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The impact of an RNA-binding protein group on regulating the RSPO-LGR4/5-ZNRF3/RNF43 module and the immune microenvironment in hepatocellular carcinoma

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Abstract

Background Hepatocellular carcinoma (HCC) is a leading cause of cancer mortality. RNA-binding proteins (RBPs) are potential therapeutic targets because of their role in tumor progression. This study investigated the interactions between specific HCC progression-associated RBPs (HPARBPs), namely, ILF3, PTBP1, U2AF2, NCBP2, RPS3, and SSB, in HCC and their downstream targets, as well as their impact on the immune microenvironment and their clinical value.

Methods Tissue samples from human HCC, collected from 28 patients who experienced recurrence following postoperative adjuvant therapy were examined. The mRNA levels of RBPs and their prospective targets were quantified through RNA isolation and quantitative real-time PCR. Data from two public datasets were scrutinized for both expression and clinical relevance. Through Student's t test and logistic regression, HPARBPs were identified. Enhanced cross-linking immunoprecipitation (eCLIP) experiments revealed RBP-RNA interactions in HepG2 cells. For functional enrichment, Metascape was used, whereas CIBERSORT was used to characterize the immune microenvironment.

Results Public database analysis confirmed widespread RBP expression abnormalities in HCC (false discovery rate < 0.00001 and fold change ≥ 1.15 or ≤ 0.85), leading to the identification of 42 HPARBPs and core modules. eCLIP data analysis revealed the specificity of downstream target genes and binding site features for core HPARBPs (signal value > 3, *P value* < 0.01). Four core HPARBPs may bind to RNAs of genes in the RSPO-LGR4/5-ZNRF3/RNF43 module,

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affecting the Wnt pathway and HCC progression. Immunoinfiltration analysis revealed changes in the HCC immune microenvironment due to altered expression of relevant genes.

Conclusion In our study, we identified core HPARBPs that might contribute to HCC progression by binding to RNAs in the RSPO-LGR4/5-ZNRF3/RNF43 module. Changes in the expression of HPARBPs affect the HCC immune microenvironment. Our findings offer novel insights into the regulatory network of Wnt pathway-related RBPs and their potential clinical value in HCC.

Keywords Hepatocellular carcinoma, RNA-binding proteins, Enhanced cross-linking immunoprecipitation, Posttranscriptional regulation, Tumor immune microenvironment

Introduction

Liver cancer, notably hepatocellular carcinoma (HCC), ranks eighth in prevalence and third in tumor-related mortality worldwide [1]. HCC accounts for 80% of liver cancer cases [2]. The landscape of liver diseases research is in continuous evolution, as researchers worldwide are delving deeper into the intricate genetic underpinnings of liver diseases development [3–6]. Large-scale HCC genome sequencing analyses have identified core driver genes, such as TERT and TP53, as initial molecular events, as well as other low-frequency driver genes [7]. In the field of tumor research, the abnormal changes in genes encoding RNA-binding proteins (RBPs) in tumors and the potential effects of downstream regulatory groups of proteins on tumor progression have gradually attracted the attention of researchers, who have focused on precision targeted therapy [8, 9].

RBPs are highly conserved and diverse proteins that participate in multiple aspects of RNA regulation, including transcription [10], translation [11], splicing [12, 13], polyadenylation [14], stability [15], and localization [16]. RBPs can form dynamic functional complexes by directly interacting with other proteins and RNAs, such as ribonucleoproteins, ultimately forming functional granules or bodies through phase separation [17]. RBPs serve as crucial regulators of cellular homeostasis, and their dysregulation is associated with various human diseases, including cancer [18].

In the study of HCC, researchers have investigated the impact and role of certain RBPs in the regulation of target RNAs. For instance, with respect to transcriptional regulation, the oncogenic factor NELFE directly binds to MYC in the nucleus, regulating its interaction with chromatin and influencing MYC-mediated transcription. NELFE also regulates MYC downstream targets in the cytoplasm, affecting their expression in posttranscriptional processes and ultimately contributing to tumorigenesis in HCC [19, 20]. In terms of alternative splicing, SRSF2 mediates alternative splicing events in HCC, promoting the generation of the GCH1-L isoform of GCH1, thus promoting HCC development [21]. In terms of stability, highly expressed HuR promotes the stability of MAT2A by binding to its 3' UTR in HCC. It can also bind to the mRNA of β-catenin to inhibit its degradation. Moreover, an imbalance in the expression of HuR and certain other RBPs plays a crucial role in HCC cell proliferation [22, 23].

The aberrant overexpression of RBPs in tumors may lead to abnormal regulation of downstream target RNAs, resulting in tumorigenesis and tumor progression. Consequently, research on targeted RNA-binding protein therapy for tumors is actively underway [24]. For example, studies have shown that the small molecule inhibitor MS-444, which targets HuR, can effectively inhibit the growth of intestinal tumors [25]. Researchers are currently developing small-molecule drugs to disrupt the interaction between the key oncogenic factor HuR and its target mRNAs, offering a novel therapeutic approach for tumors with upregulated HuR expression [26].

Overall, RBPs play a significant role and have a profound impact on HCC, with studies on their function and targets having crucial biological and clinical importance. To explore all the target RNAs bound by RBPs in the physiological or pathological states of living cells or tissues, researchers have developed high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation (HITS-CLIP) over the past two decades. This technique integrates the advantages of immunoprecipitation and high-throughput sequencing to elucidate all target RNAs via an antibody against a selected RBP, and its methodology is constantly improving [27].

In this study, we focused primarily on upregulated RBPs such as ILF3 (also known as NF90), PTBP1, U2AF2, NCBP2, RPS3, and SSB because of their differential expression and potential clinical relevance in HCC. Notably, some of these RBPs may share common downstream RNA targets, such as RNF43, ZNRF3, and LGR4. These target genes were identified through the analysis of publicly available high-throughput sequencing data specific to RBP-RNA interactions derived from enhanced cross-linking immunoprecipitation (eCLIP) experiments [28], which can be considered an advanced

iteration of HITS-CLIP. Compared to HITS-CLIP, eCLIP demonstrates superior performance by minimizing RNA loss, reducing PCR duplicates, eliminating background noise with higher efficiency, substantially increasing library construction success rates, and enhancing target recognition accuracy. In addition to investigating the downstream regulatory networks of these RBPs, we utilized public database data and our own research cohort to explore the impact of their expression levels on the immune microenvironment and their clinical value in HCC.

Materials and methods

Human HCC samples

A total of 28 paraffin-embedded tissue samples were collected from patients who were diagnosed with HCC by the Pathology Department of Nanjing Drum Tower Hospital and who experienced recurrence following postoperative adjuvant therapy between January 2020 and January 2023 (Table 1). These patients who experienced recurrence were subsequently treated with a combination of anti-angiogenic targeted therapies and immunotherapy. Efficacy was evaluated by the Response Evaluation Criteria in Solid Tumors (RECIST). This study was approved by the Ethics Committee of Nanjing Drum Tower Hospital, Affiliated Hospital of Medical School, Nanjing University, with ethical approval number 2023-646-02. All patients provided written informed consent to participate in this study.

RNA isolation and quantitative real-time PCR (qRT–PCR) analysis

The FFPE DNA/RNA extraction kit from Aperbio Technologies, Suzhou, China, was used to extract nucleic acids from formalin-fixed, paraffin-embedded tissue samples following the manufacturer's instructions. Nucleic acid quantification was performed via a Colibri microvolume spectrophotometer (Titertek-Berthold, Pforzheim, Germany) and a Qubit Fluorometer 2.0 (Invitrogen, California, USA). cDNA synthesis was carried out according to the instructions of HiScript® III RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China). QRT–PCR was performed via ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) and was repeated three times on an ABI7500 system. Changes in the expression of genes (ILF3, PTBP1, U2AF2, NCBP2, RNF43, ZNRF3, and LGR4) at the mRNA level were detected. The primers used are listed in Table S1. The differences in expression measured by qRT–PCR were compared via the Wilcoxon method for statistical analysis.

Public data sources

The gene expression and clinical data of HCC patients were obtained from the Cancer Genome Atlas (TCGA) liver hepatocellular carcinoma (LIHC) dataset via the Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov/). Furthermore, the gene expression and clinical data of HCC patients from the Liver Cancer Institute (LCI) cohort were obtained from the Gene Expression

Table 1 Characteristics of the HCC patients in our local cohort. Patients in our study had been previously diagnosed with HCC, experienced recurrence following adjuvant chemotherapy, and underwent postoperative anti-angiogenesis targeted drugs combined with immunotherapy treatment subsequent to relapse. BMI, body mass index; PD, progressive disease; PR, partial response; SD, stable disease

Characters	Cases (*N* = 28,%)
Age (range)	56 (32 ~ 76)
Sex	27 (96.4%)
Male	1 (3.6%)
Female	
BMI	1 (3.5%)
< 18.5	12 (42.9%)
18.5 ~ 23.9	10 (35.7%)
24.0 ~ 27.9	5 (17.9)
≥ 28.0	
Hepatitis B	24 (85.7%)
Yes	4 (14.3%)
No	
Liver Cirrhosis	8 (28.6%)
Yes	20 (71.4%)
No	
Alcoholic History	7 (25.0%)
Yes	21 (75.0%)
No	
RECIST	6 (21.4%)
PR	17 (60.7%)
SD	5 (17.9%)
PD	

Omnibus (GEO) GSE14520 dataset (http://www.ncbi.nlm.nih.gov/geo).

Processed data of the eCLIP experiment performed on the HepG2 cell line against six core HPARBP genes (ILF3, PTBP1, NCBP2, U2AF2, SSB, and RPS3) were downloaded from the Encyclopedia of DNA Elements (ENCODE) portal (https://www.encodeproject.org/) with the following identifiers: ENCFF071QDP, ENCFF726SQU, ENCFF692RZM, ENCFF721PWF, ENCFF848JWA and ENCFF301IJW.

Data processing

Differentially expressed RNA-binding proteins (DERBPs) were examined in both the TCGA and LCI cohorts via Student's t test, with a false discovery rate (FDR) < 0.00001 and a fold change ≥ 1.15 or ≤ 0.85, while low-abundance genes were excluded. Subsequently, hepatocellular carcinoma progression-associated RNA-binding proteins (HPARBPs) were identified by logistic regression analysis with a significant *P-value* < 0.05 for both TNM stage and alpha-fetoprotein (AFP) level. Following the screening of HPARBPs, only the TCGA-LIHC cohort was utilized for further analysis on the basis of its HCC expression data. Differential analysis of only the TCGA-LIHC cohort was performed via DESeq2 (R package), with an FDR < 0.00001 and a fold change ≥ 1.15 or ≤ 0.85, while low-abundance genes were excluded.

The upstream analysis of eCLIP sequencing data was performed in accordance with the eCLIP-seq Processing Pipeline v2.2 20200409, which was released to the public on the ENCODE website. The bed format files were obtained for further mining. These bed format files, which contain information about specific binding sites (signal value > 3, *P-value* < 0.01), were sorted and then annotated by HOMER software on the basis of the hg38 reference genome.

Functional and pathway enrichment analysis

The web tool Metascape was used for functional and pathway enrichment analysis of specific gene lists on the basis of Gene Ontology (GO) Biological Processes, GO Cellular Components, GO Molecular Functions and the Kyoto Encyclopedia of Genes and Genomes (KEGG) [29]. All genes in the genome were used as the enrichment background. Terms with a *P-value* < 0.01, a minimum count of 3, and an enrichment factor > 1.5 (the enrichment factor is the ratio between the observed counts and the counts expected by chance) were collected and grouped into clusters on the basis of their membership similarities.

Immunoinfiltration analysis

CIBERSORT (R package), which uses the principle of linear support vector regression to deconvolute the expression matrix of 22 human immune cell subtypes and the TCGA-LIHC expression matrix normalized by transcripts per million (TPM), was used to explore the estimated proportion of immune cells in HCC patients. We subsequently screened out the immune cells that showed significant differences in infiltration between patients with low and high expression levels of specific genes via the Wilcoxon method and analyzed the correlations with those genes via the Spearman method.

Statistical analysis and visualization

All the statistical analyses were implemented via R software (version 4.2.1) and Microsoft Excel. For comparisons, Student's t test (two-sided), the Wilcoxon rank-sum test and Spearman's correlation analysis were performed as indicated. Visualization of the analysis results was accomplished via R packages, TBTools [30], SRplot [31] and Integrative Genomics Viewer (IGV) [32].

Results

Abnormal expression of RBPs appears to be prevalent in HCC

On the basis of public databases and preceding reports, we successfully retrieved expression profiles and clinical data from 887 clinical samples from the TCGA-LIHC and LCI cohorts. We subsequently performed differential analysis between HCC and normal tissues to obtain a list of DERBPs (FDR < 0.00001, fold change ≥ 1.15 or ≤ 0.85). Univariate logistic regression analysis of the aforementioned DERBPs was then performed to screen out those that exhibited significant correlations with TNM stage and the AFP level (*P value* < 0.05). This analysis yielded the identification of 248 (245 upregulated) and 126 (118 upregulated) HPARBPs in the TCGA and LCI cohorts, respectively (Fig. 1A and Table S2). Notably, almost all 42 overlapping HPARBPs in both cohorts exhibited interactions via protein–protein interaction (PPI) analysis using STRING (Fig. 1B). The core module consisting of 13 core HPARBPs was discovered within these complex interactions of HPARBPs via the cytoHubba plugin of Cytoscape (Fig. 1C). Functional enrichment analysis revealed that these core HPARBPs are primarily implicated in mRNA metabolism, mRNA process regulation, RNA nuclear output, translation regulation, splicing, and are likely involved in the formation of cytoplasmic ribonucleoprotein particles (Fig. 1D). Additionally, a subset (6 out of 13 core HPARBPs) of the core module was found to have matched high-throughput sequencing data of its binding target RNAs in the liver cancer HepG2 cell line, namely, eCLIP data, available in the ENCODE database for further analysis. In conclusion, our findings indicate that RBPs are commonly upregulated in HCC, and we identified a set of interacting core HPARBPs associated with

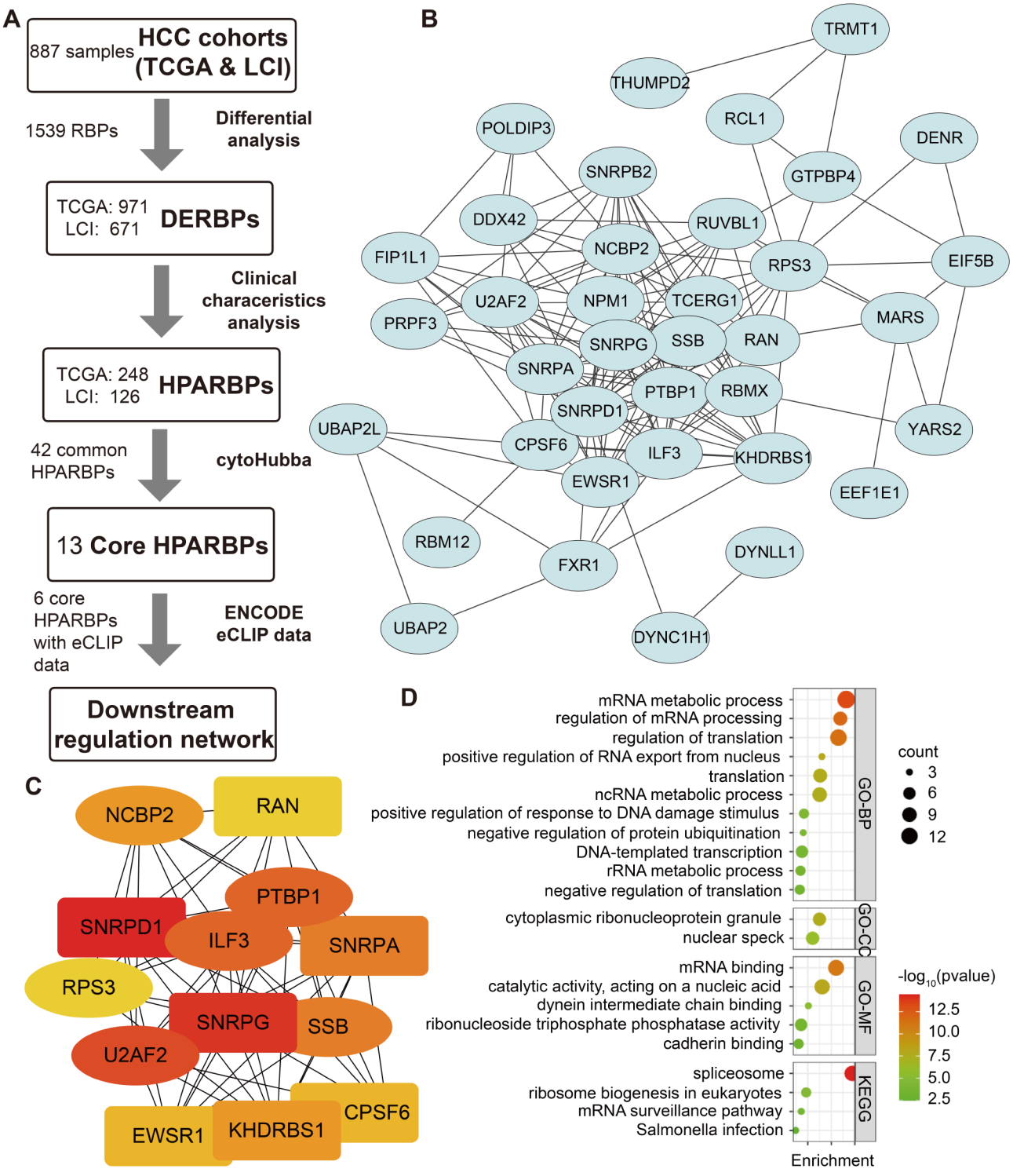

Fig. 1 Identification and characterization of the core HPARBP network. (A) Schematic representation of the study design, outlining the approaches employed to achieve the core HPARBP network. (B) PPI network comprising 42 HPARBPs, illustrating the interaction relationships among these RNA-binding proteins. (C) Topological analysis of the HPARBP PPI network via the cytoHubba MNC algorithm, revealing a core module of HPARBPs with key members highlighted in a greater degree of red, indicating their relative importance within the module. The oval shape denotes open-access eCLIP data availability from ENCODE for these members. (D) GO and KEGG functional enrichment analysis results of the core module. The size of the circle represents the number of genes involved, and the abscissa represents the significance of GO terms or KEGG pathways

the progression of HCC that may participate in various RNA processes.

The core module of HPARBPs may be extensively involved in the binding and regulation of genes related to the Wnt pathway

Through integrative analysis of eCLIP data and genome annotation of binding sites, we observed that the significant binding sites (signal value > 3, *P-value* < 0.01) of the six core HPARBPs (ILF3, PTBP1, NCBP2, U2AF2, SSB, and RPS3) presented distinct proportions of genomic regions (Fig. 2A) and limited crossover between the downstream target regulatory groups of the core HPARBPs (Fig. 2B and Table S3). A functional enrichment analysis of the shared downstream targets of the core HPARBPs referenced above, excluding SSB due to its low overlap with the others, revealed that the core HPARBPs play a significant role in the regulation of genes associated with the negative control of epithelial cell proliferation and the Wnt signaling pathway (Fig. 2C). By integrating multiple functional annotated gene sets, we identified 485 genes related to the Wnt pathway, nearly half of which are potentially regulated by one or more core HPARBPs (Fig. 2D). Differential gene expression analysis of the TCGA-LIHC cohort revealed that the majority of Wnt pathway-related target genes were not significantly differentially expressed in HCC (adjusted *P-value* < 0.05 was considered significant) (Fig. 2E). These findings indicate that in HCC, core HPARBPs may engage with the Wnt signaling pathway by modulating downstream RNA target genes, albeit predominantly through mechanisms that do not significantly alter the expression levels of those target genes.

Specific core HPARBPs may coregulate the RSPO-LGR4/5-ZNRF3/RNF43 module upstream of the Wnt pathway in HCC

Among the potential Wnt pathway-related targets of the six core HPARBPs, three members of the RSPO-LGR4/5-ZNRF3/RNF43 module upstream of the Wnt pathway, specifically RNF43, ZNRF3, and LGR4, can be commonly bound by specific core HPARBPs, including ILF3, NCBP2, PTBP1, and U2AF2, thereby exerting regulatory functions (Fig. 3). The IGV browser revealed that the binding regions of these specific HPARBPs in RNF43, ZNRF3, and LGR4 are consistent with the proportion characteristics observed in previous results (Figs. 2A and 3). ILF3, PTBP1, and U2AF2 were found to bind to the intron regions of the three targets, whereas NCBP2 bound to the promoter-TSS regions. Given their reported expression in the nucleus, we speculated that these four specific core HPARBPs (ILF3, NCBP2, PTBP1, and U2AF2) may interact with three target pre-mRNAs (RNF43, ZNRF3, and LGR4) in the nucleus, potentially influencing posttranscriptional processes.

The expression levels of four specific core HPARBPs significantly correlate with immune cell infiltration patterns in HCC, demonstrating high similarity

On the basis of the analysis of gene expression data from the TCGA-LIHC cohort and the leukocyte signature matrix (LM22), we identified the predominant immune cell types infiltrating HCC, which included M0 macrophages, $CD8^+$ T cells, M2 macrophages, and resting memory $CD4^+$ T cells (Fig. 4A). LIHC patients were then divided into a high expression group and a low expression group using the median expression level of a certain gene as a cutoff. The classification of expression levels for four specific core HPARBPs revealed common patterns: γδ T cells were significantly increased in the low-expression group for all four specific core HPARBPs (*P-value* < 0.05), follicular helper T cells were significantly elevated in the high-expression groups for three core HPARBPs (ILF3, PTBP1, and U2AF2) (*P-value* < 0.05), and activated natural killer (NK) cells were significantly more abundant in the low-expression groups for two specific core HPARBPs (ILF3 and U2AF2) (*P-value* < 0.05) (Fig. 4B-E). Spearman correlation analysis revealed a significant negative correlation between the expression levels of these four core HPARBPs and γδ T cells (*P-value* < 0.05), which aligns with previous findings (Fig. S4A-D). These comprehensive analyses shed light on the intricate interplay between the expression of specific core HPARBPs and the immune cell composition within the tumor microenvironment. We offer valuable insights into the potential regulatory functions of HPARBPs in modulating immune responses in HCC, particularly concerning γδ T cells.

The potential clinical guiding significance of core HPARBPs and their targets in the RSPO-LGR4/5-ZNRF3/RNF43 module awaits further validation

After analyzing tumor tissue samples from 28 patients who experienced HCC recurrence following post-adjuvant therapy at our institution (as detailed in Table 1) and comparing them with two normal control tissues via qRT−PCR and differential analysis, we detected no significant differences in HPARBP expression levels (*P-value* >0.05) (Fig. S5A-D and Table 2). Additionally, the expression levels of LGR4 and RNF43, which are considered as downstream targets of the specific core HPARBPs in the RSPO-LGR4/5-ZNRF3/RNF43 module, did not significantly change (*P-value* >0.05), whereas

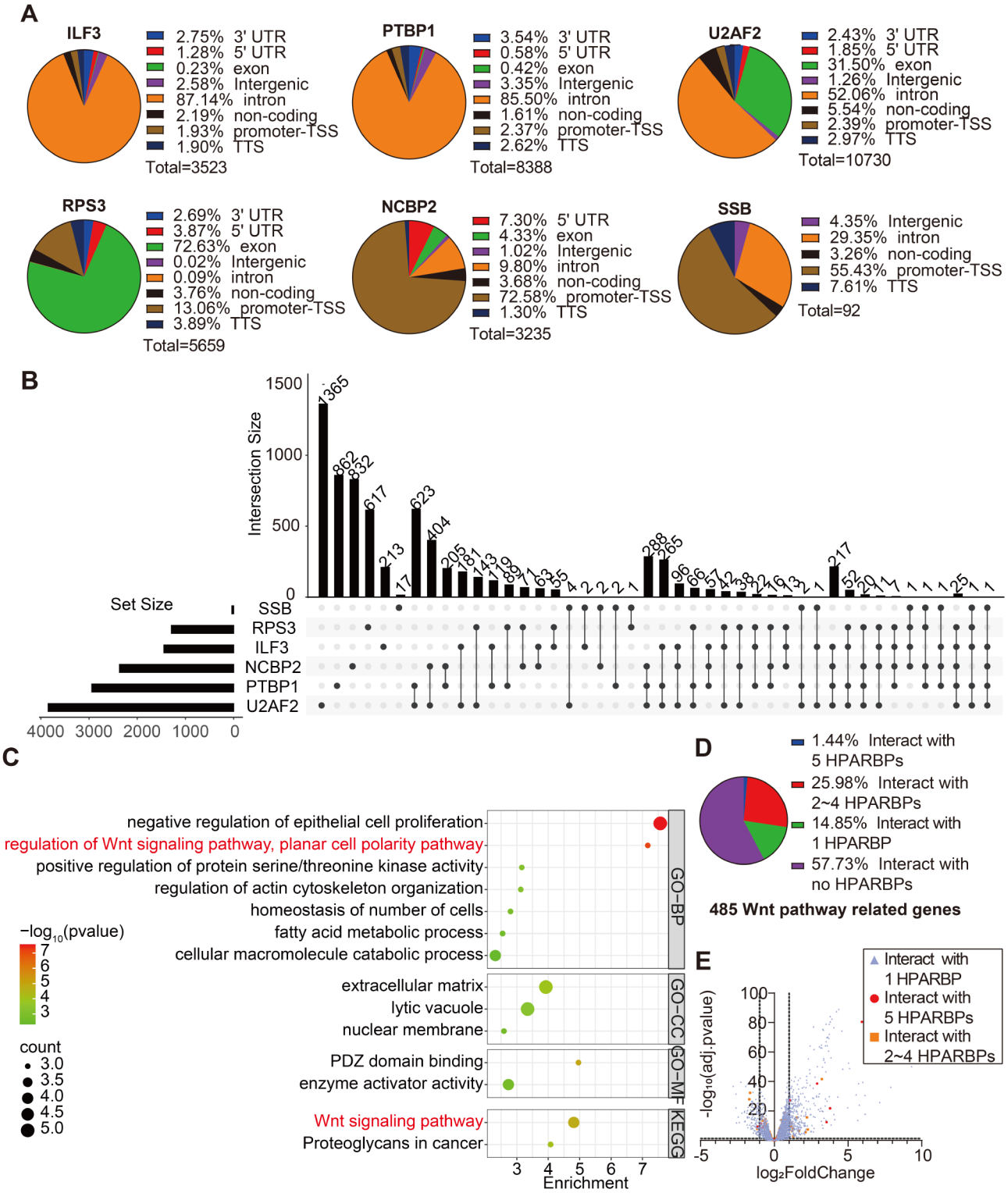

Fig. 2 Target binding profile of core HPARBPs. (**A**) Pie chart of target binding genomic regions of the six core HPARBPs. (**B**) UpSet plot depicting the target genes of the six core HPARBPs. (**C**) Results of the GO and KEGG functional enrichment analyses of the common targets of the core HPARBPs. Wnt-related terms and pathways are highlighted with red characters. (**D**) Pie chart displaying the characteristics of Wnt pathway-related genes targeted by the core HPARBPs, providing a detailed view of the regulatory landscape of these genes. (**E**) Volcano map of Wnt pathway-related genes identified via TCGA-LIHC differential analysis, together with labels of target characteristics

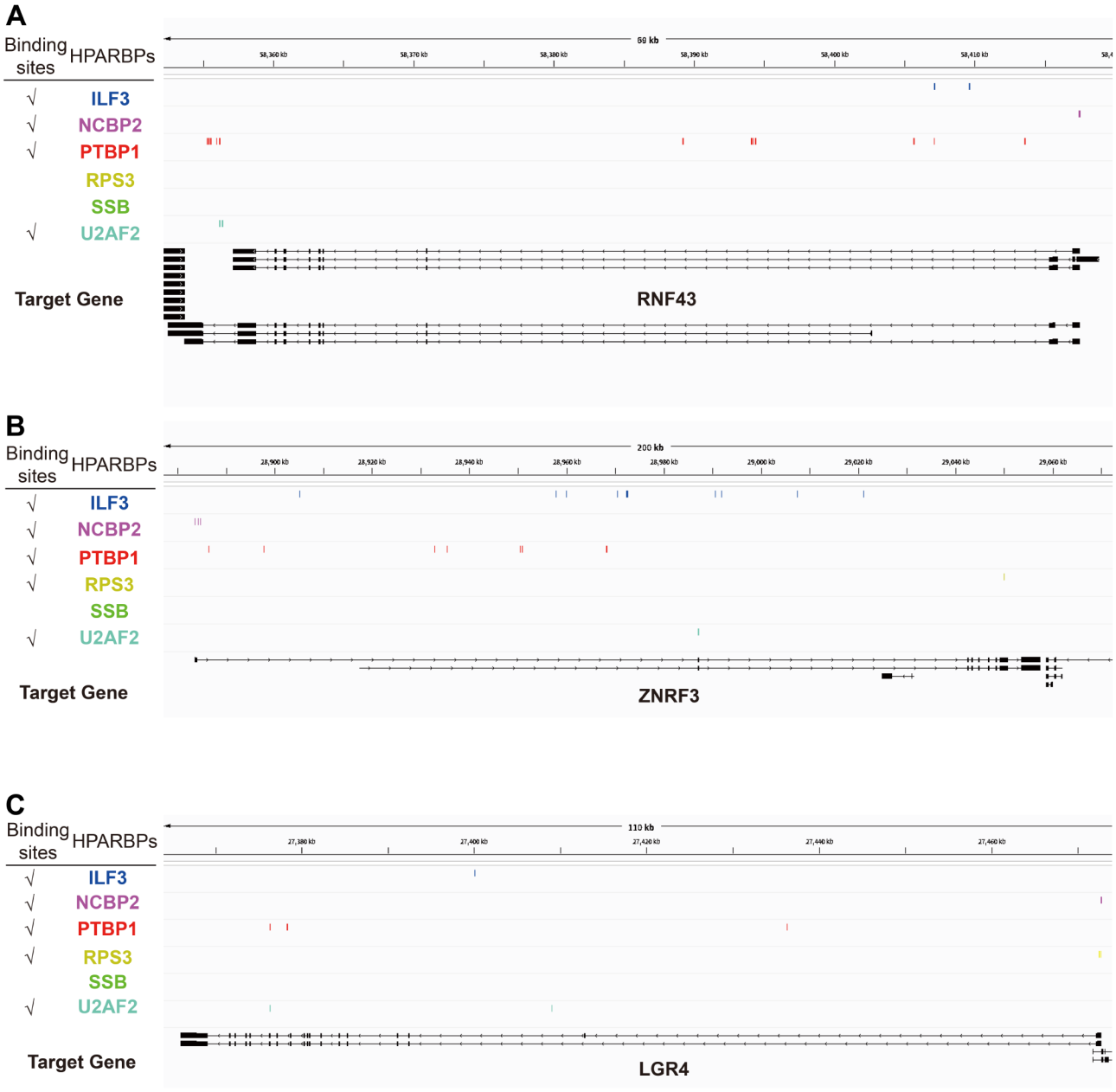

Fig. 3 Visualization of binding sites. The genomic binding sites of the specific core HPARBPs (signal value > 3, *P-value* < 0.01) on the gene structure of RNF43, ZNRF3, and LGR4 were visualized via the IGV browser without the enrichment signal information

the expression level of ZNRF3 was significantly downregulated (*P-value* <0.05) (Fig. 5A-C and Table 2). Furthermore, we performed an immunoinfiltration analysis focusing on these three target genes. By analyzing the differences between the high- and low-expression groups via correlation analysis, we discovered that the expression levels of these genes were significantly positively correlated with those of naive B cells and negatively correlated with those of activated NK cells (*P-value* <0.05) (Fig. 5D-F and S4E-G). Notably, the immune microenvironment attributes of ZNRF3 and RNF43 display substantial similarity. To delve deeper into the correlation between the expression levels of these genes and the efficacy of combining anti-angiogenesis targeted drugs with

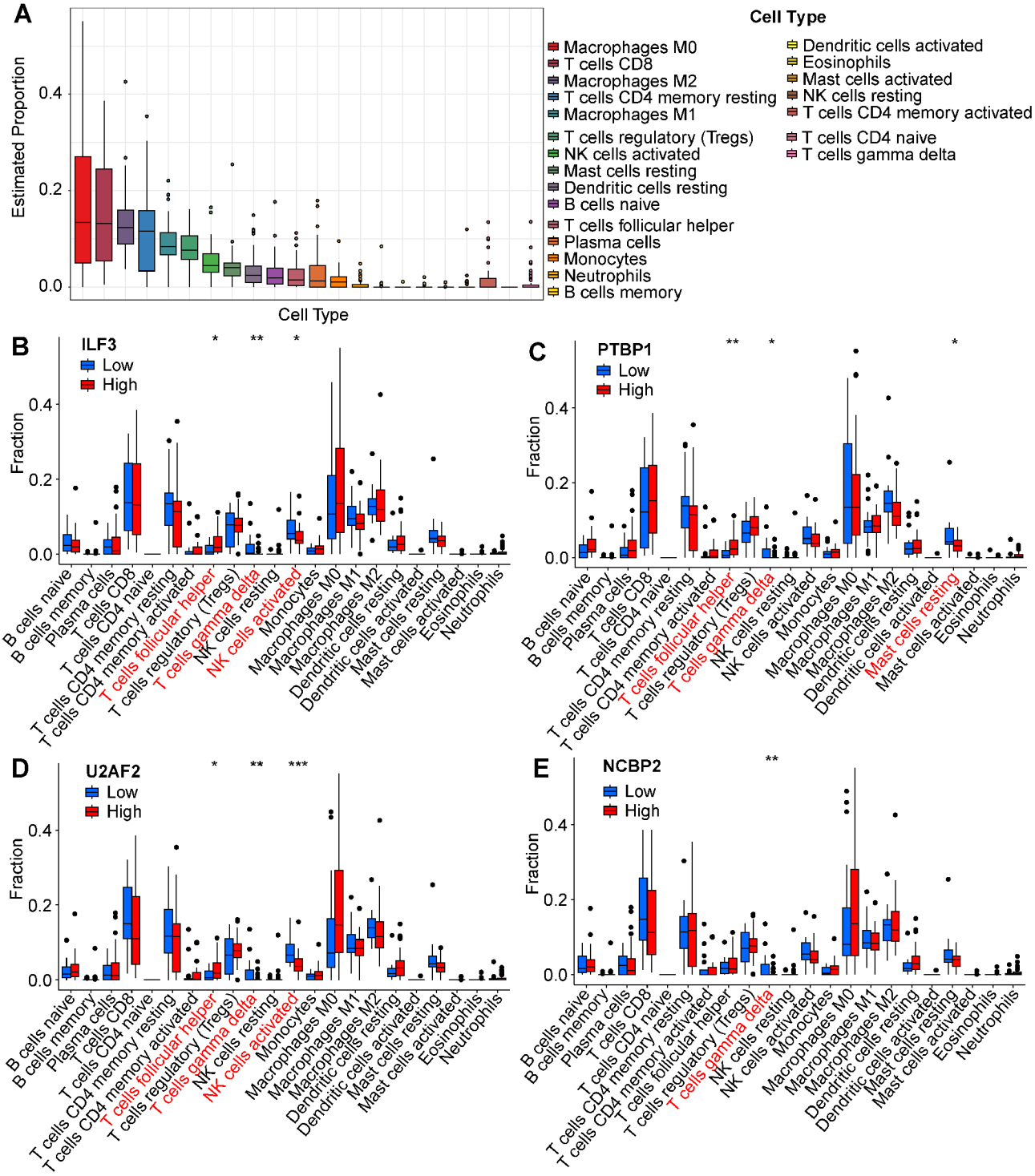

Fig. 4 Immunoinfiltration analysis in HCC patients on the basis of the expression of the four specific core HPARBPs. (**A**) Box plot illustrating the estimated proportion of immune cells in HCC patients. (**B**-**E**) Comparison of immune cell infiltration between the high- and low-expression groups of the core HPARBPs via the Wilcoxon test, with the median used as the group cutoff. Immune cell subsets with *P-values* < 0.05 are denoted in red. **P-value* < 0.05; ***P-value* < 0.01; ****P-value* < 0.001

Table 2 Summary of significant changes in the expression of specific core HPARBPs and their downstream binding targets in HCC-related samples. The list named "HCC vs. normal" includes findings from expression in tissues and/or cell lines associated with HCC, as reported in other studies [46–53]. The list named "recurrance after adjuvant HCC vs. normal" includes the results obtained by qRT–PCR in HCC and normal tissues in this study. Ns, *not significant; *P-value* < 0.05

Type	Gene	HCC vs. normal	Recurrence after adjuvant therapy HCC vs. normal
Specific Core HPARBPs	ILF3	upregulated	ns
	PTBP1	upregulated	ns
	U2AF2	upregulated	ns
	NCBP2	upregulated	ns
Downstream Binding Targets	LGR4	upregulated	ns
	RNF43	upregulated	ns
	ZNRF3	downregulated	downregulated [*]

immunotherapy, we reclassified the locally sourced HCC cohort using the RECIST guidelines and performed differential analysis of qRT-PCR data from various HCC immunotherapy response groups. The results revealed that the expression levels of these genes did not significantly differ among the various immunotherapy response groups and normal tissues (*P-value* > 0.05), except for ZNRF3, which was significantly downregulated in the stable disease (SD) group compared with the normal group (*P-value* < 0.05) (Fig. 6A-G and Table 2). Furthermore, we employed ordinal logistic regression analysis to explore the relationships between the expression levels of seven genes and treatment response outcomes. However, the model lacked statistical significance and did not demonstrate any significant correlations (*P-value* > 0.05) (data not shown). The weighted average expression levels of all seven genes also showed no significant differences (*P-value* > 0.05) (Fig. 6H). Taken together, our analysis of HCC tumor tissues subsequent to adjuvant therapy revealed no significant change in the expression levels of HPARBPs together with downstream LGR4 and RNF43. However, ZNRF3 was significantly downregulated both in patients who experienced recurrence after adjuvant therapy for HCC and in those in the SD group after immunotherapy. The expression of these three downstream targets was correlated with the infiltration levels of naive B cells and activated NK cells.

Discussion

Historically, studies of RBPs have focused primarily on one-on-one interactions with target RNAs. However, in the 1990s, Gao et al. introduced a groundbreaking method using human brain RNAs that revealed that HuB could bind multiple mRNAs in vitro [33]. This pivotal work paved the way for understanding global mRNA targeting and established the foundation for the post-transcriptional RNA regulon theory [34, 35]. RBPs play crucial roles in cancer by controlling numerous mRNAs encoding proto-oncogenes, growth factors, and cell cycle regulators. Changes in RBP expression or localization can profoundly affect gene expression patterns, as reflected by the stark differences in global RNA expression levels between cancerous and normal tissues [36–38].

We initially used data from two HCC cohorts to identify HPARBPs and core modules, followed by enrichment analysis. These findings suggest that HPARBPs likely interact with specific target groups as functional complexes, regulating various posttranscriptional processes of target RNAs. Previous studies have reported that functional complexes are formed by several RBPs and other proteins/RNAs, such as spliceosomes [39], stress granules [40], and processing bodies [41]. Our study identified potential molecular targets for future targeted therapies. However, further experiments are necessary to ascertain whether specific core HPARBPs form protein functional complexes that bind and regulate targets within the same spatial and temporal context.

The eCLIP experiment uses immunoprecipitation to isolate complexes of specific RBPs and their bound RNAs [28]. All target RNAs can be separated and sequenced after processing. The sequencing reads, followed by steps such as mapping and peak calling, provide the genomic coordinates of significant binding sites. Genome annotation on the basis of these coordinates allows us to uncover more information about binding sites. RBPs may perform diverse posttranscriptional regulatory functions by binding to different regions of their target RNAs [42]. We found that ILF3, PTBP1, and U2AF2 tend to bind to the intron regions of target RNAs, suggesting that they may play crucial roles in pre-mRNA splicing regulation

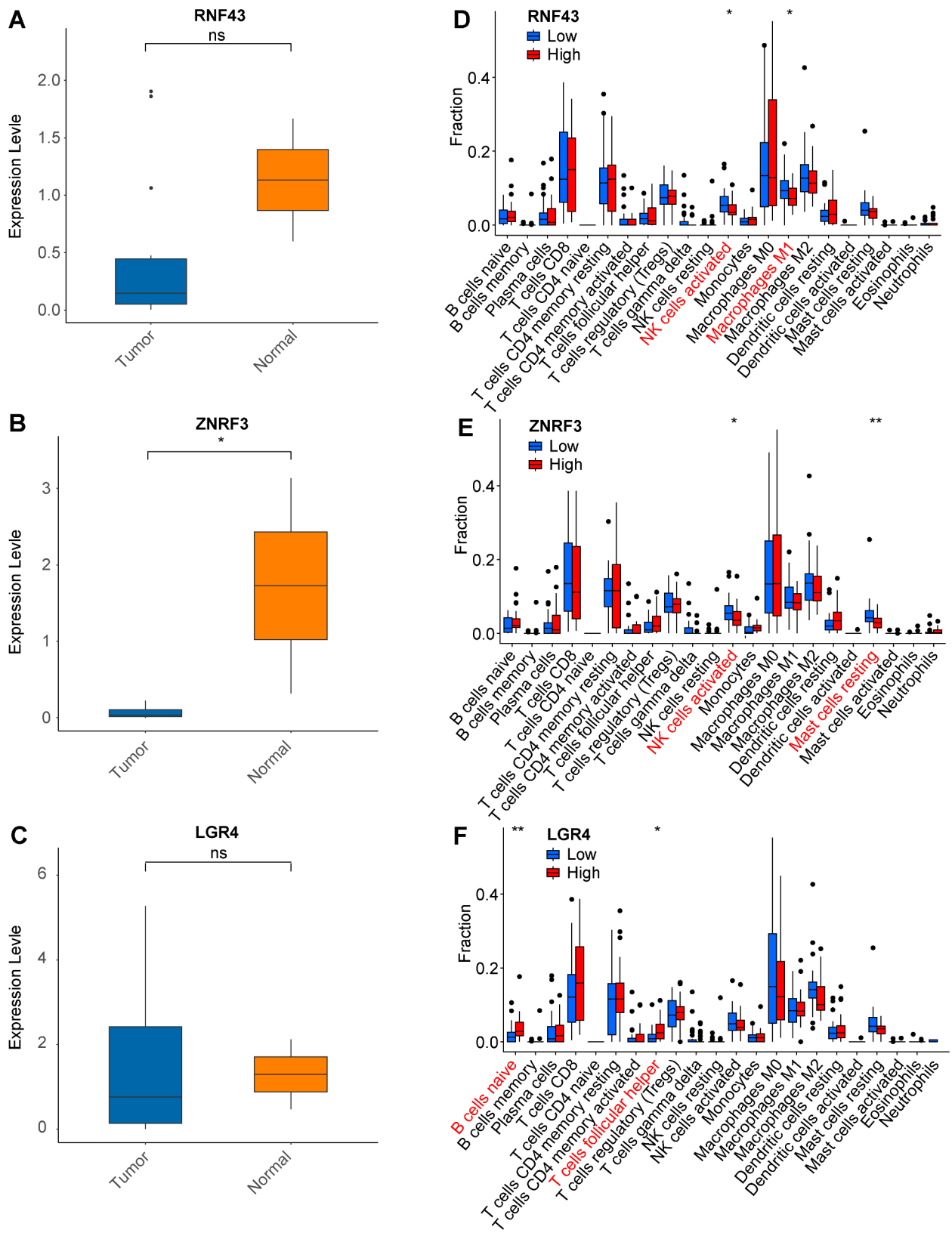

Fig. 5 Comparison of the expression levels and immune microenvironments of common target genes. (**A**-**C**) Box plot of RT–qPCR expression levels of target genes between posttreatment HCC tumor and normal samples via the Wilcoxon rank-sum test for comparison. (**D**-**F**) Comparison of immune cell infiltration between the high- and low-expression groups of target genes, using the median as the group cutoff. Immune cell subsets with *P-values* < 0.05 are marked in red. **P-value* < 0.05; ***P-value* < 0.01

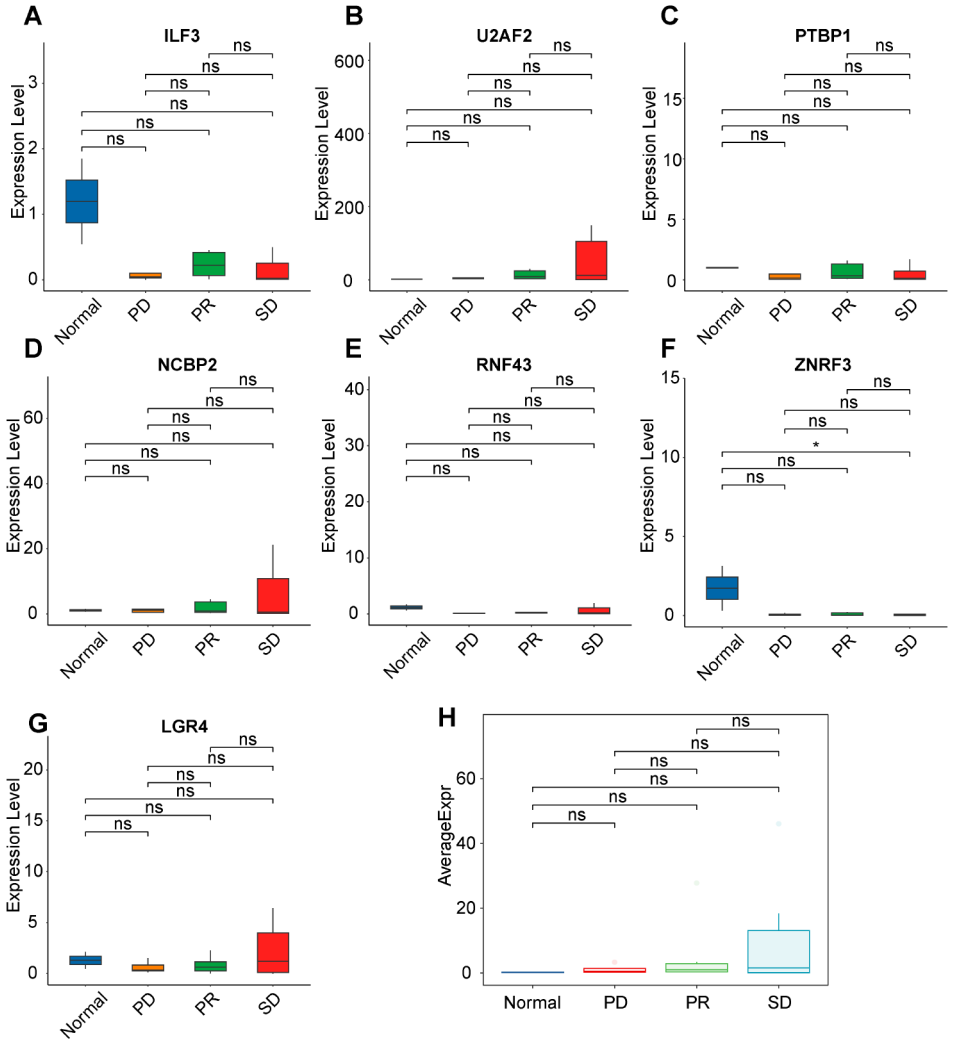

Fig. 6 Comparative analysis of gene expression levels across HCC immunotherapy response groups and normal tissues. (**A**-**G**) Box plot of the expression levels of relevant genes across the normal group and various HCC immunotherapy response groups generated via the Wilcoxon rank-sum test for comparison. (**H**) Box plot of the total expression levels of all relevant genes across the normal group and various HCC immunotherapy response groups generated via the Wilcoxon rank-sum test for comparison. AverageExpr calculates the weighted average expression of seven relevant genes for each sample within different groups. ns, *not significant;* **P-value* < 0.05; PD, progressive disease; PR, partial response; SD, stable disease

[43]. NCBP2 and SSB, which preferentially bind to promoter-TSS regions, likely play significant roles in translational control and mRNA stability [44, 45].

Although our initial screening of core HPARBPs was based on data from public databases via bioinformatics, statistics, and topology methods, the four specific core HPARBPs we ultimately focused on have been reported to have important clinical significance in HCC. ILF3, PTBP1, U2AF2, and NCBP2 are significantly upregulated in HCC and contribute to tumor progression through distinct molecular mechanisms involving nuclear RNA processing and gene posttranscriptional regulation, which affect the proliferation, migration, and invasion of tumor cells, with the expression levels of some of these genes closely related to patient prognosis [46–53]. These proteins reportedly interact with downstream target RNAs and play a role in tumorigenesis and tumor development. Specifically, ILF3 primarily acts in the nucleus by modulating the mRNAs of specific genes, such as CCNE1, IRF3, and IRF9, influencing the cell cycle and immune response [54]. PTBP1 binds to the precursor mRNAs of AXL and NUMB, leading to splicing

variations that produce aberrant transcripts, thereby promoting tumor migration and invasion [50, 55, 56]. Concurrently, PTBP1 and U2AF2 competitively interact to regulate AXL splicing, further impacting tumor development [55]. As a splicing factor, U2AF2 is overexpressed in HCC and enhances the stability of EXO1 mRNA through interaction with the lncRNA CECR7, facilitating tumor progression [57]. These findings not only reveal the complexity of multiple crucial gene expression regulatory networks within HCC but also validate the rationality of our screening methodology and highlight the significant role of these four specific core HPARBPs. However, our findings indicate that the majority of these aberrant alterations in target RNAs are not directly manifested at the transcriptional level.

According to our results, the four specific core HPARBPs mentioned above may collectively bind and regulate the RNAs of LGR4, ZNRF3, and RNF43 within the RSPO-LGR4/5-ZNRF3/RNF43 module, which in turn exerts profound effects on downstream Wnt signaling pathways. In the mature healthy liver, the Wnt pathway is predominantly inactive. However, during processes of cell renewal and regeneration, as well as in certain pathological conditions, diseases, precancerous states, and cancers, the Wnt pathway can be reactivated [58]. The Wnt/β-catenin pathway governs numerous cellular processes involved in the initiation, proliferation, survival, migration, differentiation, and apoptosis of HCC [59]. The Wnt/β-catenin pathway is activated in up to 50% of HCC cases in the liver [60, 61]. The activated Wnt pathway collaborates with multiple signaling cascades to drive the formation and development of HCC and exerts its effects through downstream effector molecules [62].

RNF43 and ZNRF3, two negative feedback regulators of the Wnt pathway, function as E3 ubiquitin ligases that specifically target Frizzled (FZD) receptors for rapid endolysosomal degradation. Conversely, R-spondins (RSPO), ligands of LGR4-5-6 receptors, interact with ZNRF3/RNF43 to counteract FZD membrane clearance mediated by these proteins, thereby increasing both the strength and duration of Wnt signaling [63–67]. LGR4 and RNF43 have been reported to be overexpressed in HCC tissues [68, 69], but the changes in ZNRF3 expression remain unclear. ZNRF3 expression was found to be decreased in liver cancer tissues and HepG2 cells, which contradicts the results reported in the TCGA database [70]. In vitro experiments revealed that LGR4 can act as an oncogene in HCC and that its expression level is positively correlated with the tumor size, microvascular invasion, TNM stage and pathological differentiation grade of HCC patients [69, 71]. As Wnt pathway-related genes, ZNRF3 and RNF43 have been reported to be mutated in HCC, and patients with ZNRF3 mutations have been reported to have a poorer prognosis [63, 72–74]. ZNRF3 and RNF43 predispose people to liver cancer by controlling the proliferative, differentiation and lipid metabolic states of hepatocytes [63].

The upregulation of RNF43 expression appears to contradict the activation of the Wnt pathway in HCC, which may be the result of the combined activation of other bypass pathways. The influence of aberrant HPARBP expression on downstream targets in HCC remains largely unexplored. Given that the regulatory impact of HPARBPs is mostly absent at the transcriptional level of their targets, we propose that HPARBP abnormalities could lead to inactivated, unstable, or oncogenic products in the RSPO-LGR4/5-ZNRF3/RNF43 module and other carcinogenic pathways, potentially hindering their inherent suppression of the Wnt pathway. Perturbations within the complex Wnt upstream network could trigger compensatory mechanisms, resulting in abnormal activation of the Wnt pathway [75]. Furthermore, epigenetic alterations and crosstalk with other signaling pathways are also conjectured to substantially contribute to this activation process [76, 77]. Our hypothesis provides new insights into the tumor progression and regulatory mechanisms of HCC and offers a potential research direction for those who are interested in the interaction between RBP groups and downstream target groups in the tumor environment. However, the mechanism by which RBPs control the RSPO-LGR4/5-ZNRF3/RNF43 module to affect the Wnt pathway still requires further validation in in vivo and in vitro models.

Additionally, some RBPs have been reported to significantly influence antitumor immunity by modulating immune cell activity or infiltration within the tumor microenvironment, ultimately affecting tumor progression [78–83]. Therefore, we explored the impact of specific core HPARBPs on the immune microenvironment. The altered expression of the four HPARBPs has a similar effect on specific immune cell infiltration patterns, suggesting that they may synergistically regulate downstream oncogenes or tumor suppressor genes through common pathways or mechanisms, ultimately affecting the tumor microenvironment. Notably, their elevated expression in HCC could impair γδ T-cell

functionality or hinder γδ T-cell production pathways. γδ T cells, which exhibit a distinctive capacity to recognize stress-associated antigens on tumor cells without major histocompatibility complex restriction, present a promising avenue for hematological and solid tumor immunotherapy. These cells circumvent immune evasion, mitigate rejection, and function as potent antigen-presenting cells (APCs), thereby increasing the prospects of cancer therapy [84]. In the context of HCC, γδ T cells contribute to the antitumor response and are linked to a favorable prognosis. However, within the tumor microenvironment, these cells may acquire protumorigenic attributes [85]. Our research sheds new light on the potential RBP regulators of γδ T cells in HCC and related immunotherapeutic strategies. Nonetheless, whether the observed alterations in γδ T cells correlate with the upstream modulation of the Wnt pathway by HPARBPs, specifically through the RSPO-LGR4/5-ZNRF3/RNF43 module, warrants additional investigation.

The expression of the HPARBP targets, ZNRF3 and RNF43 in HCC is inversely related to the infiltration of activated NK cells. Acting as E3-ubiquitin ligases, these proteins target the cytoplasmic loops of FZD receptors for ubiquitination, leading to their accelerated endolysosomal degradation [63]. This synergistic role aligns with their consistent immune microenvironment attributes in our study. Furthermore, LGR4, ZNRF3, and RNF43 each exhibited positive correlations with naive B cells, indicating a potential regulatory relationship between the RSPO-LGR4/5-ZNRF3/RNF43 axis and naive B cells. However, the immune microenvironment properties of these four HPARBPs and their downstream targets are not uniformly matched, supporting our prior findings that HPARBP-mediated modulation of target RNAs may not directly manifest at the transcriptional levels of those targets. Notably, the results of the immune infiltration analysis need to be validated in HCC tissue samples using experiments such as tissue staining to verify the key genes and associated immune markers, which is what we will be working on in the future.

As mentioned earlier, the specific core HPARBPs and their downstream target genes, with the exception of ZNRF3, exhibit widespread upregulation in HCC. However, in recurrent HCC tissues following postoperative adjuvant therapy, their expression levels were not significantly different from those in normal tissues. This finding suggests that the current clinical approach may intervene in the expression of these genes from overexpression to normal levels and highlights the importance of our research subjects for further investigation into the clinical therapy of HCC. The expression of ZNRF3 remained lower than that in normal tissues both before and after recurrence, and it continued to be downregulated in the SD group following immunotherapy for recurrent patients. These findings suggest that the expression level of ZNRF3 may serve as a potential biomarker to predict the efficacy of adjuvant therapy after hepatectomy, recurrence, and the response to immunotherapy postrecurrence in HCC patients. In addition to ZNRF3, the expression levels of other related genes were not significantly different among the various immune response groups or between the treatment groups and the control group. This finding indicates that the predictive value of these related genes is not as anticipated and that they may serve only as prognostic indicators. In our exploration, it was not possible to entirely exclude the influence of factors such as limited sample size, individual variability in response to immunotherapy, and tumor genetic expression heterogeneity on determining whether these genes can serve as predictive markers for the efficacy of immunotherapy [86, 87]. Nonetheless, clues about the close relationships between these genes and tumor immunotherapy have been discovered, particularly regarding therapeutic outcomes [88–91]. Therefore, additional studies employing larger sample sizes, particularly when combined with HCC tumor samples prior to any treatment, are necessary to validate and update these preliminary findings. In addition, we will focus more on protein expression levels rather than transcriptional expression levels when we study the relationship between the expression levels of these key genes and clinical outcomes in the future.

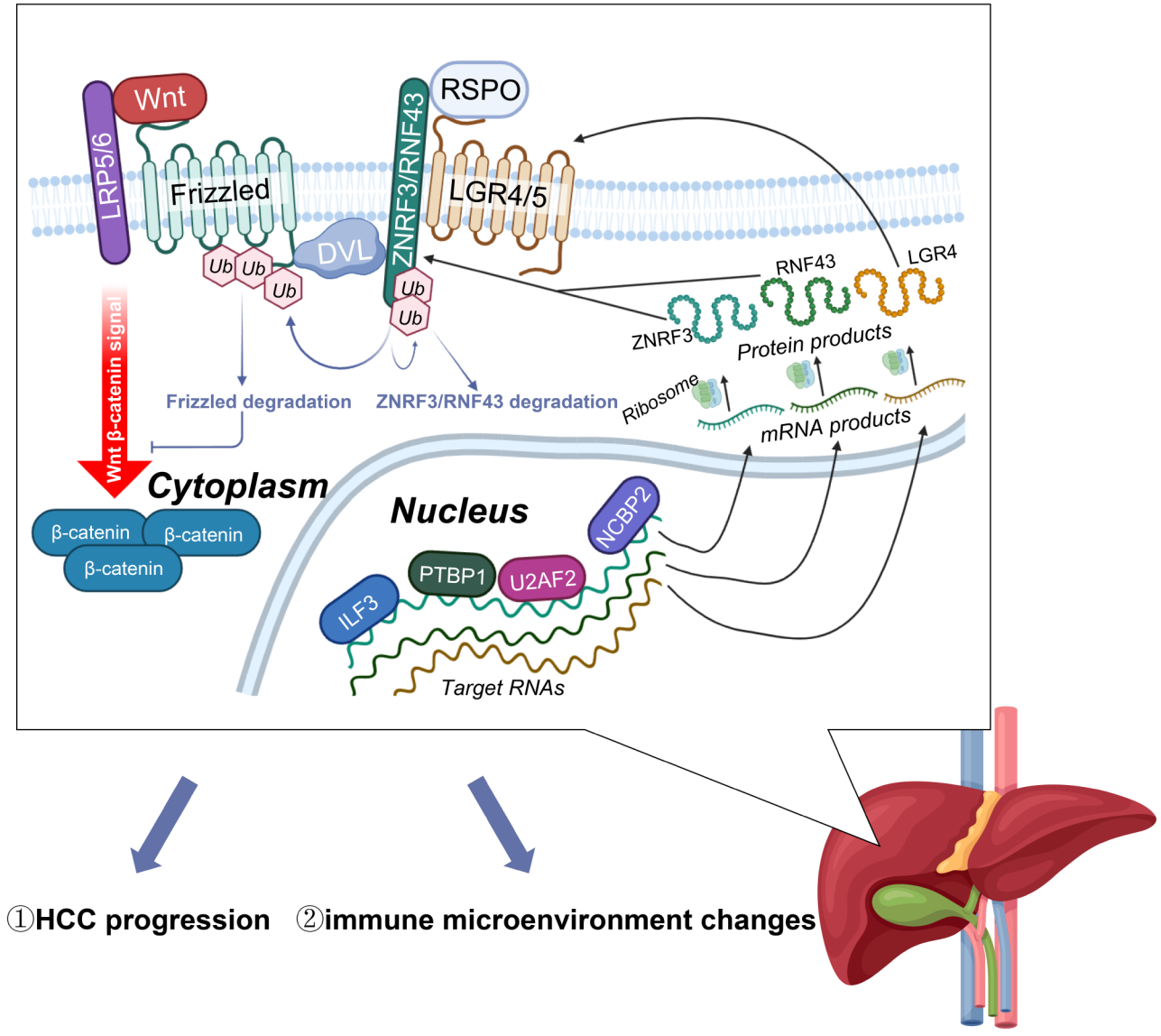

Fig. 7 A schematic representation of the underlying molecular mechanisms of the specific HPARBPs and their common binding targets regulating the Wnt signaling pathway. Under normal physiological conditions, Wnt proteins interact with FZD and LRP5/6 to initiate Wnt/β-catenin signaling. Acting as negative regulators, ZNRF3 and RNF43 impede Wnt/β-catenin signaling by promoting the ubiquitination and subsequent degradation of the Wnt receptors FZD and LRP6, thereby deactivating Wnt/β-catenin signaling. RSPO interacts with LGR4/5 and ZNRF3/RNF43, triggering their ubiquitination and degradation. In the HCC state, four highly expressed specific core HPARBPs bind to downstream target RNAs, leading to the generation of abnormal mRNA and protein products. This aberrant process results in the abnormal degradation of FZD and ZNRF3/RNF43, ultimately causing the failure of Wnt signaling shutdown. Sustained Wnt signaling furthers tumor progression and, when combined with other unidentified factors, ultimately influences the tumor immune microenvironment

Conclusions

In summary, we identified core HPARBPs that might contribute to HCC progression by binding to RNAs in the RSPO-LGR4/5-ZNRF3/RNF43 module (Fig. 7). Changes in the expression of HPARBPs affect the HCC immune microenvironment. Our findings offer novel insights into the regulatory network of Wnt pathway-related RBPs and their potential clinical value in HCC.

Abbreviations

AFP	Alpha fetoprotein
APCs	Antigen presenting cells
AXL	AXL Receptor tyrosine kinase
BMI	Body mass index
CECR7	Cat eye syndrome chromosome region candidate 7
CCNE1	Cyclin E1
DERBPs	Differentially expressed RNA-binding proteins
ENCODE	Encyclopedia of DNA Elements
eCLIP	Enhanced cross-linking immunoprecipitation
EXO1	Exonuclease 1
FDR	False discovery rate

FZD	Frizzled
GEO	Gene Expression Omnibus
GO	Gene Ontology
GCH1	GTP cyclohydrolase 1
HCC	Hepatocellular carcinoma
HPARBPs	Hepatocellular carcinoma progression-associated RNA-binding proteins
HITS-CLIP	High-throughput sequencing of RNA isolated by cross-linking immunoprecipitation
HuR	Human antigen R
IGV	Integrative Genomics Viewer
IRF3	Interferon regulatory factor 3
IRF9	Interferon regulatory factor 9
ILF3	Interleukin enhancer-binding factor 3
KEGG	Kyoto Encyclopedia of Genes and Genomes
LGR4	Leucine-rich repeat-containing G protein–coupled receptor
LCI	Liver Cancer Institute
LIHC	Liver hepatocellular carcinoma
MAT2A	Methionine adenosyltransferase 2 A
NK	Natural killer
NELFE	Negative elongation factor complex member E
NCBP2	Nuclear cap binding protein subunit 2
NF90	Nuclear factor 90
NUMB	NUMB endocytic adaptor protein
PR	Partial response
PTBP1	Polypyrimidine tract binding protein 1
PD	Progressive disease
PPI	Protein–protein interaction
qRT–PCR	Quantitative real-time PCR
RECIST	Response evaluation criteria in solid tumors
RPS3	Ribosomal protein S3
RNF43	Ring finger protein 43
RBPs	RNA-binding proteins
RSPO	R-spondins
SRSF2	Serine- and arginine-rich splicing factor 2
SSB	Single-stranded DNA binding protein
SD	Stable disease
TERT	Telomerase reverse transcriptase
TCGA	The Cancer Genome Atlas
TPM	Transcript per million
TP53	Tumor protein P53
U2AF2	U2 small nuclear RNA auxiliary factor 2
ZNRF3	Zinc and ring finger protein 3
CTNNB1	β-catenin

Supplementary information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-025-13874-x.

Additional File 1. Table S1. Primers used for qRT-PCR experiments in this study

Additional File 2. Table S2. Relevant results during the screening process of HPARBPs, related to Figure 1. DERBPs and HPARBPs in the TCGA and LCI cohort are listed in this file

Additional File 3. Table S3. The binding target gene lists for the core HPARBPs, related to Figure 2. The target genes of each core HPARBPs arelisted and gathered in this file

Addtional File 4. Figure S4. Relationship of core HPARBPs expression and immune cell subtypes in HCC patients. (**A**-**G**) The number on the abscissa represents the correlation coefficient based on Spearman's correlation analysis, and the number on the ordinate represents the P-value, where P-values < 0.05 are marked in red. They all share the legend in graph G

Additional File 5. Figure S5. Comparison of immunotherapy effectiveness of HPARBPs. (A-D) Box plot of RT-qPCR expression level of HPARBPs between post-treatment HCC tumor and normal samples using Wilcoxon rank-sum test for comparison

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Author contributions
XZY, DZY, LZY, LSY, LJJ and SJ designed the research, interpreted the data, and revised the paper. XZY, DZY and LJJ performed the data analysis. LZY, CYQ, HST and LSY collected the tissue specimen and performed validation experiments. XZY drafted the paper. All of the authors approved the submitted and final versions.

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Data availability
In this study, publicly available data sets and online tools were utilized as described in the main text. The specific details are as follows: The Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov/), Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo), the Encyclopedia of DNA Elements (ENCODE) portal (https://www.encodeproject.org/), STRING website (https://string-db.org/), Metascape website (https://metascape.org) and SRplot (https://www.bioinformatics.com.cn/en). The analyzed result data are provided in the SUPPLEMENTARY INFORMATION section.

Declarations

Ethical approval
This study was approved by the Ethics Committee of Nanjing Drum Tower Hospital, Affiliated Hospital of Medical School, Nanjing University, with ethical approval number (2023-646-02).

Human ethics declaration
The study was conducted according to the guidelines laid down in the Declaration of Helsinki.

Consent for publication
The patient's written informed consent was obtained for publication.

Competing interests
The authors declare no competing interests.

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