

HnRNPF extensively regulates expression and alternative splicing of genes associated with proliferation and migration in gastric cancer cells

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Abstract. Heterogeneous nuclear ribonucleoprotein F (hnRNPF) is an RNA-binding protein involved in alternative splicing (AS) regulation and tumorigenesis; however, its role in gastric cancer (GC) remains poorly understood. In the present study, the expression pattern of hnRNPF in stomach adenocarcinoma samples was investigated and hnRNPF knockdown using small interfering RNA in AGS cells was performed. Subsequently, a whole-transcriptome sequencing method, namely RNA-sequencing (RNA-seq) was conducted to investigate how hnRNPF regulates gene expression and AS GC cells. Reverse transcription-quantitative PCR (RT-qPCR) was performed to validate the molecular targets of hnRNPF. Results indicated that the hnRNPF expression level was significantly increased in tumor samples and associated with nodal metastasis status in patients with GC based on The Cancer Genome Analysis and was also correlated with the overall survival time of patients with GC. RNA-seq analysis identified 929 differentially expressed genes (DEGs) and 2,669 hnRNPF-regulated AS events. Functional enrichment analysis revealed that hnRNPF-regulated DEGs were primarily enriched in pathways associated with 'inflammatory response', 'extracellular matrix organization' and 'immune response',

which are notably associated with GC progression. In addition, hnRNPF depletion significantly reduced the expression levels of CD55, connective tissue growth factor, frizzled-5, keratin 80 (KRT80) and transglutaminase 2. Furthermore, hnRNPF significantly altered the splicing patterns of key GC-associated genes, including KRT80, CDC25B, CD151, docking protein 1 and ADAM metalloproteinase domain 15. These AS alternations were validated by RT-qPCR and were found to be closely associated with the prognosis result of patients with GC. Collectively, the present findings demonstrated that hnRNPF orchestrates the GC cell transcriptome at both transcriptional and post-transcriptional levels, making hnRNPF a potential candidate for further pre-clinical target evaluation in GC.

Introduction

Gastric cancer (GC) is a malignant tumor that poses a serious threat to human health and is one of the most common cancers globally (1). According to recent global cancer statistics, GC was reported to be the fifth most frequently diagnosed cancer (4.9% of all cancers globally) and the fifth leading cause of cancer-associated mortality (6.8% of all cancers globally) (2). Thus, it is important to decipher the underlying molecular mechanisms of GC to develop effective therapeutic strategies (3). In addition, *Helicobacter pylori* (*H. pylori*) infection, dietary and genetic factors have been reported to be involved in the pathogenesis of GC (4,5). A recent study demonstrated that the interaction between *H. pylori* and cancer immunomodulatory stromal cells serve important roles in GC pathogenesis (6), highlighting the key roles of cellular and molecular interactions in GC development. In summary, early detection, standardized treatment and active prevention can effectively reduce the incidence rate and mortality of GC and improve the survival rate and quality of life of patients.

Among the identified molecular regulatory mechanisms involved in GC, dysregulation of gene expression and alternative splicing (AS) serve key roles (7,8). A previous study demonstrated that circular RNA-URI1 interacts with the RNA binding protein (RBP) heterogeneous nuclear ribonucleoprotein M (hnRNPM) to modulate the AS of genes (such as

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Abbreviations: AS, alternative splicing; DEGs, differentially expressed genes; FC, fold change; GC, gastric cancer; KEGG, Kyoto Encyclopedia of Genes and Genomes; PCA, principal component analysis; RNA binding protein, RBP; siRNA, small interfering RNA

Key words: heterogeneous nuclear ribonucleoprotein F, alternative splicing, gastric cancer, transcriptome profile

VEGFA and galectin 8) involved in the process of cell migration, thereby suppressing GC metastasis, indicating the role of AS in GC metastasis (9). It has also been demonstrated that AS events (ASEs) are associated with the prognosis of patients with GC by exploring the global AS patterns using univariate Cox regression analysis (8). Another RBP, heterogeneous nuclear ribonucleoprotein U (hnRNPU), can also modulate the AS pattern of the MET proto-oncogene gene, thereby promoting GC progression; the expression level of hnRNPU is regulated by fat mass and obesity-associated-mediated m⁶A modification (10). In summary, these findings demonstrated that the AS patterns and upstream AS regulators serve key roles in regulating GC progression.

The present study focused on hnRNPF, which broadly modulates the steps of RNA metabolism and is involved in the progression of several diseases, including a number of cancer types (11). HnRNPF promotes the proliferation of bladder cancer cells and acts as a downstream target of the PI3K/Akt/FOXO1 axis (12). In GC, the transcription factor ventral anterior homeobox 2 (VAX2) promotes the expression of the long noncoding RNA (linc)-01189, which interacts with hnRNPF to enhance hnRNPF degradation through ubiquitination. This VAX2/linc01189/hnRNPF axis has been reported to contribute to GC development by regulating both GC cell invasion and migration (13). An additional study also demonstrated that inhibition of hnRNPF ubiquitination and degradation mediated by the ecdysoneless/zinc finger protein 91 regulatory axis promotes GC invasion and metastasis (14). However, the regulatory mechanisms of hnRNPF in GC remain unclear.

Based on the aforementioned findings, the present study speculated that hnRNPF may regulate gene expression and alternative splicing by directly binding to mRNA or interacting with other regulatory factors, thereby serving a key role in GC initiation and progression. To decipher the underlying mechanisms, hnRNPF was knocked down in human gastric cancer (AGS) cells by using small interfering RNA (siRNA) and transcriptome sequencing data [RNA-sequencing (RNA-seq)] was performed to identify hnRNPF-regulated changes using high-throughput sequencing technology. The potential targets of hnRNPF regulated at the transcriptional and alternative splicing levels in AGS cells, as well as their biological functions, were analyzed. Furthermore, dysregulated genes were validated through reverse transcription-quantitative PCR (RT-qPCR) experiments. In summary, the present study hypothesized that hnRNPF may contribute to GC progression by regulating key genes involved in a number of central pathways, thus aiming to extend the molecular mechanisms of hnRNPF and highlight the potential therapeutic value of hnRNPF in GC therapy in future.

Materials and methods

siRNA design and detailed sequences. All siRNA duplexes were purchased from Suzhou GenePharma Co., Ltd. Negative control siRNA (siCtrl) sequences: Sense, 5'-UUCUCCGAA CGUGUCACGUTT-3' and antisense, 5'-ACGUGACAC GUUCGGAGAATT-3'. siRNA sequences targeting hnRNPF (si-hnRNPF): sense, 5'-CCCUGUGAGAGUCCAUAUUTT-3' and antisense, 5'-AAUAUGGACUCUCACAGGGTT-3'.

AGS cell culture and siRNA transfection. si-hnRNPF and si-Ctrl siRNAs were transfected into AGS cells (Procell Life Science & Technology Co., Ltd.), which were cultured at 37°C with 5% CO₂ in F12 and 15% FBS (cat. no. 10099-141; Gibco; Thermo Fisher Scientific, Inc.) with 100 µg/ml streptomycin and 100 U/ml penicillin. The p3x-FLAG-hnRNPF (plasmid #21926; Addgene, Inc.) and empty control vector p3xFLAG-CMV-7.1 were transfected using LipofectamineTM 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Transfected cells were harvested after 48 h for subsequent RT-qPCR analysis.

RNA extraction and sequencing. Total RNA was treated with RQ1 DNase (Promega Corporation) to remove DNA. The quality and quantity of the purified RNA were determined by measuring the absorbance at 260/280 nm (A₂₆₀/A₂₈₀) using NanodropTM One (Thermo Fisher Scientific, Inc.). RNA integrity was further verified by 1.5% agarose gel electrophoresis.

For each sample, 1 µg total RNA was used for RNA-seq library preparation by KAPA Stranded mRNA-Seq Kit for Illumina[®] Platforms (cat. no. KK8544; Kapa Biosystems; Roche Diagnostics). Each group contained three biological replicates. Polyadenylated mRNAs were purified by VAHTS mRNA Capture Beads (cat. no. N401-01; Vazyme Biotech Co., Ltd.) and fragmented mRNAs were converted into double strand cDNA. Following end repair and A tailing, the cDNAs were ligated to Diluted Roche Adaptor (cat.no. KK8726; Roche Diagnostics). After purification of ligation product and size fraction to 300-500 bp, the ligated products were amplified, purified, quantified and stored at -80°C before sequencing. The strand marked with deoxyuridine triphosphate (the 2nd cDNA strand) was not amplified, allowing strand-specific sequencing.

For high-throughput sequencing, the libraries were measured using Qubit 3.0 fluorometer (Invitrogen; Thermo Fisher Scientific) with concentrations from 16.32-23.00 ng/µl. Libraries were prepared following the manufacturer's instructions and applied to an Illumina NovaSeq 6000 system (Illumina, Inc.) for 150 nt paired-end sequencing.

RNA-seq quality filtering and alignment. Raw reads containing >2-N bases were first discarded. Then adaptors and low-quality bases (<20) were trimmed from raw sequencing reads using FASTX-Toolkit (version 0.0.13, http://hannonlab.cshl.edu/fastx_toolkit). The short reads <16 nt were also dropped. After that, clean reads were aligned to the GRCh38 human genome by TopHat2 (15) with no more than 4 mismatches. Uniquely mapped reads were used for gene reads number counting and fragments per kilobase of transcript per million fragments mapped (FPKM) calculations (16).

Differentially expressed genes (DEGs) analysis. The 'edgeR' R package (17) was utilized to screen out the DEGs. False discovery rate (FDR)<0.05 and fold change >2 or <0.5 were set as the cut-off criteria to identify DEGs.

Alternative splicing analysis. ASEs and regulated alternative splicing events (RASEs) between the two groups were defined and quantified using the in-house ABLas pipeline as described previously (18). Briefly, ABLas detection of ten ASEs types was based on the splice junction reads, including exon skipping

(ES), alternative 5' splice site (A5SS), alternative 3' splice site (A3SS), intron retention, mutually exclusive exons (MXE), MXE 5'-untranslated regions (UTRs), MXE 3'-UTRs, cassette exon, A3SS&ES and A5SS&ES.

To assess hnRNPF-RASEs, an unpaired Student's t-test was performed to evaluate the significance of the ratio alteration of ASEs between the two groups. Those ASEs with a P-value cut-off corresponding to an FDR<5% were considered as RASEs.

RT-qPCR experiment for gene expression assessment. GAPDH was used as the control gene to assess the effects of hnRNPF knockdown. First strand cDNA synthesis was performed using the Hifair® II 1st-Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus; Shanghai Yeasen Biotechnology Co., Ltd.), containing 5x gDNA-digester buffer, gDNA-digester, 2x Hifair® II SuperMix plus (including M-MLV reverse transcriptase, dNTP mixture, RNase-inhibitor and oligo-dT/random-hexamer primers). The RT thermal protocol was set as: 25°C for 5 min, 42°C for 30 min, followed by enzyme inactivation at 85°C for 5 min. RT-qPCR was performed on the Bio-Rad S1000 with Hieff™ qPCR SYBR® Green Master Mix (Low Rox Plus; Shanghai Yeasen Biotechnology Co., Ltd.). The qPCR thermocycling profile consisted of: Initial denaturation at 95°C for 5 min; followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec and extension at 72°C for 30 sec. A melting-curve analysis (95°C for 15 sec, 60°C for 1 min, gradual increase to 95°C) was performed after amplification to verify amplification specificity. Primer sequence information is presented in Table SI. The concentration of each transcript was then normalized to the GAPDH mRNA level using the $2^{-\Delta\Delta C_q}$ method (19).

Functional enrichment analysis. To determine functional categories of DEGs, Gene Ontology terms (<http://geneontology.org/>) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (<https://www.kegg.jp>) were identified using the KEGG Orthology-Based Annotation System server (20). The hypergeometric test and Benjamini-Hochberg FDR controlling procedures were used to define the enrichment of each term.

The Cancer Genome Atlas (TCGA) dataset analysis and survival analysis. Expression levels and overall survival results of hnRNPF were analyzed using the public TCGA dataset (<https://www.cancer.gov/ccg/research/genome-sequencing/tcga>), all tumor samples and adjacent normal samples were compared with obtain the statistical difference of hnRNPF expression. Survival analysis was conducted by classifying patients with GC into two equal groups according to the median value of hnRNPF expression levels using Kaplan-Meier plotter method (21).

Statistical analyses. All statistical analyses were conducted using R software (version 4.0; Posit Software, PBC). Stacked bar charts, boxplots, non-metric multidimensional scaling and principal component analysis (PCA) plots were generated using the 'ggplot2' R package (<https://github.com/tidyverse/ggplot2>). All reported P-values were two-sided and calculated through

inter-group Wilcoxon rank-sum tests (Mann-Whitney U tests) with FDR adjustment using the 'rstatix' R package (<https://rpkgs.datanovia.com/rstatix/>) and Holm-Bonferroni method. All analyses were two-sided, with P<0.05 being considered to indicate a statistically significant difference.

Results

Analysis of hnRNPF expression and transcriptomic data in GC. Previous studies have explored the functions and molecular targets of hnRNPF in a number of cancer types, including bladder cancer and breast cancer (12,22,23), while its function in GC remains largely unknown. To explore the expression pattern of hnRNPF in GC, the present study systematically analyzed its expression pattern and associated with the survival time of patients with GC. The global expression pattern of hnRNPF was first analyzed across numerous cancer types using TCGA database, with results indicating that hnRNPF was highly expressed and exhibited elevated transcript per million values in the majority of cancer types (Fig. 1A; cancer types indicated in red). Further analysis demonstrated that hnRNPF expression was significantly increased in stomach adenocarcinoma compared with adjacent normal control (STAD; Fig. 1B). In addition, hnRNPF expression was found to be consistently higher in all cancer stages compared with normal samples (Fig. 1C), as well as across all nodal metastasis statuses compared with normal samples (Fig. 1D), indicating that hnRNPF expression is associated with GC progression. The present study further analyzed the association between hnRNPF expression and the survival of patients with GC. Using TCGA survival data, results indicated that patients with GC and higher hnRNPF expression exhibited improved overall survival compared with those with lower hnRNPF expression (Fig. 1E). To validate these results, additional survival analysis was performed using the Kaplan-Meier plotter method (21). Consistently, patients with GC exhibiting higher hnRNPF expression showed a more favorable prognosis (Fig. 1F), which was consistent with the previous findings (24). In summary, these results demonstrate that hnRNPF is dysregulated in GC and is associated with the prognosis of patients with GC, while the underlying regulatory mechanisms remain unclear and require further investigation.

HnRNPF regulates the transcriptome profile in AGS cells. To further explore the molecular functions of hnRNPF in GC, hnRNPF expression was knocked down by transfecting sihnRNPF into the AGS GS cell line. After transfection, the expression level of hnRNPF was assessed through RT-qPCR, with results indicating a significant decrease in sihnRNPF samples compared with control (siCtrl) samples (Fig. 2A), determining the successful knockdown of hnRNPF. The present study further performed global transcriptome sequencing (RNA-seq) of sihnRNPF and siCtrl samples to identify hnRNPF-regulated molecular targets. Based on the normalized expression levels (FPKM values) of all genes, hnRNPF expression was also found to be significantly decreased in sihnRNPF samples (Fig. 2B), consistent with the RT-qPCR results. PCA of all expressed genes showed that si-hnRNPF samples were clearly separated from siCtrl samples along the first principal component (Fig. 2C), implying that hnRNPF

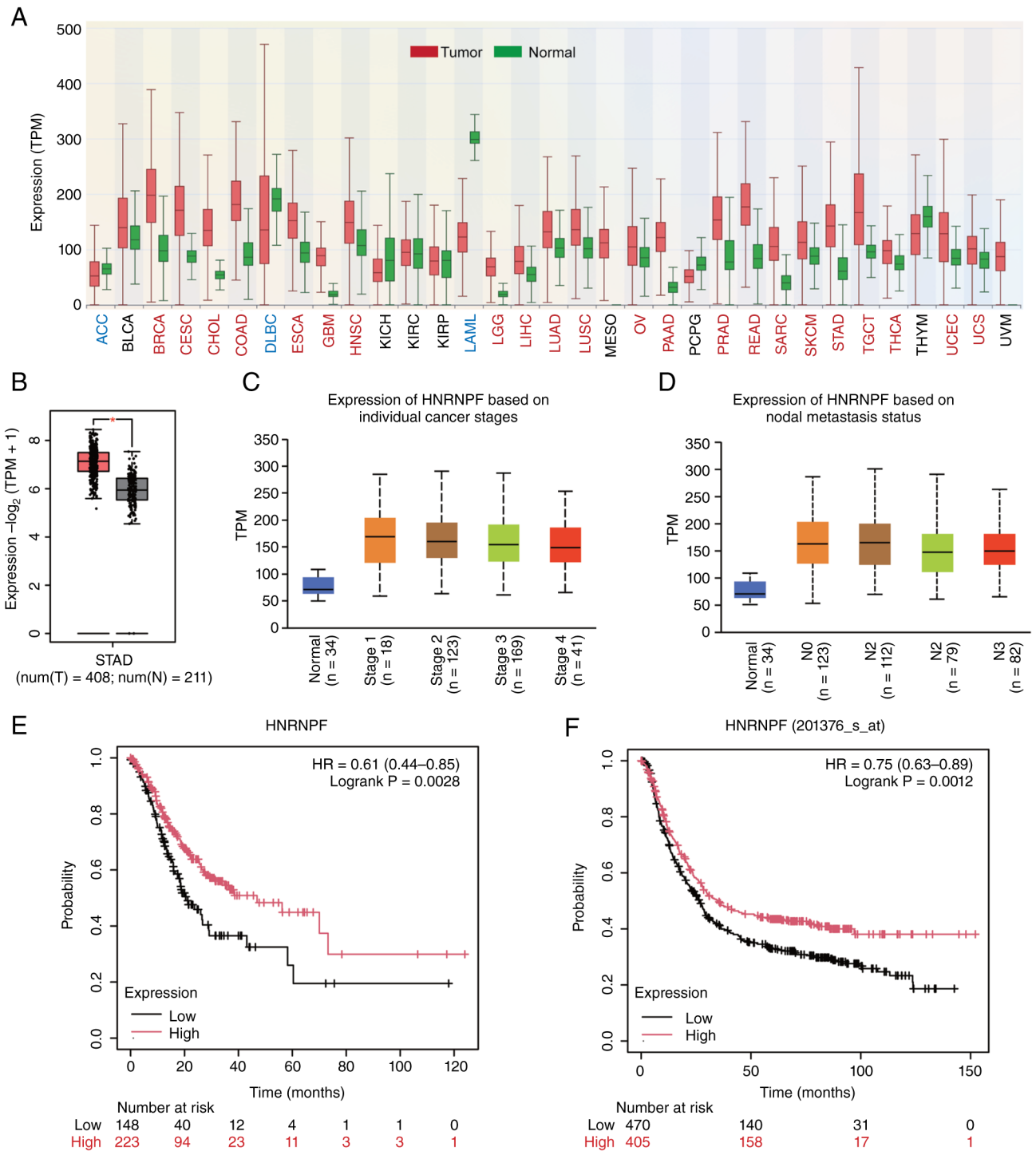


Figure 1. HnRNPF was dysregulated in GC cancer and associated with prognosis of patients with GC. (A) Bar plot showing the normalized expression level of HnRNPF in tumor and normal samples of all cancer types from TCGA database. Blue and red colors represent significantly downregulated and upregulated cancer types, respectively. (B) Box plot showing the significantly increased expression level of HnRNPF in STAD tumor samples. (C) Box plot showing the expression levels of HnRNPF in different cancer stages. (D) Box plot showing the expression levels of HnRNPF in different metastasis statuses. (E) Line plot showing the improved overall survival results of patients with STAD with higher HnRNPF expression level using TCGA dataset. (F) Line plot showing the improved overall survival results of patients with STAD and higher HnRNPF expression level using microarray data. HnRNPF, heterogeneous nuclear ribonucleoprotein F; GC, gastric cancer; TCGA, The Cancer Genome Atlas; STAD, stomach adenocarcinoma; TPM, transcripts per million; HR, hazard ratio.

knockdown globally regulated the transcriptome profile of AGS cells. DEGs analysis was subsequently performed to identify the molecular targets of HnRNPF. Using the stringent criteria, 929 DEGs were identified, including 497 upregulated and 432 downregulated DEGs in HnRNPF samples (Fig. 2D). By plotting the expression patterns of these DEGs, results

indicated that they showed consistent expression pattern among the three replicates of these two groups (Fig. 2E). Subsequently, the present study performed functional enrichment analysis for these DEGs by separating them into up and down gene sets. The up DEG set was enriched in pathways associated with 'inflammatory response', 'extracellular matrix

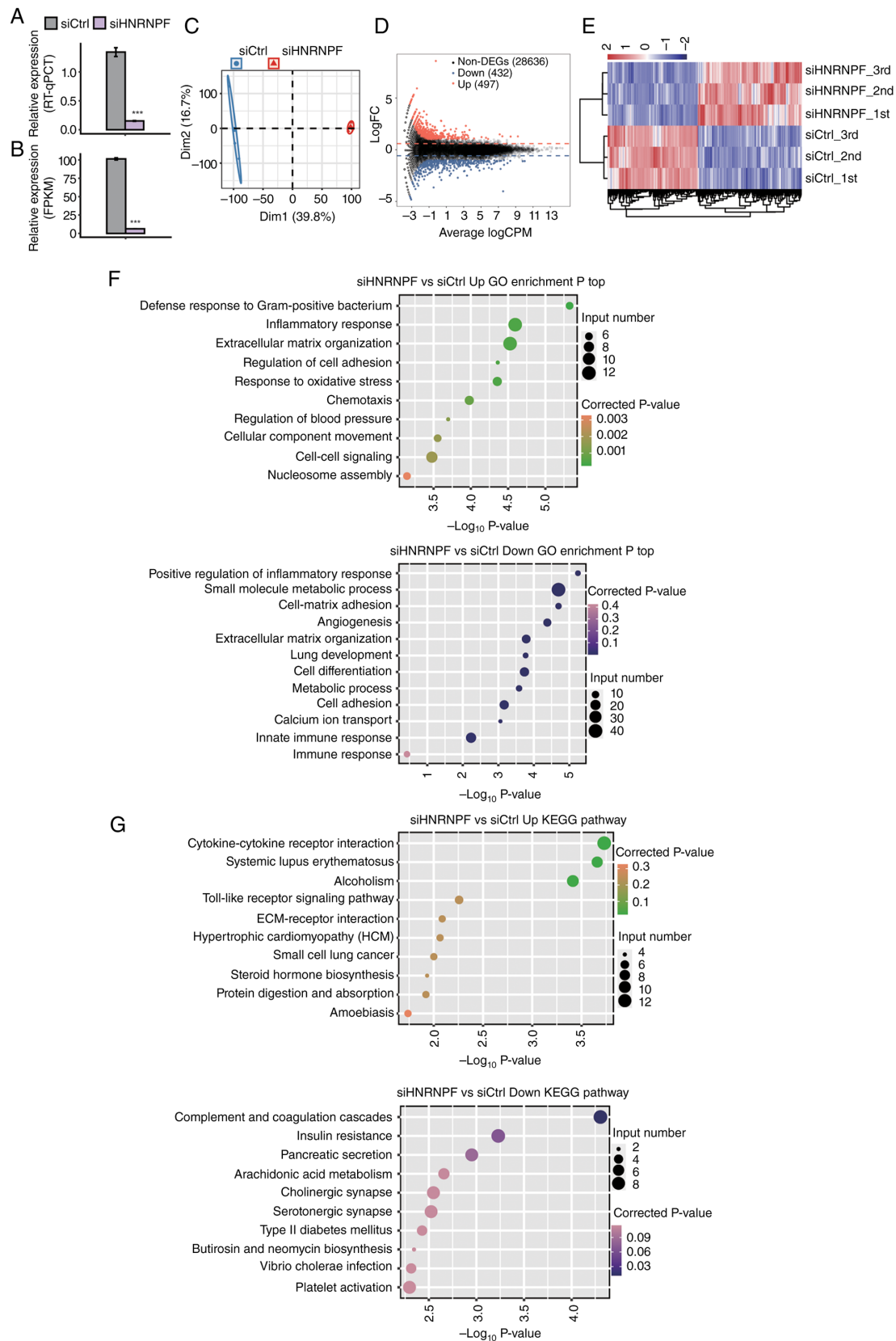


Figure 2. hnRNPF knockdown significantly regulated the transcriptome profile of AGS cells. (A) hnRNPF expression was quantified by RT-qPCR. Error bars represent the mean $\pm$ SEM. ***P<0.001. (B) hnRNPF expression was quantified by RNA-sequencing data. FPKM values were calculated as aforementioned. Error bars represent the mean $\pm$ SEM. ***P<0.001. (C) Principal component analysis of two groups of samples based on normalized gene expression level. (D) Identification of hnRNPF regulated genes. Upregulated genes are labeled in red, whereas downregulated are labeled in blue in the minus-average plot. (E) Hierarchical clustering of DEGs in control and hnRNPF knockdown samples. FPKM values are log₂-transformed and then median-centered by each gene. (F) The top 10 representative GO biological processes of up- or down-regulated genes. (G) The top 10 representative KEGG pathways of up- or downregulated genes. HnRNPF, heterogeneous nuclear ribonucleoprotein F; RT-qPCR, reverse transcription-quantitative PCR; DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; si-, small interfering RNA; Ctrl, control; FC, fold change; FPKM, fragments per kilobase million; CPM, counts per million.

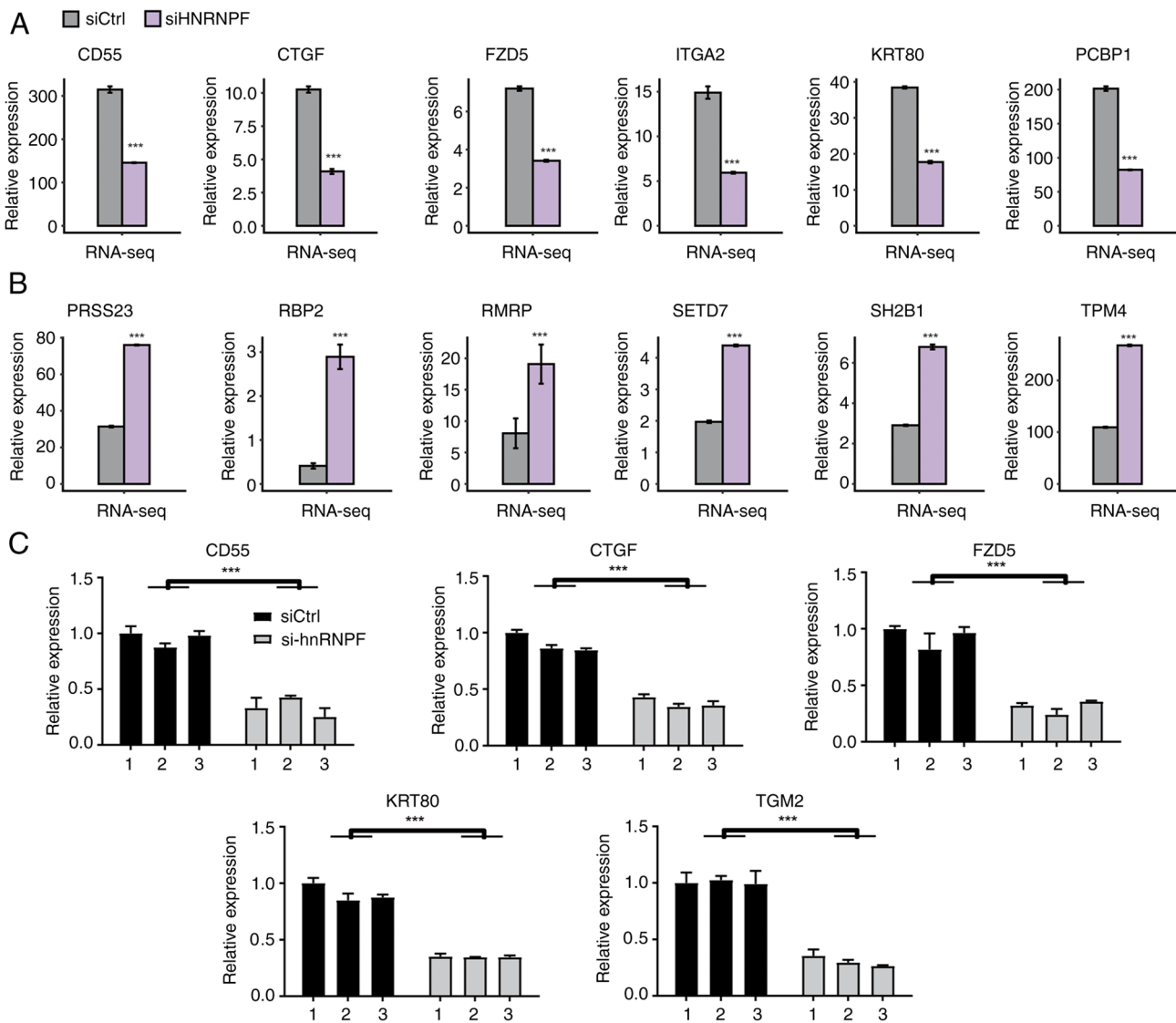


Figure 3. hnRNPF regulates the expression of genes involved in cell proliferation and migration from the RNA-seq data. (A) Bar plot showing the decreased level of selected DEGs in si-hnRNPF group. Student's t-test, ***P<0.001. (B) Bar plot showing the upregulated DEGs in the si-hnRNPF group. Student's t-test, ***P<0.001. (C) Bar plot showing the validation result of down DEGs in the si-hnRNPF group through reverse transcription-quantitative PCR. One-way ANOVA test, ***P<0.001. hnRNPF, heterogeneous nuclear ribonucleoprotein F; DEGs, differentially expressed genes, si-, small interfering RNA; Ctrl, control; RNA-seq, RNA-sequencing.

organization', 'regulation of cell adhesion' and 'small cell lung cancer' (Fig. 2F-G, upper panel). The down DEG set was primarily found to be enriched in 'immune response', 'positive regulation of inflammatory response', 'cell-matrix adhesion', 'metabolic process', 'angiogenesis' and additional associated pathways (Fig. 2F-G, lower panel). Similar pathways were enriched in both up and down DEGs, indicating that hnRNPF may systematically regulate gene expression to modulate pathways closely associated with GC development.

hnRNPF regulates the expression of genes involved in cell proliferation and migration. Based on the aforementioned results, the present study further explored the DEGs involved in important pathways associated with GC progression, including cell proliferation and migration. Among the down DEGs, six genes were selected from the cell-matrix adhesion pathway, including CD55, connective tissue growth factor (CTGF), frizzled-5 (FZD5), integrin α -2, keratin

80 (KRT80) and poly (rC) binding protein 1 (Fig. 3A). Simultaneously, six up DEGs, including serine protease 23 (PRSS23), RBP2, RNA component of mitochondrial RNA processing endoribonuclease, SET domain-containing lysine methyltransferase 7 (SETD7), SH2B adaptor protein 1 (SH2B1) and tropomyosin 4, were selected and these genes were enriched in pathways associated with cell proliferation and migration (Fig. 3B). To validate the expression changes of the selected DEGs, RT-qPCR was performed for five genes, including CD55, CTGF, FZD5, KRT80 and tissue transglutaminase 2 (TGM2), which have been reported to be associated with cell proliferation and migration (25,26). The RT-qPCR results showed that the expression levels of these genes were significantly reduced by sihnRNPF in AGS cells (Fig. 3C), consistent with the RNA-seq results. In summary, these findings demonstrate that hnRNPF may modulate GC cell proliferation and migration by regulating the expression levels of these genes.

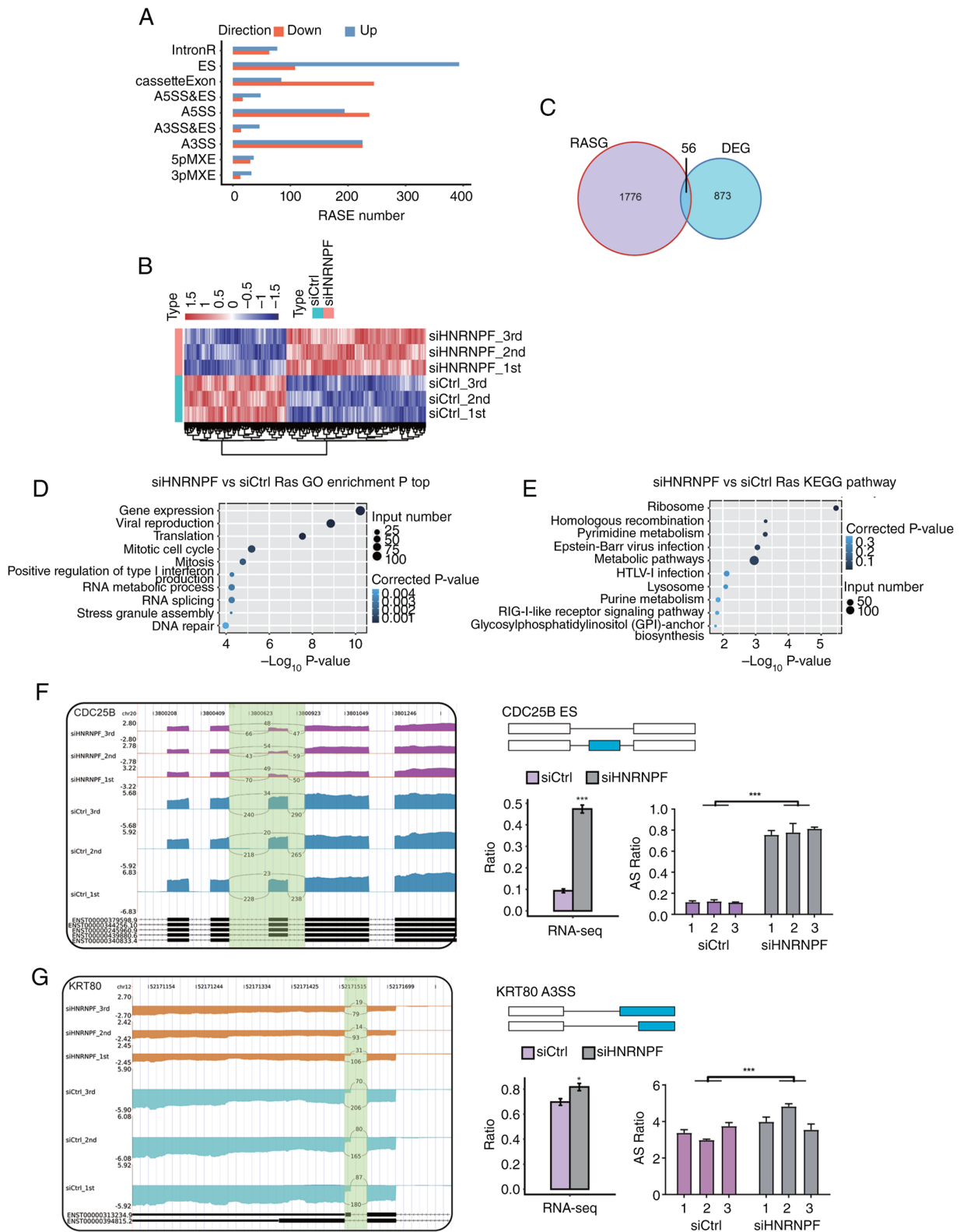


Figure 4. HnRNPF selectively regulates the alternative splicing of genes in cell proliferation and migration. (A) Frequency distribution of different types of hnRNPF-regulated alternative splicing events. (B) Hierarchical clustering of RASGs in control and hnRNPF knockdown samples. FPKM values were $\log_2$ -transformed and then median-centered by each gene. (C) The overlap analysis between DEGs and RASGs. (D) Bar plot showing the top 10 GO biological processes of RASGs. (E) Bar plot showing the top 10 KEGG pathways of RASGs. HnRNPF-regulated (F) cassette exon of CDC25B and (G) A3SS of KRT80, also validated by reverse transcription-quantitative PCR. Integrative Genomics Viewer-sashimi plots show AS changes in hnRNPF knockdown cells and control cells (left panel) and the transcripts for the gene are shown below. The schematic diagrams depict the structures of ASEs (right panel, top). The constitutive exon sequences are denoted with white boxes, intron sequences with horizontal line, while alternative exons with blue boxes. RNA-seq quantification of ASEs are shown at the bottom of the right panel. Student's t-test was performed to compare sihnRNPF and siCtrl samples with significance set at $P < 0.05$. * $P < 0.05$ and *** $P < 0.001$. hnRNPF, heterogeneous nuclear ribonucleoprotein F; RASGs, hnRNPF-regulated alternative splicing genes; DEGs, differentially expressed genes, si-, small interfering RNA; Ctrl, control; RNA-seq, RNA-sequencing; GO, Gene Ontology; FPKM, fragments per kilobase million; KEGG, Kyoto Encyclopedia of Genes and Genomes; AS, alternative splicing; ASE, AS event; KRT80, keratin 80; ES, exon skipping; A5SS, alternative 5' splice site; A3SS, alternative 3' splice site; MXE, mutually exclusive exons; RASE, regulated ASE; chr, chromosome.

HnRNPF selectively regulates the alternative splicing of genes in cell proliferation and migration. As an RBP from the hnRNP family, hnRNPF has the potential ability to modulate the AS profile (27,28). Thus, the present study systematically investigated the dysregulated AS profile induced by si-hnRNPF in AGS cells. To obtain a global view of the AS profile, the ABLAS program (18) was used to detect ASEs, including known and novel ASEs classified into nine types (as aforementioned). After obtaining the total ASEs, statistical significance analysis was performed to predict hnRNPF-RASEs. A total of 2,669 RASEs were identified, including 1,530 up RASEs and 1,139 down RASEs (Fig. 4A). Among these RASEs, ES was the dominant AS type, exhibiting a marked increase within sihnRNPF samples. In addition, the cassette exon event exhibited a notable decrease in sihnRNPF samples (Fig. 4A), indicating that hnRNPF may inhibits the ES events in AGS cells.

By plotting the distribution patterns of RASE ratios, results indicated that the up and down RASEs exhibited consistent AS ratio changes between sihnRNPF and siCtrl samples (Fig. 4B). Simultaneously, overlap analysis between RASE-associated genes (RASGs) and DEGs identified 56 co-regulated genes (Fig. 4C), indicating that these genes may be regulated by hnRNPF at both gene expression and AS levels. The enriched functions of these RASGs were further analysed, with results indicating that they were enriched in pathways associated with 'gene expression', 'mitotic cell cycle', 'RNA splicing' and 'viral reproduction' (Fig. 4D). KEGG pathway analysis showed that 'ribosome', 'metabolic pathways' and 'lysosome' pathways were significantly enriched (Fig. 4E). Among the genes enriched in top pathways, numerous genes closely associated with GC development were identified, including KRT80, ADAM metalloproteinase domain 15 (ADAM15), stimulated by retinoic acid gene 13 (STRA13), ataxin-2-like (ATXN2L), docking protein 1 (DOK1), filamin A (FLNA), CDC25B and CD151. In addition, one exon of CDC25B was significantly repressed and showed higher skipping ratio in sihnRNPF samples (Fig. 4F). In addition, one A3SS event of KRT80 was observed and showed higher alternative ratio in sihnRNPF samples (Fig. 4G). The present study also detected and presented the ES events of ADAM15 and DOK1, cassette exon events of CD151, FLNA and STRA13 and A3SS event of ATXN2L (Figs. S1 and S2). In summary, these results demonstrated that hnRNPF may contribute to GC progression by modulating the splicing patterns of key genes.

Finally, the present study explored whether these RASGs were associated with the prognosis results of patients with GC. Using the Kaplan-Meier plotter method, it was found that the validated RASGs shown in Figs. 4 and S1 and 2 were significantly associated with the overall survival time of patients with GC, with higher expression levels of these RASGs being associated with poorer prognosis (Fig. 5A). Survival analysis demonstrated that hnRNPF may influence the survival of patients with GC likely through regulating the expression or alternative splicing of downstream targets.

Discussion

Emerging evidence has demonstrated the aberrant function of RBPs in oncogenic gene regulatory networks (29); however, the extent of their influence, especially in GC, remains

incompletely understood. The present study demonstrated that the hnRNPF protein is not solely a passive post-transcriptional regulator but also an active modulator of the GC transcriptome, simultaneously shaping gene expression and AS patterns that may contribute to the proliferation and migration status of GC cells. Results indicated that hnRNPF extensively regulated the expression of genes associated with immune and inflammatory response pathways, as well as the AS patterns of genes associated with cell cycle. The present findings therefore advance the understanding of hnRNPF function in GC via three principal aspects: i) Elucidating a genome-wide hnRNPF-dependent regulatory atlas in GC cells; ii) uncovering hnRNPF-mediated splicing switches in key cancer-associated genes; and iii) providing a potential mechanistic scaffold for precision therapeutic targeting of hnRNPF or its downstream effectors.

With regard to hnRNPF as a bidirectional regulator of GC progression, previous studies have demonstrated an association between hnRNPF, bladder cancer proliferation and EMT-like programs through the stabilization of Snail-1 or targeting protein for Xklp2 mRNA (22,30). The present study extends these findings to GC by integrating RNA-seq with rigorous functional annotation analyses. Importantly, hnRNPF depletion in AGS cells resulted in 929 DEGs and 2,669 RASEs, suggesting that hnRNPF may regulate multiple pathways associated with GC progression, including cell-cycle control, extracellular matrix remodeling, angiogenesis and immune evasion (31). The extensive regulatory effects observed in the present study suggest that hnRNPF may function as a broad regulator rather than a gene-specific regulator, consistent with the reported roles of hnRNPM and hnRNPU in GC (9,10). Based the structural modules, hnRNPF can both shape alternative-splicing outcomes and indirectly influence steady-state mRNA abundance, either by altering pre-mRNA processing stability or by modulating the activity of transcriptional complexes. Despite this, the exact direct-binding targets and detailed mechanistic chain associating hnRNPF domain-level activity with the observed global transcriptome changes in AGS cells remain to be further validated by orthogonal assays such as cross-linking immunoprecipitation-seq and domain-truncation functional experiments.

Notably, it was observed that hnRNPF depletion down-regulated KRT80, CD55, CTGF, TGM2 and FZD5, while upregulating PRSS23, RBP2 and SETD7, resulting in a transcriptional imbalance that may affect cell proliferation and migration *in vitro*. KRT80 encodes a keratin intermediate-filament protein associated with oxaliplatin resistance and metastatic potential in GC; CD55 mediates complement immune evasion (32); CTGF and FZD5 participate in extracellular-matrix remodeling and Wnt-driven oncogenic signaling (33,34); and TGM2 contributes to cancer-stem-cell-like properties and chemotherapy resistance (35).

Correlative survival data from TCGA-STAD and Kaplan-Meier Plotter indicate that the expression of these genes is associated with patient prognosis, raising speculative potential for these molecules to serve as prognostic biomarkers or therapeutic effectors in GC. These findings are consistent with recent single-cell atlases of GC showing that hnRNPF-high signatures are associated with advanced tumor stages (36), indicating that hnRNPF may serve an oncogenic role in late stages of GC. In addition, it was further observed that higher

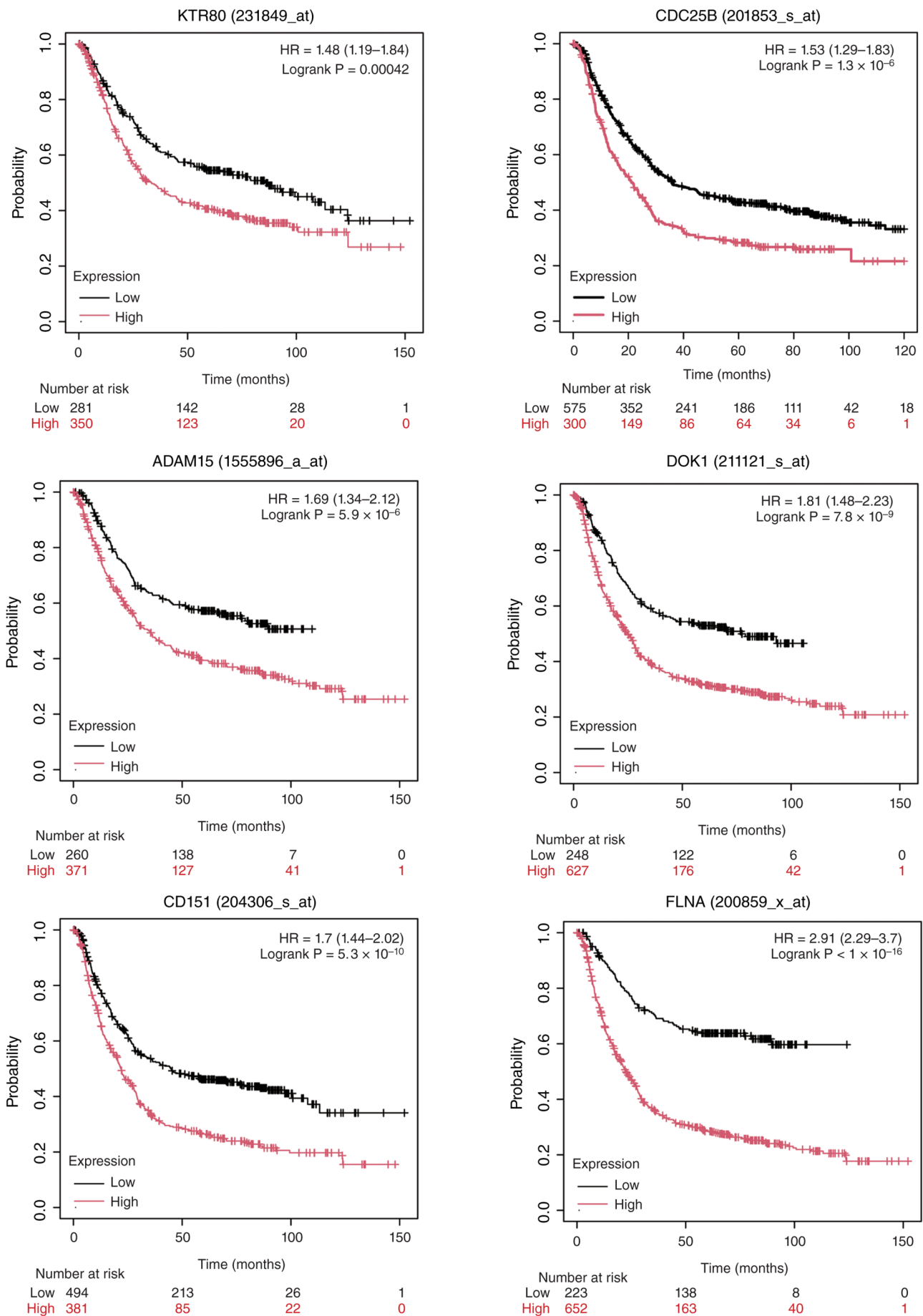


Figure 5. Line plots showing the overall survival time difference of patients with gastric cancer by dividing them into two groups according to the expression levels of target genes. HR, hazard ratio; KTR, keratin; ADAM15, ADAM metalloproteinase domain 15; DOK1, docking protein 1; FLNA, filamin A.

hnRNPF expression was associated with improved overall survival in patients with GC, suggesting that hnRNPF may serve as a potential prognostic biomarker for GC (37) and may regulate its molecular targets involved in tumor progression. The present transcriptomic and clinical survival analyses revealed that elevated hnRNPF expression was associated with lymph node metastasis and differential overall survival in patients with STAD, accompanied by transcriptional upregulation and altered alternative splicing of the pro-invasive effector KRT80. This tumor-intrinsic molecular regulation mediated by hnRNPF may be associated with preoperative nutritional and wasting phenotypes quantified by the Preoperative Cachexia Index (CXI), a validated clinical composite marker predictive of severe postoperative morbidity after gastrectomy (38).

Advanced GC with dysregulated hnRNPF-mediated splicing programs may contribute to systemic inflammation, extracellular matrix remodeling and skeletal muscle loss, which collectively reduce CXI and increase perioperative complication risks. Moving forward, combined stratification of patients with STAD using tumor hnRNPF expression and baseline CXI may help identify high-risk individuals requiring preoperative nutritional support before radical resection, potentially improving surgical outcomes in patients experiencing cachexia with aggressive hnRNPF-high tumors. Thus, further studies are warranted to comprehensively investigate the regulatory network of hnRNPF in GC using single cell and spatial transcriptomic or proteomic approaches.

With regard to alternative splicing, hnRNPF may rewire oncogenic isoforms. A notable finding of the present study was the hnRNPF-dependent modulation of specific exon inclusion or exclusion events affecting GC-relevant genes. Specifically, hnRNPF knockdown induced more ES events and repressed cassette exon events (Fig. 4A), indicating the important role of hnRNPF in regulating exon selection. For example, the present study detected a decreased A3SS event in exon 9 of *KRT80* and a skipped exon of *CDC25B* following hnRNPF knockdown. Both events may generate truncated protein isoforms lacking key functional domains. It has been reported that KRT80 has a smaller but fully functional splice variant during mammalian evolution (39,40). In GC, KRT18 serves as a biomarker and therapeutic target and can regulate the phenotypes of GC cells (41). Furthermore, higher expression of KRT80 is associated with poor prognosis in GC and promotes resistance to oxaliplatin (42). The alternative isoform of *CDC25B* has been identified and may contribute to the regulation of cancer cell proliferation or cell cycle, as summarized in a recent study (43). The present study speculates that C-terminal domain truncation caused by the A3SS switch of KRT80 may affect keratin cytoskeletal dynamics and metastatic signaling. Notably, one study has reported that key cell-cycle factors, including *CDC25B*, can be regulated at the AS level, thereby modulating the regular process of cell cycle (44). These splicing shifts theoretically may lead to truncated protein products with modified domain composition, which could perturb enzymatic activity, protein-protein interaction surfaces, cytoskeletal organization or membrane-receptor signaling, thereby theoretically attenuating G₂/M checkpoint control and restraining malignant phenotypes. Additional functional and rescue experiments based on hnRNPF knockdown are needed to determine whether si-hnRNPF can suppress the proliferation or migration levels of GC cells by modulating the

splicing pattern of these RASGs. Future studies using minigene reporters and CRISPR-Cas13-mediated splicing correction will be key in validating the causal roles of individual AS event.

Simultaneously, the present study also observed a survival paradox that high hnRNPF expression was associated with improved prognosis. Notably, despite hnRNPF having promoted aggressive behavior *in vitro*, TCGA-STAD and Kaplan-Meier plotter analyses revealed that patients with higher hnRNPF mRNA level exhibited longer overall survival. This paradox corresponds with observations in bladder cancer whereby hnRNPF loss sensitizes cells to cisplatin-induced apoptosis (30). A number of non-mutually exclusive explanations merit discussion. First, subcellular localization: HnRNPF shuttles between nucleus and cytoplasm; nuclear retention may favor pro-apoptotic splice variants (such as Bcl-xS), whereas cytoplasmic accumulation may promote oncogenic translation (45). Second, post-translational regulation: Linc01189 can trigger hnRNPF ubiquitination (13). In high-expressing tumors, hyper-ubiquitination may attenuate hnRNPF activity, resulting in a net tumor-suppressive effect. Third, the microenvironmental context: HnRNPF may enhance immune infiltration through the regulation of cytokine transcripts splicing, thereby improving patient survival despite its intrinsic oncogenic potential. The TCGA-STAD dataset is dominated by locally advanced and metastatic GC cases, a pattern amplified globally during the coronavirus disease 19 pandemic through widespread diagnostic delays and reduced routine endoscopic screening, which contributed to pronounced 'stage migration' toward late-stage presentation and worsened preoperative nutritional depletion at initial diagnosis (46). Within this context, the identification of hnRNPF as a key splicing regulator controlling metastatic programs (KRT80, *CDC25B* and *ADAM15*) highlights the potential clinical value of molecular biomarkers for stratifying aggressive tumor characteristics, particularly in patients presenting with malnutrition and advanced disease secondary to pandemic care disruptions. Early detection of hnRNPF upregulation may enable risk stratification before irreversible cachexia and metastatic spread develop. In addition to the aforementioned explanations, longitudinal single-cell and spatial transcriptomics of GC specimens are warranted to further dissect these context-dependent mechanisms.

Beyond the splicing-targeted small molecules, antisense oligonucleotides and proteolysis targeting chimera (PROTAC) degraders aforementioned, mesenchymal stem cells represent a promising regenerative approach for abdominal surgical disorders, with documented immunomodulatory, anti-inflammatory and tissue-repair capacities to relieve postoperative complications of gastrectomy (47). HnRNPF globally activates pro-inflammatory, ECM remodeling and metastatic transcriptional-splicing programs (exemplified by dysregulated KRT80 isoform patterns), which may exacerbate surgical inflammation, tissue fibrosis and cachexia-associated perioperative adverse events. The present study thus proposes a potential combinatorial therapeutic strategy involving concurrent MSC administration for mucosal and immune protection together with hnRNPF-targeted splicing suppressors to simultaneously restrain GC progression and reduce surgical morbidity in cachectic patients with low CXI. Preclinical studies are required to verify whether MSC-derived secretomes can reverse hnRNPF-mediated oncogenic splicing events. In addition, adoptive chimeric antigen receptor (CAR)-T

cell therapy has achieved notable efficacy against hematological malignancies, yet faces key limitations in solid tumors such as GC, including the scarcity of truly tumor-specific target antigens and impaired chimeric antigen receptor T-cell infiltration imposed by fibrotic stromal barriers (48). The present findings may provide potential translational insights for advancing CAR-T therapy in gastrointestinal cancers by identifying surface antigens potentially regulated by hnRNPF.

The present findings thus nominate hnRNPF as a potential druggable target in GC. Small-molecule RNA-binding cleft inhibitors or antisense oligonucleotides (ASOs) that disrupt hnRNPF-RNA interactions could, in principle, reprogram oncogenic transcriptome. The recent success of patisiran (transthyretin-targeting ASO) and splice-switching ASOs in neuromuscular disorders provides a valuable translational framework. In addition, PROTAC-mediated degradation of hnRNPF may overcome the survival paradox by achieving direct protein depletion rather than expression-level modulation. Given that hnRNPF appears to be dispensable for normal adult epithelial homeostasis (as evidenced by viable hnRNPF heterozygous mice), a potential therapeutic window may exist.

A number of caveats should be acknowledged. First, the present study was limited to AGS cells, which represent an intestinal-type GC model; therefore, validation in diffuse-type (such as HGC-27 and MKN45) and patient-derived organoids is warranted. Parallel experiments using an independent hnRNPF siRNA and ectopic hnRNPF rescue overexpression are also essential to discriminate specific hnRNPF-dependent transcriptomic changes from non-specific siRNA off-target effects. Follow-up *in vitro* assays, including Cell Counting Kit-8/MTT cell proliferation detection, wound healing migration assays and Transwell migration/invasion assays following hnRNPF knockdown or overexpression, are required to further determine whether hnRNPF depletion affects the proliferative and migratory capacities of GC cells. Second, *in vivo* CRISPR screens using GC xenograft models, minigene reporters or splice-switching oligonucleotides, are needed to test the functional relevance of hnRNPF-regulated AS events under immune-competent conditions. Third, although the present study observed hnRNPF-dependent splicing of metabolic genes (such as glycolytic enzymes), the downstream metabolic consequences remain unexplored. Integrating stable-isotope tracing with hnRNPF perturbation may help elucidate potential metabolic vulnerabilities. Finally, phosphoproteomics may clarify whether hnRNPF activity is modulated by oncogenic kinases (including PI3K/Akt), offering additional avenues for combinatorial therapies. Accordingly, the functions and translational value of hnRNPF and these hnRNPF-regulated DEGs remain preliminary. Moving from *in-vitro* transcriptomic signatures to clinical application will require further orthogonal evidence: Protein-level validation in clinical tumor specimens, functional phenotyping to separate driver vs. passenger transcript changes and well-powered clinical cohort studies to test their performance as prognostic or predictive biomarkers.

In conclusion, collectively, the present study positions hnRNPF as a key post-transcriptional regulator whose dual control of gene expression and alternative splicing contributes to GC progression. The apparent survival paradox highlights the importance of context-dependent RBP functions and cautions against simplistic interpretations of ‘upregulation equals

oncogenicity’ interpretations. By delineating the hnRNPF-RNA regulatory network and its functional outcomes, the present study provides a potential roadmap for precision therapeutics strategies targeting RNA-level vulnerabilities in GC.

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Availability of data and materials

The data generated in the present study may be found in the National Center for Biotechnology Information Gene Expression Omnibus under accession number GSE314680 or at the following URL: <https://www.ncbi.nlm.nih.gov/gds/?term=GSE314680>.

Authors' contributions

NL and JZ conceptualized the present study, designed the experiments and drafted the manuscript. FZ and YF collected clinical data and performed the statistical analysis. MT and YJ coordinated data integration and validation. YZ processed the samples and managed sequencing workflows. YX conducted the statistical analyses (R programming), calculated diversity indices and generated the figures. NL and JZ confirm the authenticity of all the raw data. All authors critically reviewed, read and approved the final version of the manuscript.

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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