Am J Transl Res 2023;15(9):5808-5825
www.ajtr.org /ISSN:1943-8141/AJTR0151608

Original Article

The cancer/testis antigen HORMAD1 promotes gastric cancer progression by activating the NF-κB signaling pathway and inducing epithelial-mesenchymal transition

Geng Bian[1,2], Weiyu Li[3,4], Dabing Huang[5], Qi Zhang[6], Xiping Ding[7], Xiaodong Zang[8], Yingquan Ye[1,2], Jie Cao[9], Ping Li[1,2]

[1]Department of Chinese Integrative Medicine Oncology, The First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui, China; [2]Department of Integrated Traditional Chinese and Western Medicine, Anhui Medical University, Hefei 230022, Anhui, China; [3]National Clinical Research Center of Digestive Diseases, Beijing 100050, China; [4]Liver Research Center, Beijing Key Laboratory of Translational Medicine in Liver Cirrhosis, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China; [5]Department of Oncology, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei 230001, Anhui, China; [6]Department of Urology, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei 230001, Anhui, China; [7]Department of Geriatrics, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei 230001, Anhui, China; [8]Department of Pediatrics, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei 230001, Anhui, China; [9]Department of Respiratory, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei 230001, Anhui, China

Received June 6, 2023; Accepted September 12, 2023; Epub September 15, 2023; Published September 30, 2023

Abstract: Objectives: HORMAD1 is a cancer/testis antigen (CTAs) that regulates DNA homologous recombination, mismatch repair, and other tumor characteristics. However, its role and regulatory mechanisms in gastric cancer remain unclear. Methods: We performed transcriptomic profiling on seven gastric cancers and paired tissues; HORMAD1 was significantly upregulated in gastric cancer samples and was related to poor prognosis survival. Furthermore, cancer pathway microarray, bioinformatic analysis, western blot, and immunochemistry assay demonstrated that HORMAD1 affected the NF-κB signaling pathway. Results: In vitro and vivo studies confirmed that HORMAD1 knockdown inhibited cell growth and invasion, whereas overexpression reversed these effects. Mechanistically, HORMAD1 regulates the epithelial-mesenchymal transition process (EMT) via the NF-κB pathway by increasing the phosphorylation levels of NF-κB (p-65) and Iκκ-β. Downstream target genes of the NF-κB signaling pathway, such as c-Myc, CyclinD1, may be involved in HORMAD1-induced tumorigenesis in gastric cancer (GC). Conclusions: HORMAD1 plays an important role in gastric cancer progression and could be a promising prognostic biomarker and therapeutic target.

Keywords: HORMAD1, gastric cancer, cell proliferation, migration and invasion, NF-κB signaling pathway

Introduction

Gastric cancer (GC) is the fifth most common cancer and the third leading cause of cancer death globally. In East Asian countries, especially China, the incidence of GC is as high as 42% [1, 2]. Presently, the diagnosis and treatment of gastric tumors have been greatly improved with advances in diagnostic and surgical techniques [3]. However, because of the low early diagnosis rate, most patients are diagnosed at an advanced GC stage, leading to a 30-50% five-year mortality rate [4]. Therefore, exploring the mechanisms underlying GC pro-

gression and identifying novel therapeutic targets and biomarkers for diagnosis and treatment is vital.

Studies suggest that the NF-κB signaling pathway has been widely demonstrated as one of the most commonly activated and essential pathways for EMT and GC progression [5, 6]. Moreover, NF-κB as a transcription factor has also been identified in many cancers and is thought to promote tumor progression [7]. Furthermore, several studies have suggested the activation of the transcription factor NF-κB by numerous regulatory proteins involved in cell cycle, proliferation, apoptosis, and migration [8] that is composed of two distinct subunits (p50 and p65) and is subject to regulation at multiple levels [9]. Under normal conditions, NF-κB remains inactive in the cytoplasm due to its binding with inhibitor proteins known as IκBs [10]. Upon activation, phosphorylation of the inhibitor by IκB kinase (IKK) triggers the degradation of IκB-α, allowing the NF-κB subunit p65 to translocate to the nucleus, where it induces its transcriptional activity [11]. Thus, activating the NF-κB signaling pathway plays an important role in tumor progression; however, the molecular function of NF-κB activation in GC remains unclear.

HORMAD1 was originally identified as cancer/testis antigen (CTA46), a member of the CTAs family, normally expressed in the testes and diverse cancers [12]. HORMAD1, like other CTAs proteins, participates in tumor initiation, progression, metastasis, and drug resistance and is closely correlated with poor prognosis. For instance, previous studies have shown that HORMAD1 promotes EMT and enhances growth and metastasis through the Wnt signaling pathway in lung cancer [13]. Another study found that HORMAD1 can induce chemoresistance and radioresistance by promoting homologous recombination to ensure DNA damage repair in lung adenocarcinoma [14, 15]. Moreover, compelling evidence shows that HORMAD1 expression and poor prognosis are associated with increased mutation load and genomic instability in many cancers [12, 16]. Furthermore, reports have indicated that the down-regulation of HORMAD1 enhances apoptosis when combined with docetaxel, leading to reduced levels of VEGF protein and microvessel density in human epithelial ovarian cancer both in vivo and in vitro [17]. Recently, it has been indicated that HORMAD1 is an independent prognostic factor in triple-negative breast cancers with cyclophosphamide treatment [18]. Notably, it has also been documented that HORMAD1 was over-expressed in > 45% of GC specimens tested [19]. However, the role of HORMAD1 in gastric cancer growth and progression and the potential underlying mechanism has not been investigated.

In our study, we focused on the clinical importance and biological function of HORMAD1 in GC. Using RNA-seq, we discovered that HORMAD1 was significantly up-regulated in GC tissues and confirmed by the TCGA database to be associated with a poor prognosis. Further experiments showed that HORMAD1 had a higher expression in GC tissues and cell lines. Additionally, loss- and gain-of-function demonstrated that HORMAD1 promotes the proliferation, migration, and invasion of GC cells in vitro and in vivo. Mechanistically, HORMAD1 promotes EMT and GC progression through activating the NF-κB signaling pathway.

Materials and methods

Clinical tissue collection

In this study, 46 GC tissue samples and matched normal adjacent gastric tissues were studied at the First Affiliated Hospital of USTC, University of Science and Technology of China (USTC), from 2019 to 2021, and none of the patients were treated with any preoperative therapy. All patients provided written informed consent for the clinical research use of their tumor tissues. This study was approved by the Ethics Committee of USTC (No. 2019-X(H)-001).

RNA-sequencing

RNA-seq analysis of cells and tissue samples was performed by Gene Denovo Biotechnology Co. (Guangzhou, China). RNA was purified and fragmented to construct the RNA-seq library for sequencing. The cDNA fragments were purified using a QiaQuick PCR extraction kit. After agarose gel electrophoresis, suitable fragments were used as templates for PCR amplification. Real-time PCR was used to characterize the sample library. Finally, the library was sequenced using Illumina HiSeq™ 4000.

Analysis of HORMAD1 gene expression and Kaplan-Meier survival analysis based on The Cancer Genome Atlas database

HORMAD1 gene expression data were obtained from The Cancer Genome Atlas (TCGA, http://tcgadata.nci.nih.gov/tcga/). To evaluate the effect of HORMAD1 expression on survival, we conducted Kaplan-Meier analysis and generated corresponding survival curves, hazard ratios (HRs), 95% confidence interval (CIs), and log-rank *p*-values (p) using the Kaplan-Meier plotter (http://kmplot.com) platform.

Immunohistochemical staining

For patient tissues, Immunohistochemical (IHC) staining was performed following standard protocols [20]. The percentage of positive cells was scored as follows: 0 (negative), 1 (≤ 10%), 2 (> 10%-50%), 3 (> 50%-80%), and 4 (> 80%). Staining intensity was rated as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). The target protein expression was determined using the following criteria: low (score < 6) and high (score ≥ 6). As previously described, IHC staining was performed for the mice plant tumor using anti-Ki67, anti-HORMAD1, and anti-p-NF-κB (p-p65) antibodies. Two pathologists independently assessed the results.

Cell culture

All human gastric cell lines were stored at the Anhui Provincial Key Laboratory of Tumour Immunotherapy and Nutrition Therapy. RPMI 1640 medium (RPMI 1640, Gibco, USA) was used, and all cells were supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 2% penicillin/streptomycin (Trans Gen Biotech, Beijing, China). For inhibition treatment, cultured cells were incubated with 10 μmol/L BAY-117082 (Selleck, USA) for 48 h.

Lentivirus infection

The lentivirus expressing short hairpin RNA (shRNA) targeting HORMAD1 was designed and synthesized by Genechem (Shanghai, China). The lentivirus overexpressing HORMAD1 was designed and synthesized by Genechem (Shanghai, China). The sh-RNA target sequence is described in Supplementary Table 1. The efficiency of the lentivirus infection was validated using western blot and qRT-PCR.

RNA extraction and RT-qPCR analysis

Total RNA was isolated from the tissues and cells using an RNA extraction kit (Analytik Jena, Germany) for qRT-PCR. The complementary DNA (cDNA) was reverse transcribed using the Trans-Script All-in-One First-Strand cDNA Synthesis kit (Trans Gen Biotech, Beijing). The analysis was performed using Green qPCR Super Mix kits (Trans Gen Biotech, Beijing). The primer sequences used in this study are listed in Supplementary Table 2.

Western blotting (WB) analysis

Total protein was extracted from the cells and tissues using the RIPA buffer. The cell and control nuclear and cytoplasmic protein extracts were prepared using nuclear and cytoplasmic protein extraction kits. Protein concentrations were detected using BCA kits and heated at 97°C for 10 min. The heated proteins were separated on a 10% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes. These membranes were then incubated for 15 min at room temperature with a protein-free rapid-blocking buffer. Subsequently, the blots were incubated with HORMAD1, CyclinD1, E-cadherin, p21, vimentin, c-Myc, NF-κB (p65), phospho-NF-κB (p-p65), phospho-IκBα, IκBα, phospho-Iκκ-β, Iκκ-β, β-actin, and GAPDH primary antibodies at 4°C overnight. After washing with TBST, membranes were hybridized with an appropriate secondary antibody at room temperature for 1 h. Finally, images of the WB bands were obtained using ChemiCapture, and the intensity in each group was measured using ImageJ software. GAPDH and β-actin were used as internal controls. The antibodies in this study are shown in the Supplementary Table 3.

Cell counting kit 8 (CCK-8) assay

For the CCK-8 assay, stably transfected cells were seeded in 96-well plates at a density of 2,000 cells per well after various treatments. The absorbance was measured at 450 nm.

Colony formation

The stably transfected cells were plated into 6-well plates and cultured in a medium containing 10% FBS and 2% penicillin/streptomycin; the medium was changed every 2-3 days. After 10-14 days, if colonies were observed, the

plates were washed with PBS, and the cells were fixed and stained with crystal violet for 20 min. After that, cells were observed under a microscope (BX51; OLYMPUS, Japan).

EDU assay

EDU staining was conducted using the Beyo-Click™ EdU Cell Proliferation Kit with Alexa Fluor 594 (Beyotime Biotechnology, China) per the manufacturer's instructions. Images were acquired using a fluorescence microscope (BX51; OLYMPUS, Japan) and quantified using ImageJ software.

Cell migration and invasion assay

The invasive potential of the GC cells was measured using Matrigel (BD, Franklin Lakes, NJ, USA) and transwell inserts (8 μm, Costar, Manassas, VA, USA) containing polycarbonate filters with 8-μm pores. The inserts were coated with 50 μl of 1 mg/ml Matrigel matrix according to the manufacturer's instructions. Images were acquired using a fluorescence microscope and quantified using ImageJ software.

Xenograft tumor formation assay

All animal experiments were approved and were performed on 12 five-week-old male BALB/c nude mice that were purchased from the Laboratory Animal Centre of Anhui Medical University. Moreover, the MKN-45 cells were stably transfected with NC-shRNA or HORMAD1-shRNA. Subsequently, 0.1 ml MKN-45 cells (2×10^7 cells/ml) were subcutaneously injected into the right and left sides of the abdomen of each nude mouse. The mice were randomly distributed to the NC-shRNA and HORMAD1-shRNA groups (n = 6). Tumour volume was measured every 3 days for 30 days and calculated using the following formula: volume = (Length × Width^2)/2. The tumor tissues were weighed, and IHC staining was used to identify tissue sections expressing Ki-67, HORMAD1, and p-p65. All animal experiments followed the institutional guidelines and were approved by the experimental Animal Ethics Committee of USTC (No. 2021-N(A)-258).

Statistical analysis

All experiments were repeated three times. Statistical analyses were conducted using SPSS 23.0, and the results were visualized using GraphPad Prism 8.0 (V8, USA). Student's t-test (two-tailed) or one-way ANOVA was used to compare the means of two or three groups, whereas the correlation between HORMAD1 expression and clinicopathological variables was calculated using either the chi-square or Fisher's exact test. Spearman's correlation analysis was used to evaluate the correlation between HORMAD1 and p-p65. Statistical significance was set at $P < 0.05$.

Results

HORMAD1 is upregulated in GC tissues

To explore the unique genes contributing to GC progression, RNA-seq analysis was performed using total RNA isolated from seven GC samples and seven paired matched adjacent normal tissues; 1,894 upregulated and 167 downregulated genes were identified using threshold fold change > 1.0 and $P < 0.05$. These samples were further classified into three subgroups: tumor vs matched adjacent tissues (n = 7), high differentiated tumor vs matched adjacent tissues (n = 4), and low differentiated tumor vs matched adjacent tissues (n = 3). Of these screened genes, 823 crossover upregulated genes were selected for further study, including HORMAD1, KLK5, MAGEA10, CST1, HOXC-10, MUC-2, DKK4, REG3G, POU3F3, and SERPINB4 (**Figure 1A**). Subsequently, following differential gene expression analysis between the three groups and upstream gene set enrichment analysis, Gene Ontology (GO) enrichment analysis indicated that the most abundant biological processes mainly included epithelial-mesenchymal cell proliferation and migration processes (**Figure 1B**). Regarding cellular components, the most enriched categories included "junction" and "passive transmembrane transporter activity" (**Figure 1C**). Next, functional enrichment of differential gene expression (DEGs) was performed using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis and Gene Ontology (GO) terminology analysis. KEGG analysis showed that these differentially expressed genes were significantly enriched in multiple cancer-related signaling pathways, such as metabolism-related and focal adhesion-related pathways (**Figure 1D**). Consistent with these findings, Gene Set

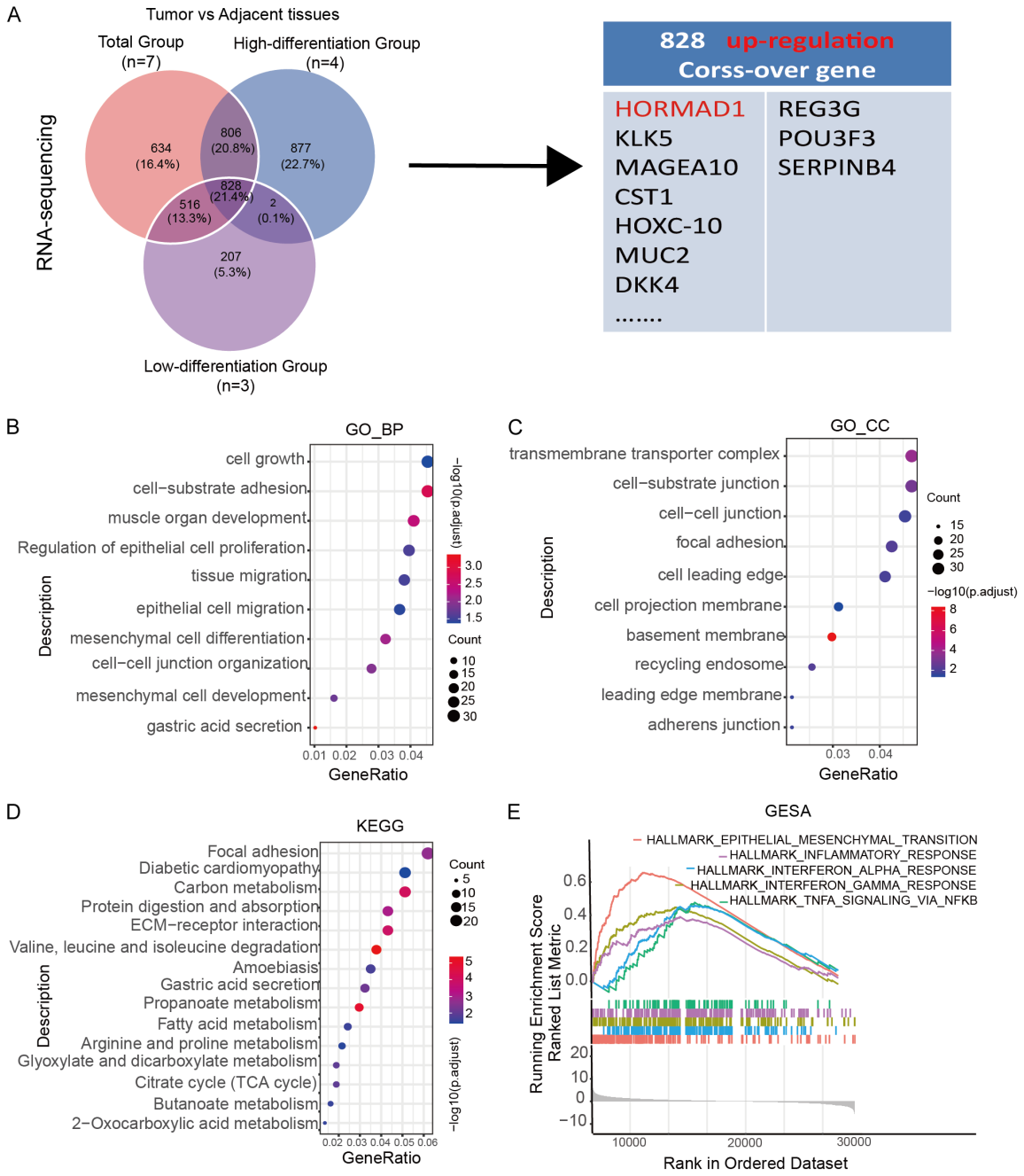

Figure 1. HORMAD1 was upregulated in GC tissues by RNA-seq. A. Venn diagrams show the overlap between the differentially expressed genes ($P < 0.05$) from GC and adjacent tissues (n = 7) with low differentiation tumour group vs matched adjacent samples and high differentiation tumour group (n = 4) vs matched adjacent samples for upregulated clusters. B, C. GO enrichment analysis of DEGs was mainly related to regulating epithelial cell proliferation, migration, and mesenchymal cell differentiation and development. D. KEGG pathway enrichment analysis of DEGs was mainly related to focal adhesion based on the high-throughput RNA sequencing database. E. GSEA analysis revealed that HORMAD1 was mainly related to epithelial-mesenchymal transition and TNF-α and NF-κB signaling and interferon-gamma response based on a high-throughput RNA sequencing database. mRNAs.

Enrichment analysis (GSEA) revealed that the epithelial-mesenchymal transition signatures, the interferon alpha response, and TNF-α via NF-κB signaling were more represented in the GC tissues than in the adjacent tissues (**Figure 1E**).

HORMAD1 overexpression was associated with clinicopathological characteristics in GC

Firstly, we analyzed HORMAD1 expression in 34 types of human cancer. HORMAD1 was upregulated in 33 cancer types and markedly increased in 12 cancer types compared with levels in normal tissues (**Figure 2A**). Subsequently, we analyzed TCGA cohort and found that HORMAD1 expression was significantly higher in 375 gastric adenocarcinoma tissues than in 34 adjacent normal tissues (**Figure 2B**). Next, we first used RT-qPCR to confirm HORMAD1 expression in GC to examine HORMAD1 mRNA levels in 46 GC and adjacent tissues. We observed significant upregulation of HORMAD1 in GC tissues compared to adjacent tissues (**Figure 2C**). Furthermore, Kaplan-Meier survival analysis showed that patients with highly-expressed HORMAD1 had significantly lower post-progression survival ($P < 0.01$) and relapse-free survival ($P < 0.05$) rates than patients with low-expressed HORMAD1 (**Figure 2D**, **2E**). To further validate HORMAD1 protein levels in GC, we detected HORMAD1 in GC tissue containing seven gastric tumor tissues and corresponding adjacent tissues by western blot. Similarly, HORMAD1 protein was significantly increased in GC samples compared to adjacent tissues (**Figure 2H**). We examined a small cohort of 46 pairs of GC and paired adjacent tissues using IHC analysis. Expression patterns of HORMAD1 in GC and adjacent tissues were classified according to IHC scores, and detailed demographic and clinicopathological parameters of these patients are presented in **Table 1**. GC tissues tended to show stronger cytoplasmic staining of HORMAD1, and adjacent tissues exhibited a lower or negative expression of HORMAD1 (**Figure 2F**). Subsequently, we analyzed the relationship between HORMAD1 expression with cancer subtypes and clinicopathological features. Elevated HORMAD1 expression was detected in tumor samples with larger tumor sizes and in older patients (**Table 1**). Moreover, patients with advanced GC showed higher levels of HORMAD1 expression than patients with early-stage GC. Overall, we found that increased HORMAD1 expression was associated with age ($P < 0.01$), tumor volumes ($P < 0.01$), and TNM stage ($P < 0.01$) but not with sex, differentiation, or lymph node metastases. Ultimately, these results indicate that HORAMAD1 plays an essential role in GC development and pathology. Meanwhile, we further revealed that HORMAD1 was observed in GC cell lines compared to the healthy gastric epithelial GES-1 cell line according to our western blotting and RT-qPCR results. We also observed that HORMAD1 was highly expressed in MKN-45 and AGS cells and less expressed in MKN-74 and HCG-27 cells (**Figure 2G**, **2I**). These results indicate that HORMAD1 is frequently upregulated in GC and may function as a tumor-promoting factor in human GC.

HORMAD1 promotes proliferation, migration, and invasion of GC cells in vitro

To determine whether HORMAD1 affects GC cell proliferation, we transduced MKN-45 and AGS cell lines with shRNAs targeting HORMAD1 or controls. We knocked down HORMAD1 efficiency using selected shRNA-1 and shRNA-2 from four distinct shRNAs in MKN-45 and AGS cells confirmed by western blot and RT-qPCR analysis (**Figure 3A-D**). Next, we used CCK-8, colony formation, and EDU assays to detect cell proliferation, colony formation capacity, and DNA synthesis. Compared with control cells, the proliferation rate (MKN-45-sh-HORMAD1, $P < 0.001$; AGS-sh-HORMAD1, $P < 0.001$) and the number of colony formations (MKN-45-sh-HORMAD1, $P < 0.001$; AGS-sh-HORMAD1 $P < 0.001$) of GC cells were significantly reduced (**Figure 3E-G**). Subsequently, EDU was performed to assess the effect of sh-HORMAD1 on DNA synthesis in AGS and MKN-45 cells (**Figure 3I**, **3J**). The results showed that HORMAD1 downregulation inhibited cell growth in both cells. Conversely, HORMAD1-overexpressing plasmids were introduced into HCG-27 and MKN-74 cells, and HORMAD1 expression was confirmed by RT-qPCR and western blot assay (**Figure 4A**, **4B**). We found that ectopic HORMAD1 expression significantly promoted HCG-27 and MKN-74 cell proliferation, colony formation capacity and viability compared to the empty vector (**Figure 4C-E**, **4H**, **4I**). Furthermore, to investigate the role of HORMAD1 in GC migration and invasion, we observed that ectopic overexpression of HORMAD1 resulted in significantly increased migration and invasion of HCG-27 and MKN-74 cells (**Figure 4F**, **4G**). Conversely, HORMAD1 depletion in MKN-45 and AGS cells significantly reduced cell migration and invasion (**Figure 3H**). These results suggest that HORMAD1 is associated with GC cell proliferation, migration, and invasion.

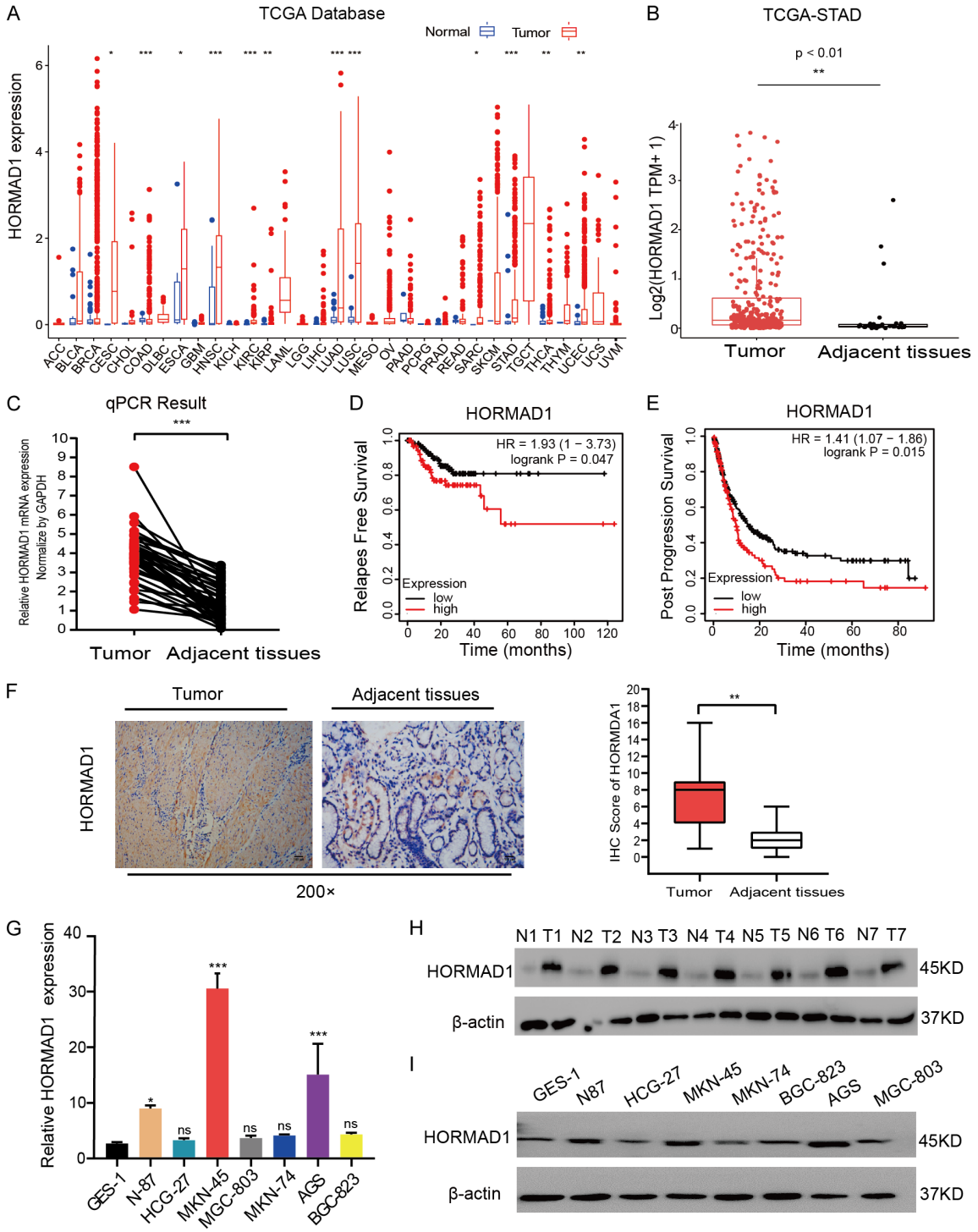

Figure 2. Expression of HORMAD1 and the prognostic role of HORMAD1 mRNA expression in GC. A. Expression of HORMAD1 across 34 cancer types and the corresponding normal tissues using data from The Cancer Genome Atlas (TCGA) database. Data were analyzed using the Wilcoxon test. B. HORMAD1 mRNA levels in gastric tissues (n = 373) and adjacent normal tissues (n = 34) were assessed using the online TCGA database. C. Relative mRNA expression of HORMAD1 was analyzed using RT-qPCR in tumor tissues and matched adjacent normal tissues (n = 46). GAPDH was used for the normalization of RT-qPCR results (***$P < 0.001$). D, E. The effect of HORMAD1 mRNA expression in GC tissue on patient's overall relapse-free survival and post progression survival was assessed based on the database of the Kaplan-Meier plotter platform. F. HORMAD1 protein expression is significantly higher in GC than in the matched normal tissues by IHC staining. IHC score analysis for HORMAD1 expression in 46 GC

tissues compared with that in the matched normal tissues (**P < 0.01). G. RT-qPCR analysis of HORMAD1 mRNA expression in gastric cell lines (MKN-45 vs GES-1, AGS vs GES-1, ***P < 0.001). H. WB analysis of HORMAD1 in seven pairs of GC (T) and the matched adjacent (N) tissues (n = 7). I. WB analysis of HORMAD1 protein expression in gastric cell lines. The number indicates the relative band intensity of HORMAD1 protein normalized against that of β-actin. Representative images and statistical plots are shown in the upper and lower panels, respectively. IHC, immunohistochemistry; GC, gastric cancer.

Table 1. Correlation between HORMAD1 expression and clinicopathologic characteristics in 46 gastric cancer patients

Clinicopathological features	Category	HORMAD1 expression (%)		χ^2	*p value*
		High (> 6)	Low (< 6)		
Total	46				
Age				10.435	0.004
< 53	8	2	6		
≥ 53	38	31	7		
Sex				0.023	0.347
Male	36	28	8		
Female	10	8	2		
Tumor size				3.622	0.007
< 5	8	4	4		
≥ 5	38	31	7		
Location				5.895	0.752
GEJ-Cardia	30	19	11		
Fundus-Body	9	2	7		
Antrum-Pylorus	6	4	2		
Remnant + multi-sites	1	1	0		
Lauren type				1.759	0.193
Intestinal	36	26	10		
Diffuse + mixed	10	5	5		
pT stage				18.892	0.150
T1 + T2	15	2	13		
T3 + T4	31	25	6		
pN stage				0.810	0.955
N0	9	6	3		
N1	12	8	4		
N2	14	10	4		
N3	11	6	5		
M stage				0.378	0.601
M0	44	31	13		
M1	2	1	1		
TNM stage				10.388	0.0001
I + II	9	2	7		
III + IV	37	29	8		

HORMAD1 promotes GC progression in xenograft mouse models

To study the in vivo function of HORMAD1 in GC tumor growth, we used a subcutaneous xenograft tumor model, wherein MKN-45 GC cells (2×10^7) were injected into the right flank of mice. Thirty days post-transplantation, we observed that tumors derived from control MKN-45 cells were significantly larger in size when compared to those from HORMAD1-depleted cells (**Figure 5A**). Furthermore, tumors

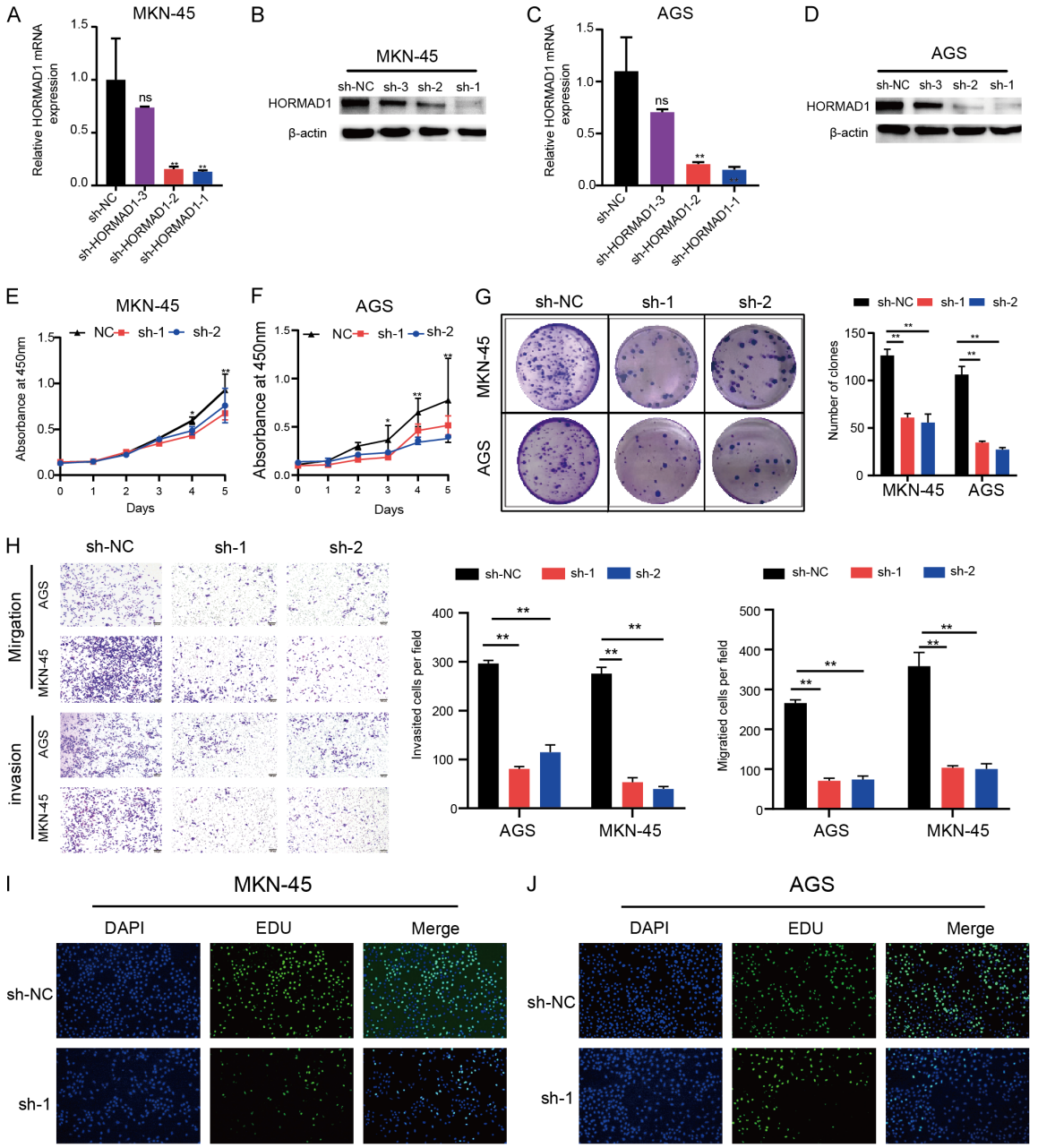

Figure 3. Knockdown of HORMAD1 inhibits cell proliferation and colony formation. A-D. WB and RT-qPCR analysis of the transfection efficiency of sh-HORMAD1 in MKN-45 and AGS cells (***P* < 0.01 vs sh-NC). E, F. Cell proliferation activities of sh-HORMAD1-transfected MKN-45 and AGS cells were measured using CCK8 analysis (**P* < 0.05 vs NC; ***P* < 0.01 vs NC). G. Analysis of cell colony formation capacity of sh-HORMAD1-transfected MKN-45 and AGS cells (***P* < 0.01 vs NC). H. The cells' invasive and migration potential was assessed using the Transwell assay in sh-HORMAD1 transfected MKN-45 and AGS cells (***P* < 0.01 vs sh-NC). All histograms represent the results of three independent experiments. I, J. The EDU assay was used to observe the effects of HORMAD1 knockdown on the DNA synthesis of MKN-45 and AGS cells.

formed in the control group had a larger tumor volume and heavier weight than those in the sh-HORMAD1 group (**Figure 5B**, **5C**). Furthermore, the IHC results showed that tumors dissected from the sh-NC group exhibited a stronger HORMAD1 staining than those from the sh-HORMAD1 group. Meanwhile, IHC analysis confirmed that sh-HORMAD1 inhibited the expression of the proliferation marker Ki-67 (**Figure 5F**). Moreover, tumors derived from sh-HOR-

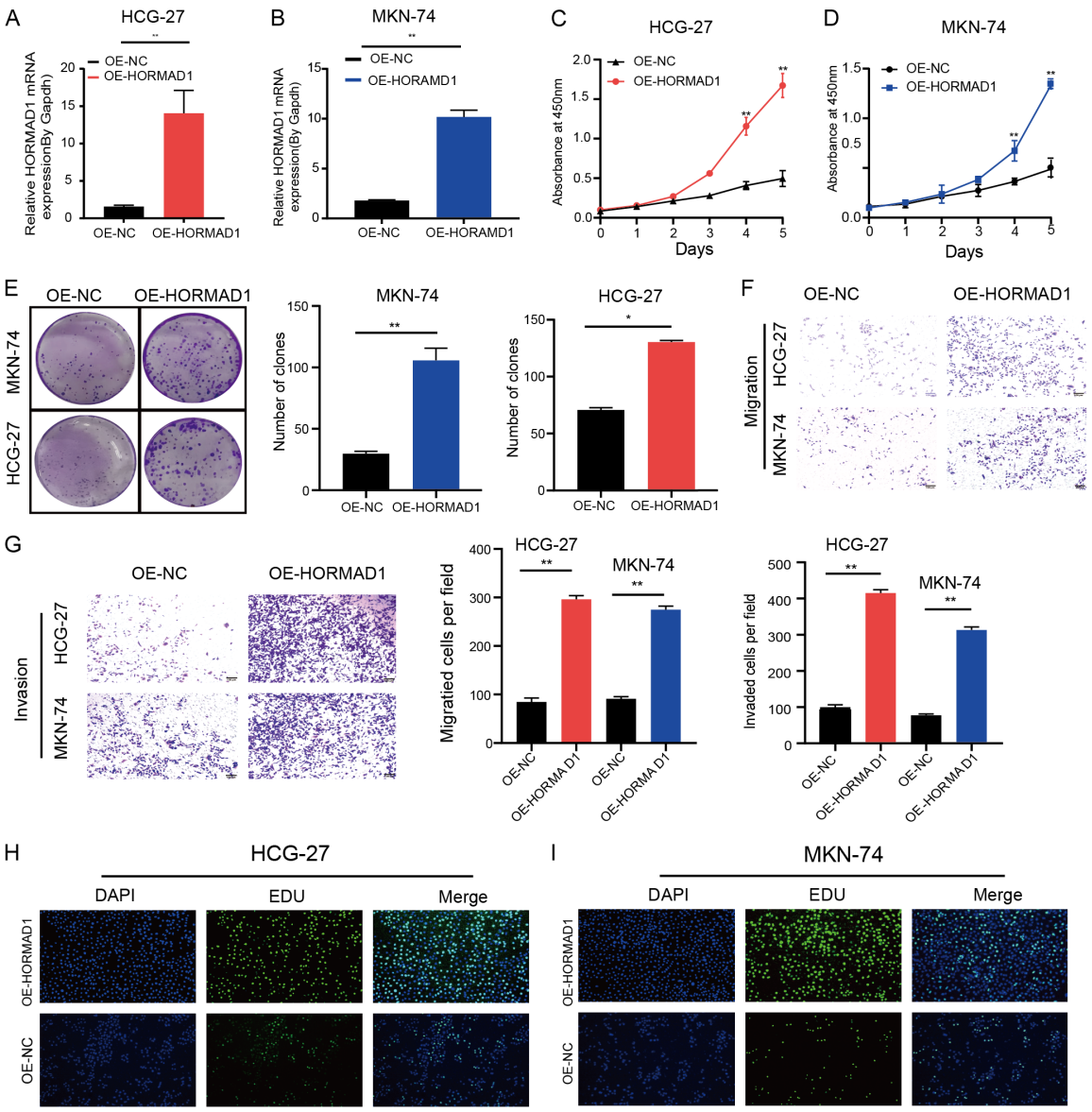

Figure 4. Overexpression of HORMAD1 promotes cell growth and invasion. A, B. RT-qPCR analysis of the transfection efficiency of HORMAD1 in MKN-74 and HCG-27 cells (***P* < 0.01 vs OE-NC). C-E. Cell proliferation activity and colony formation capability were estimated using CCK-8 and colony formation assays, respectively, in HORMAD1-transfected MKN-74 and HCG-27 cells (***P* < 0.01 vs OE-NC). F, G. Transwell invasion assay was performed to assess cell invasive and migration potential in MKN-74 and HCG-27 cells overexpressing HORMAD1 (*P < 0.05 vs OE-NC, **P < 0.01 vs OE-NC). H, I. The results of EdU assay effects on DNA synthesis of HGC-27, MKN74-cells.

MAD1 with MKN-45 cells had lower levels of p-NF-κB/p65 proteins than sh-NC cells by western blot and IHC analysis (**Figure 5D**). Furthermore, Pearson's analysis showed that p-p65 protein levels were positively correlated with HORMAD1 in xenograft tissues (**Figure 5E**). We confirmed this phenomenon in GC by assessing the p-p65 expression in GC and adjacent tissues. As shown in **Figure 5H**, p-p65 expression was stronger in GC tissues than in adjacent tissues. Furthermore, GC tissues with high HORMAD1 expression had a higher p-p65 expression than GC tissues with low HORMAD1 expression (**Figure 5G**). Further, Pearson's analysis demonstrated that p-p65 protein levels were positively correlated with HORMAD1 in GC tissues (**Figure 5I**). These results suggest that HORMAD1 promotes tumor growth, possibly through the NF-κB/p65 pathway.

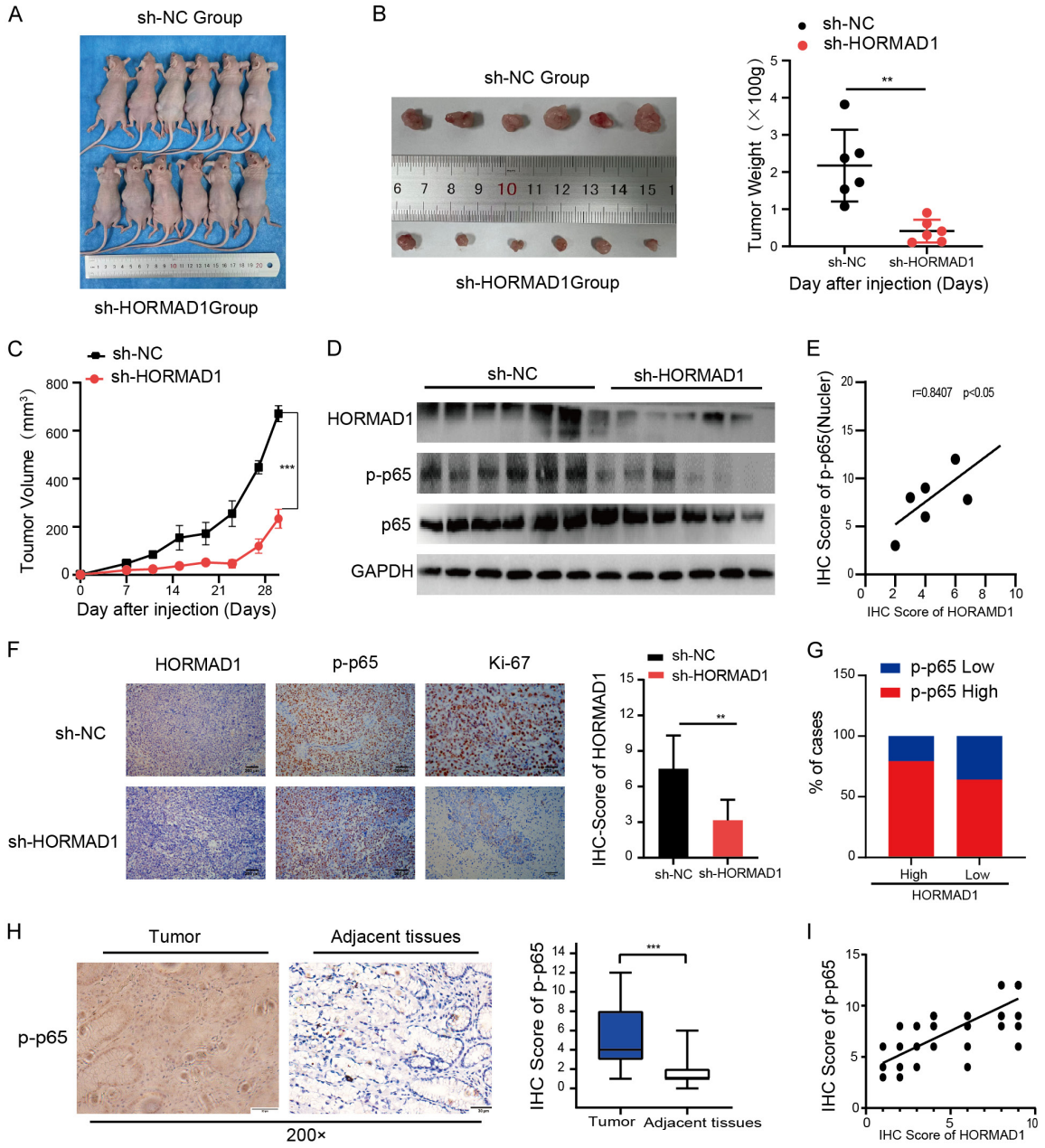

Figure 5. HORMAD1 knockdown inhibits GC tumor formation and growth in nude mice. A. Tumor xenografts were generated using HORMAD1-silencing MKN-45 cells. Images of tumors are shown (n = 6). B. The tumors formed in mice were surgically collected 30 days after the injection. Tumor tissues in sh-NC and sh-HORMAD1 groups were weighed after 30 days. C. Tumor growth curve of the xenograft tumor growth tendency in the sh-HORMAD1 and NC groups (**$P < 0.01$ vs sh-NC). D. Protein levels of HORMAD1, phospho-NF-κB (p-p65), p65, and GAPDH in tumor tissues were measured using western blotting assay of sh-HORMAD1 groups vs sh-NC (n = 6). E. Positive correlation between the expression level of HORMAD1 and the level of phosphorylated NF-κB (S473) by IHC in nude mice xenograft gastric tumors (**$P < 0.01$ vs sh-NC). F. Representative photomicrographs of IHC staining of HORMAD1, p-p65, and Ki-67 in GC nude mice tissues (Magnification: ×200). G. Percentages of specimens showing low or high HORMAD1 expression relative to the levels of p-p65. H. Significantly higher expression of p-p65 protein was observed in GC tissues compared with that in matched normal tissues, as indicated by IHC staining. IHC score analysis of p-p65 expression in 46 GC tissues revealed a significant difference (***P < 0.001) compared to that in matched normal tissues. I. Pearson's correlation coefficient (r) was calculated to assess the correlation between HORMAD1 and p-p65 in the 46 pairs of GC tissues.

HORMAD1 enhances the activity of the NF-κB signaling pathway

To further explore the molecular mechanism by which HORMAD1 promotes GC development, we analyzed differentially upregulated gene expression in 237 and 112 downregulated genes in MKN-45 sh-HORMAD1 cells using RNA sequencing. We found that HORMAD1 silencing significantly regulated the expression of 349 genes in MKN-45 cells (log2 ratio > 1 or < -1, $P < 0.05$), containing 112 downregulated and 237 upregulated mRNAs in HORMAD1-silenced GC cells, compared to that in the negative control groups (**Figure 6A**, **6B**). KEGG categorization significantly enriched these differentially expressed genes in multiple cancer-related signaling pathways, including NF-κB and TNF-α as the top-ranked signaling pathways (**Figure 6C**). Furthermore, GSEA showed that the NF-κB signaling pathway was strongly enriched in the control group (**Figure 6D**). Based on RNA-seq results, western blotting confirmed that HORMAD1 knockdown in MKN-45 cells decreased NF-κB activity. Notably, NF-κB activation requires translocation of the p65 subunit from the cytoplasm to the nucleus, during which IKK-β is a key kinase to facilitate translocation by releasing the p65 subunit from IκB-α. Therefore, we investigated whether HORMAD1 affects the status of Iκκ-β and IκB-α in GC cells. The results suggested that HORMAD1 knockdown decreased the abundance of both p-Iκκ-β (atThr188) and p-IκB-α (atSer32), while total IKK-β and IκB-α levels were unchanged (**Figure 6E**). Overall, these findings suggested HORMAD1 plays a role in transactivating the NF-κB signaling pathway.

HORMAD1 promoted EMT and proliferation in gastric cancer cells

Because EMT is considered a prominent feature of most cancers and plays a crucial role in cancer progression and invasion, it was worth investigating whether HORMAD1 was involved in regulating the EMT process of GC cells. Therefore, by western blotting assay, we examined EMT markers in HORMAD1 overexpressed in HCG-27 and MKN-74 cells. The data revealed the downregulation of the cohesive epithelial marker E-cadherin and a corresponding upregulation of the mesenchymal markers Vimentin and N-cadherin upon HORMAD1 overexpression (**Figure 6F**). As shown in (**Figure 6G**), HORMAD1 knockdown exerted the opposite effect in MKN-45 and AGS cells. Furthermore, we found that HORMAD1 could enhance NF-κB downstream proliferation-related gene expression. Moreover, the HORMAD1 knockdown group showed the downregulation of c-Myc and CyclinD1 and the upregulation of p21, whereas the HORMAD1 overexpressed group showed the opposite (**Figure 6F**, **6G**). Thus, the functional changes induced by HORMAD1 suggest that HORMAD1 may be an important facilitator of EMT and proliferation in GC.

HORMAD1 facilitates the GC progression through the NF-κB signaling pathway

To determine whether HORMAD1 promotes proliferation and invasion of GC cells via the NF-κB signaling pathway, we treated HORMAD1-overexpressed MKN-74 cells with the IKK inhibitor Bay-117082 (20 μmol/l) for 2 h. Compared to Dimethyl sulfoxide (DMSO), Bay-117082 treatment resulted in a significant reduction in cell proliferation ($P < 0.001$) and colony formation ($P < 0.001$) in MKN-74-HORMAD1 cells (**Figure 7A-C**). Similarly, migration and invasion assays showed that MKN-74-HORMAD1 cells treated with IKK inhibitor showed reduced invasion capacity compared to the DMSO group (**Figure 7D**) ($P < 0.001$). To ascertain whether EMT mediates the role of HORMAD1 in the migration and invasion of GC cells, we treated MKN-74 cells with Bay-117082; the results of the western blot assay showed that it was possible to rescue the expression changes of E-cadherin, N-cadherin, and Vimentin upon HORMAD1 overexpression (**Figure 7E**). In short, EMT was partly responsible for the promotion effect of HORMAD1 on the migration and invasion of GC cells. Furthermore, we characterized the signaling mechanism by which HORMAD1 regulates the NF-κB signaling pathway in GC cells. Our results demonstrated that overexpression of HORMAD1 increased Ser32 phosphorylation of IκB-α and Thr188 phosphorylation of Iκκ-β, which attenuated p-NF-κB degradation (p-p65) and promoted its nuclear translocation (**Figure 7F**). In addition, the IKK inhibitor Bay-117082 partially restored increased levels of p-Iκκ-β (Thr188) and p-IκB-α (atSer32), accompanied by a partial reversal of HORMAD1 overexpression-induced changes in p-NF-κB (p-p65) expression levels and transcription-

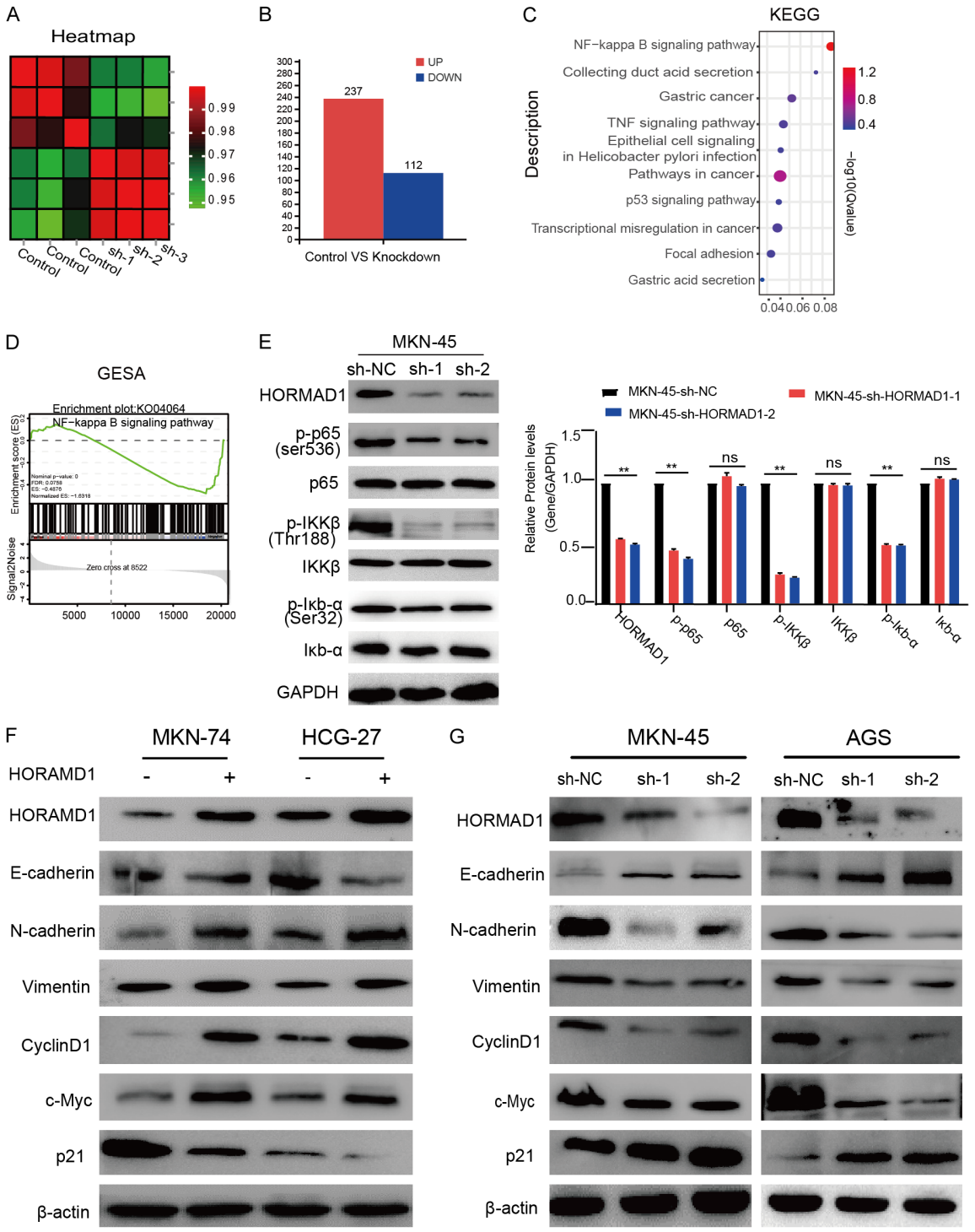

Figure 6. HORMAD1 is involved in the regulation of the NF-κB signaling pathway. A. Heatmap of the differentially expressed genes from the RNA-seq experiment following HORMAD1 knockdown. Red and blue represent upregulation and downregulation, respectively. B. The number of differentially expressed mRNAs. C. KEGG enrichment analysis of the 10 most important enriched pathways regulating HORMAD1 in GC cells. Based on KEGG signaling pathways, this analysis allowed for the functional categorization of differentially expressed mRNAs triggered by the knockdown of HORMAD. D. GSEA analysis comparing the sh-HORMAD1 group (red) to the sh-control group (blue) of MKN-45 GC transfect cells. E. Western blotting analysis of the expression of HORMAD1, p-p65, p65, p-Ikκ-β, Ikκ-β, p-IκB-α, IκB-α, and GAPDH in the cochlea. GAPDH was used as an internal control (**P < 0.01 vs OE-NC, ***P < 0.001 vs sh-NC). F. Western blot assay analysis of the expressions of EMT and proliferation relative gene (E-cadherin, N-cadherin, vimentin, p21, CyclinD1, and c-Myc) on HCG-27 and MKN-74 with overexpressed HORMAD1. G. Western blot analysis

of the expression of EMT and proliferation related gene (E-cadherin, N-cadherin, vimentin, p21, CyclinD1, and c-Myc) by HORMAD1 knockdown in MKN-45 and AGS cells. β-actin was used as an internal control.

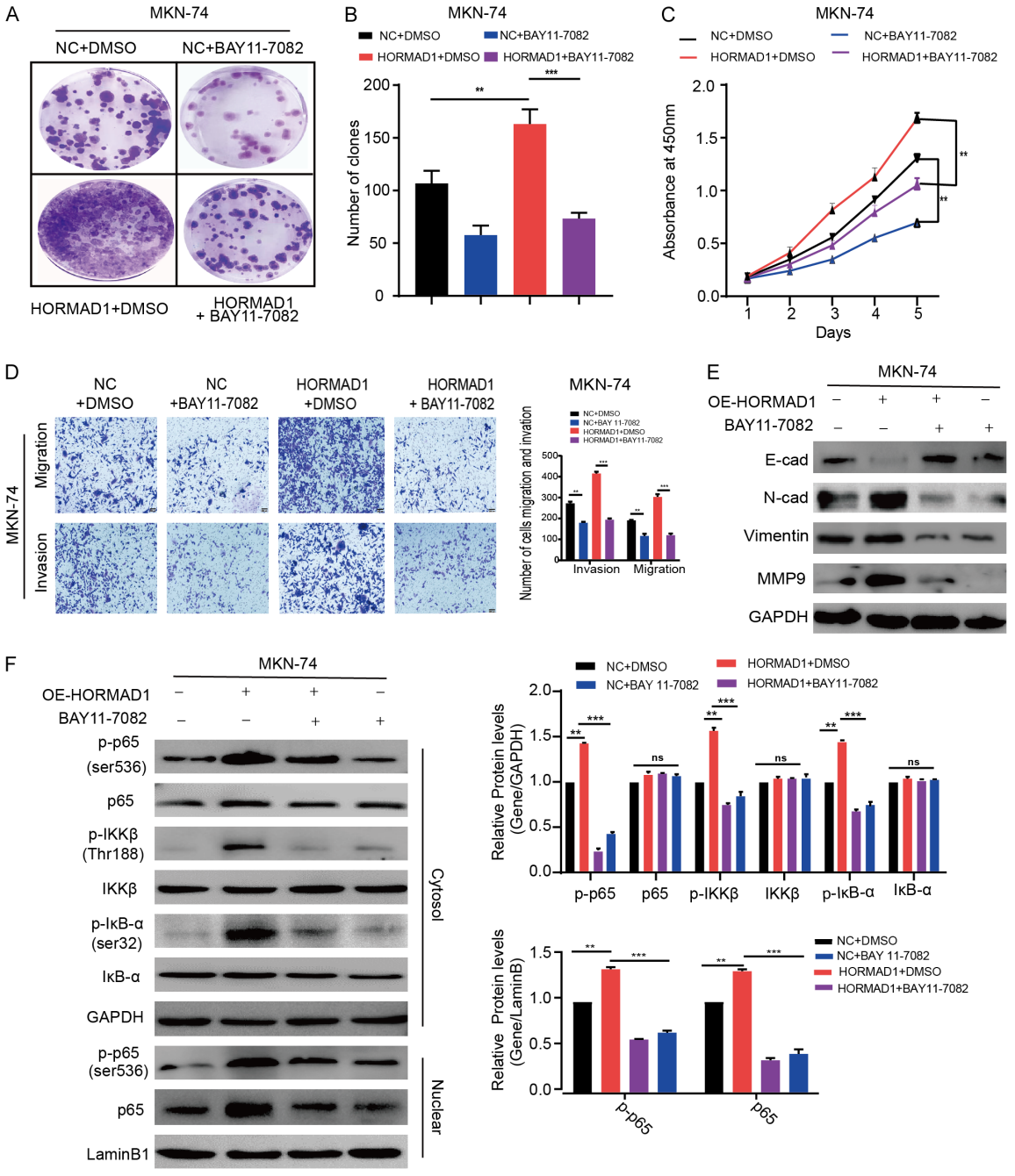

Figure 7. HORMAD1 enhances GC cells' proliferative and invasive abilities by the NF-κB signaling pathway. A. Representative image of the colony formation assay for MKN-74 cells transfected with HORMAD1 and their control cells with DMSO or Bay117082 (**P < 0.01 vs DMSO, ***P < 0.001 vs DMSO). B. The number of colonies formed by each group is shown in the histogram. C. The cell growth curve of MKN-74 cells transfected with HORMAD1 and their control cells with DMSO or Bay117082 (**P < 0.01 vs DMSO). D. Representative images of the Matrigel invasion assay for MKN-74 cells transfected with HORMAD1 and their control cells with DMSO or Bay117082 (**P < 0.01 vs DMSO, ***P < 0.001 vs DMSO). The invasive cell number for each group is shown in the histogram. E. The MKN-74 cells with HORMAD1 overexpression were treated with Bay117082 for 48 h, then the protein levels of EMT markers were measured by western blotting. F. HORMAD1-overexpressed or control MKN-74 cells were treated with Bay117082 or DMSO for 2 h. Subsequently, the protein levels were measured by western blotting. Lamin B1 or GAPDH was used as an internal control (*P < 0.05 vs DMSO, **P < 0.01 vs DMSO, ***P < 0.001 vs DMSO).

al activity. Therefore, we identified that Bay-117082 treatment significantly inhibited p-NF-κB (p-p65) and p-NF-κB (p-p65) nuclear translocation induced by HORMAD1 overexpression in MKN-74 cells, suggesting that HORMAD1-mediated p-NF-κB activation is indeed in the p-NF-κB/Ikκ-β pathway. These results demonstrated that HORMAD1 activates GC cell proliferation and invasion by regulating the activation of the NF-κB signaling pathway.

Discussion

Previous studies have shown that HORMAD1, a meiosis-specific protein, is widely expressed in various cancer types, including lung, ovarian, and breast cancer [13-17]. Furthermore, HORMAD1 has been implicated in tumour development and chemotherapeutic resistance. However, the precise biological functions of HORMAD1 in GC progression and its underlying mechanisms remain unclear. This study attempted to reveal the role and mechanisms of HORMAD1 in GC progression. We identified a significant increase in HORMAD1 expression in GC tissues and cell lines. Specifically, high levels of HORMAD1 expression correlated with poor prognosis, higher tumor stage, and increased lymph node metastasis rates. Moreover, we found that HORMAD1 facilitated the proliferation, migration, and invasion of gastric cancer cells, consistent with previous research [13, 17]. Interestingly, our study also showed a positive association between HORMAD1 expression and p-NF-κB expression based on IHC score analysis, confirmed by vivo experiments. Therefore, our findings strongly suggest that HORMAD1 may act as an oncogenic gene in GC's development and progression.

The initial stage of metastatic progression is essentially dependent on EMT [21, 22]. Specifically, the activation of EMT allows cancer cells to migrate and invade beyond the extracellular matrix of surrounding tissues in the metastatic cascade [4]. During cancer progression, EMT can alter the adhesion of epithelial cancer cells and allow them to invade and migrate to distant sites, thus contributing to tumor metastasis [23]. Recent studies have highlighted the role of CTAs in promoting tumor metastasis through the induction of EMT. For instance, Testes-specific protease 50 (TSP50) has been shown to promote invasion and metastasis in GC by inducing EMT [24]. Moreover, studies showed that HORMAD1 promotes EMT and metastasis through the Wnt/β-catenin pathway [13]. To date, no studies have investigated the role of HORMAD1 (CTA46) as a CTAs in EMT and tumor progression in GC. Moreover, the mechanism underlying its pathogenesis is rather lacking evidence. In this context, our study provided mechanistic evidence supporting HORMAD1's vital role in promoting EMT and proliferation in GC. We report that HORMAD1-knockdown GC cells have low levels of Vimentin, N-Cadherin, and c-Myc and high levels of E-cadherin, suggesting that HORMAD1 is a potent inducer of EMT, resulting in more invasive and proliferation biological behavior in GC cells.

It is well known that the NF-κB signaling pathway is involved in cell migration and invasion and plays a key role in promoting and maintaining the invasiveness of cancer cells via regulating EMT processes in different tumors, including GC [6, 25]. Multiple lines of evidence have implicated the NF-κB signaling pathway in regulating various pathophysiological processes, including inflammation, malignant transformation, transcription, cell apoptosis, metastasis, and proliferation [26]. Furthermore, a previous study showed that NF-κB is required for TSP50-induced migration and invasion of breast cancer cells [27]. Moreover, NF-κB/p65, as a transcription factor involved in EMT or proliferation, has been reported to play an important role in tumors. However, the effect of HORMAD1 on NF-κB in GC has not been investigated. Therefore, based on our RNA-seq results of GSEA and KEGG analysis, it is strongly suggested that the NF-κB pathway is highly relevant to HORMAD1. Moreover, identifying and understanding specific modulators regulating the NF-κB pathway in GC cells could offer new insights into the molecular mechanisms underlying EMT and potentially lead to the discovery of novel therapeutic targets for GC. This study revealed that HORMAD1 promotes p-p65 expression by increasing cytoplasmic accumulation and subsequent nuclear translocation, leading to NF-κB signaling activation. It is well known c-Myc as an oncogene in several carcinogenesis processes, which are involved in cell proliferation and growth, cell cycle regulation, cell adhesion, metabolism, ribosome biogenesis, protein synthesis, and mitochondrial func-

tion [28, 29]. Several studies showed that c-Myc can promote the growth and proliferation of gastric cancer cells and help them maintain malignant phenotype [30, 31]. Meanwhile, CyclinD1 acts as a growth factor drives cell cycle progression and promote cell proliferation in many different types of cancer [32]. Previous studies have indicated that CyclinD1 as a key cell cycle-related molecules are involved in the occurrence and progression of GC [33]. Consistent with our finding, upregulation of HORMAD1 was observed to increase the expression of CyclinD1 and c-Myc while decreasing the expression of p21. Conversely, the knockdown of HORMAD1 reversed these effects, resulting in decreased expression of CyclinD1 and c-Myc and increased expression of p21. Notably, both c-Myc and CyclinD1 play important roles in cell proliferation.

We report that HORMAD1 induces malignant phenotypes in GC cells by activating the NF-κB pathway in GC cells. Our results demonstrate that HORMAD1 overexpression increases the phosphorylation of NF-κB (Ser536) and Iκκ-β (Thr188), which promotes its nuclear translocation. Moreover, the Iκκ-β inhibitor partially reduced the levels of p-NF-κB and p-Iκκ-β, which were initially inhibited by HORMAD1 knockdown. Furthermore, treatment of HORMAD1-overexpressing cells with the specific Iκκ-β inhibitor, BAY-117082, partially restored the levels of p-NF-κB (Ser536) and transcriptional activity of NF-κB. Meanwhile, the treatment with Iκκ-β inhibitor, BAY-117082, partly restored the level of EMT markers in HORMAD1-overexpressed MKN-74 cells. These above results indicate that HORMAD1 overexpression activates the NF-κB signaling pathway, thus promoting EMT in GC cells. These findings collectively demonstrate the upregulation of HORMAD1 in GC and its promotion of GC proliferation. Additionally, HORMAD1 was found to partially regulate the EMT process by activating the NF-κB signaling pathway. Until now, the role of HORMAD1 in gastric cancer still has some limitations. Accordingly, in our present finding provides an effective strategy and potential the therapeutic target for GC. Moreover, we speculated that GC patients with high HORMAD1 expression may benefit more from treatment with inhibitors of the NF-κB pathway, which provide novel insights that could be used to improve the clinical efficacy of these inhibitors. However, our research has not been adequately verified, therefore, comprehensive research is needed to confirm the regulated mechanism remain to be investigated in the future.

Acknowledgements

This work was supported by the Fundamental Research Funds for the Central Universities (No. WK9110000064), Anhui Provincial Natural Science Foundation (No. 2208085QH228) and Anhui Provincial Natural Science Foundation (2208085QH230). The Special Support for the Anhui Provincial Project of the Key Laboratory of Tumor Immunotherapy and Nutrition Therapy (2019b12030026).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ping Li, Department of Chinese Integrative Medicine Oncology, The First Affiliated Hospital of Anhui Medical University, No. 120 Wanshui Road, Hefei 230022, Anhui, China. Tel: +86-0551-65908527; E-mail: liping1964@ahmu.edu.cn

References

[1] Rawla P and Barsouk A. Epidemiology of gastric cancer: global trends, risk factors and prevention. Prz Gastroenterol 2019; 14: 26-38.

[2] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68: 394-424.

[3] Patel TH and Cecchini M. Targeted therapies in advanced gastric cancer. Curr Treat Options Oncol 2020; 21: 70.

[4] Lu W and Kang Y. Epithelial-mesenchymal plasticity in cancer progression and metastasis. Dev Cell 2019; 49: 361-374.

[5] Dolcet X, Llobet D, Pallares J and Matias-Guiu X. NF-kB in development and progression of human cancer. Virchows Arch 2005; 446: 475-482.

[6] Mirzaei S, Saghari S, Bassiri F, Raesi R, Zarrabi A, Hushmandi K, Sethi G and Tergaonkar V. NF-κB as a regulator of cancer metastasis and therapy response: a focus on epithelial-mesenchymal transition. J Cell Physiol 2022; 237: 2770-2795.

[7] Ji J, Ding K, Luo T, Zhang X, Chen A, Zhang D, Li G, Thorsen F, Huang B, Li X and Wang J.

TRIM22 activates NF-κB signaling in glioblastoma by accelerating the degradation of IκBα. Cell Death Differ 2021; 28: 367-381.
[8] Kunnumakkara AB, Diagaradjane P, Guha S, Deorukhkar A, Shentu S, Aggarwal BB and Krishnan S. Curcumin sensitizes human colorectal cancer xenografts in nude mice to gamma-radiation by targeting nuclear factor-kappaB-regulated gene products. Clin Cancer Res 2008; 14: 2128-2136.
[9] Le F, Zhang JY, Liu W, Huang XM and Luo WZ. The levels of NF-κB p50 and NF-κB p65 play a role in thyroid carcinoma malignancy in vivo. J Int Med Res 2018; 46: 4092-4099.
[10] Kanarek N and Ben-Neriah Y. Regulation of NF-κB by ubiquitination and degradation of the IκBs. Immunol Rev 2012; 246: 77-94.
[11] Hayden MS and Ghosh S. NF-κB, the first quarter-century: remarkable progress and outstanding questions. Genes Dev 2012; 26: 203-234.
[12] Liu K, Wang Y, Zhu Q, Li P, Chen J, Tang Z, Shen Y, Cheng X, Lu LY and Liu Y. Aberrantly expressed HORMAD1 disrupts nuclear localization of MCM8-MCM9 complex and compromises DNA mismatch repair in cancer cells. Cell Death Dis 2020; 11: 519.
[13] Liu K, Cheng L, Zhu K, Wang J and Shu Q. The cancer/testis antigen HORMAD1 mediates epithelial-mesenchymal transition to promote tumor growth and metastasis by activating the Wnt/β-catenin signaling pathway in lung cancer. Cell Death Discov 2022; 8: 136.
[14] Gao Y, Kardos J, Yang Y, Tamir TY, Mutter-Rottmayer E, Weissman B, Major MB, Kim WY and Vaziri C. The Cancer/Testes (CT) antigen HORMAD1 promotes homologous recombinational DNA repair and radioresistance in lung adenocarcinoma cells. Sci Rep 2018; 8: 15304.
[15] Nichols BA, Oswald NW, McMillan EA, McGlynn K, Yan J, Kim MS, Saha J, Mallipeddi PL, LaDuke SA, Villalobos PA, Rodriguez-Canales J, Wistuba II, Posner BA, Davis AJ, Minna JD, MacMillan JB and Whitehurst AW. HORMAD1 is a negative prognostic indicator in lung adenocarcinoma and specifies resistance to oxidative and genotoxic stress. Cancer Res 2018; 78: 6196-6208.
[16] Tarantino D, Walker C, Weekes D, Pemberton H, Davidson K, Torga G, Frankum J, Mendes-Pereira AM, Prince C, Ferro R, Brough R, Pettitt SJ, Lord CJ, Grigoriadis A and Nj Tutt A. Functional screening reveals HORMAD1-driven gene dependencies associated with translesion synthesis and replication stress tolerance. Oncogene 2022; 41: 3969-3977.
[17] Shahzad MM, Shin YH, Matsuo K, Lu C, Nishimura M, Shen DY, Kang Y, Hu W, Mora EM, Rodriguez-Aguayo C, Kapur A, Bottsford-Miller J, Lopez-Berestein G, Rajkovic A and Sood AK. Biological significance of HORMA domain containing protein 1 (HORMAD1) in epithelial ovarian carcinoma. Cancer Lett 2013; 330: 123-129.
[18] El-Botty R, Vacher S, Mainguené J, Briaux A, Ibadioune S, Dahmani A, Montaudon E, Nematí F, Huguet L, Sourd L, Morriset L, Château-Joubert S, Dubois T, Maire V, Lidereau R, Rapinat A, Gentien D, Coussy F, Bièche I and Marangoni E. HORMAD1 overexpression predicts response to anthracycline-cyclophosphamide and survival in triple-negative breast cancers. Mol Oncol 2023; [Epub ahead of print].
[19] Aung PP, Oue N, Mitani Y, Nakayama H, Yoshida K, Noguchi T, Bosserhoff AK and Yasui W. Systematic search for gastric cancer-specific genes based on SAGE data: melanoma inhibitory activity and matrix metalloproteinase-10 are novel prognostic factors in patients with gastric cancer. Oncogene 2006; 25: 2546-2557.
[20] Seethala RR, Gooding WE, Handler PN, Collins B, Zhang Q, Siegfried JM and Grandis JR. Immunohistochemical analysis of phosphotyrosine signal transducer and activator of transcription 3 and epidermal growth factor receptor autocrine signaling pathways in head and neck cancers and metastatic lymph nodes. Clin Cancer Res 2008; 14: 1303-1309.
[21] Sánchez-Tilló E, Liu Y, de Barrios O, Siles L, Fanlo L, Cuatrecasas M, Darling DS, Dean DC, Castells A and Postigo A. EMT-activating transcription factors in cancer: beyond EMT and tumor invasiveness. Cell Mol Life Sci 2012; 69: 3429-3456.
[22] Pastushenko I and Blanpain C. EMT transition states during tumor progression and metastasis. Trends Cell Biol 2019; 29: 212-226.
[23] Spaderna S, Schmalhofer O, Hlubek F, Berx G, Eger A, Merkel S, Jung A, Kirchner T and Brabletz T. A transient, EMT-linked loss of basement membranes indicates metastasis and poor survival in colorectal cancer. Gastroenterology 2006; 131: 830-840.
[24] Cao QH, Liu F, Li CZ, Liu N, Shu M, Lin Y, Ding L and Xue L. Testes-specific protease 50 (TSP50) promotes invasion and metastasis by inducing EMT in gastric cancer. BMC Cancer 2018; 18: 94.
[25] Liu J, Wu Z, Han D, Wei C, Liang Y, Jiang T, Chen L, Sha M, Cao Y, Huang F, Geng X, Yu J, Shen Y, Wang H, Feng L, Wang D, Fang S, Wang S and Shen Y. Mesencephalic astrocyte-derived neurotrophic factor inhibits liver cancer through small ubiquitin-related modifier (SUMO)ylation-related suppression of NF-κB/snail signaling pathway and epithelial-mesenchymal transition. Hepatology 2020; 71: 1262-1278.

[26] Sheppard KA, Rose DW, Haque ZK, Kurokawa R, McInerney E, Westin S, Thanos D, Rosenfeld MG, Glass CK and Collins T. Transcriptional activation by NF-kappaB requires multiple coactivators. Mol Cell Biol 1999; 19: 6367-6378.

[27] Song ZB, Ni JS, Wu P, Bao YL, Liu T, Li M, Fan C, Zhang WJ, Sun LG, Huang YX and Li YX. Testes-specific protease 50 promotes cell invasion and metastasis by increasing NF-kappaB-dependent matrix metalloproteinase-9 expression. Cell Death Dis 2015; 6: e1703.

[28] Oster SK, Ho CS, Soucie EL and Penn LZ. The myc oncogene: MarvelouslY complex. Adv Cancer Res 2002; 84: 81-154.

[29] Calcagno DQ, Leal MF, Assumpcao PP, Smith MA and Burbano RR. MYC and gastric adenocarcinoma carcinogenesis. World J Gastroenterol 2008; 14: 5962-5968.

[30] Zhang L, Hou Y, Ashktorab H, Gao L, Xu Y, Wu K, Zhai J and Zhang L. The impact of C-MYC gene expression on gastric cancer cell. Mol Cell Biochem 2010; 344: 125-135.

[31] Shi Q, Wang W, Jia Z, Chen P, Ma K and Zhou C. ISL1, a novel regulator of CCNB1, CCNB2 and c-MYC genes, promotes gastric cancer cell proliferation and tumor growth. Oncotarget 2016; 7: 36489-36500.

[32] Liao DJ, Thakur A, Wu J, Biliran H and Sarkar FH. Perspectives on c-Myc, Cyclin D1, and their interaction in cancer formation, progression, and response to chemotherapy. Crit Rev Oncog 2007; 13: 93-158.

[33] Arici DS, Tuncer E, Ozer H, Simek G and Koyuncu A. Expression of retinoblastoma and cyclin D1 in gastric carcinoma. Neoplasma 2009; 56: 63-67.

Supplementary Table 1. sh-RNA sh-HORMAD1 and primer sequences used in the study

Sh-NC	TTCTCCGAACGTGTCACGT
Sh-1	GAAGATGAACAGGAGCATTAT
Sh-2	GCATTCTCCTCATTCGCAAGA
Sh-3	GATCTACACAGTTAGTGAAAT

Supplementary Table 2. RT-qPCR Primer sequence

Gene	Primer F	Primer R
HORMAD1	5'-GCCCAGTTGCAGAGGACTC-3'	5'-TCTTGTTCCATAAGCGCATTCT-3'
GAPDH	5'-GAGTCAACGGATTTGGTCGT-3'	5'-GACAAGCTTCCCGTTCTCAG-3'

Supplementary Table 3. Western blot antibody in this study

Gene	Ratio	Brand	Art. NO
HORMAD1	1:1000	Proteintech	#13917
p21	1:1000	Proteintech	#10355
E-Cadherin	1:5000	Proteintech	#20874
N-cadherin	1:5000	Proteintech	#22018
Vimentin	1:1000	cst	#5741
c-Myc	1:1000	cst	#9402
CyclinD1	1:10000	Proteintech	#60186
MMP-9	1:500	ZenBio	#380831
NF-κB p65	1:500	cst	#13008
phospho-NF-κB p65	1:1000	cst	#3033
IκBα	1:1000	Proteintech	#10268
IKKβ	1:1000	Abcam	#ab32135
Phospho-IKKβ	1:1000	Immunoway	#YP0637
Phospho-IκBα	1:1000	Immunoway	#YP1372