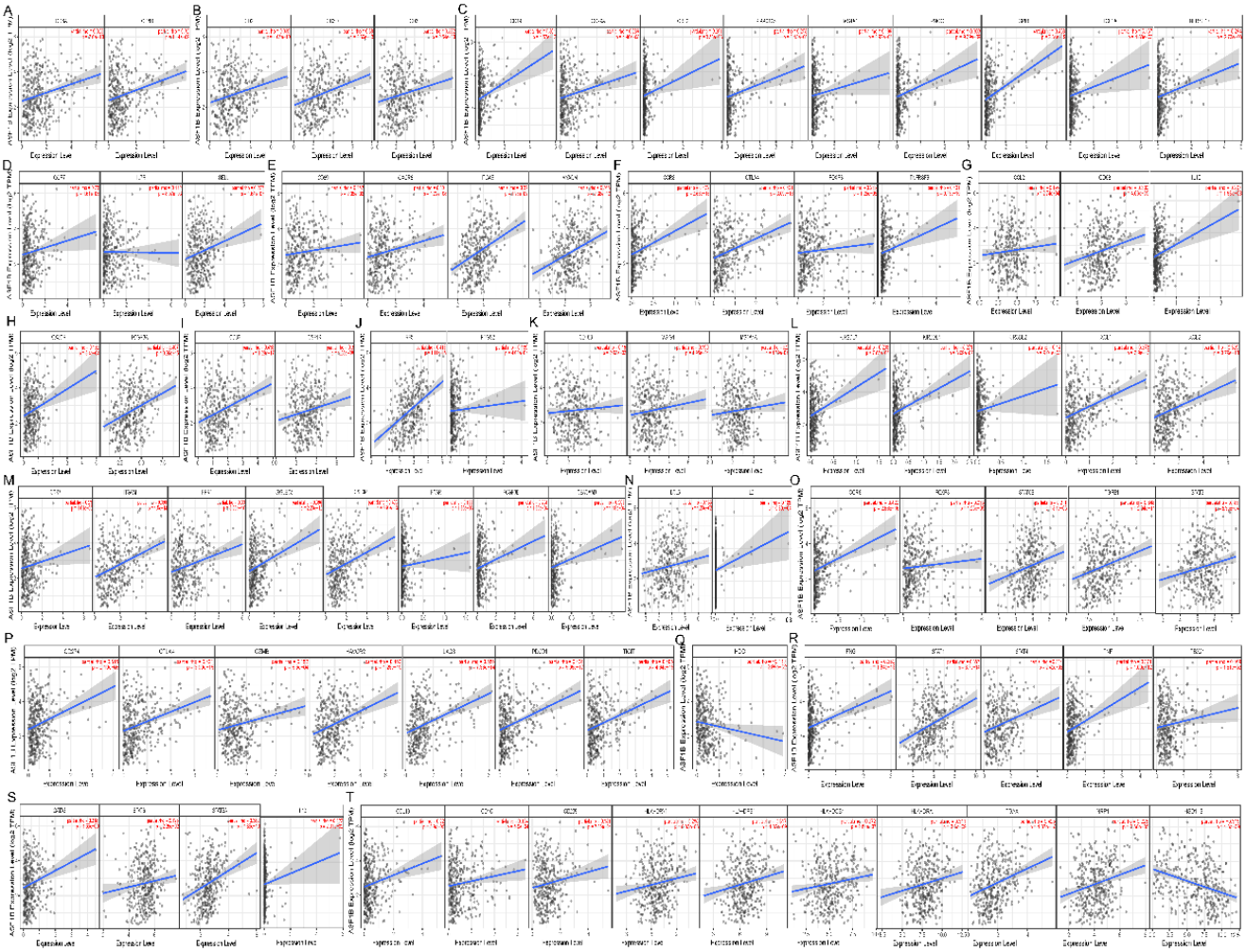

Figure 6. *ASF1B expression correlated with immune markers in HCC. Markers include: CD8A and CD8B of CD8+ T cell(A); CD2, CD3D and CD3E of T cell (general)(B); CD19, CD79A, FCRL2, KIAA0125, MS4A1, PNOC, SPIB, TCL1A, and TNFRSF17 of B cell(C); CCR7, IL7R and SELL of Central memory T cell(D); CD69, CXCR6, ITGAE, and MYADM of Resident memory T cell(E); CCR8, CTLA4, FOXP3, and TNFRSF9(F); CCL2, CD68, and IL10 of TAM(G); CX3CR1 and FCGR3A of FCGR3A(H); CD86 and CD115 (CSF1R) of Monocyte(I); IRF5 and COX2(PTGS2) of M1 Macrophage(J); CD163, VSIG4, and MS4A4A of M2 Macrophage(K); KIR2DL3, KIR2DL4, KIR3DL2, XCL1, and XCL2 of Natural killer cell(L); CCR7, CD11b (ITGAM), FPR1, SIGLEC5, CSF3R, FCAR, FCGR3B, and CEACAM3 of Neutrophils(M); BCL6 and IL21 of Tfh(N); CCR8, FOXP3, STAT5B, TGFβ (TGFB1), and STAT3 of Treg(O); PDL1(CD274), CTLA4, GZMB, TIM-3 (HAVCR2), LAG3, PD-1 (PDCD1), and TIGIT of T cell exhaustion(P); HDC of Mast cell(Q); IFN-γ (IFNG), STAT1, STAT4, TNF-α (TNF), and T-bet (TBX21) of Th1(R); GATA3, STAT6, STAT5A, and IL13 of Th2(S); CCL13, BDCA-1(CD1C), CD209, HLA-DPA1, HLA-DPB1, HLA-DQB1, HLA-DRA, CD11c (ITGAX), BDCA-4(NRP1), and HSD11B1 of Dendritic cell(T). HCC, hepatocellular carcinoma; TAM, tumor-associated macrophage; Th, T helper cell; Tfh, Follicular helper T cell; Treg, regulatory T cell.*

The above results indicate that ASF1B significantly correlates with the gene markers of B cells, T cells, CD8+ T cells, CD4+ T cells, mast cells, as well as most of the functional T cells, such as effector T cells, effector memory T cells, central memory T cells, exhausted T cells, and effector Treg cells. In-

terestingly, we found that the expression levels of most markers have strong correlations with ASF1B expression in HCC ((P <0.0001; Figure 6, Table 2). What's more, these results not only corroborated the critical relationships between ASF1B and B cells, T cells, and functional T cells as previous studies, but also demonstrated a novel close link between ASF1B and mast cells while rare relevant researches have been reported, which showed its potential value and it is worth further investigation of underlying mechanism.

Table 2. *Correlation analysis between ASF1B and relate genes and markers of immune cells in HCC through TIMER2.0.*

Description	Gene markers	None		Purity	
		cor	p	cor	p
CD8+ T cell	CD8A	0.196609	0.000138	0.323493	7.60E-10
	CD8B	0.200959	9.72E-05	0.320271	1.14E-09
T cell (general)	CD3D	0.251919	8.86E-07	0.380106	2.66E-13
	CD3E	0.163741	0.001553	0.332013	2.54E-10
	CD2	0.182919	0.000398	0.338656	1.05E-10
B cell	CD19	0.269462	1.36E-07	0.361135	4.57E-12
	CD79A	0.156253	0.002544	0.289312	4.46E-08
	BLK	-0.05908	0.256349	0.031013	0.565908
	FCRL2	0.152728	0.003186	0.291278	3.58E-08
	KIAA0125	0.133591	0.009994	0.258491	1.13E-06
	MS4A1	0.071628	0.168585	0.199453	0.000192
	PNOC	0.176043	0.000659	0.302917	9.39E-09
	SPIB	0.360987	7.34E-13	0.457771	2.84E-19
	TCL1A	0.079163	0.128002	0.16683	0.001875
	TNFRSF17	0.149971	0.003788	0.293682	2.73E-08
Central memory T cell	CCR7	0.072699	0.16229	0.229928	1.61E-05
	IL7R	0.02216	0.67052	0.142408	0.008073
	SELL	0.153153	0.003102	0.277343	1.64E-07
Resident memory T cell	CD69	0.114336	0.027661	0.252419	2.05E-06
	CXCR6	0.150834	0.003589	0.300655	1.22E-08
	ITGAE	0.304042	2.25E-09	0.31969	1.23E-09
	MYADM	0.326247	1.2E-10	0.365652	2.36E-12
Effector Treg T cell	CCR8	0.318931	3.23E-10	0.421629	2.66E-16
	CTLA4	0.299126	4.17E-09	0.420781	3.09E-16
	FOXP3	0.14694	0.004566	0.232811	1.25E-05
	TNFRSF9	0.307224	1.50E-09	0.400713	9.73E-15
TAM	CCL2	0.069595	0.181029	0.194732	0.000274
	CD68	0.204134	7.48E-05	0.308816	4.66E-09
	IL10	0.175267	0.000697	0.29718	1.83E-08
Effector T cell	CX3CR1	0.130788	0.011686	0.191782	0.00034
	FGFBP2	-0.13139	0.011303	-0.10276	0.056538

Description	Gene markers	None		Purity	
		cor	p	cor	p
	FCGR3A	0.293332	8.51E-09	0.407226	3.26E-15
Monocyte	CD86	0.270858	1.17E-07	0.438134	1.29E-17
	CD115 (CSF1R)	0.141789	0.006225	0.300485	1.25E-08
M1 Macrophage	INOS (NOS2)	0.00083	0.987291	0.012585	0.815819
	IRF5	0.403556	5.79E-16	0.413476	1.11E-15
	COX2(PTGS2)	0.05574	0.284241	0.189491	0.000402
M2 Macrophage	CD163	0.039251	0.450991	0.1597	0.002933
	VSIG4	0.072892	0.161176	0.199248	0.000195
	MS4A4A	0.057195	0.271839	0.20044	0.000179
Natural killer cell	KIR2DL1	-0.01205	0.817055	-0.02929	0.587747
	KIR2DL3	0.187391	0.000284	0.238016	7.85E-06
	KIR2DL4	0.230101	7.56E-06	0.271342	3.09E-07
	KIR3DL1	0.0203	0.696743	0.039788	0.461336
	KIR3DL2	0.090125	0.082989	0.14042	0.00901
	KIR3DL3	0.04537	0.383546	0.053374	0.322916
	KIR2DS4	0.078105	0.133191	0.0774	0.151405
	XCL1	0.327251	1.04E-10	0.377611	3.90E-13
	XCL2	0.242794	2.23E-06	0.328919	3.79E-10
	NCR1	0.03214	0.537154	0.097916	0.069298
Neutrophils	CD66b (CEACAM8)	0.054238	0.297438	0.088409	0.101137
	CCR7	0.072699	0.16229	0.229928	1.61E-05
	CD11b (ITGAM)	0.289616	1.33E-08	0.398096	1.50E-14
	FPR1	0.1829	0.000399	0.330081	3.26E-10
	SIGLEC5	0.226133	1.09E-05	0.36605	2.23E-12
	CSF3R	0.254492	6.79E-07	0.40275	6.93E-15
	FCAR	0.059534	0.252687	0.166233	0.001948
	FCGR3B	0.164692	0.001456	0.204082	0.000135
	CEACAM3	0.113215	0.029232	0.205666	0.000119
	S100A12	-0.06339	0.223182	-0.02338	0.665162
Tfh	BCL6	0.126637	0.014654	0.134679	0.012283
	IL21	0.140968	0.006535	0.185375	0.000539
Treg	CCR8	0.318931	3.23E-10	0.421629	2.66E-16
	FOXP3	0.14694	0.004566	0.232811	1.25E-05
	STAT5B	0.224771	1.24E-05	0.210581	8.10E-05
	TGFβ (TGFB1)	0.242877	2.21E-06	0.348022	2.94E-11
	STAT3	0.130141	0.01211	0.182949	0.000639
T cell exhaustion	PD-1 (PDCD1)	0.314751	5.63E-10	0.427609	9.06E-17

Description	Gene markers	None		Purity	
		cor	p	cor	p
	CTLA4	0.299126	4.17E-09	0.420781	3.09E-16
	GZMB	0.095853	0.065142	0.181839	0.00069
	TIM-3 (HAVCR2)	0.287047	1.81E-08	0.461707	1.28E-19
	LAG3	0.334483	3.78E-11	0.388298	7.33E-14
	PDL1(CD274)	0.228457	8.81E-06	0.315012	2.19E-09
	TIGIT	0.266017	1.99E-07	0.405534	4.34E-15
	TPSB2	-0.05715	0.272195	-0.00466	0.93131
Mast cell	TPSAB1	-0.07116	0.171391	-0.0019	0.971885
	CPA3	-0.02792	0.591908	0.055804	0.301339
	MS4A2	-0.00373	0.942891	0.066717	0.216427
	HDC	-0.16602	0.00133	-0.11699	0.029817
Th1	T-bet (TBX21)	0.051447	0.32303	0.169197	0.00161
	STAT4	0.241966	2.42E-06	0.314211	2.42E-09
	STAT1	0.334533	3.76E-11	0.387227	8.70E-14
	IFN-γ (IFNG)	0.284543	2.43E-08	0.382455	1.84E-13
	TNF-α (TNF)	0.23286	5.83E-06	0.370821	1.09E-12
Th2	GATA3	0.169848	0.001022	0.317823	1.55E-09
	STAT6	0.127046	0.014336	0.122759	0.022578
	STAT5A	0.312305	7.77E-10	0.382435	1.85E-13
	IL13	0.108926	0.035974	0.122335	0.023053
Dendritic cell	CCL13	0.146127	0.004799	0.219558	3.90E-05
	BDCA-1(CD1C)	0.082349	0.11331	0.184082	0.00059
	CD209	0.092936	0.073793	0.181303	0.000716
	HLA-DPA1	0.140941	0.006545	0.285453	6.83E-08
	HLA-DPB1	0.175135	0.000704	0.316544	1.82E-09
	HLA-DQB1	0.147584	0.00439	0.272155	2.84E-07
	HLA-DRA	0.172089	0.000874	0.310943	3.60E-09
	CD11c (ITGAX)	0.283199	2.85E-08	0.425308	1.37E-16
	BDCA-4(NRP1)	0.193353	0.000179	0.224526	2.56E-05
	HSD11B1	-0.32727	1.04E-10	-0.31921	1.30E-09

HCC, hepatocellular carcinoma; TAM, tumor-associated macrophage; Th, T helper cell; Tfh, Follicular helper T cell; Treg, regulatory T cell; Cor, R value of Spearman's correlation; None, correlation without adjustment. Purity, correlation adjusted by purity.

3.5. PPI and GO Biological Process Enrichment

PPI (protein-protein interaction) analysis using STRING database revealed that10 genes including HIST1H4A, CHAF1B, H3F3A, TLK2, TLK1, MCM2, NASP, HAT1, H3F3B and HIST2H4A were interacted with ASF1B (Figure 7A). In addition, the top 500 genes that expressed similar ASF1B in HCC were downloaded from the GEPIA2 database.

Enrichment analysis with these genes through Metascape database showed that the genes similar to ASF1B in HCC were mainly enriched in Cell Cycle, cell division, DNA replication, cell cycle phase transition, regulation of chromosome segregation, cell cycle signaling pathways, PID PLK1 PATHWAY, PID E2F PATHWAY, et al. (Figure 7B-C). The above results indicate that ASF1B promotes tumor progression in HCC a variety of complex regulatory mechanisms, including the cell cycle, cell cycle phase transition and DNA replication.

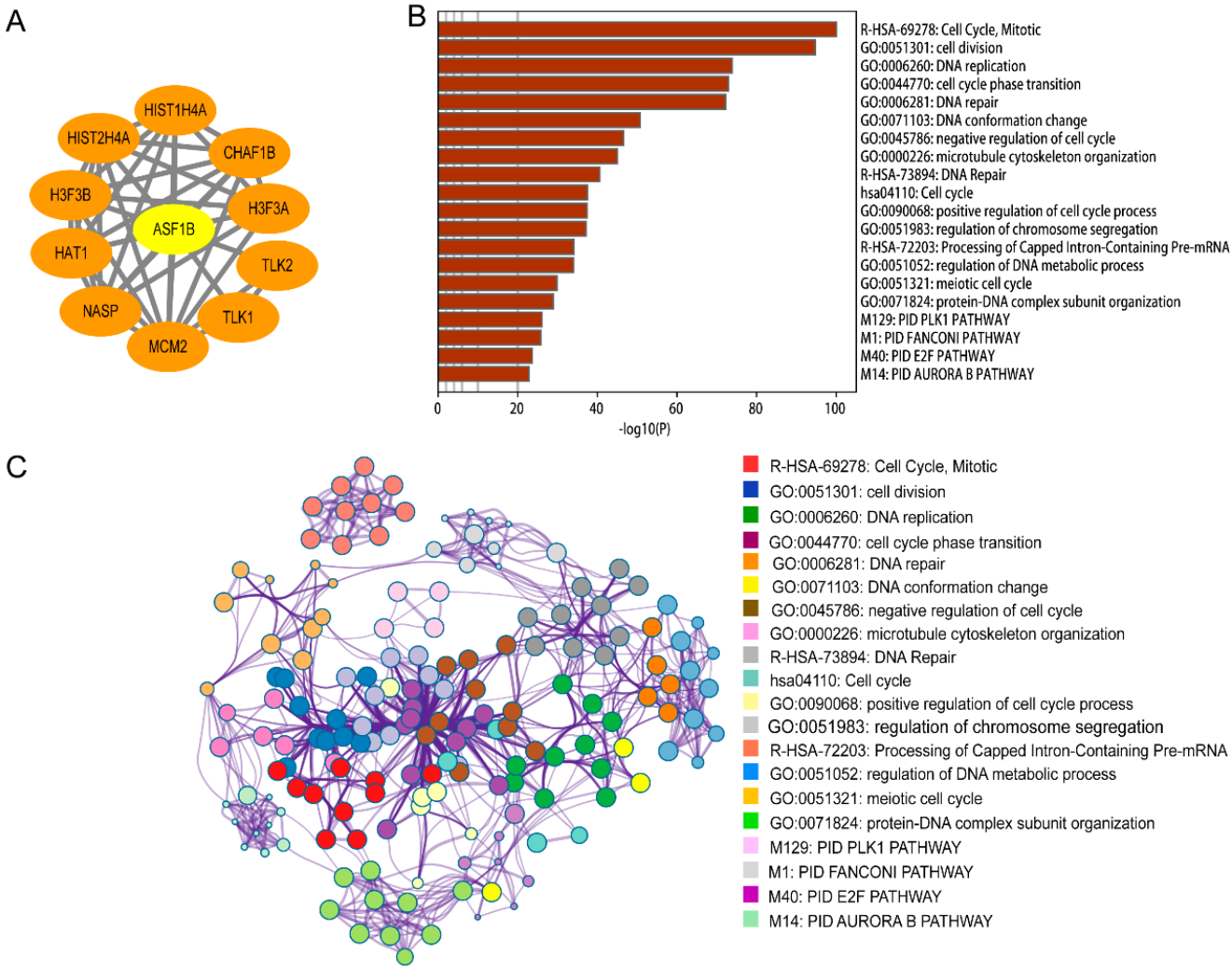

Figure 7. *Protein-protein interaction of ASF1B using String analysis (A); The visualized networks of functional enrichment of the top 500 genes that expressed similar ASF1B in HCC, then each node represents an enriched term and is colored first by its p-value(B) and by its cluster ID (C).*

4. Discussion

As one of the members of histone H3-H4 chaperone protein, ASF1(including ASF1A and ASF1B) has been demonstrated to contribute to tumorigenesis [32, 33]. ASF1B (Anti-Silencing Function 1B Histone Chaperone) is is closely related to DNA replication, DNA damage repair, and transcription regulation [34-37]. It is known to promote the progression of a variety of tumors, such as breast cancer [7] and prostate cancer [38]. What's more, a recent study has shown that ASF1B not only promotes the progression of lung adenocarcinoma, but also that high ASF1B expression indicates a poor prognosis in patients with lung adenocarcinoma [39], as well as the Clear Cell Renal Cell Carcinoma [40]. However, the role of ASF1B in HCC remains unclear. And there is no research on the relationship between ASF1B and immune infiltrates. In our study, we found that ASF1B was significantly overexpressed in HCC.And the higher the stage of HCC patients, the higher the expression of ASF1B. This result is similar to the overexpressed ASF1B in breast cancer, cervical carcinoma and prostate cancer [38]. While siRNA-ASF1B significantly reduced the viability, and promoted apoptosis, G1 phase cell cycle arrest of SNU-423 cells. And high expression ASF1B correlates

with a poorer prognosis in HCC. Furthermore, our analyses show that immune infiltration levels and diverse immune markers are correlated with levels of ASF1B expression in HCC. Thus, our study provides insights in understanding the potential role of ASF1B in tumor immunology and its use as a cancer biomarker, as well as the pathogenesis and patient prognosis of HCC.

A previous study has demonstrated that the overexpressed ASF1B markedly enhances the proliferation of breast cancer prostate cancer [38]. Thus, we hypothesized that siASF1B may decrease the growth of HCC cells. The results also demonstrated that siRNA-ASF1B significantly decreased the viability of SNU-423 cells. Previous studies have found that cell proliferation is related to cell cycle distribution [41]. In our study, we found siRNA-ASF1B induced G1 phase cell cycle arrest, and it inhibit the transition from G1 phase to S phase of the cell cycle (Figure 3D). As another important process in the progression of tumors, tumor cell apoptosis plays an important role in the mechanism of anticancer drugs and anticancer gene therapy [42]. Therefore, the effect of siASF1B on the apoptosis of HCC was analyzed. The results revealed that siRNA-ASF1B increased the apoptosis rate of SNU-423 cells (Figure 3E). Previous studies have shown that ASF1B may be a target for tumor therapy [38]. Taken together, down-regulation of ASF1B expression can induce anti-tumor effects on HCC by promoting cell apoptosis and cell cycle arrest.

Previous studies have shown that tumor infiltrating immune cells are highly correlated with prognosis and the identification of immunotherapeutic targets in HCC [43]. And immune infiltration can promote tumor progression by influencing tumor microenvironment [44, 45]. Based on the relationship between tumor microenvironment and immune infiltration, immunotherapy plays an important role in tumor therapy, such as T cell therapy [45-47]. Therefore, the relationship between the expression of ASF1B and immune infiltration level (including regulating tumor immunology, such as immune markers) in HCC was analyzed via TIMER2.0, and the results showed that ASF1B expression had significant positively correlations with infiltrating levels of CD4+ T cells, CD8+ T cells, NK cells, Tregs, B cells, myeloid dendritic cells, MDSC, monocyte, macrophages, neutrophils in HCC, as well as some signature cells of the tumor microenvironment, especially in T cells exhaustion (Figures 5-6). Osteopontin (OPN) is considered as a potential therapeutic target for glioblastoma by recruiting macrophages into glioblastoma and mediating the interaction between tumor cells and the immune system [48]. Signal transducer and activator of transcription 3 (STAT3), when activated in tumor cells and tumor-infiltrating immune cells, and it has emerged as a promising target for cancer immunotherapy [49]. Our results showed that the expression of ASF1B was positively correlated with STAT3 in HCC (Figure 6O). Regulatory T cells (Treg) promote tumor progression in malignant tumors by inhibiting effective anti-tumor immunity. In addition, increased Treg in tumor-infiltrating lymphocytes is associated with poor prognosis in various types of human cancers [50]. T cells become exhausted/dysfunctional play a very important role in tumor progression and prognosis, based on this, PD-1 is particularly important in the anti-cancer treatment of some tumors [51]. Therefore, the high expression of ASF1B promotes the progression of HCC, and is responsible for the poorer prognosis of HCC patients, which may be related to immune infiltration. It provides a preliminary theoretical basis for the development of new immunotherapy.

Previous studies also provide some possible mechanism to explain why ASF1B expression is associated with immune infiltration and poor prognosis. previous studies suggest that ASF1B may be involved in DNA replication and repair, and transcriptional regulation [34-37], as well as steady-state erythroid differentiation [52]. Overexpression of ASF1B is sufficient to induce human β-cell proliferation through binding histones H3 [53-55]. And ASF1B regulates DNA repair by participating in nucleosome assembly [35], promoting histone delivery to downstream histone chaperones [56]. The occurrence and development of cancer is a complex process. To further explore the possible mechanism of ASF1B in the progression of HCC, enrichment analysis was performed. The results showed that ASF1B may be involved in the progression of HCC through cell cycle, cell division and differentiation, DNA replication and repair, cell cycle phase transition, regulation of cell cycle process, PID E2F PATHWAY, and so on (Figure 7). Therefore, cell cycle, DNA replication and repair, transcriptional regulation, cell cycle, and signaling pathway regulation may be the potential mechanisms of ASF1B expression and HCC progression and poor prognosis. These data further suggest that ASF1B is a potential therapeutic target for cancer therapy.

5. Conclusions

In summary, overexpression of ASF1B is a marker of poor prognosis in HCC. Downregulation of ASF1B expression inhibited proliferation and induced G1/S phase arrest and cell apoptosis in SNU-423 cells. Increased immune infiltration levels in CD4+ T cells, CD8+ T cells, B cells, and T cells. In addition, ASF1B expression potentially contributes to most of the functional T cells, such as effector T cells, effector memory T cells, central memory T cells, exhausted T cells, and effector Treg cells. Therefore, ASF1B likely plays an important role in immune cell infiltration and as a prognosis biomarker in HCC. ASF1B is a potential therapeutic target for HCC patients.

Abbreviations

HCC	Hepatocellular Carcinoma
ASF1B	Anti-silencing Function 1B Histone Chaperone
PD - 1	Programmed cell Death 1
TMB	Tumor Mutational Burden
PD-L1	Programmed cell Death 1 Ligand 1
GEO	Gene Expression Omnibus

MDSC Myeloid Derived Suppressor Cells

Author Contributions

Feng Tian: Conceptualization, Data curation, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft

Qiyi Chen: Data curation, Investigation, Methodology, Visualization, Writing – original draft

Jun Li: Investigation, Methodology, Resources, Writing – original draft

Yonghui Wang: Conceptualization, Resources, Supervision, Validation, Writing – review & editing

Jianfei Tu: Investigation, Resources, Writing – review & editing

Xingdong Cai: Conceptualization, Methodology, Validation, Writing – review & editing

Daxia Cai: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing

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Data Availability Statement

The databases that downloaded from the GEO database in our study have been taken permission, and the data used and analyzed during the present study are shared from the corresponding authors with reasonable request. The other data supporting the results of this study is available in an open database including GEPIA2 database(http://gepia2.cancer-pku.cn/#index), TIMER2.0 database (http://timer.cistrome.org/), Oncomine database (https://www.oncomine.org/resource/login.html), and STRING database (https://string-db.org/). The other data during the study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that there is no conflict of interest.

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