BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Alternative Splicing Factor Heterogeneous Nuclear Ribonucleoprotein U as a Promising Biomarker for Gastric Cancer Risk and Prognosis with Tumor-Promoting Properties

Ying-ying Dong,* Meng-ya Wang,* Jing-jing Jing,* Yi-jun Wu,* Hao Li,† Yuan Yuan,* and Li-ping Sun*

From the Tumor Etiology and Screening Department of Cancer Institute and Key Laboratory of Cancer Etiology and Prevention in Liaoning Education Department,* the Key Laboratory of GI Cancer Etiology and Prevention in Liaoning Province,† and the Department of Clinical Laboratory, The First Hospital of China Medical University, Shenyang; and the Department of Radiotherapy, Zhumadian Central Hospital, Zhumadian, China

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Gastric cancer (GC) is a major global health concern with poor outcomes. Heterogeneous nuclear ribonucleoprotein U (HNRNPU) is a multifunctional protein that participates in pre-mRNA packaging, alternative splicing regulation, and chromatin remodeling. Its potential role in GC remains unclear. In this study, the expression characteristics of HNRNPU were analyzed by The Cancer Genome Atlas data, Gene Expression Omnibus data, and then further identified by real-time quantitative PCR and immunohistochemistry using tissue specimens. From superficial gastritis, atrophic gastritis, and hyperplasia to GC, the in situ expression of HNRNPU protein gradually increased, and the areas under the curve for diagnosis of GC and its precancerous lesions were 0.911 and 0.847, respectively. A nomogram integrating HNRNPU expression, lymph node metastasis, and other prognostic indicators exhibited an area under the curve of 0.785 for predicting survival risk. Knockdown of HNRNPU significantly inhibited GC cell proliferation, migration, and invasion and promoted apoptosis in vitro. In addition, RNA-sequencing analysis showed that HNRNPU could affect alternative splicing events in GC cells, with functional enrichment analysis revealing that HNRNPU may exert malignant biological function in GC progression through alternative splicing regulation. In summary, the increased expression of HNRNPU was significantly associated with the development of GC, with a good performance in diagnosing and predicting the prognostic risk of GC. Functionally, HNRNPU may play an oncogenic role in GC by regulating alternative splicing. (Am J Pathol 2024, 194: 13e29; https://doi.org/10.1016/j.ajpath.2023.10.007)

Worldwide, gastric cancer (GC) is ranked as the fifth most prevalent type of tumor, and it occupies the third position among the leading causes of tumor-related death.1 GC is a complex disease that involves multiple factors, gene abnormalities, and stages of development.2e4 The exact mechanism is not fully clear. Therefore, the identification of diagnostic/prognostic markers and potential targets of therapy for GC is urgently warranted to increase the survival rate and individualized treatment options for patients with GC.

Heterogeneous nuclear ribonucleoproteins (HNRNPs) constitute a prominent group of RNA-binding proteins.5 They are crucial in alternative splicing (AS), DNA repair, telomere biogenesis, cell signal transduction, mRNA stabilization, and the regulation of transcription and translation.6e8 Dysregulation of HNRNPs leads to alterations in the transcriptome and proteome of tumor cells, thereby impacting crucial cellular processes, such as cell growth, proliferation, invasion, and apoptosis.9e12 Previous pan-
cancer analysis showed that HNRNPs exhibit differential expression patterns and prognostic implications across various types of cancer. Heterogeneous nuclear ribonucleoprotein U (HNRNPU) is the largest member of the HNRNP family. It can bind to pre-mRNA and single-stranded DNA and participate in chromatin remodeling, transcription regulation, AS regulation, and maintenance of mRNA stability, and it is involved in the regulation of tumorigenesis and development. According to recent findings, patients with high expression of HNRNPU in clear cell renal cell carcinoma had poor prognoses. However, the association between HNRNPU expression and the risk and prognosis of GC, HNRNPU biological functions, and its potential molecular mechanisms in GC are poorly understood.

The aim of this study was to detect the expression of HNRNPU protein in human tissue, analyze its correlation with the risk and prognosis of gastric diseases, and explore the impact of HNRNPU on the biological function of GC cells and its possible molecular mechanism.

Materials and Methods

Identification of HNRNP Genes that Are Differently Expressed in GC Based on TCGA Data and Gene Expression Omnibus Data

Gene expression data of HNRNP family in GC were obtained by downloading from The Cancer Genome Atlas (TCGA—Stomach Adenocarcinoma (STAD); https://portal.gdc.cancer.gov, last accessed January 8, 2022). The Gene Expression Omnibus database (GSE174237; https://www.ncbi.nlm.nih.gov/geo), which contained the expression of the HNRNPs, was used for verification. R 4.1.3 (http://www.r-project.org) was used to normalize the expression data. Genes with the combination of adjusted \( P < 0.05 \) (Benjamini-Hochberg \( P \)-value correction) and \( \log_2 \) fold changel \( > 1 \) were regarded as significantly differentially expressed HNRNPs.

Tissue Specimen Collection

A total of 377 subjects were enrolled from The First Hospital of China Medical University (Shenyang, China) from 2012 to 2019, including 39 cases of superficial gastritis (GS), 39 cases of atrophic gastritis (GA), 63 cases of dysplasia (GD), and 236 cases of GC, which were divided into 63 cases of early gastric cancer (EGC), 131 cases of advanced gastric cancer (AGC), and 236 cases of GC, which were divided into 63 cases of early gastric cancer (EGC), 131 cases of advanced gastric cancer (AGC), and 42 cases that could not be staged. All cases were clearly diagnosed by histopathologic examination. The gastric mucosa tissues at the site of the lesion were used for the paraffin-embedded section (96 cases of gastric cancer also had adjacent nontumor tissues). RNA isolation was performed on 40 pairs GC and corresponding paracancer tissues. Cancerous tissue and normal tissue (>3 cm adjacent to the cancer) were taken.

Information on patients was collected by medical record method, including age, sex, Helicobacter pylori infection, and clinicopathologic parameters, such as tumor location, tumor maximum diameter, general classification, Lauren classification, growth pattern, vessel carcinoma embolus, perineural invasion, and TNM stage. A total of 106 patients with GC were followed up, and the follow-up end point was November 2019.

Isolation of Total RNA Followed by Reverse Transcription

Total RNA was extracted from the cells and tissues with TRIzol Reagent (TIANGEN, Beijing, China), followed by phenol-chloroform extraction, and isopropyl alcohol precipitation; the RNA was washed with 75% ethanol and then dissolved in RNase-free water. The RNA samples were stored at \(-80^\circ C\). Then, the cDNA was synthesized using a PrimeScript RT Master Mix (TaKaRa, Dalian, China). All the above procedures followed the manufacturer’s instructions.

Quantitative Real-Time PCR

mRNA expression levels were detected by quantitative real-time PCR (realplex; Eppendorf, Hamburg, Germany) using TB Green Premix Ex Taq II (TaKaRa). Primers for \( \beta \)-actin and HNRNPU are listed in Table 1. The relative mRNA expression level was determined using the \( 2^{-\Delta\Delta Ct} \) method.

Immunohistochemistry

The tissue was fixed in 10% formalin (Sinopharm Chemical Reagent Co., Ltd., Shenyang, China) and embedded in paraffin (Sinopharm Chemical Reagent Co., Ltd.), then cut into sections (4 \( \mu m \) thick). All procedures of immunohistochemistry were performed routinely in the laboratory using standard protocols. The slides were subjected to deparaffinization using xylene, followed by rehydration through a series of graded alcohol and washing with tap water. For antigen retrieval, the tissue sections were incubated in boiling sodium citrate buffer (pH 6.0) for 90 seconds in a steam pressure cooker. After cooling to room temperature, the slides were rinsed with running water and incubated in phosphate-buffered saline (pH 7.4) for 10 minutes. To block endogenous peroxidase activity, the sections were treated with an endogenous peroxidase blocking agent for 30 minutes. To prevent nonspecific binding, tissue collagen was blocked by adding 10% normal goat serum and incubating at room temperature for 30 minutes. The primary antibody, anti-HNRNPU antibody (ab10297; diluted 1:400; Abcam, Cambridge, UK), was applied and incubated overnight at 4°C. After three washes with phosphate-buffered saline, the sections were incubated with biotinylated secondary antibody (goat anti-rabbit antibody; Maixin Inc., Fujian, China) and streptavidin-biotin binding, tissue collagen was blocked by adding 10% normal goat serum and incubating at room temperature for 30 minutes. The primary antibody, anti-HNRNPU antibody (ab10297; diluted 1:400; Abcam, Cambridge, UK), was applied and incubated overnight at 4°C. After three washes with phosphate-buffered saline, the sections were incubated with biotinylated secondary antibody (goat anti-rabbit antibody; Maixin Inc., Fujian, China) and streptavidin-biotin.
peroxidase for 10 minutes. The chromogenic substrate, diaminobenzidine (Maixin Inc.), was used for 30 seconds to visualize the antigen-antibody reaction. Finally, the slides were rinsed with water, counterstained with hematoxylin in water, dehydrated, cleared with xylene, and mounted.

A scoring system with a semiquantitative approach was employed to assess the expression of HNRNPU in the nucleus. The immunohistochemistry score was accumulated by the staining intensity: the scale of 0, 1, 2, and 3 indicates negative, weak, moderate, and strong, respectively (intermediary intensity between two levels was scored as 0.5, 1.5, and 2.5), multiplied by the proportion of stained cells (0, 0% to 5%; 1, 6% to 25%; 2, 26% to 50%; 3, 51% to 75%; and 4, >75%).

Cell Culture and Transfection

The gastric adenocarcinoma cell line HGC27, AGS, and normal gastric epithelial cell GES-1 were grown in 1640 medium (Solarbio, Beijing, China) containing 10% fetal bovine serum (Biological Industries, Kibbutz Beit-Haemek, Israel). HNRNPU siRNA (si-HNRNPU#1: the target sequence was 5’-CAGUCCUCUUCCUAAAAUTTT-3’; si-HNRNPU#2: the target sequence was 5’-GCAACUGUGAGACUGAGAU-3′) and control siRNAs, synthesized by ShengGong (Sangon Biotech, Shanghai, China), were transfected with a final concentration of 50 nmol/L, according to the jetPRIME siRNA transfection protocol. For transfection, siRNA was added into 60% to 80% confluent cells per well. After 4 to 6 hours, medium was replaced with fresh complete medium to culture for another 24 to 48 hours. The total RNA and protein were isolated to further confirm the transfection efficiency.

Western Blot Analysis

Protein samples were extracted by radioimmunoprecipitation assay buffer (RO020; Solarbio), and the concentration was quantified using a bichinchonic acid protein assay kit (P0010; Beyotime, Beijing, China). HNRNPU siRNA (si-HNRNPU#1: the target sequence was 5’-CAGUCCUCUUCCUAAAAUTTT-3’; si-HNRNPU#2: the target sequence was 5’-GCAACUGUGAGACUGAGAU-3′) and control siRNAs, synthesized by ShengGong (Sangon Biotech, Shanghai, China), were transfected with a final concentration of 50 nmol/L, according to the jetPRIME siRNA transfection protocol. For transfection, siRNA was added into 60% to 80% confluent cells per well. After 4 to 6 hours, medium was replaced with fresh complete medium to culture for another 24 to 48 hours. The total RNA and protein were isolated to further confirm the transfection efficiency.

Table 1  Real-Time Quantitative PCR Primer Sequences

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>5′-ATGTGGCCGAGACCTTTGATT-3′</td>
<td>5′-AGTGGGCTGCTTCTTAGATG-3′</td>
</tr>
<tr>
<td>HNRNPU</td>
<td>5′-GAACAAGTATAGCAGAGCCCAAATC-3′</td>
<td>5′-TCTGTAACCTTCTATCCAAACACAC-3′</td>
</tr>
</tbody>
</table>

Cell Viability Assay

Following siRNA transfection, HGC27 (6 × 10³ cells/well) and AGS (3 × 10³ cells/well) cells were seeded into a 96-well plate. The plates were incubated in the condition of 37°C and 5% CO₂ for 24, 48, 72, and 96 hours. To each well, 10 µL Cell Counting Kit-8 (MedChem Express, Monmouth Junction, NJ) solution was added, and following a 2-hour incubation period, the absorbance at 450 nm was measured (BioStack Ready, PowerWave XS; BioTek Instruments, Winooski, VT). Each experiment was repeated in triplicate.

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

To assess cell proliferation, the BeyoClick EdU-555 Cell Proliferation Detection Kit (C0075S; Beyotime) was used following the manufacturer’s instructions. In brief, cells were seeded (2 × 10⁴) in a 24-well plate after being transfected with siRNA. On the following day, the cells were incubated with a 10 nmol/L EdU solution and cultured for 2 hours at 37°C and 5% CO₂ incubator. After being fixed in 200 µL 4% paraformaldehyde (LA0427; Labcoms, Los Angeles, CA) for 15 minutes and then permeabilized in 200 µL Enhanced Immunostaining Permeabilization Buffer (P0097; Beyotime) for 15 minutes at ambient temperature, the cells were washed by Immunol Staining Wash Buffer (Beyotime; P0260). Then, the cells were incubated with Click Additive Solution and Hoechst 33342 (C0075S; Beyotime) for 15 minutes at ambient temperature, the cells were washed by Immunol Staining Wash Buffer (Beyotime; P0260). Then, the cells were incubated with Click Additive Solution and Hoechst 33342 (C0075S; Beyotime). Finally, the percentage of EdU incorporation was calculated to evaluate cell proliferation.

Flow Cytometry Analysis

Cell apoptosis was detected using YF647A—annexin V and propidium iodide apoptosis kit (Y6026S; US Everbright, Suzhou, China). In brief, harvested cells were incubated
with a mix of YF647A—annexin V and propidium iodide at ambient temperature in the dark for 15 minutes following the manufacturer’s instructions. BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) was used to detect the stained cells. Flow cytometry analysis was conducted on a gated population of 10,000 events. The gating strategy is shown in Supplemental Figure S1.

Cell Migration and Invasion Assay

Following siRNA transfection, cells at a density of $4 \times 10^4$ cells/well were seeded onto the upper chamber, which contained 8-μm porous membranes (3422; Corning Life Sciences, Corning, NY), and 100 μL serum-free medium was added. Matrigel was precoated onto the upper chambers for the cell invasion assay. After incubation for 24 hours for migration and 48 hours for invasion assay, any nonmigrated or noninvaded cells present on the upper surface were eliminated with a cotton swab, whereas the migrated and invaded cells were fixed using 4% paraformaldehyde for 30 minutes and subsequently stained with 0.1% crystal violet for 20 minutes.

Cells at a density of $2 \times 10^5$ cells/well were cultured in 6-well plates for wound healing assay. While the cells reached 90% confluence after transfection, a wound was generated using a 200 μL pipette tip, which is aseptic to form standardized wound scratching. After being washed lightly with phosphate-buffered saline three times, the cells were incubated with 2 mL serum-free medium. Cell migration into the wound area at 0, 24, and 48 hours was observed and recorded by a microscope and digitally imaged (Nikon Eclipse Ti-S; Nikon, Tokyo, Japan). The wound healing percentage was calculated by using the following formula:

$$ \text{wound healing} = \frac{(0 \text{ hour scratch width} - 48 \text{ hours scratch width})}{0 \text{ hour scratch width}} \times 100\% $$

Gene Set Enrichment Analysis

The TCGA GC samples were categorized into two groups (namely, the high and low expression groups of HNRNPU) based on the median expression level of HNRNPU. Gene Set Enrichment Analysis was conducted to detect the pathways HNRNPU was involved in. Statistical significance was defined as normalized enrichment score $> 1$ and false discovery rate $< 0.05$.

Transcriptome Sequencing and Alternative Splicing Analysis

Total RNA extraction was implemented using TRIzol Reagent in AGS. After DNA digestion by DNasel, RNA concentration was quantified by NanoDrop (Thermo Fisher Scientific Inc., Waltham, MA). After verifying RNA integrity, a unique identifier mRNA-sequencing library was constructed. The 200- to 500-bp library products were subjected to enrichment, quantification, and sequencing using the PE150 model on a DNBSEQ-T7 sequencer (MGI Tech Co., Ltd., Shenzhen, China). After filtering and discarding low-quality reads, the reads that contain an identical unique molecular identifier sequence were classified into a single cluster, from which one consensus sequence was generated for each sub-cluster.

Using Spliced Transcripts Alignment to a Reference (STAR) software version 2.5.3a (https://github.com/alexdobin/STAR), the deduplicated consensus sequences were aligned to the Homo sapiens GRCh38 reference genome. The gene expression index was calculated using reads per kilobase per million reads. Differential alternative splicing events (ASEs) were identified by using replicate multivariate analysis of transcript splicing (rMATS) software version 3.2.5 (http://rnaseq-mats.org).
Figure 2  HNRNPU protein expression [immunohistochemistry (IHC)] and diagnostic efficiency. A: Representative hematoxylin and eosin (H&E)—stained images of HNRNPU protein and corresponding IHC in different gastric diseases. B–E: Box plots of IHC scores of HNRNPU. B: HNRNPU protein expression levels in gastric cancer (GC) and adjacent control tissues. C: HNRNPU protein expression levels in different gastric disease tissues. D: HNRNPU protein expression levels in superficial gastritis (GS), GC, and its precancerous lesions. E: HNRNPU protein expression levels in non-GC and GC. F: Receiver operating characteristic (ROC) curves of HNRNPU protein for diagnosing disease of the stomach (left) and gastric cancer (right). n = 96 (B–E). ***P < 0.001, ****P < 0.0001. Scale bars = 200 µm (A). AGC, advanced GC; AUC, area under the curve; EGC, early GC; GA, atrophic gastritis; GD, dysplasia.
sourceforge.net), with $P < 0.05$ and $|\Delta \psi| > 0.1$, which uses reads spanning both splice junctions and exons. KEGG Orthology Based Annotation System (KOBASE) software version: 2.1.1 (http://kobas.cbi.pku.edu.cn) was used for Gene Ontology analysis and Kyoto Encyclopedia of Genes and Genomes enrichment analysis. Statistical significance is defined as $P < 0.05$ for differences.

### Statistical Analysis

SPSS 23.0 (SPSS, Chicago, IL), GraphPad Prism 8.0 (GraphPad, San Diego, CA), and R 4.1.3 were used. If the data did not follow a normal distribution, nonparametric tests were employed for the analysis. The $U$-test was used for comparing two sets, whereas Kruskal-Wallis H test was used for comparing multiple sets. The differences in the quantitative real-time PCR and Western blot analysis data were compared using $t$-test. Receiver operating characteristic curve and area under the curve (AUC) were used for diagnostic accuracy evaluation. X-Tile software (Yale University School of Medicine, New Haven, CT; https://medicine.yale.edu/lab/rimm/research/software) was used to obtain the best cutoff value for survival prognosis. Log-rank test was used for overall and subgroup survival analysis. Univariate and multivariate Cox proportional hazards regression models were used to assess prognostic impact of various independent risk factors. Pearson correlation analysis was used for data with normal distribution, whereas Spearman correlation analysis was employed for non-normally distributed data.

### Ethical Considerations

The study was performed in accordance with institutional guidelines and authorized by the Ethics Review Committee of the First Hospital of China Medical University (2016-161). All protocols were rigorously conducted in accordance with the guidelines established in the Declaration of Helsinki. Participants who agreed to take part in the study provided informed consent. To ensure confidentiality, samples were assigned anonymous codes, safeguarding the identity of each participant.

### Results

#### Identification and Validation of Differentially Expressed HNRNPs in GC

The relevant information about HNRNP family members is listed in Supplemental Table S1. The expression levels of HNRNP1L2, HNRNPA2B1, HNRNPP1, and HNRNP2 were notably up-regulated in GC tissues compared with normal tissues, with 161). All protocols were rigorously conducted in accordance with institutional guidelines and authorized by the Ethics Review Committee of the First Hospital of China Medical University (2016-161). All protocols were rigorously conducted in accordance with the guidelines established in the Declaration of Helsinki. Participants who agreed to take part in the study provided informed consent. To ensure confidentiality, samples were assigned anonymous codes, safeguarding the identity of each participant.

### Table 2 Diagnostic Efficacy of HNRNPU Expression

<table>
<thead>
<tr>
<th>Group</th>
<th>AUC (95% CI)</th>
<th>P value</th>
<th>Cutoff</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>Youden index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease stomach vs control</td>
<td>0.911 (0.877–0.945)</td>
<td>4.31 × 10^{-17}</td>
<td>3.5</td>
<td>92.30</td>
<td>83.40</td>
<td>0.757</td>
</tr>
<tr>
<td>GC vs non-GC</td>
<td>0.847 (0.809–0.886)</td>
<td>1.62 × 10^{-29}</td>
<td>4.75</td>
<td>81.80</td>
<td>70.20</td>
<td>0.52</td>
</tr>
</tbody>
</table>

AUC, area under the curve; GC, gastric cancer.

### Table 3 Univariate and Multivariate Regression Analysis of Prognostic Factors with Overall Survival in GC ($n = 88$)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>HNRNPU (low vs high)</td>
<td>1.84</td>
<td>1.03–3.29</td>
</tr>
<tr>
<td>Sex (male vs female)</td>
<td>1.12</td>
<td>0.59–2.11</td>
</tr>
<tr>
<td>Age (&lt;60 vs ≥60 years)</td>
<td>1.16</td>
<td>0.65–2.07</td>
</tr>
<tr>
<td>T (T1 + T2 vs T3 + T4)</td>
<td>11.79</td>
<td>1.62–85.53</td>
</tr>
<tr>
<td>N (N0 vs N1)</td>
<td>7.37</td>
<td>2.27–23.94</td>
</tr>
<tr>
<td>TNM stage (I + II vs III + IV)</td>
<td>5.86</td>
<td>1.81–19.03</td>
</tr>
<tr>
<td>Tumor location (antrum only vs body only vs multifocal)</td>
<td>0.93</td>
<td>0.68–1.26</td>
</tr>
<tr>
<td>Maximum diameter (&lt;5 vs ≥5 cm)</td>
<td>3.72</td>
<td>1.15–12</td>
</tr>
<tr>
<td>General classification</td>
<td>1.23</td>
<td>0.87–1.74</td>
</tr>
<tr>
<td>(raised vs ulcer vs diffuse infiltrative vs ulcer infiltrating)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lauren classification (diffuse type vs intestine type)</td>
<td>0.29</td>
<td>0.09–0.95</td>
</tr>
<tr>
<td>Growth pattern (nested/cloidy vs infiltrative vs diffuse infiltration)</td>
<td>0.85</td>
<td>0.58–1.25</td>
</tr>
<tr>
<td>Vessel carcinoma embolus (negative vs positive)</td>
<td>1.48</td>
<td>0.79–2.76</td>
</tr>
<tr>
<td>Perineural invasion (negative vs positive)</td>
<td>5.24</td>
<td>1.26–21.73</td>
</tr>
</tbody>
</table>

All data with $P < 0.05$ are in bold. Only the factors with significant relevance in univariate analysis were considered for inclusion in the multivariable analysis.

GC, gastric cancer; HR, hazard ratio.
normal tissues (log2 fold change > 1, adjusted \( P < 0.05 \)). No notable differences were observed in the expression levels of other genes (Figure 1A).

After verification by GSE174237, among the four differentially expressed HNRNPs in TCGA-STAD, only HNRNPU had a difference between GC and control tissues, and its expression was significantly increased in GC (\( P < 0.05 \)) (Figure 1B). In 40 pairs of tissue samples, HNRNPU mRNA expression was elevated in GC tissues compared with the corresponding adjacent nontumor tissues (\( P = 0.04 \)) (Figure 1C).

The Expression of HNRNPU Increases with the Progression of Gastric Diseases, which Has a Good Diagnostic Value

HNRNPU protein showed predominant nuclear expression in gastric epithelial cells. From GS (\( n = 39 \)), GA (\( n = 39 \)), GD (\( n = 63 \)), EGC (\( n = 63 \)), and AGC (\( n = 131 \)), the expression of HNRNPU protein gradually increased significantly (Figure 2A). Except for the GA and GD groups (\( P = 0.122 \)), there was a statistically significant difference between the groups (\( P < 0.001 \)). Compared with control

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### Table A

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Hazard ratio (95% CI)</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variables</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HNRNPU Low vs high</td>
<td>1.84(1.03-3.29)</td>
<td>0.039</td>
</tr>
<tr>
<td>Sex Male vs female</td>
<td>1.12(0.59-2.11)</td>
<td>0.737</td>
</tr>
<tr>
<td>Age, years &lt;60 vs ≥60</td>
<td>1.16(0.65-2.07)</td>
<td>0.608</td>
</tr>
<tr>
<td>T</td>
<td>11.79(1.62-85.53)</td>
<td>0.015</td>
</tr>
<tr>
<td>N</td>
<td>7.37(2.77-23.94)</td>
<td>0.001</td>
</tr>
<tr>
<td>TNM stage</td>
<td>5.86(1.81-19.03)</td>
<td>0.003</td>
</tr>
<tr>
<td>Tumor location Antrum only vs body only vs multilobar</td>
<td>0.93(0.68-1.26)</td>
<td>0.631</td>
</tr>
<tr>
<td>Maximum diameter</td>
<td>3.72(1.15-12)</td>
<td>0.028</td>
</tr>
<tr>
<td>General classification Raised vs ulcer, diffuse infiltrative vs infiltrating</td>
<td>1.23(0.87-1.74)</td>
<td>0.239</td>
</tr>
<tr>
<td>Lauren classification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffuse type vs intestine type</td>
<td>0.29(0.09-0.95)</td>
<td>0.041</td>
</tr>
<tr>
<td>Growth pattern</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neured/clogged vs infiltrative vs diffuse infiltration</td>
<td>0.85(0.58-1.25)</td>
<td>0.405</td>
</tr>
<tr>
<td>Vessel carcinoma emboli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative vs positive</td>
<td>1.48(0.79-2.76)</td>
<td>0.22</td>
</tr>
<tr>
<td>Perineural invasion</td>
<td>5.24(1.26-21.73)</td>
<td>0.023</td>
</tr>
</tbody>
</table>

### Table B

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Hazard ratio (95% CI)</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variables</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HNRNPU Low vs high</td>
<td>2.19(1.16-4.15)</td>
<td>0.016</td>
</tr>
<tr>
<td>T</td>
<td>8.17(0.47-143.61)</td>
<td>0.151</td>
</tr>
<tr>
<td>T1 + T2 vs T3 + T4</td>
<td>11.01(2.47-48.75)</td>
<td>0.002</td>
</tr>
<tr>
<td>N</td>
<td>3.17(0.93-10.79)</td>
<td>0.065</td>
</tr>
<tr>
<td>TNM stage</td>
<td>0.60(0.08-4.85)</td>
<td>0.635</td>
</tr>
<tr>
<td>Maximum diameter</td>
<td>0.41(0.11-1.46)</td>
<td>0.168</td>
</tr>
<tr>
<td>Lauren classification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffuse type vs intestine type</td>
<td>3.18(0.71-14.29)</td>
<td>0.131</td>
</tr>
<tr>
<td>Positive vs positive</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3** The prognostic values of prognostic factors in gastric cancer (GC). The forest plots for the univariate (A) and multivariate (B) Cox regression analyses of prognostic factors with overall survival in GC. The red circles represent the value of hazard ratio.
tissues, the expression level of GC tissues was significantly increased ($n = 96$; $P < 0.001$) (Figure 2B). GS is not a precancerous lesion as a common lesion, so take GS as a control, the expression of HNRNPU in GC and its precancerous lesions [including GC (EGC + AGC) + gastric precancerous lesions (GA + GD)] was significantly increased ($P < 0.001$). The expression of HNRNPU was significantly higher in the GC group (EGC + AGC) compared with the noncancerous group (GS + GA + GD) ($P < 0.001$) (Figure 2, C–E).

As shown in Table 2 and Figure 2F, for diagnosing GC and its precancerous lesions, the AUC reached 0.911 (95% CI, 0.877–0.945; $P < 0.001$) with a best cutoff value of 3.5, yielding a sensitivity of 92.3% and a specificity of 83.4%. In terms of diagnosing GC, the AUC reached 0.847 (95% CI, 0.809–0.886; $P < 0.001$) with a best cutoff value of 4.75, yielding in a sensitivity of 81.8% and a specificity of 70.2%.

The correlation analysis between HNRNPU protein expression and clinicopathologic parameters in GC revealed significant differences based on sex ($P = 0.008$), with higher expression observed in male compared with female patients. In addition, there was a significant association with tumor size, where tumors $<5$ cm exhibited higher expression than tumors $>5$ cm ($P = 0.035$). No statistically significant differences were found among other groups ($P > 0.05$) (Supplemental Table S2).

Patients with High Expression of HNRNPU Protein Have a Poor Prognosis in GC

The best cutoff value of immunohistochemistry score for judging the prognosis of patients with GC was 8. The HNRNPU high expression group (immunohistochemistry score $>8$) exhibited a worse overall prognosis trend compared with the HNRNPU low expression group (immunohistochemistry score $\leq 8$) ($P = 0.055$). The results of subgroup analysis showed that, in female patients ($P < 0.001$), those aged $\geq 60$ years ($P = 0.048$), those who were *H. pylori* positive ($P = 0.002$), those with a multifocal tumor ($P = 0.009$), those with tumor size $\geq 5$ cm

Figure 4  The prognostic values of prognostic factors in gastric cancer (GC). A: Nomogram for predicting the 1-, 3-, and 5-year overall survival (OS) of patients with GC. B: Calibration curves of the nomogram for OS prediction at 1, 3, and 5 years. C: Receiver operating characteristic curves in predicting OS. D: Decision curve analysis.
(P = 0.001), those with a diffuse infiltrating type (P = 0.014), those with Lauren diffuse type (P = 0.034), those with T3 to T4 stage (P = 0.02), those with positive lymph node metastasis (P = 0.02), and those with TNM stage II to IV (P = 0.028) of patients with GC, those with high expression of HNRNPU had a significantly poor prognosis (Supplemental Figure S2).

In the univariate Cox regression model, HNRNPU protein expression, T stage, N stage, TNM stage, tumor maximum diameter, Lauren classification, and peripheral nerve invasion were significantly associated with GC prognosis (P < 0.05). Furthermore, multivariate Cox regression analysis showed that HNRNPU (P = 0.016) and lymph node metastasis (P = 0.002) were both independent risk factors affecting the prognosis of patients with GC, with an adjusted hazard ratio of HNRNPU of 2.19 (95% CI, 1.16–4.15) (Table 3 and Figure 3). To enhance the clinical relevance, a nomogram was developed that integrates prognostic indicators, such as HNRNPU expression and N stage, for predicting survival risk (Figure 4A). The calibration curves for overall survival rates at 1, 3, and 5 years demonstrated close alignment with the ideal reference line (Figure 4B). The nomogram exhibited a higher AUC of 0.785, whereas the HNRNPU expression demonstrated an AUC of 0.611 for 3-year overall survival prediction (Figure 4C). In addition, decision curve analysis demonstrated that the use of the nomogram model provided higher clinical benefits than the extreme curves (Figure 4D).

HNRNPU Exhibits Increased Expression in GC Cells and the Construction of the HNRNPU Knockdown GC Cell Model

Given the high expression of HNRNPU in GC tissues, it is speculated that HNRNPU might act as an oncogene in GC. To investigate the molecular function of HNRNPU, cell models were established in vitro. The initial analysis evaluated the expression level of HNRNPU in common GC cells by the Cancer Cell Line Encyclopedia database (Figure 5A). Then, two cell lines were selected that exhibited the highest HNRNPU expression levels. The study found a significant elevation of HNRNPU expression at both the mRNA and protein levels in HGC27 and AGS cells compared with the normal stomach cell line GES-1 (Figure 5B and C). Next, two different siRNAs were transfected to down-regulate HNRNPU in HGC27 and AGS cells. Quantitative real-time PCR and Western blot analysis verified that both si-HNRNPU#1 and si-HNRNPU#2 successfully down-regulated HNRNPU expression (Figure 5, D–F).

Figure 5  HNRNPU mRNA and protein expression in gastric cancer (GC) cell lines. A: HNRNPU mRNA expression in common GC cells by Cancer Cell Line Encyclopedia (CCLE) database. B and C: Box plots of HNRNPU expression in GES-1, AGS, and HGC27 cells at the mRNA (B) and protein (C) levels. D–F: The mRNA (D) and protein (E and F) knockdown efficiency of HNRNPU in AGS and HGC27 cells after being transfected with two siRNAs (si-HNRNPU#1 and si-HNRNPU#2). *P < 0.05, **P < 0.01, and ***P < 0.001. si-NC, control siRNA.
HNRNPU Promotes GC Cell Proliferation and Inhibits GC Cell Apoptosis

Cell Counting Kit-8 and EdU experiments were performed to detect the impact of HNRNPU on cell viability and cellular growth, respectively. The Cell Counting Kit-8 experiment revealed a notable decrease in cell viability at different time points in si-HNRNPU cells compared with control cells (Figure 6A). Similarly, the inhibition of cell proliferation in HGC27 and AGS cells was notably observed on the knockdown of HNRNPU (Figure 6, B and C). Flow cytometry was used to explore whether HNRNPU promotes GC cell viability and proliferation by regulating apoptosis. After HNRNPU knockdown, the population of late apoptotic cells increased, but the number of early apoptotic cells increased only in HGC27 cell line (Figure 7). These in vitro results revealed that HNRNPU could promote GC cell growth.

HNRNPU Promotes GC Cell Migration and Invasion

To further evaluate the migration and invasive ability of HNRNPU in GC cells, transwell experiments with or without Matrigel and wound healing experiments were performed. Subsequently, the proportion of cells that had been infected with siRNA was evaluated for invasiveness and metastasis in transwell assays at 24 hours, and the width of the wound was measured at 0, 24, and 48 hours after siRNA infection in wound healing assays. Inhibition of HNRNPU expression resulted in a significant reduction in the migratory and invasive abilities of both HGC27 and AGS cells compared with the control groups (Figure 8A). The wound healing assay also confirmed the conclusion that HNRNPU up-regulated the migration ability of GC cells (Figure 8B).

Identification of HNRNPU-Dependent AS Events by Transcriptome Analysis

As RNA-binding proteins, the main functions of HNRNPs are participating in alternative splicing, DNA damage repair, regulating telomerase activity, and stabilizing mRNA. To investigate the pathways through which HNRNPs perform their molecular function, Gene Set Enrichment Analysis was performed (Supplemental Table S3). The top two pathways of HNRNPU in Gene Set Enrichment Analysis were aminoacyl tRNA biosynthesis and spliceosome, as mentioned.
Therefore, the focus is on how HNRNPUs regulate alternative splicing to take a part in the occurrence and development of GC.

An analysis of unique identifier RNA-sequencing data was conducted to investigate the differential ASEs between control siRNA and si-HNRNPU AGS cells. With $P < 0.05$, $|\Delta \psi| > 0.1$, a total of 2893 ASEs were identified in 1898 genes that were significantly altered in the si-HNRNPU cells. The five main subtypes of ASEs are classified as follows: skipped exon, mutually exclusive exon, alternative 5' splice site, alternative 3' splice site, and retained intron: 2206 skipped exons in 1559 genes, 433 mutually exclusive exons in 351 genes, 98 alternative 5' splice sites in 95 genes, 132 alternative 3' splice sites in 125 genes, and 24 retained introns in 24 genes. Skipped exon was the predominant type, followed by mutually exclusive exon (Figure 9 and Supplemental Table S4). The alternative splicing genes were enriched in biological processes involved in many metabolic pathways, such as cellular metabolic process, primary metabolic process, and macromolecule metabolic process (Figure 10). Enriched Kyoto Encyclopedia of Genes and Genomes pathways included human T-cell lymphotropic virus type 1 infection, endocytosis, phosphatidylinositol signaling system, ubiquitin-mediated proteolysis, spliceosome, RNA transport, cell cycle, hippo signaling pathway, and so on (Figure 11). Specifically, the phosphatidylinositol signaling system is a common signaling pathway that plays a role in numerous cellular processes, including cell growth and apoptosis. The cell cycle refers to the entire process of cell growth and division, and it is strongly associated with the malignant biological behavior of GC cells. RNA sequencing of differential ASEs in GC cell line AGS with knockdown or non-knockdown of HNRNPU revealed that HNRNPU regulates AS in many important cancer-related genes and pathways, which were experimentally validated. Thus, HNRNPU possibly plays an important role in gastric tumorigenesis.

Figure 7  HNRNPU inhibits gastric cancer cell apoptosis in vitro. A and B: The percentage of apoptotic cells was demonstrated by flow cytometry in AGS (A) and HGC27 (B) cells following the knockdown of HNRNPU. C: The bar diagram showed percentage of cells in early and late apoptosis. ** $P < 0.01$. PI, propidium iodide; si-HNRNPU#1 and si-HNRNPU#2, HNRNPU siRNAs; si-NC, control siRNA.
Discussion

The HNRNPs, as important regulators involved in alternative splicing, can participate in the initiation and progression of cancers. To our knowledge, although there have been numerous studies demonstrating the significance of HNRNPs in tumor progression, the potential pro-oncogenic role of HNRNPU in GC progression is yet to be explored. Bioinformatic analysis and tissue sample verification indicated a significant increase in HNRNPU expression with the progression of gastric diseases. It can be used as an effective marker to distinguish GC and its precancerous lesions, as well as an independent risk factor affecting the prognosis of patients with GC. The in vitro cell models demonstrated that HNRNPU promoted cell proliferation, invasion, and migration and inhibited apoptosis. Through RNA sequencing, many up-regulated and down-regulated ASEs were identified in HNRNPU-knockdown GC cells, which enriched in many pathways related to the malignant biological behavior of GC cells, which provided multiple candidate targets for further mechanism research.

Initially, the expression of HNRNPs was verified in GC by bioinformatics analysis. Only HNRNPU was up-regulated in both TCGA and Gene Expression Omnibus data sets. Subsequent verification at the mRNA and protein levels confirmed that HNRNPU was highly expressed in GC tissues. HNRNPU is the largest member of the HNRNP family and is mainly distributed in the nucleus. According to the previous reports, HNRNPU is differentially expressed in a variety of cancers. It is highly expressed in neuroblastoma, lung cancer, and liver cancer, whereas its expression is low in pancreatic cancer. This study was the first to find that HNRNPU was significantly overexpressed in GC. The classic Correa model demonstrates that the occurrence of GC is a multistage model process, undergoing processes such as atrophic gastritis, intestinal metaplasia, and dysplasia, and eventually developing into GC. Early detection of precancerous lesions and early diagnosis of GC are important to improve patients’ survival rate and reduce the mortality rate. This study found that from GS, GA, GD, EGC, to AGC, HNRNPU expression gradually increased with the progression of gastric diseases.
suggesting that it might contribute to the occurrence and development of GC as a dysregulated oncogene. The diagnostic efficacy of HNRNPU was evaluated, and the expression of HNRNPU has ideal efficacy as a diagnostic marker for GC and its precancerous lesions. With the progression from healthy liver, cirrhosis, to liver cancer, the expression of HNRNPU increases gradually.\(^2^7\) On the basis of the results of bioinformatics analysis, some studies suggest that HNRNPU may be used as a molecular marker for the early diagnosis of pancreatic ductal adenocarcinoma.\(^2^8\) In the future, the diagnostic efficiency can be clarified by expanding the case detection, so as to better serve the screening and early diagnosis of GC.

In terms of the correlation of clinicopathologic parameters and the prognostic analysis, this study found that HNRNPU expression was closely related to pathologic features, such as sex and tumor size. Univariate and multivariate Cox regression analysis, high expression of HNRNPU, and lymph node metastases were observed to be independent prognostic factors in patients with GC. HNRNPU expression is significantly correlated with poor prognosis in various types of cancer, such as hepatocellular carcinoma,\(^3^4\) lung cancer,\(^3^5\) and breast cancer.\(^8\) The use of nomograms incorporating HNRNP expression levels and other clinical pathologic factors may improve the accuracy of prognostic predictions in patients with cancer. These tools may assist clinicians in identifying patients who are more susceptible to poor outcomes and developing personalized treatment strategies accordingly.\(^3^6\) For example, a nomogram incorporating HNRNPC and RNA binding motif protein 15 (RBM15) expression levels and T stage was developed to predict the survival of patients with adrenocortical carcinoma.\(^2^7\) This study established a nomogram based on HNRNPU expression and lymph node metastasis that may be a simple and efficient method to predict the overall survival rate of patients with GC, thereby facilitating informed clinical decision-making. However, more studies are required to confirm the effectiveness of the nomogram and to further explore the clinical utility of HNRNPU as a prognostic biomarker.

The dysregulation of HNRNPU plays a crucial role in regulating malignant biological behaviors in various cancers.\(^3^8,3^9\) Zhou et al\(^8\) reported the proliferation, migration, and invasion of invasive breast carcinoma cells were significantly suppressed \textit{in vitro} on HNRNPU knockdown. HNRNPU knockdown in hepatocellular carcinoma cells can lead to decreased cell growth and chromatin accessibility.\(^1^4\) In this study, HNRNPU knockdown GC cell lines were constructed to assess their changes in the malignant biological behavior. This study confirmed, for the first time, that HNRNPU may act as a regulatory factor to promote GC cell proliferation, migration, and invasion and inhibit GC cell apoptosis. This study further substantiated the significance of HNRNPU in contributing to the behavior of...
malignant tumors, indicating its involvement in tumor initiation and progression. However, the specific roles of HNRNPU in diverse tumor types and its underlying regulatory mechanisms remain incompletely understood.

The regulation of tumor cell biology behavior involving HNRNPU may be mediated through downstream target gene function or activity. For example, homeostatic factor *TM7SF3*, which is regulated by the tumor suppressor gene *TP53*, can form complexes with splicing factors, such as HNRNPU, to regulate its splicing activity. Xiao et al suggested that HNRNPU primarily regulated alternative splicing by modulating U2 small nuclear ribonucleoprotein maturation. Revealing the regulatory characteristics of downstream genes caused by HNRNPU in GC cells may help us further understand the molecular mechanisms of its functional role. AS of pre-mRNA produces multiple mature mRNAs and protein subtypes with different structural and functional characteristics. However, the dysregulation of AS will lead to the occurrence of abnormal protein subtypes, which may contribute to the development of tumors and the difficulty of treatment. In this study, RNA sequencing identified many up-regulated and down-regulated ASEs regulated by HNRNPU, and explored the potential pathways through which HNRNPU exerts its molecular function. Pathways associated with the experimentally confirmed malignant biological behaviors of GC cells were enriched, such as phosphatidylinositol signaling system, cell cycle, adherens junction, regulation of intrinsic apoptotic signaling pathway by p53 class mediator, and so on. In gastric cancer cells, abnormal activation of phosphatidylinositol signaling system pathway can promote cell proliferation and invasion by regulating gene AS, then proceed to affect drug resistance. In addition, function analysis also indicated that the dysregulation of these differential ASE genes may impact some metabolic pathways. As reported, HNRNPU, together with HNRNP and HNRPB, forms a protein complex that mediates RNA metabolism and gene regulation. Further research is needed to investigate how HNRNPU influences RNA metabolism levels and further promotes the progression of GC. On the whole, the results suggested that HNRNPU might play a malignant biological role by regulating ASEs. The mechanism of this post-transcriptional regulation requires investigation in future studies.

In conclusion, this investigation conducted a comprehensive analysis of HNRNPU expression patterns in GC. The findings demonstrated a notable increase in HNRNPU expression as the gastric diseases advanced, establishing its
potential as a valuable indicator for distinguishing GC and its precancerous lesions. Furthermore, it emerged as an autonomous prognostic factor with an impact on the outcomes of patients with GC. Functionally, HNRNPU displayed the capability to enhance the proliferation, migration, and invasion of GC cells, while concurrently inhibiting apoptosis. These observations suggest its potential malignant involvement in driving GC progression may be through the regulation of alternative splicing mechanisms.

Limitations of the Study

This study was limited to in vitro cell experiments, which restricts the generalization of the findings to the in vivo phenotype. Therefore, further investigations are necessary to determine the relevance and applicability of the findings in vivo. Because RNA splicing dysregulation has been identified as a promising target for cancer treatment, it is necessary to perform in-depth molecular mechanism studies to clarify the relevant mechanism of HNRNPU as a splicing factor in GC in the future.

Author Contributions

L.-p.S. designed the study and revised the manuscript; Y.Y. supervised the experiments and revised the manuscript; Y.-y.D. and M.-y.W. performed experiments and wrote the manuscript; J.-j.J. revised the manuscript; Y.-j.W. interpreted data; and H.L. interpreted data and performed statistical analysis. All authors analyzed data, wrote or revised the article, gave final approval of the version to be

Figure 11  Function enrichment analysis: Kyoto Encyclopedia of Genes and Genomes pathway analysis of differential alternative splicing event corresponds to genes. HTLV-I, human T-cell lymphotropic virus type 1.
published, and agreed to be accountable for all aspects of the work.

**Disclosure Statement**

None declared.

**Supplemental Data**

Supplemental material for this article can be found at http://doi.org/10.1016/j.ajpather.2023.10.007.

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