NAPSB as a Predictive Marker for Prognosis and Therapy Associated with an Immuno-Hot Tumor Microenvironment in Hepatocellular Carcinoma

Qiu Zhao (✉ qiuzhao@whu.edu.cn)

Zhongnan Hospital of Wuhan University

Yu-Mei Ning

Zhongnan Hospital of Wuhan University

Kun Lin

Zhongnan Hospital of Wuhan University

Xiao-Ping Liu

Zhongnan Hospital of Wuhan University

Yang Ding

Zhongnan Hospital of Wuhan University

Xiang Jiang

Zhongnan Hospital of Wuhan University

Zhang Zhang

Zhongnan Hospital of Wuhan University

Yu-Ting Xuan

Zhongnan Hospital of Wuhan University

Lan Liu

Zhongnan Hospital of Wuhan University

Fan Wang

Zhongnan Hospital of Wuhan University

Hai-Zhou Wang

Zhongnan Hospital of Wuhan University

Jun Fang

Zhongnan Hospital of Wuhan University

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Abstract

Background: Napsin B Aspartic Peptidase, Pseudogene (NAPSB) was associated with CD4+ T cell infiltration in pancreatic ductal adenocarcinoma. But the biological role of NAPSB in hepatocellular carcinoma (HCC) remains to be determined.

Methods: The expression of NAPSB in pan-cancer and HCC as well as its clinicopathological association were analyzed using data from several public datasets. qRT-PCR was used to verify the relative expression of NAPSB in HCC using Zhongnan cohort. Kaplan-Meier analyses, univariate and multivariate Cox regression were conducted to determine the predictive value of NAPSB on HCC prognosis. Then enrichment analyses were performed to identify the possible biological functions of NAPSB. Subsequently, the immunological characteristics of NAPSB in the HCC tumor microenvironment (TME) were demonstrated comprehensively. The role of NAPSB in predicting hot tumors and its impact on immunotherapy and chemotherapy responses was also analyzed.

Results: NAPSB was downregulated in HCC and high NAPSB expression showed an improved survival outcome. Enrichment analyses showed that NAPSB was related to immune activation. NAPSB was positively correlated with immunomodulators, tumor-infiltrating immune cells (TILs), T cell inflamed score, and cancer immunity cycles and highly expressed in immuno-hot tumors. High expression of NAPSB was sensitive to immunotherapy and chemotherapy, possibly due to its association with pyroptosis, apoptosis and necrosis (PANoptosis).

Conclusions: NAPSB was correlated with an immuno-hot and inflamed TME and tumor cell death. It can be utilized as a promising predictive marker for prognosis and therapy in HCC.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most frequent malignancy worldwide and the third leading cause of cancer-related deaths, accounting for almost 90% of primary liver cancers [1]. Systemic treatments are the important options for HCC patients as a large portion of HCC patients have already reached advanced stage when they first diagnosed and lost the opportunity of surgery [2]. In addition, emerging immunotherapies involving the use of immune checkpoint inhibitors (ICIs) are currently the focus of research in many advanced cancers [3–6]. However, both systemic chemotherapy and immune checkpoint therapy have limitations of drug resistance or response in only some patients [7–9]. Therefore, identifying new biomarkers is urgent and benefits for the individual therapy.

In HCC, the tumor microenvironment (TME) composed of cancer cells, immune cells and extracellular matrix has an immunosuppressive effect, promoting immune tolerance and avoidance [10]. However, recent studies have shown that abundant infiltration of CD8+, CD4+, regulatory T cells and dendritic cells (DCs) can shape an inflamed TME to play a potential anticancer role and influence the efficacy of ICIs [11–13]. Based on the characteristics of the TME, tumors can be divided into hot and cold tumors. Hot tumors are characterized by T-cell infiltration, molecular characteristics of immune activation and

responsive to cancer immunotherapy, while cold tumors are characterized by the opposite [14]. In HCC, such molecules and pathways, including β -catenin, PPARG (peroxisome proliferator activated receptor gamma)-network, and IDH1 (isocitrate dehydrogenase 1) pathways, have been shown to promote the formation of a non-inflamed TME [15–17], while IFN- γ (interferon-gamma), APM (antigen processing and presenting machinery), EGFR (Epidermal growth factor receptor), and hypoxia pathways contributed to shape an inflamed TME [18–21].

Pseudogenes were considered to be “junk genes” for many years because of its abrogated transcription due to harbor mutations [22]. However, over the past decade, non-coding RNAs including pseudogenes, long non-coding RNAs (lncRNAs), and microRNAs have been demonstrated to play key roles in TME [23, 24]. NAPSB is a pseudogene that had been identified to be associated with the infiltration of CD4 + T immune cells in pancreatic ductal adenocarcinoma (PDAC) [25]. In the study of Anirban et.al [26], NAPSB was highly expressed and identified as a hub gene in pre-eclampsia with systemic inflammatory response. In HCC, NAPSB was found to be downregulated [27] but its biological role has not been elucidated. Thus in this study, the potential biological functions of NAPSB were comprehensively explored in HCC, including its differential expression, prognosis value and immunological role. We also reported that high NAPSB expression was related to an immuno-hot TME and sensitive to immunotherapy/chemotherapy possibly on account of affecting pyroptosis, apoptosis and necrosis (PANoptosis) in HCC.

Materials And Methods

Public data collection

TIMER database (<https://cistrome.shinyapps.io/timer/>) was used to analyze expression levels of NAPSB in pan-cancer. HCC patients with the transcriptomic RNA-sequencing data ($\log_2$ (FPKM+1) value) of The Cancer Genome Atlas (TCGA) datasets were obtained from the online data portal UCSC Xena (<https://xenabrowser.net/datapages/>). Patients with insufficient or missing clinical and prognosis information were excluded. Then the resting 369 TCGA-HCC samples were used. Meanwhile, LIRI-JP (n=231) retrieved from International Cancer Genome Consortium (ICGC) database (<https://icgc.org/>) were chosen for primary external validation. In addition, we used multiple cohorts from Gene expression omnibus (GEO) (<https://www.ncbi.nlm.nih.gov/geo/>), including GSE55092, GSE54236, and GSE121248, to verify the relative expression of NAPSB in HCC and normal tissues.

Two immunotherapy-related cohorts, GSE78220 and GSE91061 (melanoma), were downloaded from GEO database. GSE104580, a dataset of transcatheter arterial chemoembolization (TACE) for HCC patients was also downloaded.

Tissue specimens acquisition

13 hepatocellular carcinoma tissues and paired adjacent normal tissues were obtained from Zhongnan Hospital of Wuhan University between February 2021 and September 2021 with informed consents. The patients were diagnosed with hepatocellular carcinoma based on histopathological evaluation, and no local or systemic treatment was conducted before surgery. The protocols used in the study were approved by the Research Ethics Committee of Zhongnan Hospital of Wuhan University.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays

Total RNA was extracted from hepatocellular carcinoma and paired adjacent normal tissues using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). RNA quantity was determined by NanoDrop2000c (Thermo Scientific, Waltham, MA, USA). For qRT-PCR, 1 µg RNA was reverse transcribed to cDNA using a Reverse Transcription Kit (Toyobo, Osaka, Japan). The qRT-PCR assays were conducted on LightCycler® 96. Target gene expression was normalized against GAPDH. The primer sequences were: NAPS-B-Forward: CATCCAGTTTGCTCAGGGT; NAPS-B-Reverse: TCGAAGACGGTCACATACGC; GAPDH-Forward: CCCAGCAAGAGCACAAGAG; GAPDH-Reverse: GCACAGGGTACTTTATTGATGGTAC.

Evaluate the prognostic value of NAPS-B

Kaplan-Meier (K-M) analyses, univariate and multivariate Cox regression were conducted to explore the influence of NAPS-B on the survival of patients in HCC using R package “*survminer*” and “*survival*” to investigate the optimal cutoff of NAPS-B expression and draw the K-M survival curve, while the log-rank test was applied to estimate statistical significance. Overall survival (OS), disease-free interval (DFI), and progression-free interval (PFI) were evaluated (p-value < 0.05 as significant).

Analysis of NAPS-B co-expressed genes and differential expressed genes

Genes potentially positively co-expressed with NAPS-B were predicted using R software. Those genes with a thresholds of p-value < 0.01 and |Spearman's correlation| ≥ 0.45 were selected for further analysis. Patients were classified into two groups based on the median NAPS-B expression. We screened differentially expressed genes (DEGs) between NAPS-B subgroups using the “*edgeR*” package in the R software. An adjusted p-value < 0.05 and |log2 (fold change)| value ≥ 1.3 was considered significant. We took the intersection of the co-expressed genes and the upregulated DEGs as the genes most related to NAPS-B for further analysis.

Biological function, pathway annotation, gene set enrichment analysis (GSEA) and gene set variation analysis

(GSVA)

We conducted gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses to explore the possible biological function of the genes most related to NAPSB (mentioned above) via the R package “*clusterProfiler*”, with a cutoff value of adjusted p-value < 0.05. To investigate the difference in biological process terms between the NAPSB-high and NAPSB-low groups, GSEA was applied using the R package “*clusterProfiler*” and GSVA was applied using the R package “*GSVA*”. The gene sets of “h.all.v7.4.symbols” and “c5.cp.kegg.v7.4.symbols” were downloaded from MSigDB for GSEA and GSVA respectively.

Evaluation of relationship between NAPSB expression and the immunological characteristics of the TME

Immunological characteristics of the TME in HCC include the expression of immunomodulators, infiltration level of TIICs and activity of the cancer immunity cycle. We first analyzed the correlation of NAPSB expression with immune-related genes, including major histocompatibility complex (MHC), immunomodulators, chemokines and receptors. A total 50 immunomodulators were collected from the study of Charoentong et al [28] (Supplementary Table 1). We applied the “Estimation of Stromal and Immune cells in Malignant Tumors using Expression data” (ESTIMATE) algorithm to assess the immune scores, stromal scores, estimate scores, and tumor purity for each HCC sample [29]. Thereafter, we used several algorithms to comprehensively calculate the infiltration level of TIICs to avoid calculation errors: ssGSEA, TIMER [30], CIBERSORT [31], quanTIseq [32], EPIC [33], xCell [34], and MCP-counter [35]. We also identified the effector genes of TIICs from previous studies [36, 37] (Supplementary Table 2). The cancer immunity cycles included seven critical steps: cancer antigen release and presentation (Steps 1 and 2), anticancer immune priming and activation (Step3), immune cell trafficking (Step 4), immune cell infiltration into the TME (Step 5), T cell recognition of cancers (Step 6), and killing of cancer cells (Step 7) [38]. Moreover, we calculated the activities of these steps as described previously [39]. Finally, T cell–inflamed gene expression profiles score was calculated as an average value of log2-scale normalized expression of the 18 signature genes [40].

Unsupervised clustering

Unsupervised clustering was implemented to classify HCC tissues into hot or cold tumors on the basis of hot tumor signature genes according to previous literature [41]. The number of clusters and their stability were determined by the consensus clustering algorithm. We used the “*ConsensusClusterPlus*” package to perform this algorithm and 1000 times repetitions were conducted for guaranteeing the stability of classification [42].

Calculation of the enrichment scores of various gene signatures and prediction of immunotherapy response

We analyzed the oncogenic pathways that were associated with targeted therapy, and immunotherapy responses according to previous research [36] (Supplementary Table 3). The enrichment scores of these signatures were calculated using the R package “GSVA” [43]. To analyze the efficacy of immunotherapy, two immunotherapy-related cohorts, GSE78220 and GSE91061 (melanoma) were obtained.

Prediction of chemotherapeutic response

We downloaded the transcriptional expression data and drug response of more than 1000 cancer cell lines from Genomics of Drug Sensitivity in Cancer (GDSC, <http://www.cancerrxgene.org/downloads>) [44] and Cancer Therapeutics Response Portal (CTRP) [45] respectively. The Spearman correlation between the NPSB of each cell line and half maximal inhibitory concentration (IC50) of each cell line to particular drugs was calculated. Correlations with adjusted p-value were filtered < 0.01 as significant ones. In addition, GSE104580 was used to analyze the correlation between NPSB expression and TACE response in HCC patients.

Calculation of the enrichment scores of cell death gene sets

We collected signatures of several forms of cell death, including pyroptosis, apoptosis, necroptosis, autophagy, and ferroptosis, from previous literatures [46-50] (Supplementary Table 4). The enrichment scores of these signatures were also calculated using the R package “GSVA” as mentioned above.

Statistical analysis

Statistical analyses were performed using R software (version 4.1.1) and SPSS software (version 25.0). Paired Student's t-test was performed to detect the differential expression of NPSB in paired HCC and adjacent normal tissues. The significant difference in continuous variables between the two groups was measured by the Wilcoxon rank sum test, while categorical variables were compared using the chi-square test. Correlations between variables were explored using Pearson or Spearman coefficients. For all analyses, a two-sided p-value < 0.05 was considered statistically significant if not noted. Statistical significance was defined as: ns, no significance; *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001; ****, p-value < 0.0001.

Results

Expression levels analysis and high NAPS B inferred a better prognosis for HCC

NAPS B transcription levels in different human tumors were showed in Fig. 1a. Compared with adjacent normal tissues, expression of NAPS B in BLCA (bladder urothelial carcinoma), COAD (colon adenocarcinoma), LIHC (liver hepatocellular carcinoma), LUAD (lung adenocarcinoma), LUSC (lung squamous cell carcinoma) and READ (rectal adenocarcinoma) was significantly decreased but in BRCA (breast invasive carcinoma), ESCA (esophageal carcinoma), KICH (kidney chromosome) and KIRP (kidney renal papillary cell carcinoma) was significantly increased. For TCGA cohort, we analyzed paired samples by paired Student's t-test to verify the above results in HCC (Fig. 1b). To fully demonstrate this expression difference, we validated it with multiple datasets, including ICGC, GSE55092, GSE54236, and GSE121248, finding that NAPS B was indeed significantly decreased in HCC tissues (Fig. 1c).

Moreover, NAPS B expression was examined in 13 paired HCC and adjacent normal tissues of Zhongnan cohort from our hospital by RT-PCR and we obtained consistent results. (Fig. 1d).

The correlation between NAPS B and clinicopathologic characteristics for TCGA and ICGC cohorts were presented in Supplementary Table 5 and 6. In addition, we investigated its survival value. K-M survival analysis showed that high expression of NAPS B was linked to better overall survival than its low expression (Fig. 1e) and more significantly associated with better disease-free survival (Fig. 1f) and progression-free survival (Fig. 1g). Its overall survival value was also verified in the ICGC cohort (Fig. 1h). Given that there are multiple factors affecting survival, univariate Cox regression analysis was performed using age, tumor stages, gender, and NAPS B expression as inputs; results showed that NAPS B expression were significantly associated with better DFI and PFI outcomes (Supplementary Fig. 1a) and multivariate Cox regression analysis further validated it (Supplementary Fig. 1b). Therefore, NAPS B expression was beneficial to overall survival and could serve as an independent predictor of disease-free survival and progression-free survival in HCC.

Enrichment analyses inferred NAPS B was related to immune activation

To investigate the potential biological function of NAPS B, we firstly performed a correlation analysis between NAPS B and other genes using RNA-seq data of HCC patients from the TCGA cohort. The results showed that there were 930 genes significantly associated with NAPS B ($p\text{-value} < 0.01$, $|\text{Spearman's correlation}| \geq 0.45$; Supplementary Table 7). The correlation of NAPS B and the top 50 co-expressed genes was showed in Fig. 2a, which contained some immune-related molecules like CD48, CD37, IL6, HLA-DQA1, etc. Meanwhile, we analyzed the differentially expressed genes (DEGs) between the NAPS B high and low expression groups and found that there were 993 upregulated in the NAPS B-high group compared with the NAPS B-low group (adjusted $p\text{-value} < 0.05$ and $|\log_2(\text{fold change})| \geq 1.3$; Supplementary Table 8). The top 10 upregulated genes contained immune-related molecules, such as

CD48, CD37, CCR5 (Fig. 2b). Both co-expressed genes and upregulated DEGs contained well-known molecules associated with immunity, suggesting that NPSB may also be involved in immunity.

Thereafter, the intersection of co-expressed genes and upregulated DEGs including 476 common genes were obtained as the most closely related genes to NPSB (Fig. 2c; Supplementary Table 9) and enrichment analyses were conducted in an attempt to reflect biological functions of NPSB indirectly. The GO analysis showed that the common genes were enriched in processes such as T cell activation, leukocyte cell-cell adhesion, regulation of T cell activation, and regulation of immune effector process, etc (Fig. 2d; Supplementary Table 10). The KEGG pathway analysis demonstrated that the common genes were associated with chemokine signaling pathway, Th17 cell differentiation and T cell receptor signaling pathway (Fig. 2e; Supplementary Table 11). Most biological functions and signaling pathways were immune-related, strongly implying that NPSB may mediate the TME in HCC.

Even further, we conducted GSEA and GSVA between NPSB high- and low- groups and also identified many significant pathways related to immunity (Fig. 2f, g; Supplementary Table 12, 13) in the enrichment of MSigDB Collection (gene sets of “c5.cp.v7.4.symbols.gmt” and “h.all.v7.4.symbols”). These findings paralleled the above results.

NPSB shaped an immuno-hot and inflamed TME in HCC

Given that the enrichment analyses indicated NPSB was associated with immune activation in HCC, we subsequently comprehensively explored its immunological role using TCGA and ICGC cohorts. NPSB was found to upregulated the expression of critical immunomodulators (including MHC, immunostimulator, chemokine, and receptor) (Fig. 3a), which may upregulate the activities of the cancer-immunity cycle subsequently. Additionally, ESTIMATE algorithm was applied to calculate the immune score, stromal score, estimated score and tumor purity. We found these scores were significantly increased NPSB-high group (Fig. 3b). On the contrary, tumor purity was negatively correlated with the expression of NPSB (Fig. 3c). Subsequently, TME immune cell infiltration was assessed by ssGSEA and a significant difference between NPSB-high and NPSB-low groups was observed. Almost all immune cells were significantly enriched in NPSB-high group (Fig. 3d). Furthermore, we calculated the infiltration level of TIICs using other six independent algorithms (Fig. 3e). Consistent with the result of ssGSEA analysis, the infiltration levels of CD8+ T cells, CD4+ T cells, NK cells, B cells, DCs and macrophages were almost positively correlated with NPSB in different algorithms. In particular, CD8+ T cells were positively correlated with NPSB with statistical significance in all algorithms. In line with these, NPSB was positively correlated with the marker genes of these six major types of immune cells (Fig. 3f). These results suggested NPSB was associated with an inflamed TME. Even further, we observed the NPSB expression positively correlated with the T cell inflamed score (TIS) established using IFN- γ -related mRNA profiles [40] and all of genes within this signature (Fig. 3g, h), further confirming its roles in shaping a hot inflamed TME. These findings were all verified in ICGC cohort and obtained consistent results (Supplementary Fig. 2).

Finally, we evaluated the correlation between NAPS B and seven steps of cancer-immunity cycle, which conceptualized the anti-cancer immune response [38]. Overall, In the NAPS B-high group, the activities associated with the majority of the steps in the cycle were notably upregulated (Fig. 3i), including the release of cancer cell antigens (Step 1), priming and activation (Step 3), trafficking of immune cells to tumors (Step 4), and infiltration of immune cells into tumors (Step 5). In summary, these data consistently indicated that high expression of NAPS B was to transform a non-inflamed TME into an immuno-hot and inflamed microenvironment, consequently triggering anti-cancer immune response.

NAPS B highly expressed in hot tumors and may enhance immunotherapy response

As mentioned earlier, solid tumors can be divided into hot tumors and cold tumors, and hot tumors respond to cancer immunotherapy [14, 51]. Unsupervised clustering was conducted to classified HCC samples into hot tumors and cold tumors based on the hot tumor signature genes (Supplementary Table 14; Fig. 4a-d) [41]. Then, the expression of NAPS B was compared between hot and cold tumors and we found that it was overexpressed significantly in hot tumors (Fig. 4e), suggesting that NAPS B could play a role in distinct hot/cold tumor states and be associated with therapeutic response to immunotherapy. The same methods were used to validate above results in the ICGC cohort (Supplementary Fig. 3a-e).

In addition, NAPS B expression was found to be positively correlated with BTLA (B and T lymphocyte associated), CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), IDO1 (indoleamine 2,3-dioxygenase 1), LAG-3 (lymphocyte activating 3), PD-1(programmed cell death 1), PD-L1 (programmed cell death 1 ligand 1), TIGIT (T cell immunoreceptor with Ig and ITIM domains), and TIM-3 (T-cell immunoglobulin and mucin domain-containing protein 3) expression (Fig. 4f), which were well-known predictors of response to immunotherapy. Also, the enrichment scores of therapeutic signatures, predicting clinical response, were compared in NAPS B subgroups. As exhibited in Fig. 4g, i, NAPS B was negatively correlated with the enrichment scores of PPARG network, β -catenin signaling pathway, VEGFA and IDH1, which were all immunosuppressive gene signatures [15-17, 52]. However, in the NAPS B-high group, immunotherapy-positive pathways such as IFN- γ -signature, APM-signal, EGFR-ligands, hypoxia and KDM6B (lysine demethylase 6b) were activated (Fig. 4h) [18-21, 53], indicating immune-activated state and beneficial to immunotherapy response. These observations were also validated using ICGC samples (Supplementary Fig. 3f-h).

The last but important, the role of the NAPS B in predicting the immune checkpoint blockade (ICB) response was explored in tow immunotherapy-related melanoma cohorts. In GSE91061, we found the ICB response rates were obviously higher in the NAPS B-high group than in the NAPS B-low group (Fig. 4j) and the expression of NAPS B was significantly high in response group (Fig. 4k). Similar results were observed in the GSE78220 cohort (Supplementary Fig. 3i). These evidences reconfirmed that NAPS B may be a valuable predictor of immunotherapy response across cancers.

NAPSB was associated with increased sensitivity to chemotherapy

As for whether NAPSB plays a role in chemotherapy sensitivity, the correlation between NAPSB expression and IC50 of multiple drugs in cell lines was analyzed using data from GDSC and CTRP. Intriguingly, NAPSB expression was negatively associated with IC50 of most agents in GDSC and in CTRP (Fig. 5a and Supplementary Fig. 4; Supplementary Table 15), supporting that NAPSB can enhance the therapeutic response to chemotherapy. Two heat maps showed that the IC50 of some commonly used drugs is lower in the NAPSB-high group in GDSC and CTRP databases, respectively (Fig. 5b, c). Results above speculated that high expression of NAPSB is beneficial to the sensitive response of chemotherapy.

Thereafter, by analyzing GSE104580, a HCC cohort of transarterial chemoembolization (TACE), we found the expression of NAPSB was significantly higher in TACE response group (Fig. 5d) and the response rates were obviously higher in the NAPSB-high group than in the NAPSB-low group (Fig. 5e). This data further illustrated that high expression of NAPSB may be beneficial to chemotherapy response.

Association of NAPSB with cell death of tumor cells

Given that cell death had been reported in recent years to play a significant role in tumor therapy [54], we investigated the association between NAPSB and various forms of cell death, including pyroptosis, necroptosis, apoptosis, autophagy and ferroptosis, using ssGSEA. As showed in Fig. 6a-c, and E, NAPSB expression was markedly correlated with pyroptosis, apoptosis and necroptosis, but negatively correlated with ferroptosis. Autophagy had no correlation with NAPSB expression (Fig. 6d). We also verified these discoveries with ICGC cohort. In line with these findings, the correlations between NAPSB and several cell death forms were consistent with that in the ICGC cohort (Fig. 6f). In addition, the enrichment scores of pyroptosis, apoptosis and necroptosis in NAPSB-high groups were markedly higher than NAPSB-low groups (Fig. 6g). However, similar to results of TCGA, ferroptosis scores were lower in NAPSB-high group than in NAPSB-low group and autophagy scores had no significant differences in NAPSB subgroups. Among the above results, the correlation between NAPSB and pyroptosis was the most significant. Results above inferred that NAPSB may have a beneficial effect on immunotherapy and chemotherapy responses by promoting pyroptosis, necroptosis and apoptosis (PANoptosis) in tumor therapy.

Discussion

In this study, the potential biological functions of NAPSB has been comprehensively explored for the first time in HCC. We revealed NAPSB was downregulated and related to a better prognosis in HCC patients. Enrichment analyses suggested NAPSB was closely related to immune activation and further analysis demonstrated that NAPSB was associated with immunomodulators, higher T cell infiltration, hot tumor states, and corresponding better therapeutic efficacy. Mechanistically, we speculated that NAPSB might interact with PANoptosis thus improving the sensitivity of immunotherapy/chemotherapy in HCC.

By analyzing the data from multiple public databases and Zhongnan cohort, our study obtained consistent results as previous research: NAPS B was downregulated in HCC [27]. Tan et al.'s study showed NAPS B was upregulated in PDAC and related to CD4 + T Cell infiltration [25]. Additionally, upregulation of NAPS B was also found in pre-eclampsia, a state of highly inflammatory activity [26]. It was expected that NAPS B overexpressed under inflammatory conditions. In line with these, enrichment analyses in this study showed the genes most related to NAPS B were enriched in immune cell receptor signaling pathway and inflammatory response in our study.

A more important part of this study was to comprehensively clarify the immunological role of NAPS B in HCC immune microenvironment. MHC molecules represent antigen presentation and processing capacity, while chemokines and receptors recruit effector TIICs such as CD8 + T cells, TH17 cells, and antigen-presenting cells [55, 56], which may upregulate the activities of the cancer-immunity cycle subsequently [57]. In our study, NAPS B was found to be positively correlated with these immunomodulators, suggesting that NAPS B promotes immune activation, which is consistent with the results of enrichment analyses above. In addition, NAPS B expression had a positive correlation with the abundance of infiltrating CD8 + T cells, CD4 + T cells, B cells, NK cells, macrophages, and DCs in HCC, verified by different algorithms and external validation cohort. Currently, the prognosis of HCC is known to be related to the infiltration and activation of immune cells [58, 59], whose presence participate in a hot (inflamed) TME [60, 61], supporting the observation that NAPS B can stimulate the immune response in the TME and play an anti-tumor role in HCC, thereby prolonging survival. This could also be used to explain the results of this study: high expression of NAPS B was associated with better prognosis of HCC. Additionally, we observed NAPS B was positively related to the T cell inflamed score, as well as several critical steps of the cancer immunity cycle. Since both TIS and cancer immunity cycle reflect the T cell infiltration and anti-cancer immune response of our body [38, 40], these results reaffirmed and extended the close relationship between NAPS B and an immune-hot and inflamed TME.

T-cell infiltration, molecular characteristics of immune activation, and anti-tumor response are characteristics of hot tumors [51, 62], so we speculated NAPS B can play a role in distinct hot/cold tumor states based on the above results. Here, NAPS B was highly expressed in hot tumor samples consistently. Not only that, NAPS B was significantly positively correlated with ICB therapeutic targets, such as PD-L1, PD-1, and CTLA-4. Better clinical response to ICB is another character of hot tumors due to more active immune molecules [63]. Together, NAPS B could distinguish between hot and cold tumors and facilitate immunotherapeutic responses. Meanwhile, we discovered that NAPS B high group was activated in immune-activated pathways, such as the IFN- γ signature, APM signal, EGFR ligands, and hypoxia [18–21]. These pathways had been revealed to stimulate the infiltration of TIICs by increasing the expression of immunomodulators, contributing to an inflamed TME and resulting in better clinical responses to immunotherapy. On the contrary, NAPS B was opposite to several reported immunosuppressive oncogenic pathways, such as the β -catenin, PPARG, and IDH1 pathways [15–17]. These outcomes not only demonstrated that NAPS B can improve the immunotherapy response, but also reconfirmed the role of NAPS B in activation of immune activity as discussed above.

The main treatments for advanced HCC are still chemotherapy and targeted drugs, among which first-line drugs include doxorubicin, fluorouracil, and sorafenib, etc [2], improving the five-year survival rates of HCC patients [64]. TACE is a treatment for liver cancer often applying doxorubicin or cisplatin as intra-arterial injection of cytotoxic agents [65]. In our study, we proved that NPSB was negatively correlated with IC50 of a variety of drugs in GDSC and CTRP databases, but overexpressed in TACE responders as expected, strongly inferring high NPSB expression can improve the sensitivity of chemotherapy. NPSB may be utilized as a promising predictive marker for chemotherapy as drug resistance is common at present [9, 66]. Recently, studies have focused on the interactions between tumor cell death and sensitivity or resistance of anticancer therapy. For instance, Makin et al. created that apoptosis was considered as the predominant form of regulated cell death responsible for tumor therapies [67]. And Carina et al.'s study revealed sorafenib therapy induced pyroptosis in MΦ and thereby unleashing an NK-cell response against HCC tumors [68]. Instead, autophagy, this cell death form participates in the progression of HCC and the resistance of HCC cells to sorafenib [49, 69]. In our study, we revealed NPSB was positively correlated with pyroptosis, apoptosis and necroptosis (PANoptosis), but had no correlation with autophagy, suggesting that NPSB may promote cell death synergistically with drugs to improve the sensitivity of chemotherapy.

Despite these findings, there are existing limitations. First, the study was primarily carried out using bioinformatics methods; further experiments are needed to support these findings. To remedy this deficiency, the main conclusions of this study were confirmed by several methods and external validation. For instance, the differential expression of NPSB was compared across multiple cohorts and the association of NPSB with immune infiltration was demonstrated by ssGSEA and six other independent algorithms. Second, although this study showed NPSB could serve as an independent predictor of disease-free and progression-free survival outcomes of HCC patients from the TCGA cohort, we did not validate this in another cohort due to a lack of such datasets with complete clinical information. Third, further in-depth studies are required to explore the interactions of NPSB, PANoptosis and chemotherapy.

In conclusion, our study is the first comprehensive analysis to demonstrate that NPSB expression shaped an immuno-hot and inflamed TME in HCC; NPSB could be considered a predictor of disease-free and progression-free survival outcomes in patients with HCC; NPSB can also predict the clinical response to ICB and chemotherapy. These findings will provide important insights for the development of cytokine-based therapy for cancer treatment.

Abbreviations

APM	Antigen processing and presenting machinery
BLCA	Bladder urothelial carcinoma
BRCA	Breast invasive carcinoma
BTLA	B and T lymphocyte associated
COAD	Colon adenocarcinoma
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CTRP	Cancer therapeutics response portal
DCs	Dendritic cells
DEGs	Differentially expressed genes
DFI	Disease-free interval
EGFR	Epidermal growth factor receptor
ESCA	Esophageal carcinoma
GDSC	Genomics of drug sensitivity in cancer
GEO	Gene expression omnibus
GO	Gene ontology
GSEA	Gene set enrichment analysis
GSVA	Gene set variation analysis
HCC	Hepatocellular carcinoma
IC50	Half maximal inhibitory concentration
ICGC	International cancer genome consortium
ICIs	Immune checkpoint inhibitors
IDH1	Isocitrate dehydrogenase 1
IDO1	Indoleamine 2,3-dioxygenase 1
IFN- γ	interferon-gamma
KDM6B	Lysine demethylase 6b
KEGG	Kyoto encyclopedia of genes and genomes
KICH	Kidney chromosome
KIRP	Kidney renal papillary cell carcinoma
K-M	Kaplan-Meier

LAG-3	Lymphocyte activating 3
LIHC	Liver hepatocellular carcinoma
LncRNAs	Long non-coding RNAs
LUAD	Lung adenocarcinoma
LUSC	Lung squamous cell carcinoma
MHC	Major histocompatibility complex
NAPSB	Napsin b aspartic peptidase, pseudogene
OS	Overall survival
PANoptosis	Pyroptosis, apoptosis and necrosis
PDAC	Pancreatic ductal adenocarcinoma
PD-1	Programmed cell death 1
PD-L1	Programmed cell death 1 ligand 1
PFI	Progression-free interval
PPARG	Peroxisome proliferator activated receptor gamma
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
READ	Rectal adenocarcinoma
TACE	Transcatheter arterial chemoembolization
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIICs	Tumor-infiltrating immune cells
TIM3	T-cell immunoglobulin and mucin domain-containing protein 3
TIS	T-cell inflammation score
TCGA	The cancer genome atlas
TME	Tumor microenvironment
VEGFA	Vascular endothelial growth factor a

Declarations

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Author Contributions

Q.Z. H.W. and J.F. designed the study. X.L., K.L. and F.W. collected the data. Y.N. and K.L. analyzed the data. Y.N., H.W. and K.L. prepared the figures. X.L. and F.W. prepared the tables. X.J., Z.Z. and L.L. collected the human specimens. Y.D., K.L. and Y.X. carried out the experiments. H.W. Q.Z. and J.F. supervised the data and provided statistical advice. Y.N., K.L. and X.L. wrote the article. F.W. and H.W. reviewed the article. All authors read and approved the final manuscript.

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Data Availability

Publicly available datasets were analyzed in this study. These data can be found here: <https://portal.gdc.cancer.gov/>, <https://icgc.org/>, and <https://www.ncbi.nlm.nih.gov/geo/>. The supplementary material for this article can be found online. All processed data and R codes used in this study can be obtained from the corresponding author on reasonable request.

Conflict of interests

The authors declare that they have no competing interests.

Ethical approval

The study was approved by the Research Ethics Committee of Zhongnan Hospital of Wuhan University, and the written informed consent was obtained from all patients.

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Figures

Figure 1

Differential expression and prognosis value of NAPS in pan-cancer and liver hepatocellular carcinoma (LIHC). **a** NAPS expression levels in different tumor types were measured using TIMER. **b** Paired Student's t-test analysis of NAPS expression in TCGA paired samples. **c** NAPS expression was significantly higher in normal tissues than in hepatocellular carcinoma in ICGC, GSE55092, GSE54236, and GSE121248 cohorts. **d** In Zhongnan cohort, lower NAPS expression was seen in hepatocellular

carcinoma compared with adjacent normal tissues (N=13). The expression of NAPS B was compared with a standard reference control and relative quantities (RQ) were calculated based on the $\Delta\Delta C_t$ method. **e-h** Kaplan–Meier analysis of NAPS B expression based on overall survival (OS), disease-free interval (DFI), progression-free interval (PFI) in TCGA cohort and overall survival (OS) in ICGC cohort. ns, no significance; *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001; ****, p-value < 0.0001.

Figure 2

Enrichment analysis of NPSB biological function in hepatocellular carcinoma (HCC). **a** The heat map shows the top 50 genes positively related to NPSB in HCC. **b** Volcano plot of DEGs between NPSB-high and NPSB-low HCC tissues. **c** Venn diagram of co-expressed genes and upregulated DEGs. **d** The top 20 gene ontology (GO) terms for the most closely related genes to NPSB. **e** The top 20 Kyoto Encyclopedia of Genes and Genomes (KEGG) terms for the most closely related genes to NPSB. **f** Gene Set Enrichment Analysis (GSEA) shows significant signaling pathways between NPSB-high and NPSB-low HCC tissues (The gene sets of "c5.cp.kegg.v7.4.symbols"). **g** Gene Set Variation Analysis (GSVA) between NPSB-high and NPSB-low HCC tissues (The gene sets of "h.all.v7.4.symbols").

Figure 3

NAPSBSB shapes an inflamed TME in HCC. **a** Expression levels of 50 immunomodulators (MHC, immunostimulators, chemokines, and receptors) in the NAPSBSB-high and NAPSBSB-low groups in HCC. **b** Distribution of stromal score, immune score, and ESTIMATE score calculated using the ESTIMATE algorithm in the NAPSBSB-high and NAPSBSB-low groups. **c** Correlation between NAPSBSB and tumor purity using the ESTIMATE algorithm. **d** Different expression of 28 tumor-associated immune cells calculated

with the ssGSEA algorithm between NAPSB subgroups. **e** Correlation between NAPSB and the infiltration levels of six types of TIICs (CD8+ T cells, CD4+ T cells, NK cells, B cells, dendritic cells, and macrophages), which were calculated using six independent algorithms. **f** Correlation between NAPSB expression and the effector genes of the above immune cells. (**g, h**) Correlations between NAPSB and the T cell inflamed score and the individual genes included in the T cell inflamed signature. **i** The activities of the various steps of the cancer immunity cycle in the NAPSB-high and NAPSB-low groups. ns, no significance; *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001; ****, p-value < 0.0001.

Figure 4

NAPSB was correlated with hot tumor state and improved response to immunotherapy. **a** Consensus clustering cumulative distribution function (CDF) for k = 2-9. **b** Relative change in area under CDF curve for k = 2-9. **c** Consensus clustering heat map for k = 2 in HCC samples. **d** Heat map plot showed hot tumor signature genes were enriched in hot tumor samples. **e** NAPSB was significantly overexpressed in hot tumors. **f** The expression of NAPSB was positively correlated with immune checkpoint molecules expression level. **g** Correlations between NAPSB and the enrichment scores of several therapeutic signatures. **h** Differences in enrichment scores of IFN- γ -signature, APM-signal, EGFR-ligands, hypoxia for NAPSB subgroups patients. **i** Differences in enrichment scores of PPARG network, β -catenin signaling pathway, VEGFA and IDH1 for NAPSB subgroups patients. **j** The proportion of immune response to immunotherapy of NAPSB subgroups in GSE91061. **k** NAPSB was highly expressed in CR/PR group in GSE91061. CR/PR: Complete and partial response. ns, no significance; *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001; ****, p-value < 0.0001.

Figure 5

Potential predictive chemotherapy value of NAPS expression. **a** Bar plot exhibiting Spearman correlation between NAPS and the IC50 of drugs in GDSC. **b** Correlations between NAPS and the IC50 of the frequently used drugs for advanced HCC patients in CTRP, including doxorubicin, fluorouracil, carboplatin, gemcitabine, lenvatinib, sorafenib, sabozantinib, axitinib, sunitinib, etoposide, and linifanib. **c** Correlations between NAPS and the IC50 of the frequently used drugs for advanced HCC patients in GDSC, including

fluorouracil, gemcitabine, cisplatin, sorafenib, erlotinib and grfatinib. **d** NAPSb was highly expressed in CR/PR group of transarterial chemoembolization (TACE) therapy in GSE104580. **e** The proportion of response to TACE of NAPSb subgroups in GSE104580. ns, no significance; *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001; ****, p-value < 0.0001.

Figure 6

Correlations between NAPS B and the enrichment scores of several cell death signatures in TCGA (**a-e**) and ICGC (**f, g**) cohort. NAPS B expression was positively correlated with pyroptosis (**a**; $R = 0.72$, $P < 0.001$), apoptosis necroptosis (**b**; $R = 0.21$, $P < 0.001$) and necroptosis (**c**; $R = 0.53$, $P < 0.001$). **d** Autophagy had no correlation with NAPS B expression ($R = -0.017$, $P = 0.795$). **e** NAPS B was negatively correlated with ferroptosis ($R = -0.24$, $P < 0.001$). **f** Correlations between NAPS B and several forms of cell death in ICGC cohort. **g** The enrichment scores of pyroptosis, necroptosis and apoptosis in NAPS B-high groups were markedly higher than NAPS B-low groups. However, ferroptosis scores were lower in NAPS B-high group than in NAPS B-low group and autophagy scores had no significant differences in NAPS B subgroups. Spearman coefficients were used to explore the correlations. ns, no significance; *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001; ****, p-value < 0.0001.

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