AGTRAP Is a Prognostic Biomarker Correlated With Immune Infiltration in Hepatocellular Carcinoma

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Background: Recently, it has been reported that angiotensin II receptor-associated protein (AGTRAP) plays a substantial role in tumor progression. Nevertheless, the possible role of AGTRAP in hepatocellular carcinoma (HCC) remains unrecognized.

Methods: The metabolic gene rapid visualizer, Cancer Cell Line Encyclopedia, Human Protein Atlas, and Hepatocellular Carcinoma Database were used to analyze the expression of AGTRAP in HCC tissues and normal liver tissues or adjacent tissues. Kaplan-Meier plotter and UALCAN analysis were used to assess the prognostic and diagnostic value of AGTRAP. LinkedOmics and cBioPortal were used to explore the genes co-expressed with AGTRAP in HCC. To further understand the potential mechanism of AGTRAP in HCC, Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment pathway analyses were performed using R software, the protein-protein interaction (PPI) network was established using the STRING database, and the immune infiltration and T-cell exhaustion related to AGTRAP were explored via Timer and GEPIA. In addition, immunohistochemistry was used to detect the expression of AGTRAP protein in HCC tissues and paired adjacent tissues from clinical specimens.

Results: This study found that the mRNA and protein levels of AGTRAP in HCC tissues were higher than those in normal liver tissues and adjacent tissues, and higher mRNA levels of AGTRAP were associated with higher histological grade and a poor overall survival in HCC patients. The area under the receiver operating characteristic curve (AUC) of AGTRAP was 0.856, suggesting that it could be a diagnostic marker for HCC. Moreover, the alteration rate of AGTRAP in HCC was 8%, and AGTRAP was involved in HCC probably through the NF-κB and MAPK signaling pathways. Furthermore, AGTRAP was positively correlated with the infiltration of CD8+ T cells, CD4+ T cells, B cells, macrophages, dendritic cells, and neutrophils, and the levels of AGTRAP were significantly correlated with T-cell exhaustion biomarkers. The immunohistochemistry results confirmed that the protein levels of AGTRAP were consistently higher in HCC tissues than in paired adjacent tissues.
Conclusion: The clinical value of AGTRAP and its correlation with immune infiltration in HCC was effectively identified in clinical data from multiple recognized databases. These findings indicate that AGTRAP could serve as a potential biomarker in the treatment of HCC, thereby informing its prognosis, diagnosis, and even immunotherapy.

Keywords: hepatocellular carcinoma, AGTRAP, prognosis, immune infiltration, biomarker

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fourth most common malignant tumor worldwide (1) with low overall survival (OS) of patients and poor prognosis (2). The majority of patients are diagnosed with HCC at an advanced stage, usually accompanied by intra- and extra-hepatic invasion and metastasis, with no specific symptoms at the early stage (3). Although an increasing number of adjuvant therapeutic methods have been found to improve the treatment efficacy of HCC, the recurrence and metastasis of HCC remain difficult obstacles. Therefore, there is an urgent need to identify novel specific biomarkers to improve the clinical diagnosis and prognosis in HCC patients.

Angiotensin II receptor-associated protein (AGTRAP) is localized in the perinuclear vesicle structure and the plasma membrane (4). AGTRAP, which inhibits the activation of angiotensin II, has been broadly studied in cardiovascular diseases and metabolic disorders (5, 6). Recently, Sanz-Pamplona et al. (7) found that AGTRAP is overexpressed in colon cancer and positively correlates with a poor prognosis. Figueiredo et al. (8) reported that AGTRAP-BRAF gene fusion was detected in gastric cancer, indicating a possible role of AGTRAP in tumor progression. However, little is known about the function of AGTRAP in HCC and the oncogenic mechanisms involved.

Bioinformatics is a combination of life science and computer science that aims to identify the sequence, structure, and function of biological macromolecules and their interrelationships. In the last several years, bioinformatics has proved to be a valuable tool in screening tumor biomarkers (9, 10). In this study, we used bioinformatics to explore the expression of AGTRAP in HCC tissues and normal tissues or adjacent tissues, the value of AGTRAP in HCC patient prognosis and diagnosis, and its correlation with clinicopathological parameters, and performed enrichment analyses of AGTRAP alterations and its co-expressed genes using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). Finally, immunohistochemistry (IHC) was used to verify the levels of AGTRAP in HCC tissues and paired adjacent tissues. Our study aimed at defining the potential role of AGTRAP in the occurrence and progression of HCC and whether it could be used as a diagnostic and prognostic biomarker.

MATERIALS AND METHODS

TCGA Database

The gene expression RNA-Seq (HTSeq-FPKM), clinical data, and survival data were downloaded from the The Cancer Genome Atlas (TCGA) database from UCSC Xena (https://xenabio.wser.net/datapages/), which are processed uniformly by the TOIL process, free of computational batch effects (11).

Metabolic Gene Rapid Visualizer and Cancer Cell Line Encyclopedia

The Metabolic Gene Rapid Visualizer (MERAV, http://merav.wi.mit.edu) database was used to investigate the expression of AGTRAP (12). The gene expression data are normalized across these arrays to provide a means of consistent comparison. The Cancer Cell Line Encyclopedia (CCLE, https://portals.broadinstitute.org/ccle) integrates genetic information, including chromosome copy number, gene expression, and DNA mutations. CCLE can be applied to the evaluation of cellular targets, small molecules, genetic variations, and therapeutic approaches, allowing the recognition of new biomarker drivers of tumor dependence. The CCLE database provides a resource for cancer studies using models of cancer cell lines in vitro (13).

Hepatocellular Carcinoma Database and the Human Protein Atlas

In addition, the expression of AGTRAP in different liver tissues was studied using the Hepatocellular Carcinoma Database (HCCDB, http://lifeome.net/database/hccdb) (14). Simultaneously, co-expression networks of adjacent tissues and HCC tissues were assessed through HCCDB. Datas were normalized by log2 FC, and P-values were adjusted with Benjamini & Hochberg correction.

The protein expression of AGTRAP in both LIHC and normal tissues was obtained from the Human Protein Atlas database (HPA, version 20.1) (https://www.proteinatlas.org/), which is a program with the aim to map all the human proteins in cells, tissues. Next, the HPA was used to analyze the expression of AGTRAP in human tissues and cells (15).

Kaplan-Meier Plotter and UALCAN

The Kaplan-Meier plotter (http://kmplot.com/analysis/) (16) was used to analyze the prognostic value of AGTRAP in HCC samples based on TCGA (https://www.cancer.gov/) datasets. UALCAN (ualcan.path.uab.edu) (17) is an open database for the analysis of gene expression data (TPM) from TCGA, which has been normalized. We used UALCAN to analyze the difference in AGTRAP expression in normal and HCC samples, the relationships between AGTRAP expression and the clinicopathological parameters of each sample (tumor grade, tumor stage, TP53 mutant, weight, age, sex, nodal metastasis), and its potential prognostic value.
LinkedOmics and cBioPortal
LinkedOmics (https://linkedomics.org/) is an online analysis tool that can be used to compare multi-omic cancer datasets from the Clinical Proteomic Tumor Analysis Consortium (CPTAC) and TCGA with an array of tumor types (32 cancers and 11,158 patients) and proteomics data (18). LinkedOmics involves three important analysis modules (LinkCompare, LinkFinder, and LinkInterpreter) and plots data as heat maps, scatter plots, and volcano plots. We selected RNAseq data from the TCGA-LIHC dataset for analysis, which are RSEM format data that has been converted by log2, using the Pearson correlation test, and they corrected for false positives (False Discovery Rate, FDR) using the Benjamini-Hochberg (BH) correction.

The cBio Cancer Genomics Portal (cBioPortal, version 3.7.1, http://cbioportal.org) was principally applied to explore multidimensional cancer genomics datasets with resources from 20 cancer studies including more than 5,000 tumor samples (19). We selected the log RNA Seq V2 RSEM data from TCGA-LIHC on this website for mutation analysis and co-expression gene analysis, which has been normalized.

STRING
The Search Tool for the Retrieval of Interacting Genes (STRING, version 11.5, http://string-db.org) provides references for studying the mechanism of disease occurrence or progression and provides a basis for exploring the functional interactions between proteins (20). STRING was used to demonstrate the functional networks of AGTRAP in HCC.

TIMER and GEPIA
The Tumor Immune Estimation Resource (TIMER, version 1.0, https://cistrome.shinyapps.io/timer/) was applied to analyze the association between AGTRAP gene expression and immune infiltration in HCC, as well as T-cell exhaustion (21). The relevant immune cells include B Cells, CD8+ T Cells, CD4+ T Cells, macrophages, neutrophils, and dendritic cells. The scatterplots will be generated and displayed after inputs are submitted successfully, showing the purity-corrected partial Spearman’s rho value and statistical significance.

The Gene Expression Profiling Interactive Analysis (GEPIA, version 1.0, http://geopia.cancer-pku.cn/) is a newly developed interactive web server for analyzing the RNA sequencing expression data of 9,736 tumors and 8,587 normal samples from the TCGA and the GTEx projects, using a standard processing pipeline. The RNA-Seq datasets GEPIA used is based on the UCSC Xena project (http://xena.ucsc.edu), which are computed by a standard pipeline. The database provides a customizable and interactive function, including differential expression analysis, correlation analysis, profiling plots, gene analysis, and survival analysis. The associations between AGTRAP levels and T-cell exhaustion biomarkers (PD-1 (PDCD1), GZMB, LAG-3, CTLA-4, and HAVCR2 (TIM-3)) were explored using the GEPIA database (22). The data uses Log2 (TPM+1) for normalization. We performed a survival analysis of the TCGA-LIHC dataset with a median cutoff, Kaplan–Meier (KM) plots are presented with the hazard ratio (HR), the 95% confidence interval (CI), the log-rank p-value (p). Pearson correlation was chosen for gene correlation analysis.

Immunohistochemistry Analysis
The study was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. All participants provided informed consent in writing. The pathological sections of 10 HCC patients were re-analyzed by professional pathologists via IHC staining of AGTRAP in paired tumors and adjacent tissues using the streptavidin-peroxidase (SP) method. Paraffin-embedded tumor tissues were collected and sliced into 3 mm sections. The slides were baked at 60°C for 2 h to be fully dewaxed and then rehydrated through a graded alcohol series and washed with phosphate buffered saline. Hydrogen peroxide (3%) was used to block endogenous peroxidase activity for 10 min at 25°C. Antigen retrieval was performed by microwaving in 10 mM citrate buffer (pH = 6.0) for 15 min. The slides were then blocked and sealed with 5% normal goat serum for 30 min at room temperature. The rest of the procedures are in accordance with the kit instructions (SP-9000, OriGene, China). Fifty microliters of anti-AGTRAP antibody (1:100, Sangon Biotech, China) was added to these tissue sections, which were then incubated at 4°C overnight. The sections were then incubated with the corresponding secondary antibody, OriGene, for 30 min at room temperature. Finally, the IHC score for each sample was calculated as the intensity of immunostaining (0, colorless; 1, light-yellow; 2, brownish-yellow; 3, dark brown) multiplied by the percentage of positive cells (0: 0%, 1: 1%–10%, 2: 11%–50%, 3: 51%–75%, and 4: 76%–100%).

Western Blotting
Radioimmunoprecipitation assay buffer (RIPA) buffer purchased from Beyotime Biotechnology (China) was used to extract total protein from tissues, and the supernatant was collected after centrifugation (12,000×g, 15 min, 4°C). BCA Protein Assay Kit (Biyuntian, China) was used to quantify the protein concentration. Equal protein quantities were separated by 12.5% SDS-PAGE gel and then transferred to PVDF membranes (0.22 um, PVDF membrane, GE healthcare life science, Germany). Membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS) for 1 h and was incubated with primary antibodies against AGTRAP (1:2000, Sangon Biotech, China) and β-actin (1:2000, Abclonal, China) at 4°C overnight. The membrane was then incubated with secondary antibodies (1:2000, Proteintech, China) for 1 h the next day. Finally, the signals were developed by an ECL detection system (Enhanced chemiluminescence system kit). Subsequently, gray scale quantifications of bands were performed using Image J software (National Institutes of Health, USA).

Statistical Analysis
All statistical analyses were performed using the SPSS software (version 26.0, IBM, USA) and the GraphPad Prism software (version 7.0, USA, www.graphpad.com) was used for graphing the heatmap and analyze the different grayscale values between adjacent tissues and tumor tissues of western blot bands. The
Receiver operating characteristic curve (ROC) was analyzed using the “pROC” package (version 1.17.0.1) and “ggplot2” (version 3.3.3) in R Studio (version 3.6.3). The GO and KEGG enrichment analyses were visualized using the R packages “ggplot2” (version 3.3.3) and “clusterProfiler” (version 3.15.3), the threshold for statistically significant differences was set at $p \text{ adjust} \leq 0.05$. A chi-square test was used to assess the correlation between AGTRAP expression and clinicopathological parameters. Logistic regression and Spearman correlation analyses were used to analyze the correlation between clinical parameters and AGTRAP expression. Univariate and multivariate Cox regression analyses were performed to determine the association between AGTRAP expression and OS. Differences were considered statistically significant at $p < 0.05$. False discover rate (FDR) < 0.05 was regarded statistically credible.

RESULTS

The mRNA and Protein Expression of AGTRAP in Different Databases

First, we explored the different expression levels of AGTRAP mRNA in HCC cells and HCC tissues as well as normal tissues in the MERAV (Figure 1A). AGTRAP expression was significantly higher in HCC cells than in normal tissue samples. The CCLE database was then used to visualize the gene expression data. As assessed through an array of cancer cell lines, AGTRAP was highly expressed in HCC cells (Figure 1B). Next, we reviewed the HCCDB to identify whether there were significant differences in AGTRAP mRNA expression levels between HCC datasets. As shown in Figure 1C, the AGTRAP level in HCC tissues was higher than in adjacent tissues (HCC/adjacent: logFC = 0.30). Subsequently, we compared AGTRAP levels in 12 different HCC datasets of which 9 supported our results (Figure 1D).

AGTRAP expression in normal liver tissues was lower compared to majority of human tissues in HPA (Figure 2A). Additionally, the level of AGTRAP in HCC tissues was the lowest among the cancer samples (Figure 2B). However, AGTRAP levels were higher in HCC cell lines (Hep G2) than in most cancer cell lines (Figure 2C). Intriguingly, the expression of AGTRAP in liver cells was mainly concentrated in Kupffer cells, and its expression was also high in T cells (Figure 2D). Although the expression of AGTRAP was relatively low in normal human liver tissues (Figure 2E), the HPA database showed that AGTRAP protein was moderately expressed in HCC tissues by IHC, mainly localized in cell plasma (Figure 2F).
High AGTRAP Expression Is Correlated With Poor OS in HCC Patients and May Serve as a Diagnostic Biomarker Based on TCGA Datasets

To explore the association between AGTRAP level and OS in HCC patients, we used the Kaplan-Meier plotter tool and found a significant correlation between high expression of AGTRAP and poor prognosis (Figure 3A, hazard ratio (HR) = 2.13, 95% CI = 1.45–3.03, p = 7.8e-5). Next, we conducted a correlation analysis in the GEPIA database. The resultant violin plots showed that expression of AGTRAP was significantly correlated with the pathological stage of patients (Figure 3B).

AGTRAP mRNA Expression Is Associated With Clinicopathological Characteristics in HCC Patients Based on TCGA Datasets

The UALCAN database was used to explore whether AGTRAP mRNA expression is associated with clinicopathological parameters. As illustrated in Figure 4A, a positive trend was found between expression of AGTRAP in HCC and the progression of tumor grade. The analysis of additional parameters found AGTRAP mRNA levels were positively correlated with tumor stage, patient weight, and TP53 mutation (Figures 4B–D). Furthermore, the expression of AGTRAP mRNA in cancer tissues was higher than that in normal tissues (Supplementary Figure 1A). There were no statistical differences between the expression of AGTRAP and different age periods, gender, and lymph node metastasis status (Figures 4E, F and Supplementary Figure 1B).

The Correlation Between Clinical Features and AGTRAP Expression in HCC From TCGA Data

To further analyze the clinical relevance of AGTRAP expression in HCC, we used TCGA clinical parameter data related to AGTRAP expression and deleted cases with missing data. The data was divided into two patient groups based on the median AGTRAP expression level (high or low) and the differences in group clinical features were analyzed using a chi-square test (Table 1). We found that histological grade varied significantly between the low and high expression groups. Logistic regression analysis and Spearman correlation analysis were used to analyze the correlation between these clinical parameters and the expression level of AGTRAP (Tables 2, 3) and indicated a strong correlation between OS time and AGTRAP level. Univariate Cox regression analyses showed
that age, race, pathologic stage, histologic grade, and T stage were related to OS. Finally, significant factors were analyzed by multivariate Cox regression which indicated that AGTRAP was an independent risk factor affecting the OS of patients with HCC (Tables 4, 5).

**GO and KEGG Analyses of AGTRAP and Its Co-Expressed Genes in HCC**

To better understand the biological role of AGTRAP, the LinkedOmics database was used to identify co-expressed genes that are both positively and negatively correlated with AGTRAP.
Heat maps were used to visualize the 50 genes with the strongest correlation to AGTRAP (Figures 5B, C). Next, using the HCCDB, we analyzed the co-expression networks of AGTRAP and found significant differences between HCC tissues and adjacent liver tissues (Figures 5D, E). In addition, we performed GO and KEGG enrichment analyses of the co-expressed genes in HCC tissues and adjacent liver tissues (24). The main biological processes these genes were involved in were neutrophil degranulation, neutrophil activation involved in immune response, and neutrophil activation. These genes were located in the primary lysosome, azurophil granule, and tertiary granule. Additionally, they also play crucial roles in cadherin binding and cell adhesion molecule binding. Lastly, KEGG pathway analyses indicated that these genes were mainly enriched in glycolysis/gluconeogenesis, bacterial invasion of epithelial cells, and endocytosis (Figure 5F).

Table 1: Associations between AGTRAP mRNA expression and clinicopathological characteristics in HCC.

| Characteristics | cases | Low expression of AGTRAP (%) 134 (50.2) | High expression of AGTRAP (%) 133 (49.8) | P value |
|-----------------|-------|----------------------------------------|----------------------------------------|---------|
| Age <=60       | 126   | 64 (24.0)                              | 62 (23.2)                              | 0.762   |
| >60            | 141   | 69 (25.8)                              | 72 (27.0)                              |         |
| Gender Female  | 92    | 46 (17.2)                              | 46 (17.2)                              | 0.965   |
| Male           | 175   | 87 (32.6)                              | 88 (33.0)                              |         |
| Weight <=70    | 141   | 64 (24.0)                              | 77 (28.8)                              | 0.126   |
| >70            | 126   | 69 (25.8)                              | 57 (21.3)                              |         |
| Race Asian     | 120   | 61 (22.8)                              | 59 (22.1)                              | 0.942   |
| Black or African American | 11 | 5 (1.9) | 6 (2.2) |         |
| White          | 128   | 64 (24.0)                              | 64 (24.0)                              |         |
| Pathologic stage |      |                                        |                                        |         |
| Stage I        | 250   | 181 (72.0)                             | 69 (25.8)                              | 0.145   |
| Stage II       | 60    | 22 (8.2)                               | 38 (14.2)                              |         |
| Stage III      | 53    | 28 (10.5)                              | 25 (9.4)                               |         |
| Stage IV       | 4     | 2 (0.7)                                | 2 (0.7)                                |         |
| T stage T1     | 152   | 82 (30.7)                              | 70 (26.2)                              | 0.146   |
| T2             | 60    | 22 (8.2)                               | 36 (14.2)                              |         |
| T3             | 51    | 27 (10.1)                              | 24 (8.9)                               |         |
| T4             | 4     | 2 (0.7)                                | 2 (0.7)                                |         |
| N stage N0     | 265   | 132 (49.4)                             | 133 (49.8)                             | 0.996   |
| N1             | 2     | 1 (0.4)                                | 1 (0.4)                                |         |
| M stage M0     | 264   | 131 (49.1)                             | 133 (49.8)                             | 0.994   |
| M1             | 4     | 2 (0.7)                                | 2 (0.7)                                |         |
| Histologic grade |      |                                        |                                        | 0.032*  |
| G1             | 22    | 13 (24.0)                              | 9 (3.4)                                |         |
| G2             | 133   | 76 (25.8)                              | 57 (21.3)                              |         |
| G3             | 100   | 40 (13.6)                              | 60 (22.5)                              |         |
| G4             | 12    | 4 (1.1)                                | 8 (3.0)                                |         |
| AFP (ng/ml)    |       |                                        |                                        |         |
| <=400          | 202   | 104 (41.1)                             | 98 (36.7)                              | 0.335   |
| >400           | 125   | 29 (10.7)                              | 96 (36.0)                              |         |
| Albumin (g/dl) |       |                                        |                                        | 0.084   |
| <3.5           | 60    | 24 (13)                                | 36 (13.5)                              |         |
| >=3.5          | 207   | 109 (39.3)                             | 98 (36.7)                              |         |

* means p<0.05.

GO and KEGG Analyses of AGTRAP and Its Neighboring Genes Associated With AGTRAP Alterations in HCC

Considering that genomic alterations of AGTRAP are pathogenic, the CBioPortal database was used to investigate genetic alterations of AGTRAP based on TCGA sequencing data. AGTRAP was altered in 28 of the 360 (8%) patients. From the results there was 1 patient with high AGTRAP mRNA expression (0.29%), 1 patient with amplification (0.29%), 6 patients with deep deletion (1.67%), 1 patient with missense mutation (0.29%), 17 patients with high mRNA mutation (4.72%), and only one patient with two alterations (0.29%) (Figure 6A).

To further analyze the biological function of the AGTRAP gene and its neighboring genes, we performed GO and KEGG analyses on the genes that were positively correlated with AGTRAP gene alteration. The results showed that these genes...
were mainly enriched in Salmonella infection, pathogenic *Escherichia coli* infection, regulation of actin cytoskeleton, tight junction, and Yersinia validated infection (Figures 6B–E).

**Establishment of the PPI Network**

To further understand the mechanisms of this gene, we used the STRING database to conduct a protein-protein interaction network analysis and found the strongest interactions with MAPK3, B-RAF, ARRB2, CLCN6, GNB2L1, and AKAP9 proteins (Figure 7A). We then performed enrichment analysis of the genes associated with these interacting proteins. The results showed that they were mainly enriched in parathyroid hormone synthesis, secretion and action, chemokine signaling pathway, and MAPK signaling pathway (Figure 7B).

**AGTRAP Overexpression Is Associated With Immune Infiltration and T-Cell Exhaustion**

As shown in the HPA database, AGTRAP was highly expressed in Kupffer cells and T cells. We suggested that this was related to immune infiltration and T-cell exhaustion. The relationship between AGTRAP level and immune infiltration and T-cell exhaustion in HCC was explored using the TIMER online tool based on TCGA data. AGTRAP expression was positively correlated with the infiltration of B cells (correlation = 0.225, \( p = 2.43 \times 10^{-5} \)), CD8+ T cells (correlation = 0.321, \( p = 1.29 \times 10^{-9} \)), CD4+ T cells (correlation = 0.292, \( p = 3.52 \times 10^{-8} \)), macrophages (correlation = 0.417, \( p = 8.18 \times 10^{-16} \)), neutrophils (correlation = 0.45, \( p = 4.22 \times 10^{-11} \)), and dendrites (correlation = 0.406, \( p = 6.14 \times 10^{-15} \)) (Figure 8A). It also correlated with the 3-year (\( p = 0.007 \)) and 5-year survival rates (\( p = 0.001 \)) (Figures 8B, C).

Moreover, the correlations between AGTRAP and T-cell exhaustion markers, such as PD-1 (PDCD1), GZMB, LAG-3, CTLA-4, and HAVCR2 (TIM-3) in HCC tissues were explored using the GEPIA database. The results demonstrated a positive relationship between AGTRAP expression and the five biomarkers of T-cell exhaustion (Figure 8D, all \( p < 0.001 \)).

**Expression of AGTRAP Protein in HCC Tumor Tissues and Adjacent Tissues From Clinical Samples**

We assessed the expression of AGTRAP protein in HCC tumor tissues (T) and paired adjacent tissues (A). The protein expression of AGTRAP was higher in tumor tissues than in adjacent tissues (Figure 9A) and was mainly localized in cell plasma. Moreover, the heat map showed that AGTRAP expression was higher in HCC tumor tissues than in adjacent tissues (\( p < 0.001 \)) (Figure 9B). Western blot were also performed to validate the different expression of AGTRAP between adjacent tissues and tumor tissues in eight couple of paired samples, and the results (Figures 9C, D) showed that AGTRAP has a higher expression in tumor tissues than that in adjacent tissues (\( p < 0.001 \)), which is consistent with our bioinformatic analysis and IHC results.

**TABLE 2 | Logistic regression analysis of the risk factors of AGTRAP high expression in HCC.**

| Characteristics | OR      | 95% CI      | P value |
|-----------------|---------|-------------|---------|
| Age             | 1.015   | 0.99-1.04   | 0.185   |
| Gender          | 0.815   | 0.43-1.54   | 0.530   |
| Weight          | 0.988   | 0.97-1.00   | 0.151   |
| Race Asian      | 0.604   | 0.29-1.25   | 0.174   |
| Race White      | 0.808   | 0.21-3.14   | 0.758   |
| T stage T1      | 0.619   | 0.06-6.52   | 0.690   |
| T stage T2      | 1.336   | 0.12-14.55  | 0.812   |
| T stage T4      | 0.728   | 0.07-7.60   | 0.791   |
| N stage 0.667   | 0.04-11.89 | 0.783   |
| M stage 1.147   | 0.11-12.18 | 0.909   |
| Histologic grade G1 | 0.296   | 0.06-1.56   | 0.151   |
| G2              | 0.384   | 0.08-1.26   | 0.104   |
| G3              | 0.729   | 0.19-2.73   | 0.639   |
| G4              | 1.000   | 1.00-1.00   | 0.466   |
|AFP (ng/ml)      | 1.001   | 0.99-1.01   | 0.795   |
|Albumin (g/dl)   | 0.999   | 1.00-1.00   | 0.001   |

* means \( p < 0.05 \), ** means \( p < 0.01 \).

**TABLE 3 | Spearman analysis of correlation between AGTRAP and clinicopathological parameters.**

| Characteristics | AGTRAP expression level | Spearman Correlation | p value |
|-----------------|-------------------------|----------------------|---------|
| Age             | -0.017                  | 0.781                |         |
| Weight          | -0.107                  | 0.082                |         |
| Albumin         | -0.056                  | 0.363                |         |
|AFP             | 0.125                   | 0.041**              |         |
|OS time         | -0.293                  | 0.000**              |         |

* means \( p < 0.05 \), *** means \( p < 0.001 \).
HCC continues to cause a serious public health burden worldwide. Most patients with HCC are diagnosed at an advanced stage when the tumor is unresectable (25). A need exists for the development of reliable biomarkers that would allow for early diagnosis of HCC and the accurate prognosis of long-term outcomes. In this study, we identified the diagnostic and prognostic value of AGTRAP in HCC, the mechanisms that might affect the development of HCC, and its association with immune infiltration and T-cell exhaustion.

A growing amount of research suggests that AGTRAP may play an important role in tumor progression (7, 26). Sanz-Pamplona et al. (7) performed an exome sequence of 42 cases of colon tumors and their paired mucosa and discovered that AGTRAP was overexpressed in colon cancer with poor

TABLE 4 | Univariate Cox Regression Analysis on Survival of 267 HCC patients in TCGA dataset.

| Variables                      | HR   | 95% CI       | P value  |
|--------------------------------|------|--------------|----------|
| Age                            | 1.023| 1.00-1.04    | 0.018**  |
| Gender                         | 1.528| 0.98-2.37    | 0.080    |
| Weight                         | 0.994| 0.99-1.01    | 0.979    |
| Race                           |      |              | 0.002**  |
| Asian                          |      |              |          |
| Black or African American      | 0.451| 0.27-0.75    | 0.002**  |
| White                          | 1.829| 0.72-4.63    | 0.203    |
| Pathologic stage               |      |              | 0.001**  |
| Stage I                        |      |              |          |
| Stage II                       | 0.152| 0.05-0.43    | 0.000*** |
| Stage III                      | 0.144| 0.05-0.44    | 0.001**  |
| Stage IV                       | 0.267| 0.09-0.78    | 0.016*   |
| T stage                        |      |              | 0.025*   |
| T1                             | 0.255| 0.08-0.83    | 0.023*   |
| T3                             | 0.233| 0.07-0.82    | 0.023*   |
| T4                             | 0.436| 0.13-1.47    | 0.180    |
| N stage                        | 0.339| 0.08-1.39    | 0.133    |
| M stage                        | 0.173| 0.06-0.48    | 0.001**  |
| Histologic grade               |      |              | 0.199    |
| G1                             |      |              |          |
| G2                             | 0.246| 0.06-0.48    | 0.039*   |
| G3                             | 0.503| 0.20-1.29    | 0.152    |
| G4                             | 0.584| 0.23-1.51    | 0.266    |
| AFP (ng/ml)                    | 1.000| 1.00-1.00    | 0.212    |
| Albumin (g/dl)                 | 0.998| 0.91-1.09    | 0.966    |
| AGTRAP                         | 1.450| 1.13-1.87    | 0.004**  |

* means p<0.05, ** means p<0.01, *** means p<0.001.

TABLE 5 | Multivariate Cox Regression Analysis on Survival of 267 HCC patients in TCGA dataset.

| Variables                      | HR   | 95% CI       | P value  |
|--------------------------------|------|--------------|----------|
| Age                            | 1.022| 1.00-1.04    | 0.029*   |
| Race                           |      |              | 0.007**  |
| Asian                          |      |              |          |
| Black or African American      | 0.559| 0.33-0.96    | 0.035*   |
| White                          | 2.455| 0.95-6.38    | 0.065    |
| Pathologic stage               |      |              | 0.625    |
| Stage I                        |      |              |          |
| Stage II                       | 0.583| 0.14-2.51    | 0.469    |
| Stage III                      | 2.022| 0.21-9.58    | 0.543    |
| T stage                        |      |              |          |
| T1                             | 4.544| 0.35-59.25   | 0.248    |
| T4                             | 4.024| 0.46-35.26   | 0.209    |
| M stage                        | 0.094| 0.01-0.64    | 0.016*   |
| AGTRAP                         | 1.448| 1.10-1.90    | 0.007**  |

* means p<0.05, ** means p<0.01.
FIGURE 5 | Genes in correlation with AGTRAP in HCC (LinkedOmics). (A) Genes correlated with AGTRAP showing in volcano plot; red (positive), green (negative). (B, C) Heat maps showing the top 50 genes positively and negatively correlated with AGTRAP in HCC. (D) The co-expression networks of AGTRAP in HCC tissues. (E) The co-expression networks of AGTRAP in adjacent liver tissues. (F) GO and KEGG enrichment analysis of genes co-expressed in HCC tissues and adjacent liver tissues.

FIGURE 6 | Genetic alterations of AGTRAP (cBioPortal) and the GO and KEGG analyses of the neighboring genes associated with AGTRAP alterations in HCC. (A) The mutation rate of AGTRAP was 8%. The significantly enriched GO annotations: (B) biological processes, (C) cellular components, (D) molecular functions, and (E) KEGG pathways of AGTRAP neighboring genes.
prognosis. Zeng et al. (26) used weighted gene co-expression network analysis (WGCNA) and found that AGTRAP was overexpressed and was related to poor prognosis in tongue squamous cell carcinoma. Palanisamy et al. (27), through paired-end transcriptome sequencing, detected AGTRAP-BRAF gene fusion in gastric cancer. In concurrence with these studies, we found that mRNA and protein levels of AGTRAP were higher in HCC tissues compared to normal tissues, and our results were verified using clinical samples. In addition, our results showed that high expression of AGTRAP mRNA was associated with poor OS. Furthermore, the ROC curve indicated that AGTRAP is a sensitive diagnostic marker. Xiong et al. (28) identified AGTRAP as one of the 10 most notable prognosis genes in lower-grade glioma by WGCNA and survival analysis. Given these results, AGTRAP likely plays an important role in the occurrence and development of HCC.

Next, to better understand whether AGTRAP could affect the progression of HCC, we explored the association between

![Figure 7](image7.png)

**FIGURE 7** PPI of AGTRAP in HCC patients and the GO and KEGG analyses of these genes in HCC. (A) PPI of AGTRAP and other interacting proteins. (B) GO and KEGG enrichment analyses of genes interacted with AGTRAP in the String database.

![Figure 8](image8.png)

**FIGURE 8** Association between AGTRAP and immune infiltration and T cell exhaustion in HCC. (A) AGTRAP is correlated with immune infiltrations in HCC. (B, C) Analysis of 3-year and 5-year survival of immune infiltration and AGTRAP expression in patients with HCC. (D) The correlation between AGTRAP expression and T cell exhaustion in HCC analyzed by the GEPIA database.
AGTRAP and clinicopathological parameters in HCC. Consistent with our expectations, we found that patients with higher tumor grades had higher AGTRAP levels, accompanied by worse survival outcomes. However, this result was based on a small sample size of patients in stage T4 (n = 6). Additionally, our results demonstrated that the level of AGTRAP was significantly correlated with patient weight, especially BMI $\geq$ 30 kg/m$^2$, suggesting that obese patients may have a higher risk of HCC. Indeed, obesity has already been associated with several types of cancer (29). Additionally, our results showed that patients with TP 53 mutation also displayed higher AGTRAP levels in HCC. Unexpectedly, there was no statistical difference in lymph node metastasis. This could be attributed to the small number of cases and therefore increased sample size would benefit future research. Although current knowledge suggests that AGTRAP plays an important role in tumor occurrence and development, more research is required to elucidate its effects on tumors.

Genetic alterations and dysregulated amplification are critical for the development of many tumors (30). There was an 8% genetic alteration rate of AGTRAP in HCC patients in the cBioPortal database, and its related genes were mainly involved in ubiquitin-like protein binding and the regulation of the actin cytoskeleton. Protein ubiquitination, which is a type of post-translational protein modification, has been shown to be widely involved in the invasion and metastasis of malignant tumors and that multiple ubiquitin-activating enzymes regulate the expression of key factors in various tumor metastasis-related pathways (31–33). However, changes in the cytoskeleton could affect the activity of NF-kB, which is known to affect the immune response and carcinogenic response (34, 35). Interestingly, our PPI results showed that BRAF and MAPK3 interact with AGTRAP, suggesting that AGTRAP may be related to the MAPK signaling pathway. This is also in accordance with AGTRAP-BRAF gene fusion detected in gastric cancer (8).

Immune infiltration is known to correlate with the prognosis of numerous cancers (36). Our KEGG analysis of genes co-expressed with AGTRAP in HCC tissues and adjacent tissues also showed that its co-expressed genes were mainly enriched in neutrophil degranulation and neutrophil activation. Our analyses, based on datasets from multiple recognized databases, found a positive correlation between AGTRAP expression and all immune cells as well as T-cell exhaustion in HCC patients based on TCGA data. Similarly, Xiong et al. (28) found that AGTRAP displayed potential as an immunotherapy target in brain lower-grade glioma because of its association with different immune infiltrating cells. Here, we found that AGTRAP might affect the tumor microenvironment by regulating immune cells, especially neutrophils and T cells, and may serve as an immunotherapy target in HCC.

![Figure 9](image-url)
Based on our bioinformatics analysis, it suggested that AGTRAP could be involved in the immune microenvironment of hepatocellular carcinoma, and related to MAPK signaling pathway. Many studies have implicated the MAPK pathway in both the development and progression of HCC and indicated that activation of the MAPK pathway correlates with a poor prognosis in human HCC (37, 38). In addition, Liao et al. (38) reported that CXCL8 secreted by HCC cells makes a tumorigenic inflammatory microenvironment to promote epithelial-mesenchymal transition and HCC invasion by MAPK pathway. Better understanding the immune subtypes of HCC could guide personalized immunotherapy and clinical management of HCC (39). And a recent study published in the journal of GUT has been identified that administration of p38 MAPK inhibitor could restrain monocyte myeloid-derived suppressor cell to suppress the proliferation of HCC, indicating that MAPK signaling pathway was closely related to immune microenvironment of HCC (40). Therefore, we speculate that AGTRAP may regulate immune microenvironment of hepatocellular carcinoma through MAPK signaling pathway. Besides, MAPK signaling pathway was known to affect the immune microenvironment of tumor by affecting macrophage polarization (41, 42). Yang et al. (43) illustrated that compound kushen injection activates macrophages from M2 to M1 via triggering TNFR1 and its downstream MAPK p38 signaling cascades, which induces HCC cells apoptosis and suppress HCC tumor growth and recurrence. Furtherly, we also design further mechanism research to verify whether AGTRAP could be involved in immune microenvironment of hepatocellular carcinoma by regulating MAPK signal pathway-induced macrophage polarization from M2 to M1.

In this study, we described the preliminary expression and possible mechanisms of action of AGTRAP in HCC. Further studies on the molecular mechanism of AGTRAP in HCC would be designed both in vivo and in vitro, as well as verified in a large number of clinical samples.

CONCLUSION

The mRNA and protein levels of AGTRAP in HCC tissues were higher than those in normal liver tissues or adjacent tissues, and higher mRNA levels of AGTRAP were associated with higher histological grade and poor overall survival (OS) time in HCC patients. AGTRAP is involved in HCC, probably through the NF-κB and MAPK signaling pathways. Furthermore, AGTRAP positively correlated with immune infiltration and T-cell exhaustion. According to these results, AGTRAP could serve as a potential biomarker for prognostic, diagnostic, and even immunotherapy target for HCC.

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DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SL and NJ conceived and designed this study. SL and WZ collected and analyzed the relative data. XL collected the clinical samples. LZ and YG performed the IHC experiments. SL wrote the paper. QP and CD revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.713017/full#supplementary-material

Supplementary Figure 1 | (A) The mRNA expression level of AGTRAP was significantly higher in HCC tissues than in normal tissues. (B) The expression level of AGTRAP was positively correlated with nodal metastasis in HCC patients.

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