NAPSB as a predictive marker for prognosis and therapy associated with an immuno-hot tumor microenvironment in hepatocellular carcinoma NAPSB predicts prognosis and therapy in HCC

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
Li Dong
Zhongnan Hospital of Wuhan University
Lan Liu
Zhongnan Hospital of Wuhan University
Fan Wang
Zhongnan Hospital of Wuhan University
Qiu Zhao (qiuzhao@whu.edu.cn)
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 (TIICs), 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.

1. 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 [2] and emerging immunotherapies involving the use of immune checkpoint inhibitors (ICIs) are currently the focus of research in many advanced cancers [3–5]. However, both systemic chemotherapy and immune checkpoint therapy have limitations of drug

resistance or response in only some patients [6, 7]. 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 [8]. 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 [9, 10]. 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 [11].

Over the past decade, non-coding RNAs including pseudogenes, long non-coding RNAs, and microRNAs have been demonstrated to play key roles in TME [12, 13]. NAPSB is a pseudogene that had been identified to be associated with the infiltration of CD4 + T immune cells in pancreatic ductal adenocarcinoma (PDAC) [14]. In HCC, NAPSB was found to be downregulated [15] 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 PANoptosis in HCC.

2. Materials And Methods

2.1. Public data collection

TIMER database (https://cistrome.shinyapps.io/timer/) was used to analyze expression levels of NAPSB in pan-cancer. HCC patients (n = 369) with the transcriptomic RNA-sequencing data (log2 (FPKM + 1) value) of The Cancer Genome Atlas (TCGA) datasets were obtained from UCSC Xena (https://xenabrowser.net/datapages/). 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.

Tow 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.

2.2. 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

protocols used in the study were approved by the Medical Ethics Committee of Zhongnan Hospital of Wuhan University (grant no.20200110).

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

Total RNA was extracted from HCC 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: NAPSB-Forward: CATCCAGTTTGCTCAGGGT; NAPSB-Reverse: TCGAAGACGGTCACATACGC; GAPDH-Forward: CCCCAGCAAGAGCACAAGAG; GAPDH-Reverse: GCACAGGGTACTTTATTGATGGTAC.

2.4. Evaluate the prognostic value of NAPSB

Kaplan-Meier (K-M) analyses, univariate and multivariate Cox regression were conducted to explore the influence of NAPSB on the survival of patients in HCC using R package *"survminer"* and *"survival".* 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 ($p\text{-value} < 0.05$ as significant).

2.5. Analysis of NAPSB co-expressed genes and differential expressed genes

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

2.6. 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"*. To investigate the difference in biological process terms in NAPSB subgroups, 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.

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

A total 50 immunomodulators (including MHC, immunomodulators, chemokines and receptors) were collected from the study of Charoentong et al [16] (Supplementary Table 1). We applied the ESTIMATE algorithm to assess the immune scores, stromal scores, estimate scores, and tumor purity for each HCC sample [17]. Several algorithms were used to calculate the infiltration level of TIICs to avoid calculation errors: ssGSEA, TIMER [18], CIBERSORT [19], quanTIseq [20], EPIC [21], xCell [22], and MCP-counter [23]. We identified the effector genes of TIICs from previous studies [24, 25] (Supplementary Table 2). Also, we calculated the steps of cancer immunity cycles as described previously [26]. Finally, T cell–inflamed score was calculated as an average value of log2-scale normalized expression of the 18 signature genes [27].

2.8. 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 [28]. We used the *"ConsensuClusterPlus"* package to perform this algorithm and 1000 times repetitions were conducted for guaranteeing the stability of classification [29].

2.9. 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 [24] (Supplementary Table 3). The enrichment scores of these signatures were calculated using the R package *"GSVA"* [30]. To analyze the efficacy of immunotherapy, tow immunotherapy-related cohorts, GSE78220 and GSE91061 (melanoma) were obtained.

2.10. 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) [31] and Cancer Therapeutics Response Portal (CTRP) [32] respectively. The Spearman correlation between the NAPSB 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 NAPSB expression and TACE response in HCC patients.

2.11. 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 [33–37] (Supplementary Table 4). The enrichment scores of these signatures were also calculated using the R package *"GSVA"* as mentioned above.

2.12. Statistical analysis

Statistical analyses were performed using R software (version 4.1.1). Paired Student's t-test was performed to detect the differential expression of NAPSB in paired HCC and adjacent normal tissues. One-way ANOVA test were used for comparison multiple groups. Correlations between variables were explored using Pearson or Spearman coefficients. For all analyses, a two-paired 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.

3. Results

3.1. Expression levels analysis and high NAPSB inferred a better prognosis for HCC

NAPSB transcription levels in different human tumors were showed in Fig. 1A. Compared with adjacent normal tissues, expression of NAPSB 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. 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 NAPSB was indeed significantly decreased in HCC tissues (Fig. 1C). Moreover, NAPSB expression was examined in 13 paired HCC and adjacent normal tissues of Zhongnan cohort by RT-PCR and we obtained consistent results. (Fig. 1D).

The correlation between NAPSB and clinicopathologic characteristics for TCGA and ICGC cohorts were presented in Supplementary Tables 5 and 6. In addition, K-M survival analysis showed that high expression of NAPSB 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). Univariate Cox regression analysis showed that NAPSB 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, NAPSB expression was beneficial to overall survival and could serve as an independent predictor of disease-free survival and progression-free survival in HCC.

3.2. Enrichment analyses inferred NAPSB was related to immune activation

Correlation between NAPSB and other genes was analyzed using TCGA-HCC data and there were 930 genes significantly associated with NAPSB (p-value < 0.01, |Spearman`s correlation| ≥ 0.45; Supplementary Table 7). The correlation of NAPSB with the top 50 co-expressed genes was showed in Fig. 2A, which contained some immune-related molecules like CD48, CD37, IL6, HLA-DQA1. Meanwhile, DEGs analysis between NAPSB subgroups showed that there were 993 upregulated in the NAPSB-high group compared with the NAPSB-low group (adjusted p-value < 0.05 and | log2 (fold change) | value ≥ 1.3; Supplementary Table 8). The top 10 upregulated genes also contained immune-related molecules, such as CD48, CD37, CCR5 (Fig. 2B), suggesting that NAPSB may 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 NAPSB (Fig. 2C; Supplementary Table 9). The GO analysis for these common genes demonstrated they were enriched in processes such as T cell activation, regulation of T cell activation, and regulation of immune effector process (Fig. 2D; Supplementary Table 10). The KEGG analysis showed they 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 NAPSB may mediate the TME in HCC.

Even further, we conducted GSEA and GSVA between NAPSB subgroups and also identified many significant pathways related to immunity (Fig. 2F, G; Supplementary Table 12, 13). These findings paralleled the above results.

3.3. NAPSB shaped an immuno-hot and inflamed TME in HCC

The immunological role of NAPSB was comprehensively explored subsequently using TCGA and ICGC cohorts. NAPSB 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. Then ESTIMATE algorithm was applied to calculate the immune score, stromal score, estimated score and tumor purity. We found these scores were significantly increased NAPSB-high group (Fig. 3B) while the tumor purity was negatively correlated with the expression of NAPSB (Fig. 3C). As for TME immune cell infiltration, almost all immune cells were significantly enriched in NAPSB-high group (Fig. 3D). Consistent with these, the infiltration levels of CD8 + T cells, CD4 + T cells, NK cells, B cells, DCs and macrophages were almost positively correlated with NAPSB in six different algorithms (Fig. 3E). In line with these, NAPSB was positively correlated with the marker genes of these six major types of immune cells (Fig. 3F). These results suggested NAPSB was associated with an inflamed TME. Even further, we observed the NAPSB expression positively correlated with the T cell inflamed score (TIS) 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 NAPSB and seven steps of cancer-immunity cycle, which conceptualized the anti-cancer immune response [38]. Overall, In the NAPSB-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 NAPSB was to transform a non-inflamed TME into an immuno-hot and inflamed microenvironment, consequently triggering anti-cancer immune response.

3.4. NAPSB highly expressed in hot tumors and may enhance immunotherapy response

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) [28]. The expression of NAPSB was compared between hot and cold tumors and we found that it was overexpressed in hot tumors (Fig. 4E), suggesting that NAPSB 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, NAPSB expression was found to be positively correlated with BTLA, CTLA-4, IDO1, LAG-3, PD-1, PD-L1, TIGIT, and TIM-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 NAPSB subgroups. As exhibited in Fig. 4G, I, NAPSB was negatively correlated with the enrichment scores of PPARG network, β-catenin signaling pathway, VEGFA and IDH1, which were all immunosuppressive gene signatures [39–42]. However, in the NAPSB-high group, immunotherapy-positive pathways such as IFN-γ-signature, APM-signal, EGFR-ligands, hypoxia and KDM6B were activated (Fig. 4H) [43–47], 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 NAPSB 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 NAPSB-high group than in the NAPSB-low group (Fig. 4J) and the expression of NAPSB was significantly high in response group (Fig. 4K). Similar results were observed in the GSE78220 cohort (Supplementary Fig. 3I). These evidences reconfirmed that NAPSB may be a valuable predictor of immunotherapy response across cancers.

3.5. NAPSB was associated with increased sensitivity to chemotherapy

Using data from GDSC and CTRP, the role of NAPSB in chemotherapy sensitivity was analyzed. Intriguingly, NAPSB expression was negatively associated with IC50 of most agents in GDSC and 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 for HCC was 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 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.

3.6. 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 [48], we investigated the association between NAPSB and various forms of cell death, including pyroptosis, necroptosis, apoptosis, autophagy and ferroptosis. 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, G). 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 PANoptosis in tumor therapy.

4. Discussion

In this study, the potential biological functions of NAPSB has been comprehensively explored for the first time in HCC. By analyzing the data from multiple public databases and Zhongnan cohort, our study obtained consistent results as previous research: NAPSB was downregulated in HCC [15]. Tan et al.'s study showed NAPSB was upregulated in PDAC and related to CD4 + T Cell infiltration [14]. Additionally, upregulation of NAPSB was also found in pre-eclampsia, a state of highly inflammatory activity [49]. It was expected that NAPSB overexpressed under inflammatory conditions. In line with these, enrichment analyses in this study showed the genes most related to NAPSB 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 NAPSB in HCC immune microenvironment. MHC molecules represent antigen presentation and processing capacity, while chemokines and receptors recruit effector TIICs [50, 51], which may upregulate the activities of the cancer–immunity cycle subsequently [52]. In our study, NAPSB was found to be positively correlated with these immunomodulators, suggesting that NAPSB promotes immune activation, which is consistent with the results of enrichment analyses above. In addition, NAPSB expression had a positive correlation with the abundance of immune cells. Currently, the prognosis of HCC is known to be related to the infiltration and activation of immune cells[53, 54], whose presence participate in a hot (inflamed) TME [55, 56], supporting the observation that NAPSB 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 NAPSB was associated with better prognosis of HCC. Additionally, we observed NAPSB was positively related to the TIS, 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 [27, 38], these results reaffirmed and extended the close relationship between NAPSB and an immune-hot and inflamed TME.

T-cell infiltration, molecular characteristics of immune activation, and anti-tumor response are characteristics of hot tumors [57, 58], so we speculated NAPSB can play a role in distinct hot/cold tumor states based on the above results. Here, NAPSB was highly expressed in hot tumor samples consistently. Not only that, NAPSB 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 [59]. Together, NAPSB could distinguish between hot and cold tumors and facilitate immunotherapeutic responses. Meanwhile, we discovered that NAPSB high group was activated in immune-activated pathways, such as IFN-γ signature, which had been revealed to contributing to an inflamed TME and resulting in better clinical responses to immunotherapy. These outcomes not only demonstrated that NAPSB can improve the immunotherapy response, but also reconfirmed the role of NAPSB 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 [60]. TACE is a treatment for liver cancer often applying doxorubicin or cisplatin as intra-arterial injection of cytotoxic agents [61]. In our study, we proved that NAPSB was negatively correlated with IC50 of a variety of commonly used drugs, but overexpressed in TACE responders as expected, strongly inferring high NAPSB expression can improve the sensitivity of chemotherapy. NAPSB may be utilized as a promising predictive marker for chemotherapy as drug resistance is common at present [7, 62]. 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 [63]. Carina et al.'s study revealed sorafenib therapy induced pyroptosis in MΦ and thereby unleashing an NK-cell response against HCC tumors [64]. Instead, autophagy, this cell death form participates in the progression of HCC and the resistance of HCC cells to sorafenib [36, 65]. In our study, we revealed NAPSB was positively correlated with PANoptosis, but had no correlation with autophagy, suggesting that NAPSB 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. Second, further in-depth studies are required to explore the interactions of NAPSB, PANoptosis and chemotherapy.

5. Conclusions

In conclusion, our study is the first comprehensive analysis to demonstrate that NAPSB could shape an immuno-hot and inflamed TME in HCC; NAPSB could be considered a predictor of disease-free and progression-free survival outcomes in patients with HCC; NAPSB 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.

Declarations

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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 (grant no.20200110). All the methods were carried out in accordance to relevant guidelines" under the ethical approval and consent to participate section.

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 FIG.s. X.L. L.D. 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.

Disclosure statement

The authors declare that they have no competing interests.

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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.

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Figures

Figure 1

Differential expression and prognosis value of NAPSB in pan-cancer and liver hepatocellular carcinoma (LIHC). (**A)** NAPSB expression levels in different tumor types were measured using TIMER. **(B)** Paired Student's t-test analysis of NAPSB expression in TCGA paired samples. **(C)** NAPSB expression was significantly higher in normal tissues than in hepatocellular carcinoma in ICGC, GSE55092, GSE54236, and GSE121248 cohorts. **(D)** In Zhongnan cohort, lower NAPSB expression was seen in hepatocellular carcinoma compared with adjacent normal tissues (N=13). The expression of NAPSB was compared with a standard reference control and relative quantities (RQ) were calculated based on the ΔΔCt method. (**E-H)** Kaplan–Meier analysis of NAPSB 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 NAPSB biological function in hepatocellular carcinoma (HCC). **(A)** The heat map shows the top 50 genes positively related to NAPSB in HCC. **(B)** Volcano plot of DEGs between NAPSB-high and NAPSB-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 NAPSB. **(E)** The top 20 Kyoto Encyclopedia of Genes and Genomes (KEGG) terms for the most closely related genes to NAPSB. **(F)** Gene Set Enrichment Analysis (GSEA) shows significant signaling pathways between NAPSB-high and NAPSB-low HCC tissues (The gene sets of “c5.cp.kegg.v7.4.symbols”). **(G)** Gene Set Variation Analysis (GSVA) between NAPSB-high and NAPSB-low HCC tissues (The gene sets of “h.all.v7.4.symbols”).

Figure 3

NAPSB shapes an inflamed TME in HCC. **(A)** Expression levels of 50 immunomodulators (MHC, immunostimulators, chemokines, and receptors) in the NAPSB-high and NAPSB-low groups in HCC. **(B)** Distribution of stromal score, immune score, and ESTIMATE score calculated using the ESTIMATE algorithm in the NAPSB-high and NAPSB-low groups. **(C)** Correlation between NAPSB 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. PD/SD: Progressive and stable disease. 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 NAPSB expression. **(A)** Bar plot exhibiting Spearman correlation between NAPSB and the IC50 of drugs in GDSC. **(B)** Correlations between NAPSB 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 NAPSB and the IC50 of the frequently used drugs for advanced HCC patients in GDSC, including fluorouracil, gemcitabine, cisplatin, sorafenib, erlotinib and grfitinib. **(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 NAPSB and the enrichment scores of several cell death signatures in TCGA (**A**-**E**) and ICGC (**F, G**) cohort. NAPSB 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 NAPSB expression (R = -0.017, P = 0.795). **(E)** NAPSB was negatively correlated with ferroptosis (R = -0.24, P < 0.001). **(F)** Correlations between NAPSB and several forms of cell death in ICGC cohort. **(G)** The enrichment scores of pyroptosis, necroptosis and apoptosis in NAPSB-high groups were markedly higher than NAPSB-low groups. However, ferroptosis scores were lower in NAPSB-high group than in NAPSB-low group and autophagy scores had no significant differences in NAPSB 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.

Supplementary Files

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