Amplification of heterogeneous nuclear ribonucleoprotein A/B aids in immune infiltration regulation and breast cancer tumorigenesis

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Abstract. Breast carcinoma ranks among the most prevalent malignant tumors affecting women worldwide. The discovery of biomarkers is important in the diagnosis of breast cancer and the prediction of clinical outcomes in afflicted patients. Heterogeneous nuclear ribonucleoprotein (HNRNP)-AB belongs to the extensive HNRNP superfamily; however, its exact role in the progression of breast carcinoma has not yet been fully clarified. The present study drew upon breast cancer sample datasets retrieved from The Cancer Genome Atlas and Human Protein Atlas databases to examine the expression patterns and prognosis-associated data of HNRNPAB in clinical specimens. Reverse transcription-quantitative PCR was employed to validate the efficiency of HNRNPAB knockdown in breast cancer cell lines. Transwell assays and Cell Counting Kit-8 tests further demonstrated alterations in migratory, invasive and proliferative capacities following small interfering RNA-mediated HNRNPAB knockdown. Bioinformatics was utilized to investigate numerous aspects of HNRNPAB in cancerous tissues, including its mRNA expression profile, prognostic value, signaling pathway remodeling, interaction with cancer stem cells and regulatory effects on the tumor immune microenvironment, as well as immunotherapeutic responses. Compared with normal breast tissues, HNRNPAB exhibited elevated expression in breast cancer tissues, upregulation that appeared to be associated with overall survival outcomes, sex characteristics, lymph node metastasis staging and adverse prognostic profiles in patients. Functionally, HNRNPAB depletion was found to suppress the proliferative activity, migratory potential and invasive capabilities of breast cancer cells. Mechanistically, bioinformatics analyses indicated that high HNRNPAB expression was associated with the tumor immune infiltration status in affected individuals. In summary, the present study determined that HNRNPAB exerted a key regulatory effect on the development and progression of breast cancer and therefore its upregulation holds promise as a diagnostic and prognostic marker. In addition, HNRNPAB serves a notable role in cancer immunotherapy, supporting its potential as a novel therapeutic candidate for the early detection and prognostic evaluation of breast cancer.

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Introduction

Breast carcinoma stands as a prevalent malignancy impacting women across the globe (1). Every year, there are 2.3 million new cases and 685,000 deaths. North America, Europe and Australia have the highest incidence rate, while Asia and parts of Africa have lower incidence rates (2). It is worth noting that although relatively rare, BC can also affect men. This malignant tumor typically presents as a lump or lump in the breast, often accompanied by changes in shape, size or skin texture (3). The intricacy of molecular mechanisms that govern tumorigenesis and progression accounts for the heterogeneity inherent in breast cancer. From a molecular standpoint, such variability creates challenges regarding the choice of therapeutic strategies and the disease prognosis (4). The genomic landscapes of HR^+, $HER2^+$ and triple-negative cancer cells, as well as the tumor immune microenvironment, often lead to different immune infiltrations and functions (5). Immune cells play a crucial role in the microenvironment of breast cancer, affecting the growth, metastasis and response to the treatment of tumors. The in-depth study of the interaction between immune cells and breast cancer reveals new therapeutic targets, opening up broad possibilities for developing new therapeutic strategies (6,7). The ultimate goal of tumor immunotherapy with specific targets is to trigger antitumor immune responses, leading to clinical regression and/or recurrence of tumors. In Bergman Phase I to III trials, there are many types of specific tumor immunotherapies involving a wide range of tumor types. Due to slow progress, the response to cancer vaccines and other cancer immunotherapies may take several months or longer to emerge (8). Although immune-targeted therapies have achieved early successes in cancers such as melanoma

and lung cancer, the progress of immune-targeted treatment for breast cancer has been relatively slow (9). Therefore, it is necessary to explore the immune infiltration-associated molecular mechanisms of the progression of breast cancer to improve the survival rate of patients with breast cancer.

Heterogeneous nuclear RNAs are the primary transcripts of RNA polymerase II in eukaryotes (10). Heterogeneous nuclear ribonucleoproteins (HNRNPs) are the most abundant nuclear proteins in higher eukaryotes and are a class of commonly recognized RNA-binding proteins (11). HNRNPs play important roles in key cellular processes such as transcription, post-transcriptional modifications and translation. The dysregulation of HNRNPs is a key factor in cancer development and drug resistance. HNRNPs promote the diversity of tumor and immune-related abnormal proteomes by controlling alternative splicing and translation; they can also promote the expression of cancer-related genes by regulating transcription factors, directly binding to DNA or promoting chromatin remodeling (12). The HNRNPAB subfamily is the core member of HNRNPs. In recent years, the biological value of HNRNPAB has been discussed to some extent (13). The biogenesis mechanism of HNRNPAB, which penetrates into all aspects of cellular RNA metabolism, involves DNA binding, RNA splicing and transport and translation and stability of mRNA (14). The expression of HNRNPAB changes depending on cancer type, suggesting its role in tumorigenesis (15-17). However, the role and immune mechanism of HNRNPAB in breast cancer are still unclear. Thus, the present study aimed to investigate the role of HNRNPAB in breast cancer progression. Publicly accessible databases and cellular assays were employed to assess the prognostic and predictive importance of HNRNPAB expression, with a concurrent investigation into its potential as a diagnostic biomarker and therapeutic candidate for breast cancer.

Materials and methods

Cell culture protocol. MDA-MB-231, the breast cancer cell line, was procured from Wuhan Servicebio Technology Co., Ltd. These cells were maintained in DMEM medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. The cell cultures were incubated in a humidified 5% CO_2 environment at a constant temperature of 37˚C.

HNRNPAB gene knockdown. A 6-well plate was prepared by plating $5x10^5$ MDA-MB-231 breast cancer cells per well, followed by a 24 h stabilization period in the incubator. The siRNA sequences used in the present study were as follows: HNRNPAB-siRNA sense strand: 5'-GGAGAGGUCGUU GACUGUAdTdT-3', antisense strand: 5'-UACAGUCAA CGACCUCUCCdAdA-3'; negative control (NC)-siRNA sense strand: 5'-UUCUCCGAACGUGUCACGUdTdT-3', antisense strand: 5'-ACGUGACACGUUCGGAGAAdTdT-3'. Subsequently, NC-siRNA and HNRNPAB-siRNA (50 nm) (Tianjin Sheweisi Biotechnology Co., Ltd.) were individually introduced into the MDA-MB-231 cells at 37˚C for 24-72 h using Lipofectamine™ 3000 reagent (Thermo Fisher Scientific, Inc.) in strict accordance with the manufacturer's guidelines. At 8 h post-transfection, the transfection medium was replaced with fresh DMEM containing 10% FBS. The efficiency of siRNA transfection was quantified and the results are presented with corresponding SD values.

Reverse transcription-quantitative PCR (RT-qPCR). For this assay, MDA-MB-231 cells following siRNA transfection were selected. The culture medium was aspirated and lysis buffer (Wuhan Servicebio Technology Co., Ltd.) was added to the cells. Total RNA was isolated using a commercial RNA extraction kit (Tiangen Biotech Co., Ltd.). After quantifying the RNA concentration, reverse transcription into cDNA was performed using the ABScript II RT Master Mix for qPCR with gDNA Remover (ABclonal Biotech Co., Ltd.) as per the manufacturers' instructions. The synthesized cDNA was stored long-term at -80˚C. Fluorescence qPCR was conducted on a LightCycler480 real-time fluorescence quantitative PCR instrument (Roche Diagnostics GmbH), with cDNA loaded according to the protocol of the Genious 2X SYBR Green Fast qPCR Mix kit (ABclonal Biotech Co., Ltd.). The standardized thermal cycling conditions were set as follows: Initial pre-denaturation at 95˚C for 3 min; followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 20 sec and extension at 72˚C for 20 sec. Melting curve analysis was subsequently performed from 65˚C to 95˚C to verify the specificity of amplification products. The primer sequences used were as follows: HNRNPAB forward, 5'-TTTGGCGAG TTTGGGGAGATT-3' and reverse, 5'-GCCATACTGCTG CTGATAGAC-3'; GAPDH forward, 5'-GGAGCGAGATCC CTCCAAAAT-3' and reverse, 5'-GGCTGTTGTCATACT TCTCATGG-3'. GAPDH served as the internal reference gene and the $2^{-\Delta\Delta Cq}$ method was applied to calculate the relative mRNA expression levels (18). All experimental reactions were conducted in triplicate to ensure reproducibility.

Cell Counting Kit-8 (CCK-8) assay. Logarithmic-phase MDA-MB-231 cells transfected with either siRNA or NC-siRNA were digested with trypsin for 2 min and then seeded into a 96-well plate at a density of $8x10^4$ cells per ml. After incubation for 24, 48 and 72 h in a 5% CO_2 incubator at 37˚C, the 96-well plate was removed. The culture medium was discarded and a mixture of DMEM and CCK-8 solution (Jiangsu Kaiji Biotechnology Co., Ltd.) at a 9:1 ratio was added to each well. After 1 h, the optical density value of the cells was measured at a wavelength of 450 nm. The cell proliferation rate was calculated, with the blank knockdown group designated as the control.

Cell migration and invasion assay (Transwell chambers). Firstly, the upper chamber of a Transwell insert (Corning, Inc.) was coated at 37˚C for 2 h with 100 μl 10% Matrigel (BD Biosciences) diluted in serum-free DMEM, followed by a 2 h incubation in a cell culture incubator. Cells were then seeded into the upper chamber of the 8 μm pore-sized Transwell at a density of $5x10^4$ cells per well. The lower chamber was filled with 750 μl culture medium containing 10% FBS and the cells were incubated for 48 h at 37˚C in a 5% CO_2 environment. After incubation, the medium in the upper chamber was discarded. Cells remaining in the chamber were fixed at room temperature with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet at room temperature for 15 min. The number of cells was counted in three randomly

selected fields of view per chamber using an inverted light microscope.

Public database data acquisition. HNRNPAB expression levels in BRCA tissues from The Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov/) and Genotype-Tissue Expression (GTEx) databases were retrieved through the Gene Expression Profiling Interactive Analysis 2 platform (19-21). TCGA pan-cancer (PANCAN) dataset was acquired from the UCSC Xena browser (https://xenabrowser.net/), which offers a standardized and comprehensive compilation of pan-cancer datasets. Immunohistochemistry data for HNRNPAB was obtained from The Human Protein Atlas (HPA) database (https://www.proteinatlas) (22) which characterizes the expression pattern of HNRNPAB protein in both BRCA tissues and normal breast tissues. Prognostic survival analysis of BRCA sample data was conducted using the Kaplan-Meier Plotter online tool (http://kmplot.com/analysis/) (23) to explore the association between HNRNPAB and the prognosis of patients with BRCA. The cBioportal (https://www.cbioportal.org) (24) and TCGA databases were utilized to investigate HNRNPAB-associated gene alterations in patients with breast cancer. Immunotherapy outcomes and immune cell infiltration profiles of patients with breast cancer were collected from The Cancer Immunome Database (TCIA; https://tcia.at) and the influence of HNRNPAB on immune checkpoint inhibitor efficacy was predicted using the Immune Phenotype Score (IPS) (25). In addition, the StromalScore, ImmuneScore and ESTIMATEScore for BRCA samples in TCGA database was computed using the 'estimate' package in R software (26) and the immune cell types associated with HNRNPAB expression levels were summarized.

Gene Set Enrichment Analysis (GSEA). To further characterize differentially expressed genes and elucidate the functions of potential target genes, gene function enrichment analyses were performed using HALLMARK (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp), REACTOME (https://reactome.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.kegg.jp/) databases. The 'ClusterProfiler' package (version 3.18.0) in R software was employed to analyze pathway enrichment, thereby enhancing the understanding of mRNA-associated carcinogenic mechanisms (27-29). Gene matrix transposed files were downloaded from the Molecular Signatures Database (https://www.gsea-msigdb.org/gsea/msigdb/) and GSEA was applied to conduct enrichment analyses for HALLMARK, REACTOME and KEGG gene sets (30-32). Genes with $|\log_2$ fold change$| >1$ and adjusted $P<0.05$ were defined as differentially expressed genes, and enriched terms with adjusted $P<0.05$ were considered statistically significant.

Statistical analysis. A total of three independent experiments were performed for each assay (n=3) to guarantee the reliability and reproducibility of the experimental findings. Experimental data are presented as the mean ± SD, which is used to illustrate the central tendency and degree of dispersion of the results. For comparisons among multiple groups, one-way ANOVA was employed and Tukey's post hoc test was subsequently conducted to identify the specific differences between individual groups. The paired Student's t-test was utilized for comparisons between two groups. Pearson's correlation analysis was applied to evaluate the linear correlation between relevant indicators. All statistical analyses were carried out with SPSS (version 26.0; IBM Corp.) software with $P<0.05$ considered to indicate a statistically significant difference.

Results

Association between HNRNPAB mRNA expression and clinicopathological parameters/clinical prognosis in patients with BRCA. To comprehensively characterize HNRNPAB expression patterns across diverse tissues of patients with BRCA and its association with clinicopathological features, the standardized pan-cancer dataset TCGA TARGET GTEx was retrieved (PANCAN; n=19,131; genes=60,499) from the UCSC Xena browser. HNRNPAB was found to be significantly upregulated in 24 tumor types, whereas downregulation was detected in 2 tumor types (Fig. 1A). Differential analysis of normal and tumor tissues from TCGA and GTEx databases further demonstrated that HNRNPAB expression was significantly higher in BRCA tissues compared with normal breast tissues (Fig. 1B). Immunohistochemical data of HNRNPAB from the HPA database demonstrated that at the protein level, HNRNPAB expression was elevated in BRCA tissues compared with normal breast tissues (Fig. 1C and D). Overall survival analysis revealed that high HNRNPAB mRNA expression was closely associated with unfavorable prognosis in patients with BRCA (Fig. 1E). As breast cancer progressed to advanced stages, the median expression level of HNRNPAB exhibited a declining trend (Fig. 1F). The number of lymph node metastases in patients with breast cancer showed a positive association with increased median HNRNPAB expression (Fig. 1G). Patients with BRCA aged 21-40 years displayed a marked increase in median HNRNPAB expression, with elevated levels also observed in other age groups compared with normal tissue (Fig. 1H). HNRNPAB expression was found to be notable higher in invasive ductal carcinoma and medullary carcinoma compared with normal tissues, while a mild elevation was noted in other histological subtypes (Fig. 1I). In addition, Pearson's correlation was calculated between HNRNPAB expression and ploidy across a number of tumors and thus identified a significant positive correlation in BRCA. The expression of HNRNPAB positively correlated with tumor ploidy in breast cancer, suggesting that further investigation is warranted (Fig. 1J).

Function of HNRNPAB in proliferation, migration and invasion of breast cancer cells. Pre-transfection with siRNA targeting HNRNPAB effectively reduced HNRNPAB levels in MDA-MB-231 cells, leading to a significant decrease in intracellular HNRNPAB content (Fig. 2A). Proliferation, migration and invasion assays were performed to compare two groups, namely the MDA-MB-231 siRNA control group and the MDA-MB-231 HNRNPAB-siRNA group. The CCK-8 assay results indicated that knockdown of HNRNPAB expression significantly inhibited the proliferation of breast cancer cells (Fig. 2B). Transwell migration and invasion experiments

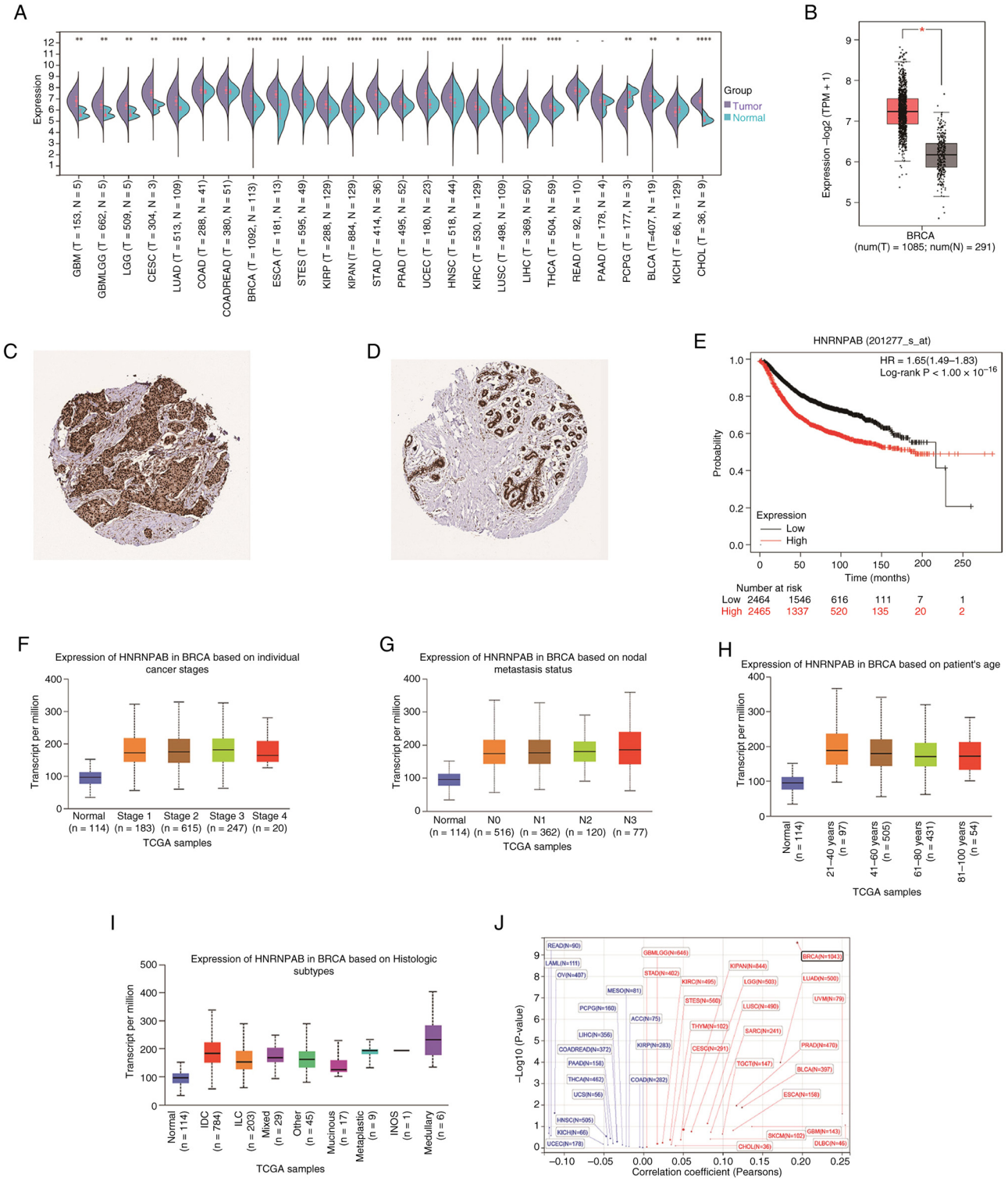

Figure 1. Expression of HNRNPAB and its association with clinical prognosis of patients with breast cancer. (A) Differential expression of HNRNPAB in different cancer types based on pan-cancer analysis. (B) Expression of HNRNPAB in BRCA tissue samples in TCGA database is higher than compared with paracancerous tissues of breast cancer. HNRNPAB immunohistochemistry images obtained from the Human Protein Atlas database show that the expression level of HNRNPAB protein in (C) breast cancer tissues is higher compared with that in (D) paracancerous tissues of breast cancer. (E) The influence of HNRNPAB expression on the prognosis of patients with breast cancer analyzed by the Kaplan-Meier method. Box plots describing the expression analysis of HNRNPAB according to (F) stage, (G) lymph node metastasis, (H) age and (I) breast cancer histology. (J) Scatter plot showing the correlation between HNRNPAB expression and ploidy in various tumors, with red indicating a positive correlation and blue indicating a negative correlation. HNRNPAB, heterogeneous nuclear ribonucleoprotein A/B; TPM, transcripts per million; HR, hazard ratio; TCGA, The Cancer Genome Atlas; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; INOS, infiltrating carcinoma not otherwise specified. *P<0.05, **P<0.01 and ****P<0.0001.

further demonstrated that HNRNPAB promoted a significant decrease in cancer cell migration and invasion (Fig. 2C and D).

Genetic alteration landscape of HNRNPAB. To explore the genetic architecture and transcriptional variations of

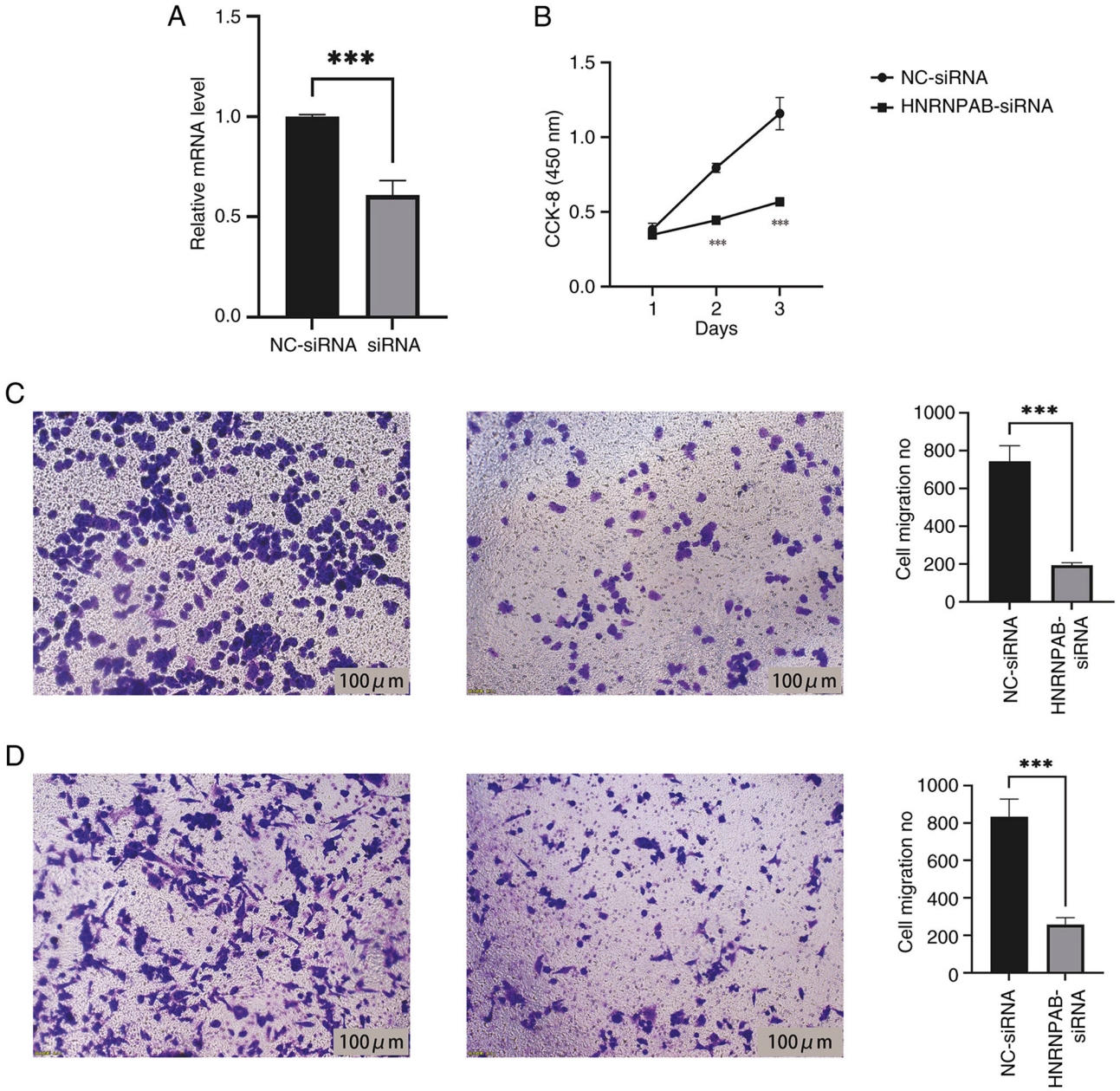

Figure 2. Role of HNRNPAB in breast cancer cells. (A) Reverse transcription-quantitative PCR was used to detect the expression level of mRNA in stably knocked-down cells of HNRNPAB. GAPDH was used as the internal reference. (B) The proliferation capacity of MDA-MB-231 breast cancer cells transfected with HNRNPAB siRNA in CCK8 is lower compared with that of the MDA-MB-231 siRNA blank group. siRNA transfection into MDA-MB-231 breast cancer cells to reduce the expression level of HNRNPAB. Transwell experiment showing that reducing the expression of HNRNPAB can significantly inhibit the (C) migration and (D) invasion ability of cancer cells. ***P<0.001. HNRNPAB, heterogeneous nuclear ribonucleoprotein A/B; siRNA, small interfering RNA; CCK-8, Cell Counting Kit-8; NC, negative control.

HNRNPAB, the cBioportal database was utilized. With regard to genetic alterations of HNRNPAB, 1.3% of patients exhibited modifications, with amplification being the predominant type of alteration in cancer (Fig. 3A). Among varying breast cancer subtypes, invasive ductal carcinoma exhibited the highest frequency of HNRNPAB mutations, followed by invasive lobular carcinoma. By contrast, invasive mucinous breast carcinoma and breast invasive carcinoma (not otherwise specified) displayed minimal HNRNPAB mutation rates (Fig. 3B). Analysis of the breast tumor cohort landscape revealed that distinct HNRNPAB expression levels were associated with specific genetic alterations. The high HNRNPAB expression group exhibited significant differences in the mutation status of PIK3CA, titin (TTN) and tumor protein p53 (TP53), whereas the low expression group exhibited higher mutation frequencies of these three genes. Therefore, mutations in PIK3CA, TP53 and TTN may affect the progression of breast cancer regulated by HNRNPAB (Fig. 3C and D). In the high HNRNPAB expression group, C>G mutations ranked second and C>A mutations ranked third. Conversely, in the low expression group, C>A mutations were the second most common, surpassing C>G mutations (Fig. 3E). Differences were also observed in variation classifications between the two groups: In the high HNRNPAB group, 'Nonstop_Mutation' ranked fifth (surpassing

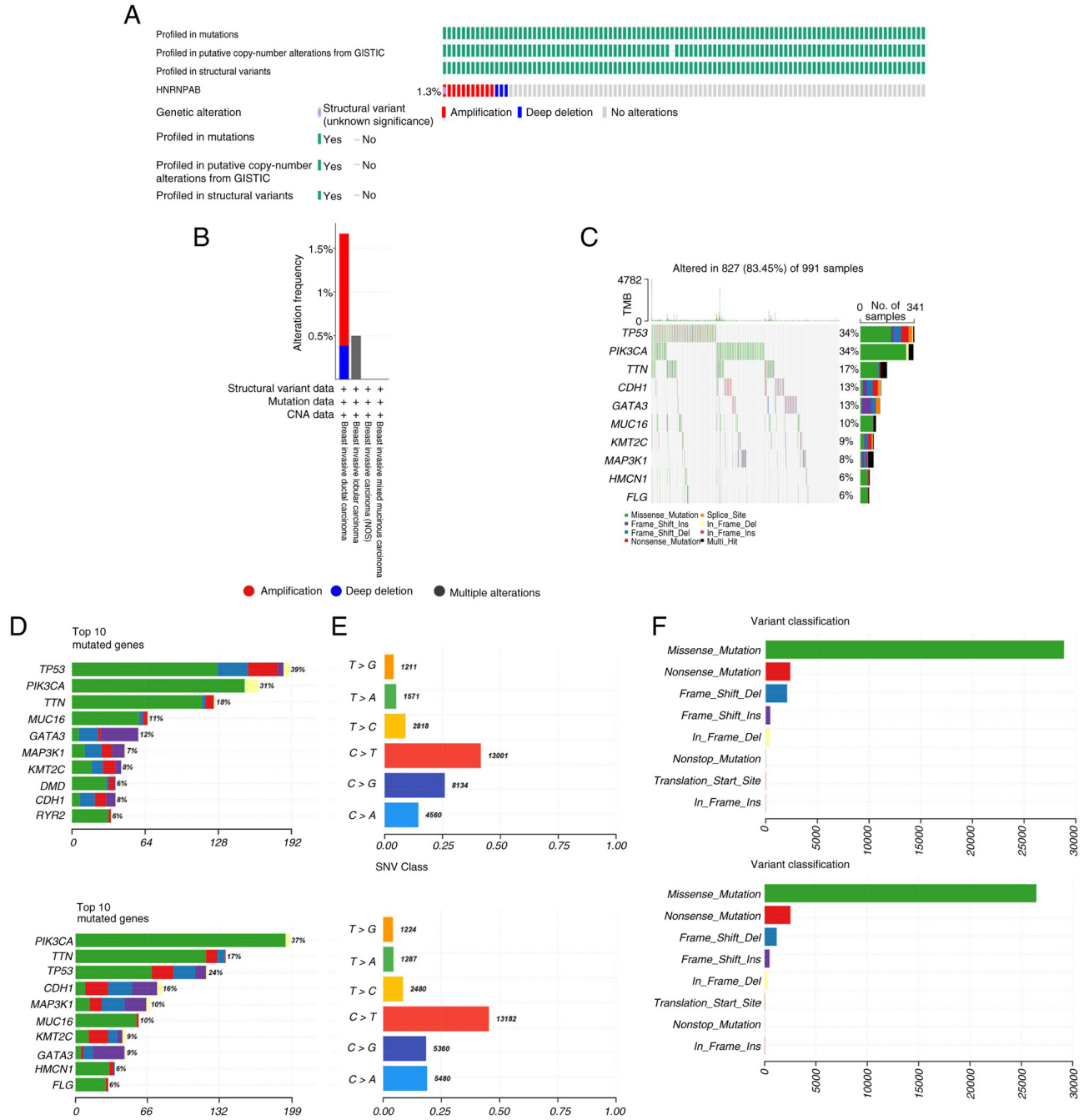

Figure 3. Genetic alteration landscape of HNRNPAB. Schematic diagrams depicting the (A) type and (B) proportion of genetic alterations of HNRNPAB in patients with breast cancer and breast cancer subclasses, using the cBioportal database. (C) Oncoplot showing the somatic landscape of breast tumor cohort. Genes are ordered by their mutation frequencies and samples are ordered by expression of HNRNPAB, as indicated by the annotation bar (bottom). Side bar plot shows the -$\log_{10}$-transformed q-values, as estimated using MutSigCV. Waterfall plot showing mutation information for each gene for each sample. Color annotation of various cancer types are shown at the bottom. The barplot above the legend shows the mutation burden. (D) Stacked bar graph showing the top 10 mutated genes. (E and F) Cohort summary plot showing the distribution of variants according to (E) SNV class and (F) variant classification. HNRNPAB, heterogeneous nuclear ribonucleoprotein A/B; SNV, single nucleotide variant; GISTIC, Genomic Identification of Significant Targets in Cancer; CNA, copy number alteration; NOS, not otherwise specified; TMB, tumor mutational burden.

'Translation_Start_Site' mutations at sixth), while in the low expression group, 'Translation_Start_Site' mutations occupied the fifth position (surpassing 'Nonstop_Mutations' at sixth; Fig. 3F). These discrepancies may be attributed to altered HNRNPAB expression; however, further studies are required to clarify their impact on breast cancer progression, which will enhance the general understanding of HNRNPAB function.

HNRNPAB-associated enrichment analyses. HALLMARK enrichment analysis suggested that HNRNPAB may participate in 'OXIDATIVE_PHOSPHORYLATION', 'G2M_CHECKPOINT', 'PI3K_AKT_MTOR_SIGNALING', 'FATTY_ACID_METABOLISM' and 'WNT_BETA_CATENIN_SIGNALING' (Fig. 4A). KEGG enrichment analysis indicated that HNRNPAB is primarily involved in 'CELL_CYCLE', 'DNA_REPLICATION',

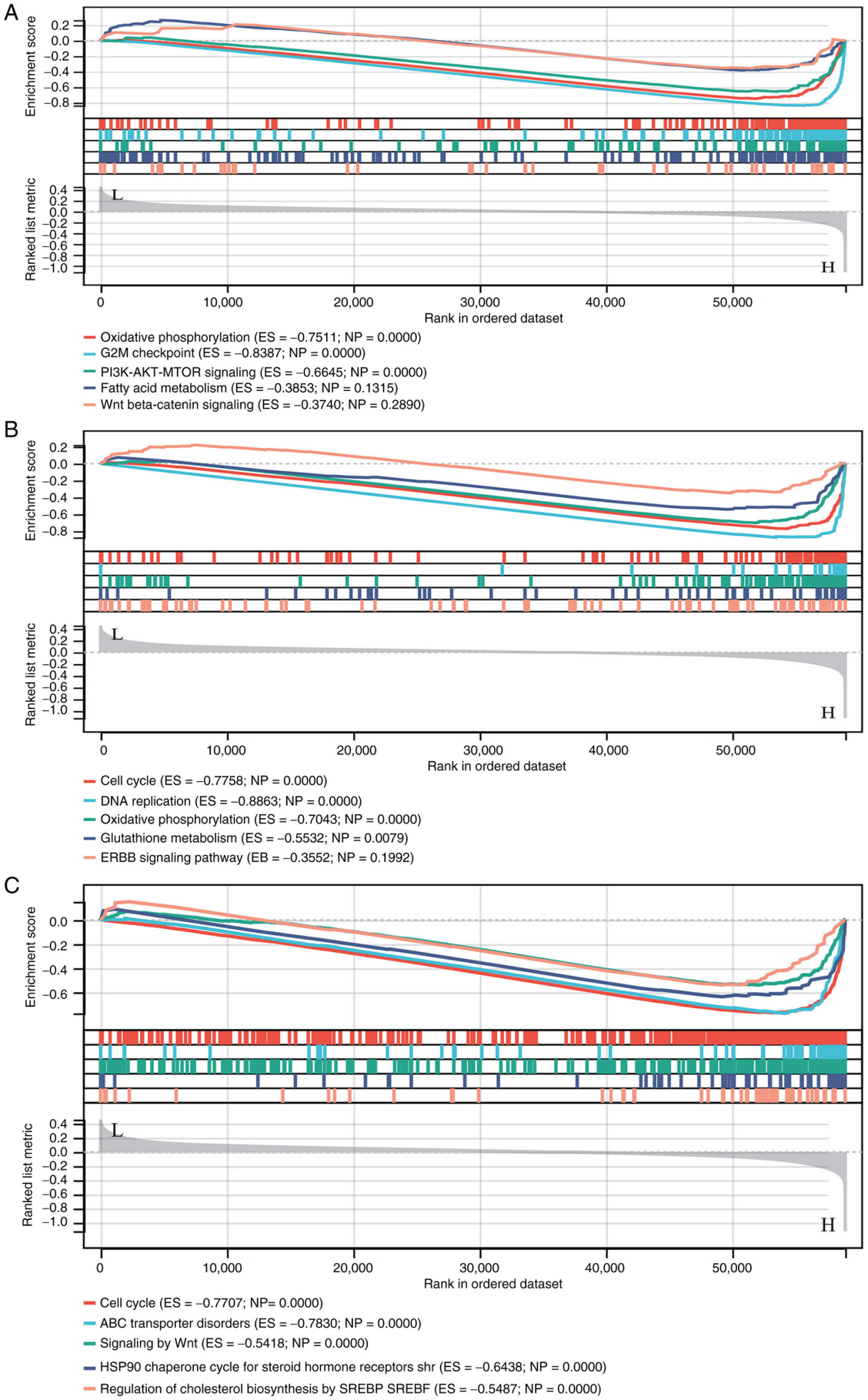

Figure 4. Enrichment analysis of differentially expressed genes in heterogeneous nuclear ribonucleoprotein A/B. (A) GSEA analysis based on HALLMARK analysis. (B) GSEA analysis based on Kyoto Encyclopedia of Genes and Genomes analysis. (C) GSEA analysis based on REACTEMO analysis. GSEA, Gene Set Enrichment Analysis; ES, Enrichment Score; NP, nominal P-value.

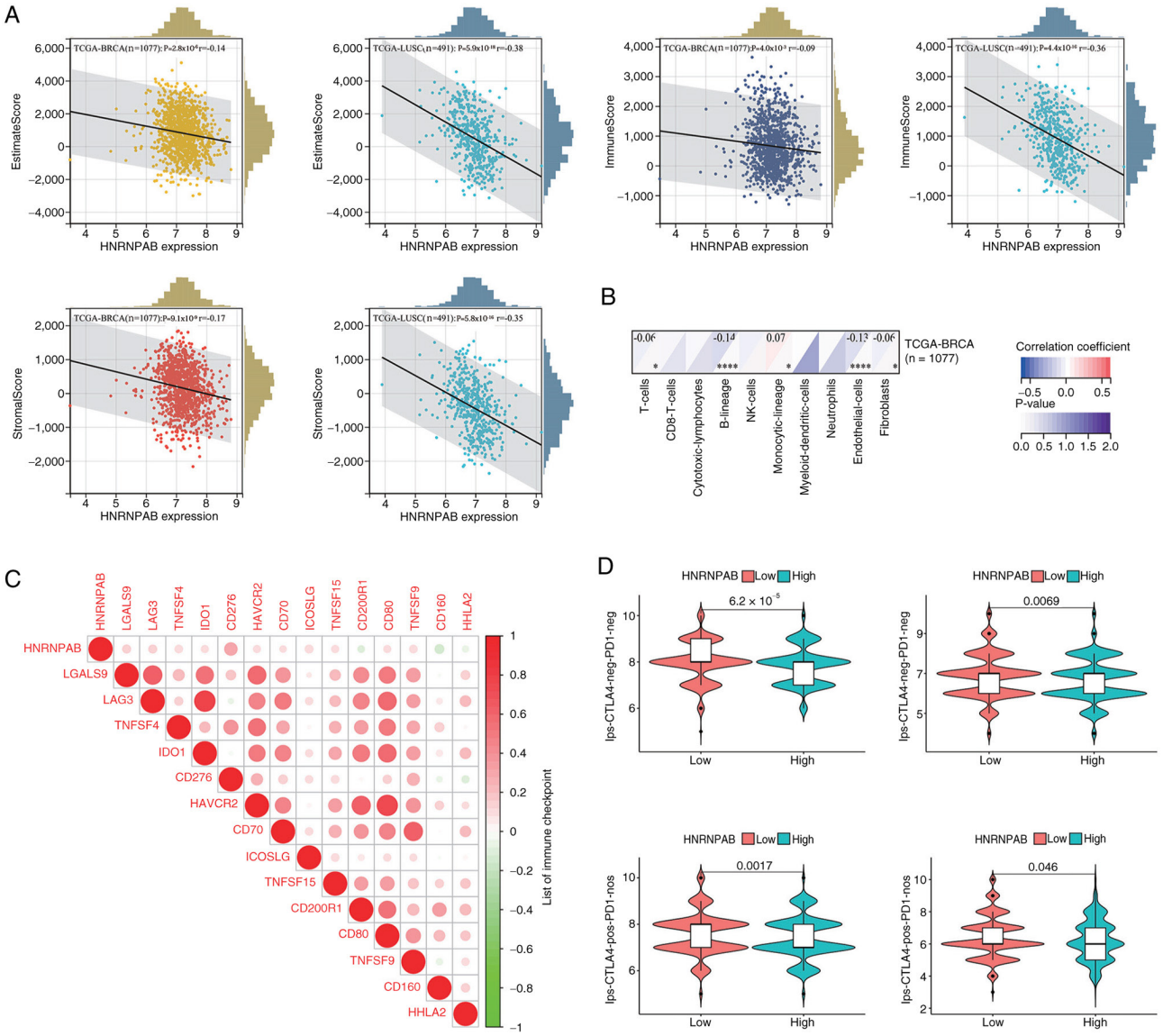

Figure 5. Tumor microenvironment score and immune cell infiltration. (A) The StromalScore, ImmuneScore and ESTIMATEScore of the low expression group are significantly higher compared with the high expression group. (B) Heat map displaying the correlation between HNRNPAB expression and immune cell infiltration in patients with breast cancer. The square above each cell type is divided into two triangles. The color of the top left triangle represents the correlation, where red indicates a positive correlation and blue indicates a negative correlation. The color of the bottom right triangle represents statistical significance, with lighter colors indicating greater statistical significance. (C) In breast cancer, HNRNPAB is shown to be significantly correlated with 11 immune checkpoint genes (blue represents a negative correlation and red represents a positive correlation). (D) The Immune Phenotype Score of the low expression group is higher compared with the high expression group. *P<0.05 and ****P<0.01. HNRNPAB, heterogeneous nuclear ribonucleoprotein A/B; TCGA, The Cancer Genome Atlas.

'OXIDATIVE_PHOSPHORYLATION', 'GLUTATHIONE_METABOLISM' and the 'ERBB_SIGNALING_PATHWAY' (Fig. 4B). REACTOME enrichment analysis results demonstrated that HNRNPAB may be involved in 'CELL_CYCLE', 'ABC_TRANSPORTER_DISORDERS', 'SIGNALING_BY_WNT','HSP90_CHAPERONE_CYCLE_FOR_STEROID_HORMONE_RECEPTORS_SHR' and 'REGULATION_OF_CHOLESTEROL_BIOSYNTHESIS_BY_SREBP_SREBF' (Fig. 4C).

HNRNPAB association with the tumor microenvironment and tumor immunotherapy. ESTIMATE algorithm analysis results showed that the StromalScore, ImmuneScore and ESTIMATEScore of the low HNRNPAB expression group was significantly higher compared with those of the high expression group (Fig. 5A). Although reduced immune cell infiltration was observed, the present findings revealed a phenomenon whereby the abundance of 'Monocytic_lineage' cells increased with elevated HNRNPAB expression (Fig. 5B). Correlation analysis between HNRNPAB and immune checkpoint gene expression levels indicated that HNRNPAB was significantly positively correlated with 11 immune checkpoint genes and negatively correlated with 3 immune checkpoint genes (Fig. 5C). IPS data of patients with BRCA from TCIA database demonstrated that the low HNRNPAB expression group exhibited a higher IPS and improved responsiveness to immune checkpoint inhibitors (Fig. 5D).

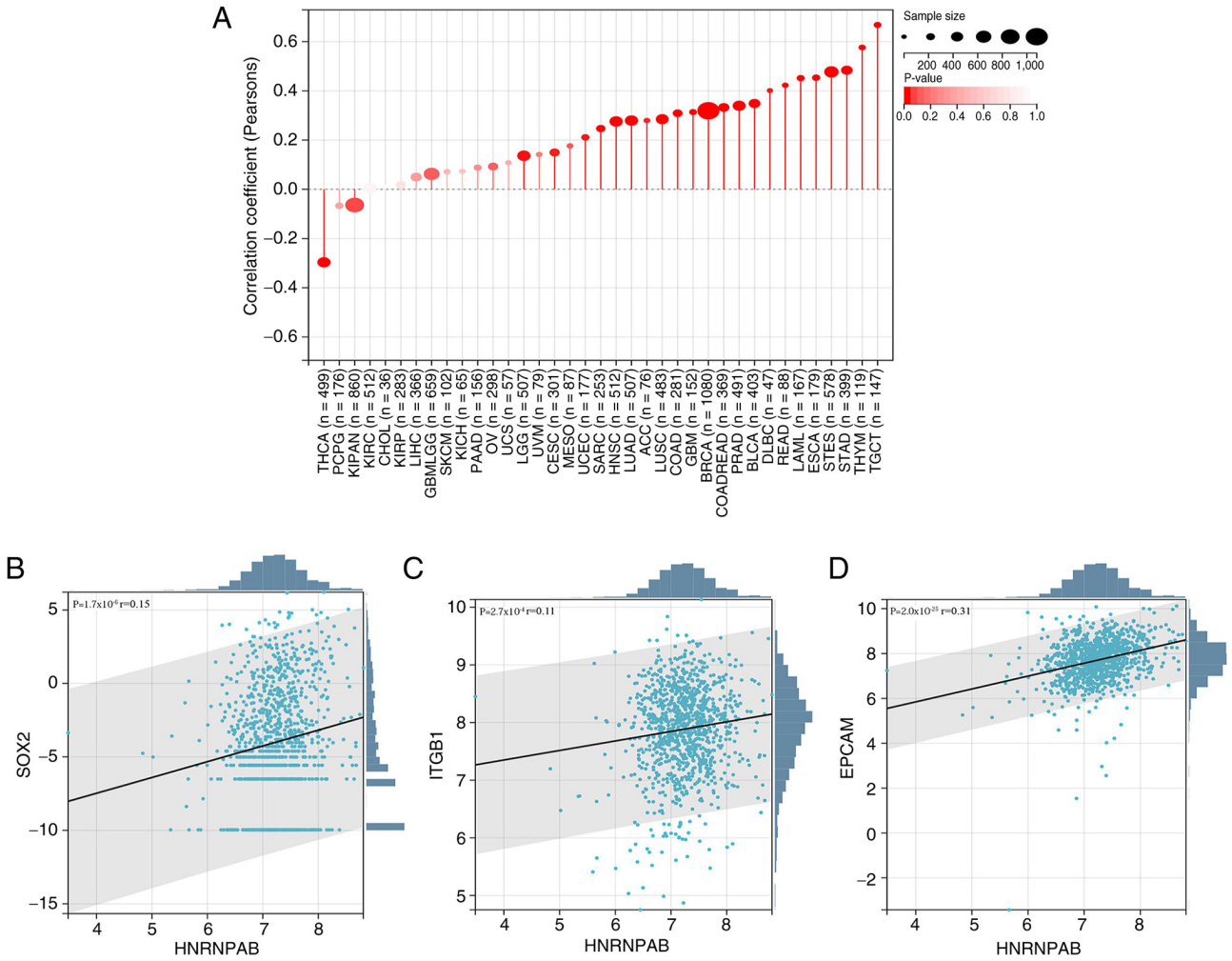

Figure 6. Association between HNRNPAB and cancer stem cells. (A) Lollipop plot depicting the correlation between HNRNPAB and tumor stemness in pan-cancer. The x-axis represents different types of tumors, while the y-axis illustrates the correlation between HNRNPAB expression and RNA stemness scores. Correlation scatter plots illustrating the association between HNRNPAB expression and (B) SOX2, (C) ITGB1 and (D) EPCAM in breast cancer. A value of 0 denotes a positive correlation, while a value of r<0 indicates a negative correlation. P<0.05 was considered to indicate a statistically significant difference. HNRNPAB, heterogeneous nuclear ribonucleoprotein A/B; ITGB1, integrin subunit β-1; EPCAM, epithelial cell adhesion molecule.

This result suggests that HNRNPAB downregulation is associated with enhanced immunotherapeutic potential, highlighting an important prognostic and predictive implication for BRCA immunotherapy.

Correlation between HNRNPAB and breast cancer stem cells. Based on the aforementioned enrichment results, it was hypothesized that HNRNPAB participates in biological processes such as cell cycle regulation, DNA replication and metabolic control, all of which are associated with stem cell functions. Therefore, the standardized TCGA pan-cancer dataset was retrieved from the UCSC Xena browser. Specifically, HNRNPAB expression data was extracted from each sample and the RNA stemness score for each tumor was calculated based on mRNA signatures. Pearson correlation coefficients were computed for each tumor type, revealing significant correlations in 26 tumors. Notably, 24 tumor types (including breast cancer) exhibited a positive correlation, while 2 tumor types exhibited a negative correlation (Fig. 6A). In addition, the correlation between HNRNPAB and common breast cancer stem cell markers was explored, identifying significant positive correlations with SOX2, integrin subunit β-1 (ITGB1), epithelial cell adhesion molecule (EPCAM) (Fig. 6B-D). Collectively, these preliminary results suggested that HNRNPAB may positively regulate breast cancer stem cells.

Discussion

In summary, the present study utilized online databases to elucidate the variations in HNRNPAB mRNA expression and its associations with clinical prognosis, cancer stem cell biomarkers, genetic mutation profiles and HNRNPAB-associated signaling pathway alterations, with a particular focus on immune infiltration during breast cancer progression. Furthermore, *in vitro* experiments were performed to validate the impact of HNRNPAB on the proliferative, migratory and invasive capacities of breast cancer cells.

Through analyses of public repositories including TCGA and HPA, it was determined that HNRNPAB is markedly upregulated in breast cancer cells and tissues relative to normal breast cells and adjacent non-tumor tissues. Concurrently,

significant upregulation of HNRNPAB in 24 tumor types (including endometrial carcinoma) and downregulation in 2 tumor types (including renal chromophobe carcinoma) was observed. This pattern aligns with the upregulation of HNRNPAB reported in a number of malignancies by previous research (33-35), indicating its potential utility as a diagnostic biomarker for tumors, particularly breast cancer. CCK-8 and Transwell assays further demonstrated that HNRNPAB facilitates the proliferation, migration and invasion of breast cancer cells, suggesting it acts as an oncogenic risk factor promoting breast cancer initiation and progression. HNRNPAB serves key roles in both normal biological processes and cancer development (36-39). Beyond its involvement in Harvey rat sarcoma viral oncogene homolog oncogene inactivation, observed elevated HNRNPAB levels in solid tumor metastases have demonstrated its pivotal function in tumor progression (40,41). Prognostic analyses in the current study have shown that high HNRNPAB expression impacts the survival and clinical outcomes of patients with breast cancer. The present study revealed that the median HNRNPAB expression level was significantly higher in breast cancer patients aged 21-40 years compared with normal tissues, indicating close associations with age and disease classification.

As well-established cancer hallmarks, ~75% of solid tumors exhibit aneuploidy and chromosomal instability, resulting in complex and heterogeneous karyotypic profiles (42,43). Assessing tumor ploidy provides valuable insights into cancer genome evolution and tumor heterogeneity. In the present study, HNRNPAB expression exhibited a positive correlation with tumor ploidy in breast cancer, indicating its close association with polyploidy and chromosomal instability in this malignancy, thus deepening the understanding of its role in disease progression. In addition, it was found that the majority of HNRNPAB alterations during breast tumorigenesis stem from mRNA expression amplification or mutation, a novel finding that, to the best of our knowledge, has not been previously reported.

However, it has been documented that HNRNPAB can enhance stemness characteristics in human stem cells and reduce their sensitivity to colorectal cancer chemotherapeutics (44). Yet, research regarding the associations and regulatory mechanisms of HNRNPAB in breast cancer stem cell modulation remains limited. Previous studies have indicated a regulatory association between HNRNPAB and the Wnt/β-catenin pathway: Decreased expression of the upstream factor microRNA-8063 attenuates its inhibitory effect on HNRNPAB, leading to activation of the Wnt/β-catenin signaling cascade and subsequent suppression of tumor metastasis (17,45). Based on these bioinformatics findings, the present study hypothesized that HNRNPAB may be involved in numerous stem cell-associated biological processes, such as cell cycle progression, DNA replication and metabolic regulation.

In KEGG and other databases, it was identified that the primary active pathways in the upregulated HNRNPAB group were associated with cell cycle signaling transduction pathways, which are aberrant pathways common to all malignancies (46). A number of components of cell cycle signaling pathways trigger uncontrolled cell division when mutated and these are closely associated with the mutation frequencies of numerous genes. These enrichment analysis findings indicate that HNRNPAB may precisely regulate the metabolic reprogramming process of tumor cells, break the normal metabolic homeostasis of tumor cells, reshape the energy supply mode and material anabolism network of tumor cells, before then regulating the proliferation activity, invasion and metastasis ability as well as the anti-apoptosis potential of breast cancer cells; thus, ultimately participating in and regulating the occurrence, development and malignant progression of breast cancer. This hypothesis may be further determined and improved in future research through targeted metabolomics detection techniques, combined with multidimensional research methods such as *in vitro* cell function experiments, *in vivo* animal model validation and clinical sample correlation analysis.

A number of studies have reported that HNRNP family proteins may promote PI3K/AKT/FOXO1-mediated bladder cancer cell proliferation (47,48). Previous research has also demonstrated an association between the PI3K/AKT/mTOR pathway and immune responses (49). The present HALLMARK-based GSEA analyses demonstrated a strong linear association between HNRNPAB and the PI3K/AKT/mTOR pathway, indicating that HNRNPAB may promote breast cancer development by activating this pathway. Building upon this finding, further immune-associated bioinformatics investigations of HNRNPAB were conducted. From the perspective of clinical practice, although the current immunotherapy for breast cancer has brought survival benefits to a number of patients. The immunocheckpoint inhibitor pabolizumab combined with chemotherapy has been approved by the FDA for the treatment of PD-L1-positive metastatic and early triple-negative breast cancer, while the ongoing clinical trial aims to expand the current treatment pattern of immunocheckpoint inhibitors in hormone receptor-positive and HER2-positive breast cancer. Antibody-conjugated drugs have been approved by the FDA for triple-negative and HER2-positive diseases, and their combination with immune checkpoint inhibitors is currently being studied. Vaccines and bispecific antibodies are active research areas. There are still problems such as limited response rate and marked individual differences (50). HNRNPAB, as a potential marker for predicting the responsiveness of immunotherapy, if its specificity and sensitivity can be further determined through large sample clinical verification, may serve as an important reference for clinicians to develop individualized immunotherapy strategies for breast cancer and also provide new research targets and ideas for further exploring the immune escape mechanism of breast cancer and developing new combined immunotherapy programs.

In conclusion, while dedicated mechanistic studies are lacking and limited in-depth explorations of how HNRNPAB modulates immune cell infiltration and immune checkpoint regulation have been translated into clinical settings, the unique expression patterns of HNRNPAB, as well as it's transcriptional profile, interactions with cancer stem cells and associations with lipid metabolism and tumor immunity, provide a novel perspective for HNRNPAB-targeted molecular therapy in patients with breast cancer. These findings also contribute to a deeper understanding of the molecular mechanisms driving breast cancer tumorigenesis. However, further

experiments are required to elucidate the specific mechanisms by which HNRNPAB regulates tumor immune infiltration and its role in immune checkpoint blockade responses. This will be the aim of future in-depth investigations conducted on this topic.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

JX and SZ designed the research and confirmed the authenticity of all the raw data. QH performed experiments. LC, MY and JH performed statistical analysis. JX and SZ wrote the manuscript. JX and QH prepared figures. LC, MY, JH and SL interpreted the data. All authors have read and approved the final manuscript. JX and QH confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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