Original Article

Identification of a five m6A-relevant mRNAs signature and risk score for the prognostication of gastric cancer

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Background: N6-methyladenosine (m6A) is the most abundant form of methylation modification in eukaryotic cell messenger RNA (mRNA). However, the role of m6A in gastric cancer (GC), which is one of the most common gastrointestinal malignancies, is unclear. In this study, m6A-relevant mRNA signatures and risk scores were determined to predict the prognosis of GC.

Methods: The expression profiles and clinical information of 367 patients were downloaded from The Cancer Genome Atlas (TCGA). Cluster analysis and univariate Cox analysis were performed to identify the regulatory factors of RNA methylation associated with GC prognosis. A co-expression network was constructed using the WGCNA package in R. The correlations between module eigengenes and clinical traits were then calculated to identify the relevant modules. We used univariate Cox analysis to screen for genes that are significantly associated with prognosis in the module. We identified hub genes by least absolute shrinkage and selection operator (LASSO) and multivariate analysis and developed a Cox prognostic model. Finally, the hub gene expression values weighted by the coefficients from the LASSO regression were applied to generate a risk score for each patient, and receiver operating characteristic (ROC) and Kaplan-Meier curves were used to assess the prognostic capacity of the risk scores. The asporin (*ASPN*) gene in GC cell lines was verified via quantitative polymerase chain reaction (qPCR) and Western blot. Moreover, 5-ethynyl-2'-deoxyuridine (EdU) and transwell assays were applied to evaluate the effects of the proliferation, migration, and invasion abilities in GC cells after ASPN knockdown. Western blot verified the effects of *ASPN* on the phosphoinositide 3-kinase (PI3K)/serine/threonine kinase (AKT)/mechanistic target of rapamycin kinase (mTOR) pathway and epithelial-mesenchymal transition (EMT) pathway-related gene expression.

Results: Our results indicated that *AARD*, *ASPN*, *SLAMF9*, *MIR3117* and *DUSP1* were hub genes affecting the prognosis of GC patients. Besides, we found that *ASPN* expression was upregulated in GC cells. The knockdown of *ASPN* expression suppressed GC cell proliferation, migration, and invasion by deactivating the PI3K/AKT/mTOR and EMT pathways, respectively.

Conclusions: Our findings indicated that *ASPN* participates in the biological process of GC as an oncogene and may be a promising biomarker in GC.

Keywords: Gastric cancer (GC); N6-methyladenosine (m6A); asporin (ASPN)

Submitted Sep 19, 2022. Accepted for publication Oct 18, 2022.
doi: 10.21037/jgo-22-962
View this article at: https://dx.doi.org/10.21037/jgo-22-962

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Introduction

Global cancer statistics have shown that gastric cancer (GC) is considered one of the most invasive cancers and the third leading cause of tumor-related deaths (1). Over the past few decades, various strategies have been developed for GC treatment, which have provided significant improvements for the early diagnosis and treatment of GC (2). However, due to the atypical and insidious nature of early clinical symptoms of GC, only a small number of patients can be clearly diagnosed, while more than 60% of patients already have local or distant metastasis at the time of diagnosis (3). Therefore, there is an urgent need to develop an effective and effective strategy for the early diagnosis and treatment of GC.

N6-methyladenosine (m6A) modification refers to the methylation of the adenosine base at the nitrogen-6 position of the mRNA. It is a rich nucleotide modification that was first discovered in eukaryotic messenger RNA in 1974 (4). Increasing evidence demonstrated that dysregulated expression and genetic changes of m6A regulators were correlated with the disorders of multiple biological process in GC progression, including dysregulate cell death and proliferation, developmental defects, tumor malignant progression, impaired self-renewal capacity, and immunomodulatory abnormality (5). Three types of enzyme-modified m6A are subject to regulation, namely writers [methyltransferases, including Wilms tumor 1 associated protein (WTAP), KIAA1429, RNA binding motif protein 15 (RBM15), and methyltransferase like (METTL)3/14], readers [YTH domain-containing RNA binding proteins, and heterogeneous nuclear ribonucleoprotein including YTH N6-methyladenosine RNA binding protein 1/2/3 (YTHDF1/2/3), YTH domain containing 1 (YTHDC1), and heterogeneous nuclear ribonucleoprotein C (HNRNPC)], and erasers [demethylases, including ALKB homolog 5 (ALKBH5) and fat mass and obesity-associated protein (FTO)] (6,7).

In recent years, it has been found that the expression of HNRNPC is related to the development of malignant tumors and gliomas and is involved in the occurrence of glioblastoma multiforme, which can predict the prognosis (8). HNRNPC has been shown to promote oral squamous cell carcinoma carcinogenesis and can be an independent prognostic biomarker (9). Studies have also demonstrated the potential value of HNRNPC as a prognostic and therapeutic marker for GC and highlighted its important role in promoting the translation of human GC genes (10).

In this study, we downloaded the expression profile and clinical data of The Cancer Genome Atlas (TCGA). We determined the prognostic performance of m6A RNA, a methylation regulator, using the univariate Cox analysis method. In addition, we performed a weighted gene co-expression network analysis (WGCNA), least absolute shrinkage and selection operator (LASSO) regression analysis, and multivariate Cox analysis to identify the pivotal genes that might be regulated by the m6A RNA methylation regulators and are related to the prognosis of GC. Finally, according to the selected combination of pivot genes, a risk-scoring model was constructed to evaluate its application in the prognosis of GC. These hub genes are closely related to the m6A RNA methylation regulators, which provides new ideas for GC research. Asporin (ASPN), a new member of the leucine-rich small proteoglycan family, is a key component of the tumor stroma and has been reported to be abnormally expressed in some types of tumors. Based on the previous findings, in this study, we aimed to further investigate the biological function and molecular mechanism of ASPN in GC.

Methods

Patient datasets and m6A regulators

The mRNA expression data and corresponding clinical information of patients with GC were downloaded from TCGA. This study included the expression profiles of 309 patients with complete follow-up data in TCGA database. The Tcgabiollinks package was used to download TCGA data. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

In this study, we included numerous m6A methylation regulators, including writers [RBM15, KIAA1429, METTL3, METTL14, zinc finger CCCH domain-containing protein 13 (ZC3H13), and WT1-associated protein], readers [YTH m6A RNA-binding protein 1 (YTHDF1), YTH m6A RNA-binding protein 2 (YTHDF2), YTHDC1, YTH domain-containing 2 (YTHDC2), and HNRNPC], and erasers (α-ketoglutarate dependent dioxygenase ALKBH5 and FTO). To study the differential expression of m6A RNA methylation regulators in tumor and normal tissues, we analyzed the mRNA expression profile of TCGA-GC (including 58 normal samples and 309 tumor samples). Cluster analysis was applied to the m6A RNA methylation regulators, and heatmaps and violin maps were presented to display the differences. The pheatmap package and the vioplot package in R software were used to draw the plots. In addition, we performed a univariate Cox analysis to identify m6A-related

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genes related to the prognosis of GC (m6A regulatory genes with a P value <0.05 were considered statistically significant).

Co-expression network construction and identification of clinically significant modules

The co-expression network was constructed using the WGCNA package in R (11). Genes with variances greater than all variance quartiles were selected, and those genes with larger variances and mean variations in different samples were considered. The expression data profile of the selected genes was qualified and the samples were clustered to detect outliers. Gene clustering modules were identified based on the clinical features (including the expression of the m6A regulatory genes that we selected before) and topological overlap matrix-based dissimilarity (12). Next, the relevance between clinical features and module eigengenes was used to identify the correlated modules. Highly correlated modules were considered to be very significant for our research.

Identification of hub genes and construction of the risk score model

We selected the modules of interest where the genes in the modules were defined as highly relevant to certain clinical traits. Next, univariate Cox analysis and LASSO were used to screen for genes that were significantly correlated to prognosis in the module (P<0.01 was considered significant). The Cluster Profiler R package was used for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of the screened genes, and P<0.05 was considered a statistically significant difference. TCGA samples were randomly divided into two groups: 153 samples were tested, and 156 samples were verified. There was no statistically significant difference in the expression of HNRNPC and other clinicopathological variables between the two groups. LASSO regression was performed in the training set (n=156), to screen out GC prognosis-related hub genes based on the lambda.min. LASSO was analyzed using the "glmnet" R package. The expression values of hub gene weighted by the coefficients from the LASSO regression generated a risk score for each GC patient. Finally, the "Survminer" package in R was performed to identify the optimal cutoff for the risk score, while ROC and Kaplan-Meier curves were applied to evaluate the prognostic power of risk score.

Cell culture

The normal gastric epithelium cell line (GES-1) and human GC cell lines (MKN45, MKN28, MGC-803, SGC-7901, and BGC-823) were purchased from the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified eagle medium (DMEM) or (Roswell Park Memorial Institute) RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) in a humidified incubator with 5% carbon dioxide (CO_2) at 37 ℃.

Cell transfection

The recombinant lentiviruses of Asporin (*ASPN*) knockdown (shASPN) and the negative control (shCON) were synthesized by GeneChem (Shanghai, China). MKN45 and SGC-7901 cells were infected with lentivirus at a multiplicity of infection (MOI) of 10 using enhanced infection solution (Eni.S) + polybrene. The target sequences of *ASPN* are as follows: shRNA1, 5'-GCTGTATCTGTCCCACAATCA-3'; shRNA2, 5'-GCTTACCACCAACTTTATTGG-3'; shRNA3, 5'-GCTCTGCCAAACCCTTCTTTA-3'; GAPDH forward, 5'-GGCAAATTCAACGGCACAGT-3', and reverse, 5'-AGATGGTGATGGGCTTCCC-3'.

Western blot analysis

Protein exaction and Western blot were conducted as previously described. The antibody against *ASPN* (Abcam, ab31303) was purchased. The following antibodies were used: antibodies against E-cadherin (Proteintech, 20874-1-AP), Vimentin (Proteintech, 10366-1-AP), N-cadherin (Proteintech, 22018-1-AP), matrix metalloproteinase-9 (MMP9) (CST, #13667), phosphoinositide 3-kinase (PI3K) (Proteintech, 20584-1-AP), phosphorylated phosphoinositide 3-kinase (p-PI3K) (CST, #17366), phospho-mechanistic target of rapamycin kinase (p-mTOR) (Proteintech, 67778-1-Ig), mTOR (Proteintech, 66888-1-Ig), p-AKT (Proteintech, 66444-1-Ig), and AKT (Proteintech, 60203-2-Ig). The protein bands were visualized using an efficient chemiluminescence (ECL) detection kit (Thermo).

Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted by TRIzol (Invitrogen). qPCR was subjected to the SYBR Master Mixture (TaKaRa) and the LightCycler 480 II Detection System (Roche). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative RNA levels (GAPDH

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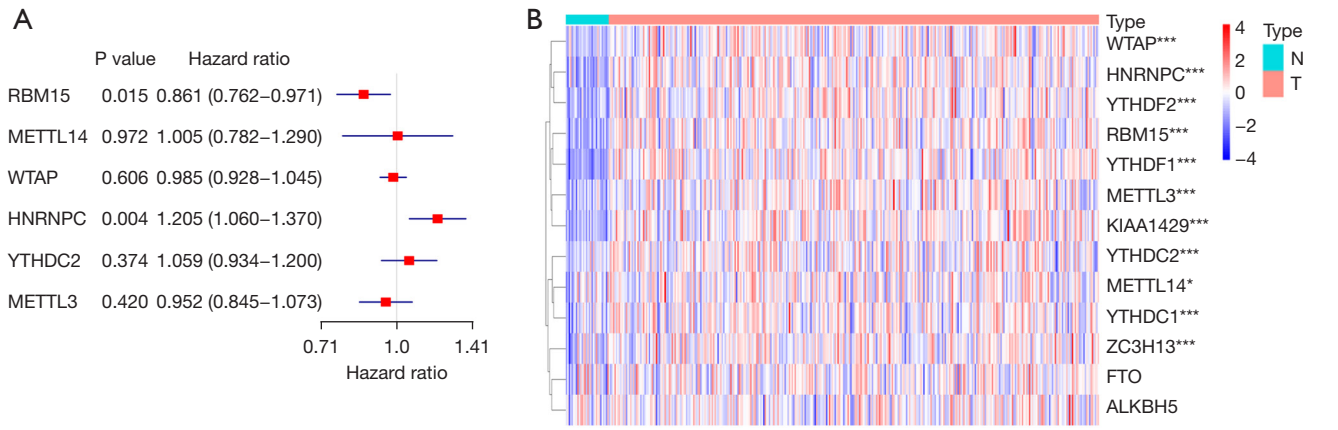

Figure 1 Identification of prognostic m6A RNA methylation regulators. (A) Forest plot of univariate Cox analysis in gastric cancer (HNRNPC is related to prognosis and is shown as a pathogenic factor in TCGA). (B) Heatmap exhibiting the mRNA expression alteration of m6A-associated genes in TCGA datasets. The expression levels of different genes in normal samples and tumor samples were significantly different, and the HNRNPC expression in tumor samples was significantly higher than that in normal samples. *, P<0.05; ***, P<0.001. N, normal tissue; T, tumor tissue; TCGA, The Cancer Genome Atlas; m6A, N6-methyladenosine; RBM15, RNA binding motif protein 15; METTL14, methyltransferase like 14; WTAP, Wilms tumor 1 associated protein; HNRNPC, heterogeneous nuclear ribonucleoprotein C; YTHDC1, YTH domain containing 1; YTHDC2, YTH domain containing 2; METTL3, methyltransferase like 3; YTHDF2, YTH N6-methyladenosine RNA binding protein 2; YTHDF1, YTH N6-methyladenosine RNA binding protein 1; ZC3H13, zinc finger CCCH domain-containing protein 13; FTO, fat mass and obesity-associated protein; ALKBH5, ALKB homolog 5.

as internal control). The sequences of primer are listed: *ASPN*, 5'-CATGGACTAATCTGTGGGAGC-3' (forward) and 5'-CAAAGCCAGGAATAATAGGAGC -3' (reverse).

Cell proliferation assay

For the EdU assay *in vitro*, the positive cells in each group were calculated and analyzed by a fluorescence microscope (Olympus) with the Cell-Light^{TM} EdU Apollo567 kit (Ribo) following the manufacturer's instructions.

Transwell assays

Migration and invasion assays were performed in 24-well plates with inserts (8-μm pore size, Corning Inc., Corning, NY, USA) without or with Matrigel. Cells (1×10^5 cells/well) were added into the upper chambers in serum-free media. Then, a FBS culture medium was added to the lower chambers. After incubation at 37 ℃ in 5% CO_2, the upper chamber was cleaned with a cotton swab and the lower chamber was fixed with 4% paraformaldehyde, dyed with 0.1% crystal violet, and then washed with water three times. An inversion microscope (Leica) was used to photograph the cells under a microscope. The number of cells per field was calculated using Image J (National Institutes of Health).

Statistical analysis

We used SPSS 20.0 (SPSS Inc., Chicago, IL, USA) to conduct statistical analyses. The differences between the control and experimental groups were analyzed using the Student's *t*-test and one-way analysis of variance (ANOVA). P<0.05 was considered statistically significant.

Results

Identification of the prognostic m6A RNA methylation regulators

Univariate Cox analysis was performed to identify the gene map of m6A related to the prognosis of liver cancer patients (forest) to identify m6A regulators with P<0.05. Based on this analysis, we found that the high expression of HNRNPC is more closely related to the prognosis of the following patients: HNRNPC of GC in TCGA data set showed P<0.05 and hazard ratio (HR) >1, which can be considered pathogenic factors that negatively affect the prognosis of GC (*Figure 1A*).

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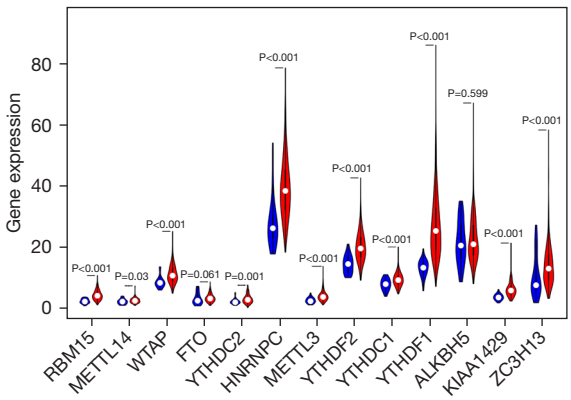

Figure 2 Differential expression of the m6A RNA methylation regulators. Vioplot visualizes the differential m6A RNA methylation regulators in gastric cancer (blue is normal and red is gastric cancer). HNRNPC showed higher expression in tumor tissues. RBM15, RNA binding motif protein 15; METTL14, methyltransferase like 14; WTAP, Wilms tumor 1 associated protein; FTO, fat mass and obesity-associated protein; YTHDC2, YTH domain containing 2; HNRNPC, heterogeneous nuclear ribonucleoprotein C; METTL3, methyltransferase like 3; YTHDF2, YTH N6-methyladenosine RNA binding protein 2; YTHDC1, YTH domain containing 1; YTHDF1, YTH N6-methyladenosine RNA binding protein 1; ALKBH5, ALKB homolog 5; ZC3H13, zinc finger CCCH domain-containing protein 13; m6A, N6-methyladenosine.

Differential expression of the m6A RNA methylation regulators

TCGA data set included 58 normal samples and 309 tumor samples. Heatmaps and violin maps were drawn according to the different gene expression levels. According to the results, we could conclude that HNRNPC exhibited higher expression in the tumor samples compared to the normal samples. RBM15, WTAP, METTL3, YTHDF2, YTHDF1, YTHDC1, YTHDC2, KIAA1429, ZC3H13, and HNRNPC were found to have significantly higher expression in tumors than in normal tissues (*Figure 1B*). As shown in the violin plot (*Figure 2*), the expressions of YTHDC2, RBM15, ZC3H13, METTL3, YTHDC1, KIAA1429, WTAP, YTHDF1, YTHDF2, and HNRNPC in normal tissues were significantly lower than those in tumor tissues, and the differences were statistically significant ($P<0.05$).

Co-expression network construction

As mentioned above, this study calculated the variance of the expression of each gene in all samples, and taking the variance value greater than the quartile as the standard, a total of 6,685 genes were screened out. A hierarchical clustering tree was constructed from these 6,685 genes in 309 tumor samples. Next, the 309 samples and sample clinical information were hierarchically clustered (*Figure 3A*). To construct a scale-free network, we needed to select the appropriate weighting factor, β, while moderately retaining the average connectivity of each gene node. We finally chose $\beta=5$ to construct the co-expression network (*Figure 3B*). After determining the β value, a total of 15 modules were identified (*Figure 3C*).

Correlation between modules and phenotypes

According to the correlation between each module and the clinical phenotype, we selected the modules that were significantly associated with prognosis and HNRNPC expression. The turquoise and magenta modules were significantly highly associated with HNRNPC expression (positive values indicate a positive correlation, while negative values indicate a negative correlation) and had a stronger correlation with the pathologic stage. This indicates that the genes in the two modules may be regulated by HNRNPC and play a role in the prognosis of GC patients (*Figure 4*).

Identification of hub genes

To further determine the prognostic genes regulated by HNRNPC, we selected the turquoise and magenta modules to conduct further research on 1,538 genes. A preliminary selection of prognostic genes was made by univariate Cox,

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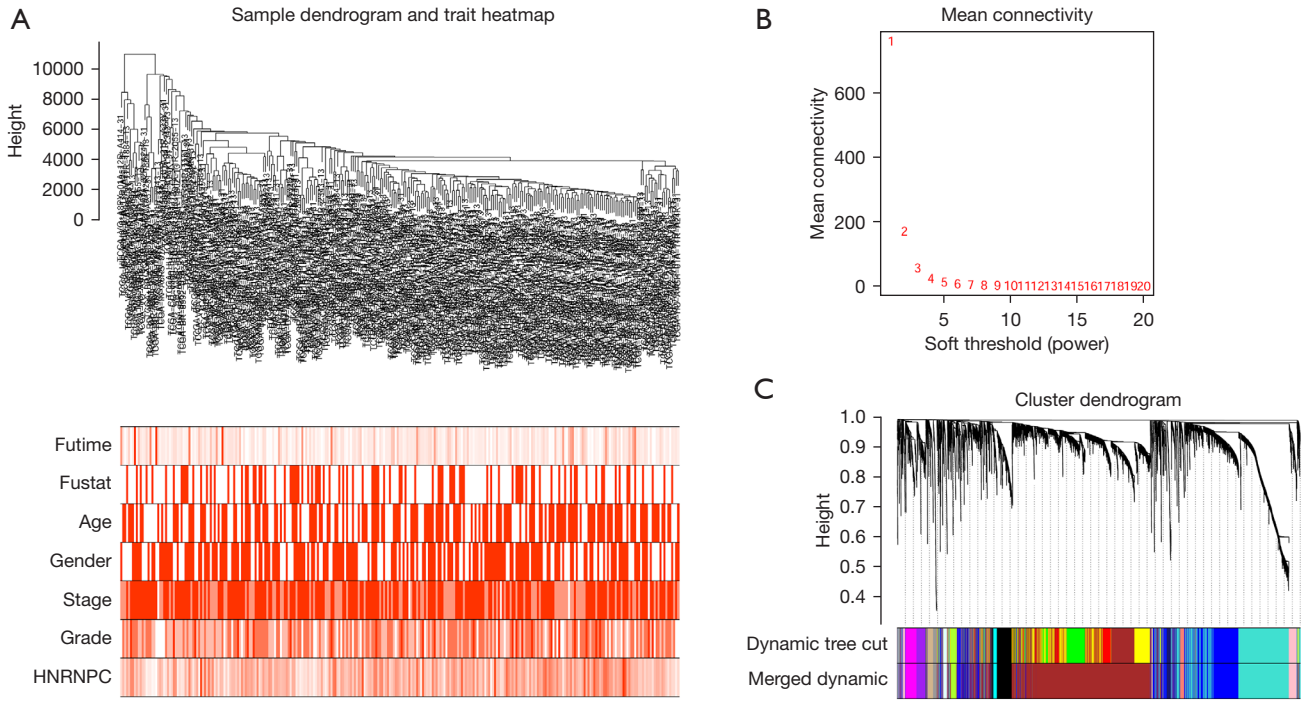

Figure 3 Co-expression network construction. (A) Clustering of 309 tumor samples and clinical information (where the number/stage is larger, a darker color is shown). (B) The scale-free index is calculated under different β and the average connectivity is calculated under different β (the numbers in the figure indicate the corresponding soft threshold power. The approximate scale-free topology can be achieved at a soft threshold power of 5). (C) Gene clustering tree diagram. Based on the common topological overlap, each color module represents a module that contains a set of highly connected genes.

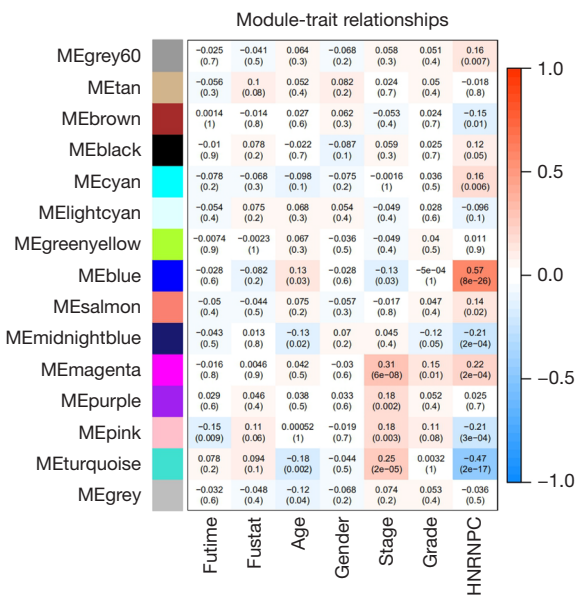

Figure 4 Correlation between modules and phenotypes. Each row in the figure corresponds to a gene module, and each column corresponds to a clinical phenotype. The numbers in brackets indicate the P value, and the numbers without brackets indicate the correlation. ME, module eigengene; HNRNPC, heterogeneous nuclear ribonucleoprotein C.

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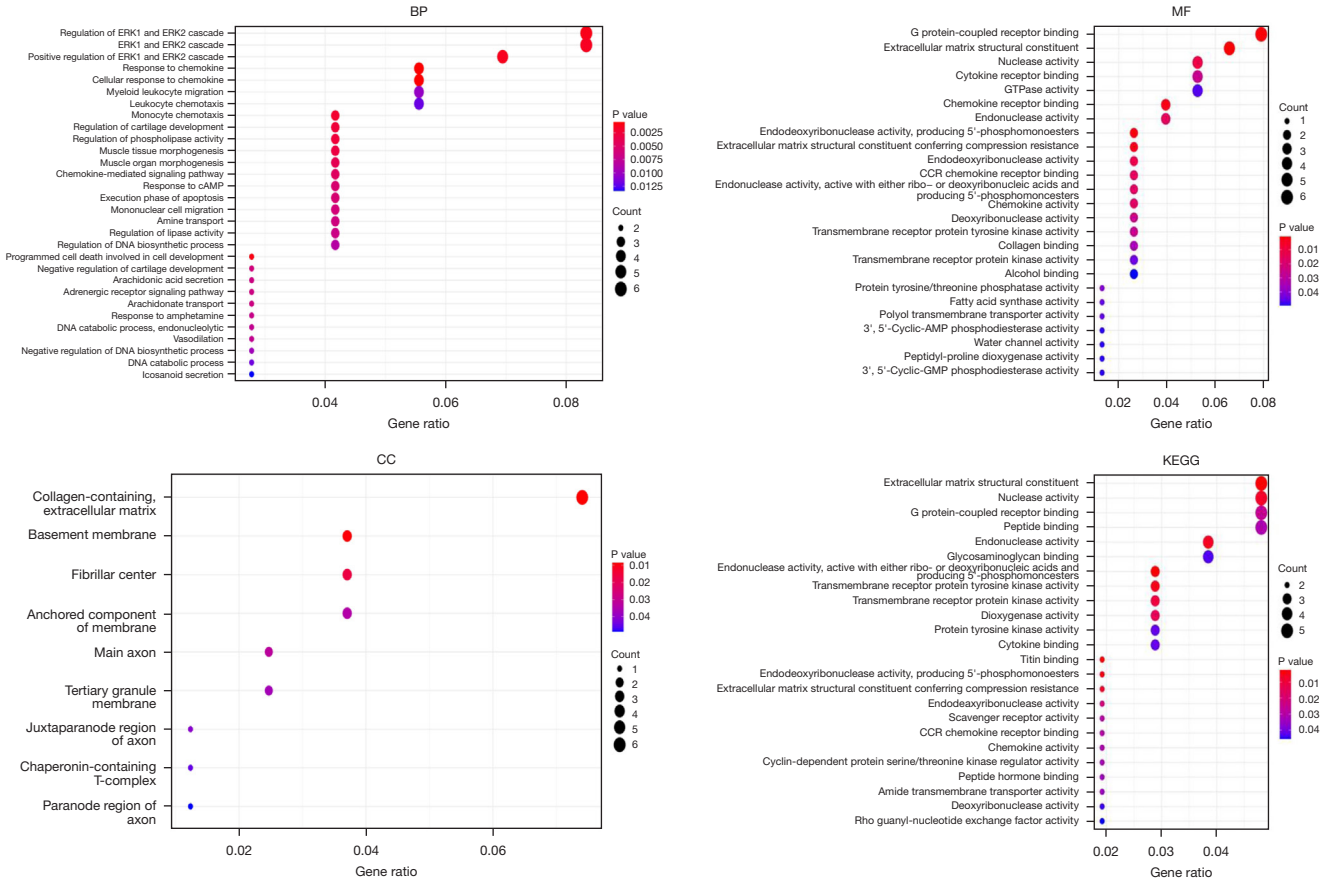

Figure 5 Functional enrichment analysis of 98 selected genes. KEGG, Kyoto Encyclopedia of Genes and Genomes; ERK, extracellular signal-regulated kinase; cAMP, cyclic adenosine monophosphate; CCR, CC chemokine receptor; GMP, guanosine monophosphate; MF, molecular function; BP, biological process; CC, cellular component.

where P<0.05 was used as a cutoff for screening prognostic genes, and 98 genes were selected. These 98 genes were analyzed using the clusterProfiler R package for GO and KEGG pathway analyses.

In terms of biological processes in the GO analysis, the genes were mainly enriched in the "positive regulation of extracellular regulated protein kinases (ERK1 and ERK2) cascade", "cellular response to chemokine", "monocyte chemotaxis", "regulation of cartilage development", "regulation of phospholipase activity", "programmed cell death involved in cell development", and "chemokine-mediated signaling pathway". In terms of cell components, the differentially expressed genes (DEGs) were mainly enriched in the "collagen-containing extracellular matrix", "basement membrane", and "fibrillar center". In terms of molecular functions, the DEGs were mainly enriched in the "extracellular matrix structural constituent", "G protein-coupled receptor binding", "endodeoxyribonuclease", "activity, producing 5'-phosphomonoesters", "chemokine receptor binding", and "extracellular matrix structural constituent conferring compression resistance".

KEGG pathway analysis demonstrated that the 98 selected genes were significantly enriched in "endonuclease activity, active with either ribo or deoxyribonucleic acids and producing 5'-phosphomonoesters", "extracellular matrix structural constituent", "titin binding", "endodeoxyribonuclease", "activity, producing 5'-phosphomonoesters", "extracellular matrix structural constituent conferring compression resistance", "endonuclease activity", "nuclease activity", "transmembrane receptor protein kinase activity", "dioxygenase activity", etc. (*Figure 5*).

Next, the 309 TCGA samples were randomly divided into a training set and a testing set. The tableone R package was used to describe the clinical information difference between the internal training and testing sets. The results showed that the expression of HNRNPC and the other

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Table 1 There was no significant difference between the two groups of clinical phenotypes

Parameters	Training set	Testing set	P value
N	156	153	
Age >65 years (%)	54%	58%	0.494
Gender, male (%)	49%	48%	0.106
Grade			0.257
1	2	3	
2	53	60	
3	101	90	
Stage			0.098
I	24	21	
II	56	44	
III	64	68	
IV	12	20	
OS time, median [IQR]	404.5 [0, 3,720.0]	383.0 [0, 2,171.0]	0.273
OS event =0/1 (%)	106/50	96/57	0.337
HNRNPC, mean (SD)	39.94 (9.98)	39.35 (10.51)	0.507

OS, overall survival; IQR, interquartile range; HNRNPC, heterogeneous nuclear ribonucleoprotein C; SD, standard deviation.

clinicopathological variables was not significantly different between the two groups (*Table 1*).

In the experimental group, a total of 98 prognostic genes were screened for the two modules using LASSO and multivariate Cox analyses. The results showed that alanine and arginine-rich domain-containing protein (*AARD*), *ASPN*, SLAM family member 9 (*SLAMF9*), *MIR3117*, and dual specificity phosphatase 1 (*DUSP1*) are real hub genes that are associated with patient prognosis (*Figure 6A-6C*). In TCGA datasets, a significant correlation was observed between the expression of HNRNPC and that of the hub genes (*Figure 6D*).

Risk scores

Five genes were identified and subsequently used to construct a prognostic gene signature. The risk score = −(0.166195281 × *AARD* + 0.016850602 × *ASPN* + 0.591607997 × *SLAMF9* + 0.591607997 × *MIR3117* + 0.00276337 × *DUSP1*), and we used the Survminer R package to find the optimal cutoff for the risk score, while ROC and Kaplan-Meier curves were used to assess the prognostic ability of the risk scores. We plotted the risk score distribution, time-dependent ROC curve, and survival analysis of the training and testing sets (*Figure* 7). Our results indicated that the five-gene signature had a poor performance in terms of survival prediction (P<0.05).

ASPN overexpression in GC

The expression of *ASPN* was remarkably elevated in GC tissues compared to that in paracancerous normal tissues in TCGA database. To further confirm the expression of *ASPN*, we used qPCR and Western blot to verify its expression in different GC cell lines. The mRNA and protein expression levels of *ASPN* were significantly improved in the GC cell lines (MKN45, MKN28, MGC-803, SGC-7901, and BGC-823) compared to that in GES-1 (*Figure 8A,8B*). These results demonstrated that ASPN is overexpressed in GC.

ASPN knockdown inhibits GC cell proliferation, migration, and invasion in vitro

To investigate the role of *ASPN* in GC, three kinds of shRNA against *ASPN* were transfected into MKN45 and

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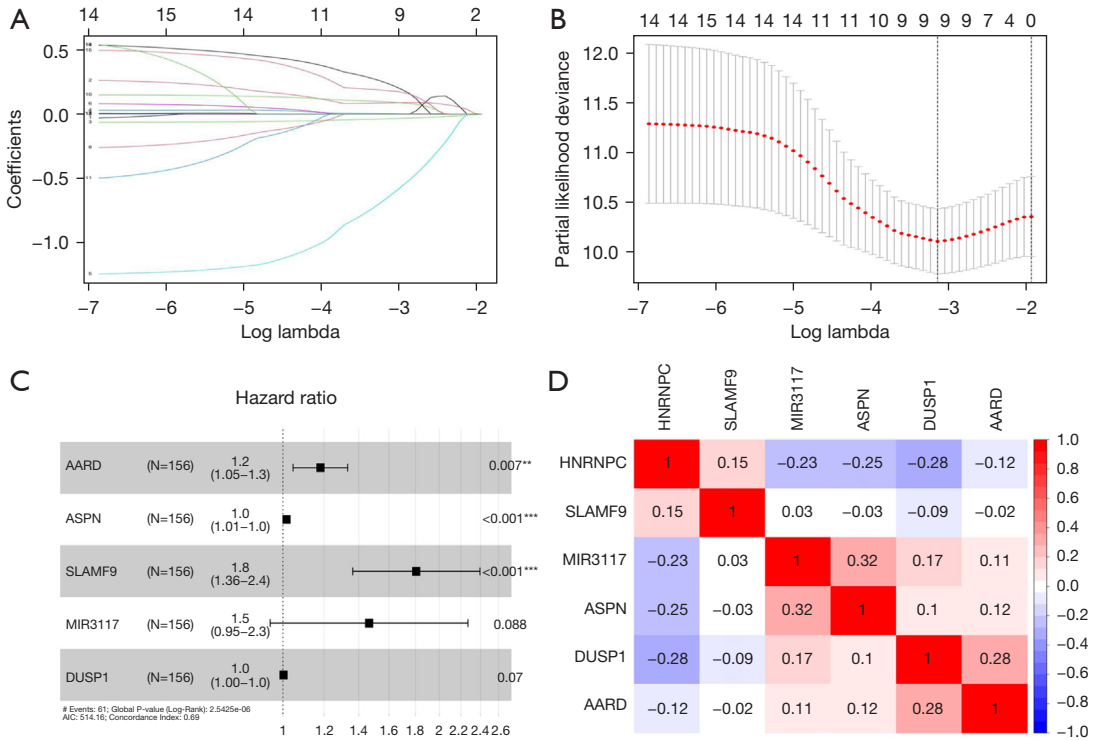

Figure 6 Identification of hub genes. (A) Distribution of LASSO coefficients for 98 genes. (B) Partial likelihood deviation of the LASSO coefficient distribution. Vertical dashed lines indicate the lambda.min and lambda.1se. (C) Forest plot of the multivariate Cox analysis in gastric cancer. (D) HNRNPC expression and hub genes showed a significant correlation. **, P<0.01; ***, P<0.001. TCGA, The Cancer Genome Atlas; AARD, alanine and arginine-rich domain-containing protein; ASPN, asporin; SLAM9, SLAM family member 9; MIR3117, microRNA 3117; DUSP1, dual specificity phosphatase 1; HNRNPC, heterogeneous nuclear ribonucleoprotein C; LASSO, least absolute shrinkage and selection operator; AIC, Akaike information criteria.

SGC-7901 cells, and the mRNA and protein expression levels of *ASPN* were decreased by qPCR and Western blot. The efficiency of shASPN#2 and shASPN#3 knockdown *ASPN* was higher than shASPN#1 in MKN45 and SGC-7901 cells (*Figure 9A-9D*).

Also, EdU proliferation assays were performed to assess the effects of *ASPN* on the DNA replication of GC cells. We found that knockdown of *ASPN* significantly inhibited cell proliferation ability compared to their corresponding control cell lines in MKN45 and SGC-7901 cells (*Figure 10*).

Transwell assays were used to detect the effect of *ASPN* on the migration and invasion abilities of GC cells. We observed that *ASPN* knockdown inhibited the migration and invasion abilities of GC cells (*Figure 11*). Taken together, these results indicate that *ASPN* participates in the regulation of proliferation, migration, and invasion of GC.

ASPN may participate in mediating the activation of the PI3K/AKT/mTOR and epithelial-mesenchymal transition (EMT) pathways

As previously reported, the PI3K/AKT/mTOR pathway is important for cell proliferation and development under physiological and pathological conditions. In this study, we investigated the effects of *ASPN* on the PI3K/AKT/mTOR pathway. It was demonstrated that *ASPN* depletion downregulated the phosphorylation of PI3K, AKT, and mTOR protein levels (*Figure 12A*). There was only a slight change in the expressions of PI3K, AKT, and mTOR in each group.

Furthermore, our study confirmed that *ASPN* could affect the migration and invasion functions of GC cells; therefore, we probed the levels of proteins related to the EMT pathway. *ASPN* downregulation elevated the protein expressions of E-cadherin protein expression and decreased

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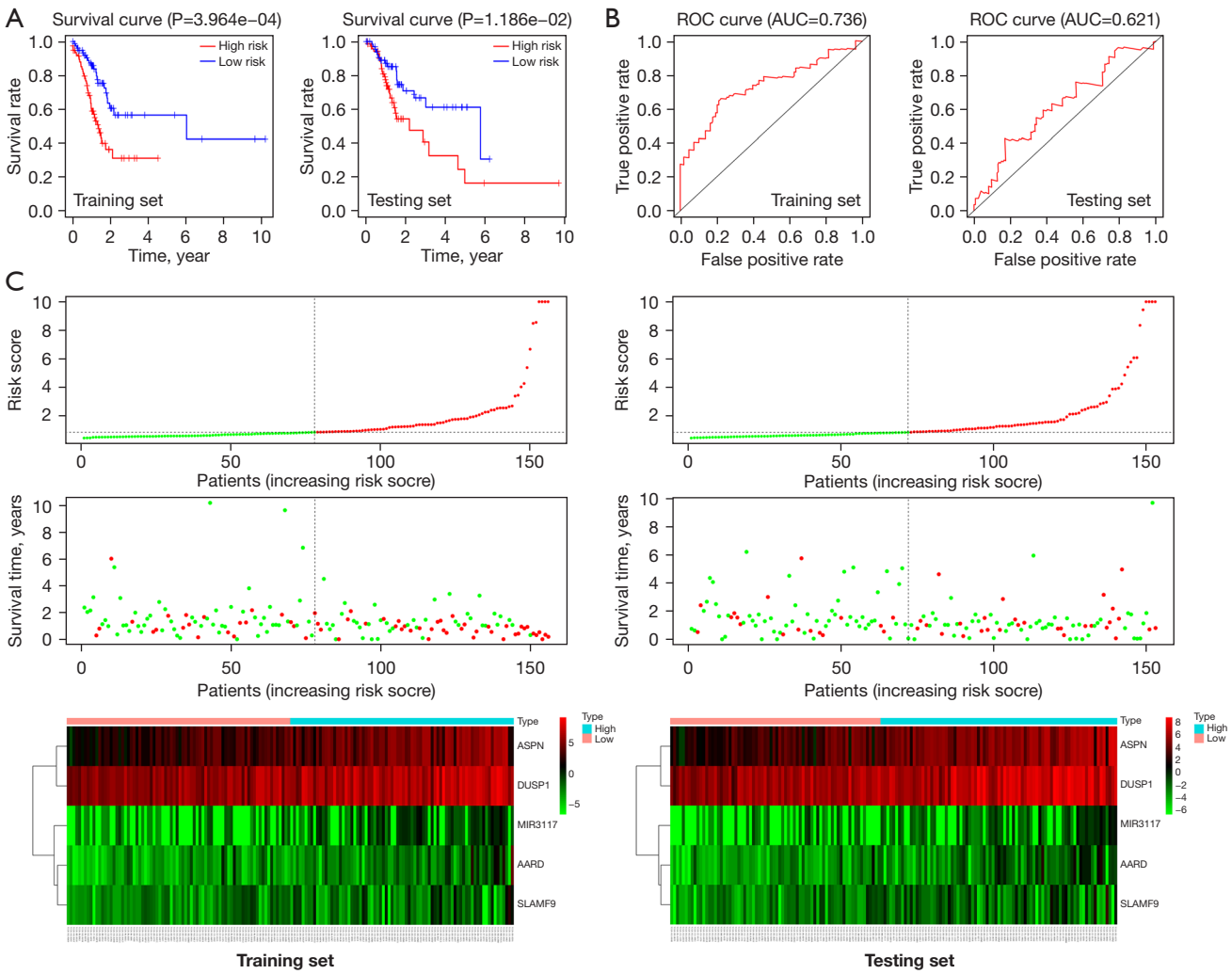

Figure 7 Risk score, heatmap of mRNA expression, time-dependent ROC analysis, and Kaplan-Meier curve of the five-gene signature in the training and testing sets. (A) Kaplan-Meier curve of the five-gene signature. (B) Time-dependent ROC analysis. (C) Risk score, heatmap of mRNA expression. AUC, the area under the ROC curves; ROC, receiver operating characteristic; ASPN, asporin; DUSP1, dual specificity phosphatase 1; MIR3117, microRNA 3117; AARD, alanine and arginine-rich domain-containing protein; SLAMF9, SLAM family member 9.

Vimentin, N-cadherin, and MMP9 (*Figure 12B*). Overall, the results above indicated that *ASPN* overexpression could promote GC cell progression and aggression by regulating the PI3K/AKT/mTOR and EMT pathways.

Discussion

m6A modification is the most common modification in human mRNA (13) and is considered to be a new epigenetic regulator of mRNA processing and translation. Numerous studies have revealed that the maladjustment of m6A is closely related to abundant physiological and pathological phenomena, including carcinogenesis (14), obesity, immune maladjustment, and so on (15,16). In recent years, mounting evidence has confirmed that m6A-related genes play a vital role in the genesis and development of GC (17,18). For example, Lin *et al.* reported that METTL3 inhibits the mobility and proliferation of human GC cells and leads to the inactivation of the AKT signaling pathway, indicating that it may be a meaningful and potential target for the treatment of human GC (19). Moreover, it has been found that the transfer of HNRNPC location may be related to the chemoresistance of GC, suggesting the potential usefulness of HNRNPC as a prognostic and therapeutic

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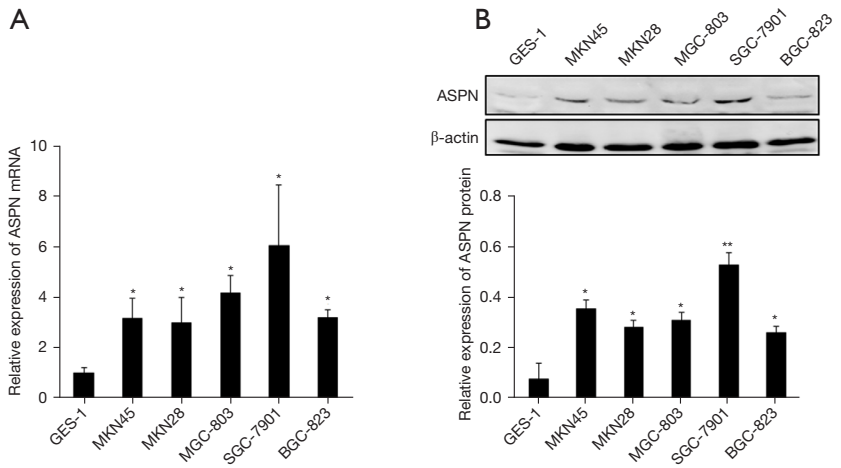

Figure 8 *ASPN* expression in GC cells. (A,B) *ASPN* is highly expressed in GC cells at the mRNA and protein levels. *, P<0.05; **, P<0.01. ASPN, asporin; GC, gastric cancer.

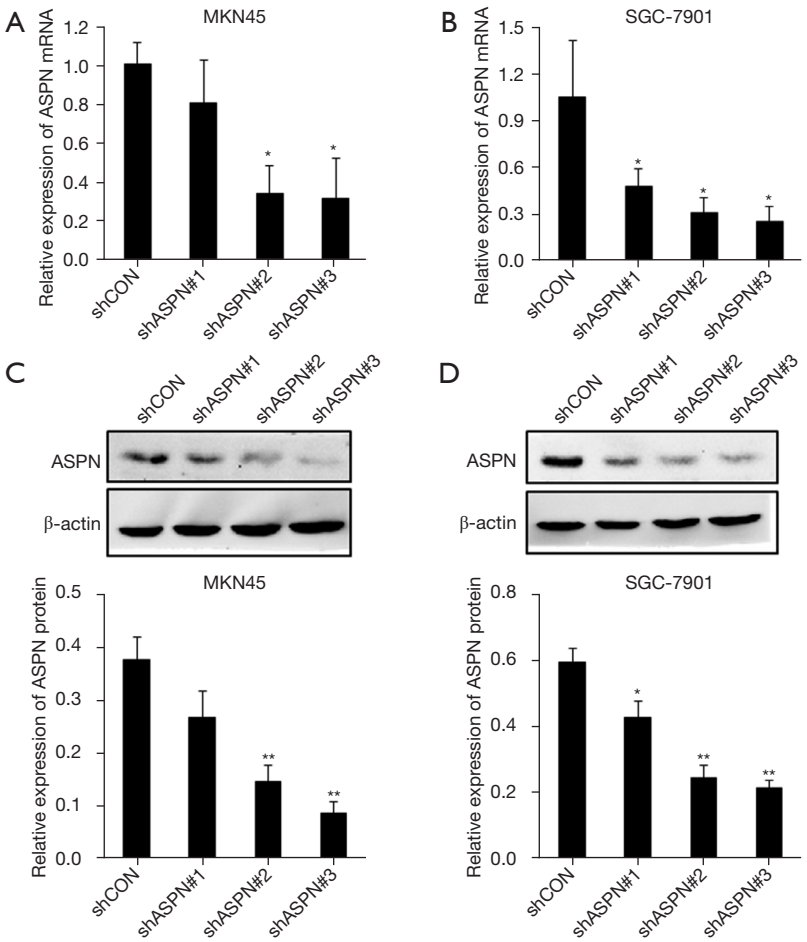

Figure 9 *ASPN* affected cell proliferation in GC. (A-D) The efficiency of *ASPN* knockdown (shASPN#1, shASPN#2, and shASPN#3) in MKN45 and SGC-7901 cells was measured by qPCR and Western blot. *, P<0.05; **, P<0.01. ASPN, asporin; GC, gastric cancer; CON, control; qPCR, quantitative PCR.

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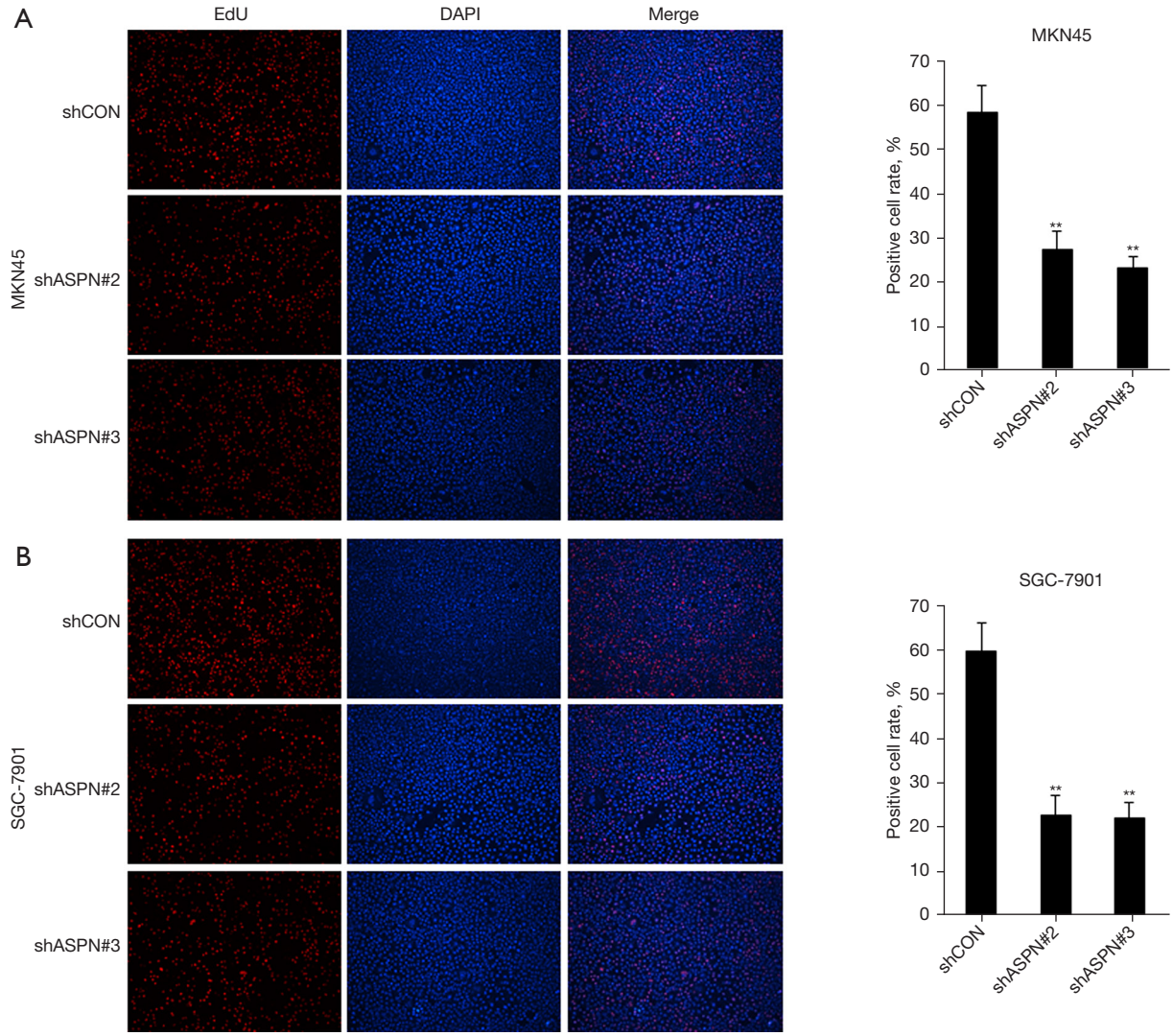

Figure 10 EdU assay was performed to detect the DNA replication abilities of MKN45 (A) and SGC-7901 (B) cells after infection of shASPN or shCON lentiviruses. Magnification, ×200. **, P<0.01. CON, control; ASPN, asporin.

marker of GC (20). Pi *et al.* reported that YTHDF1 directly promotes the translation of the key Wnt receptor frizzled7 (FZD7) in an m6A-dependent manner, so that the mutant YTHDF1 enhances the expression of FZD7, which ultimately leads to the over-activation of the Wnt/β-catenin pathway and promotes the occurrence of GC (21).

In this study, we first evaluated the expression of HNRNPC in GC and found that the expression of HNRNPC in tumor samples increased significantly. As an effective internal ribosome entry site (IRES) activator, HNRNPC is related to the establishment and maintenance of a malignant phenotype. It is regulated by increasing the level of IGF1R and ultimately promotes the occurrence of GC (22). As an important m6A methyltransferase, HNRNPC has been found to play a potentially crucial role in a variety of physiological and biochemical functions, and it is also related to the occurrence and development of many cancers.

We identified five hub genes (*AARD*, *ASPN*, *SLAMF9*, *MIR3117*, and *DUSP1*) that may be regulated by HNRNPC. In previous studies, these five genes were found to be involved in the development of several diseases. *ASPN* promotes the migration and invasion of colorectal cancer cells via the transforming growth factor β/the 2/3 members

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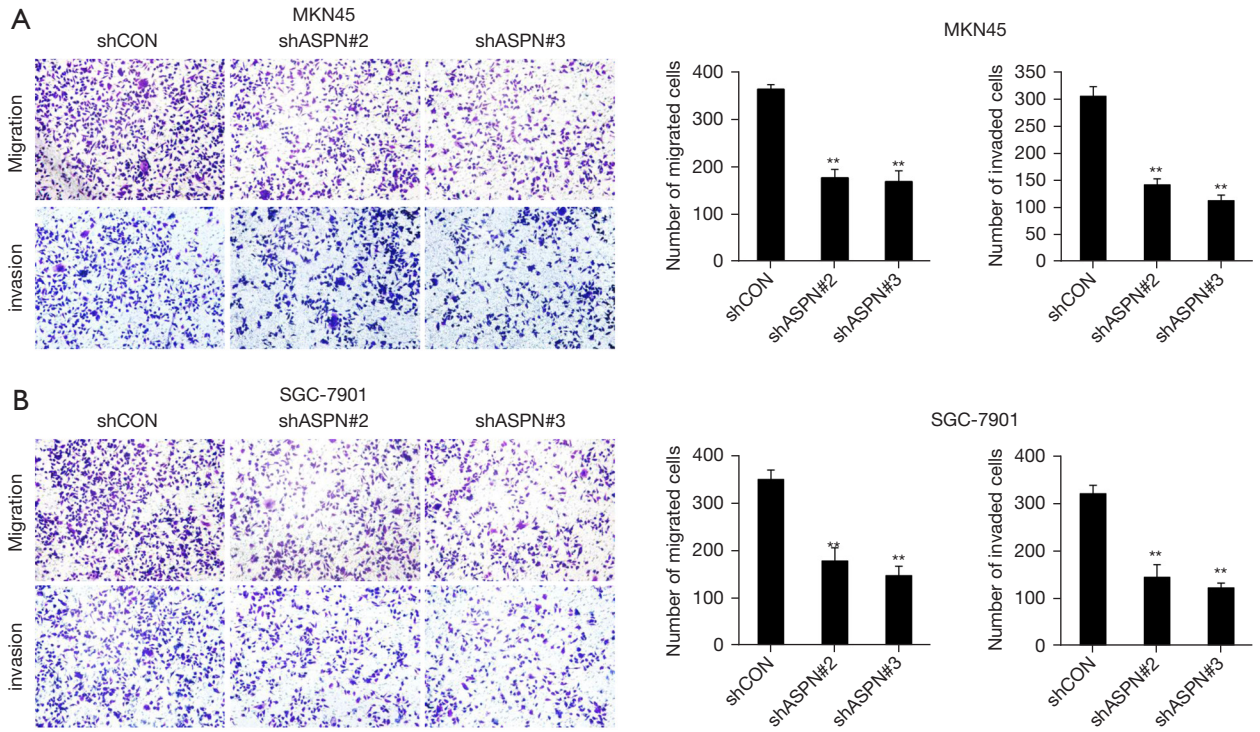

Figure 11 Transwell assay was used to evaluate the effects of ASPN knockdown on the cell migration and invasion of MKN45 (A) and SGC-7901 (B) cells. Crystal violet staining; magnification, ×200. **, P<0.01 ASPN, asporin; CON, control.

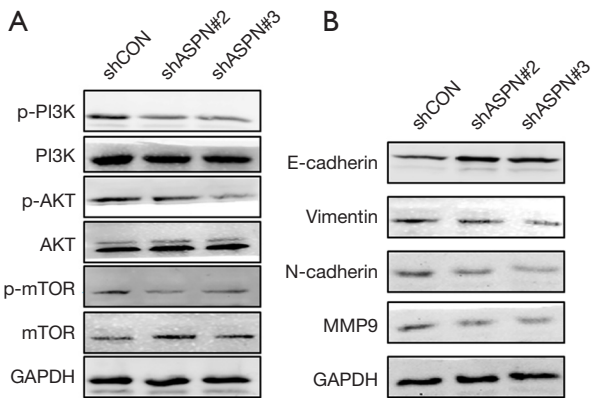

Figure 12 *ASPN* affects the expression of proteins related to the PI3K/AKT/mTOR (A) and EMT (B) pathways. ASPN, asporin; CON, control; PI3K, phosphoinositide 3-kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mTOR, mechanistic target of rapamycin kinase; MMP9, matrix metalloproteinase-9; EMT, epithelial-mesenchymal transition.

of the Smad family (TGF-β/Smad2/3) pathway and could serve as a potential prognostic biomarker in colorectal cancer patients (23). It has also been found that *ASPN* promotes cell proliferation by interacting with proteasome 26S subunit ubiquitin receptor, non-ATPase 2 (PSMD2) and down-regulation of its effectors, and serves as a potential therapeutic target in GC (24). The expression of *SLAMF9* in melanocyte lesions may indicate genetic susceptibility to the development of malignant melanoma, which suggests that *SLAMF9* plays an important role in melanoma biology (25). It has been reported that miR-3117 participates in the proliferation of HepG2 cells by targeting PH domain and leucine-rich repeat protein phosphatases (PHLPP)-like (PHLPPL), thereby participating in the occurrence and development of liver cancer (26). Elevated *DUSP1* expression is related to tumor progression, drug resistance, and poor prognosis, and can be used as a predictive biomarker for apatinib treatment (27). However, there are few studies on *AARD*.

Our research indicated that there is a certain connection between these five hub genes and HNRNPC; however, this remains to be verified by further experiments. In this study, we performed experiments to show that *ASPN* expression is upregulated in GC cells. Also, we found that the knockdown of *ASPN* expression suppressed GC cell proliferation, migration, and invasion by deactivating the PI3K/AKT/mTOR and EMT pathways, respectively.

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Conclusions

In summary, our research revealed a risk model consisting of five m6A-relevant genes, which may be useful for the prediction and diagnosis of GC. This discovery also provides a foundation for basic medical research on m6A methylation in GC. In addition, our findings suggested that *ASPN* might play a vital role in GC as a possible therapeutic biological target.

Acknowledgments

Funding: This study was supported by grants from the Nantong Science and Technology Project (Nos. MS12018060, QA2020008, MB2020008, and JC2020057) and the Scientific Research Fund of Jiangsu Provincial Health Committee (No. H2019049).

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-962/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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(English Language Editor: A. Kassem)

Cite this article as: Yin L, Feng S, Sun Y, Jiang Y, Tang C, Sun D. Identification of a five m6A-relevant mRNAs signature and risk score for the prognostication of gastric cancer. J Gastrointest Oncol 2022;13(5):2234-2248. doi: 10.21037/jgo-22-962

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