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RESEARCH ARTICLE

Chronic stress promotes gastric cancer progression via the adrenoceptor beta 2/PlexinA1 pathway

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Abstract

Chronic stress is a common emotional disorder in cancer patients. Chronic stress promotes progression of gastric cancer (GC) and leads to poor outcomes. However, the underlying mechanisms remain not clear. Herein, we explored the possible mechanisms of chronic stress in GC progression. The Cancer Genome Atlas (TCGA) datasets were analyzed for differentially expressed genes. Clinical data of GC were evaluated for their association with PlexinA1 using TCGA and Kaplan–Meier-plotter databases. Chronic stress of GC patients was evaluated using the Self-Rating Anxiety Scale and Self-Rating Depression Scale. Chronic unpredictable mild stress (CUMS) was used to induce chronic stress in mice. Gastric xenograft tumor was constructed using the sewing method. Chronic stress-like behaviors were assessed using light/dark box and tail suspension tests. Protein expression was detected using immunohistochemistry and Western blot analysis. Analyses of TCGA and the Kaplan–Meier-plotter databases showed that patients with high levels of PlexinA1 in GC had worse overall survival than those with low levels of PlexinA1. A total of 36 GC patients were enrolled in the study, and about 33% of the patients had chronic stress. Compared with patients without chronic stress, higher expression levels of adrenoceptor beta 2 and PlexinA1 were observed in patients with chronic stress. The tumor size in mice under CUMS was significantly increased compared with the control mice. Adrenoceptor beta 2, PlexinA1, N-cadherin, and alpha-smooth muscle actin, as well as Ki67 were highly expressed in the tumors of CUMS group. However, E-cadherin was lowly expressed in the tumors of CUMS group. Importantly, chemical sympathectomy with 6-hydroxydopamine or treatment with a selective β2 adrenergic receptor antagonist (ICI118,551) could reverse these effects. Our findings suggest that chronic stress plays an important role in GC progression and there is a potential for blocking the epinephrine-β2AR/PlexinA1 pathway in the treatment of GC.

Keywords Chronic stress · Gastric cancer · Epinephrine · Adrenoceptor beta 2 · PlexinA1

Abbreviations: GC, gastric cancer; CUMS, Chronic unpredictable mild stress; TCGA, The Cancer Genome Atlas; ADRB2, Adrenoceptor Beta 2; α-SMA, alpha-smooth muscle actin; 6-OHDA, 6-hydroxydopamine; HPA axis, hypothalamus-pituitary-adrenal axis; SNS, sympathetic nervous system; β2AR, Adrenoceptor beta 2; SAS, Self-Rating Anxiety Scale; SDS, Self-Rating Depression Scale; TST, tail suspension test; ROC, receiver operating characteristic; EMT, epithelial-to-mesenchymal transition

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Introduction

Gastric cancer has high incidence and mortality globally.[1,2] With the advances in gastric cancer (GC) research, the treatment methods of GC have been improved.[3–5] However, the role of chronic stress in GC pathogenesis remains to be addressed. Chronic stress causes anxiety, depression, and psychosomatic disease. There is a significant positive association between early life distress and cancer development.[6] Furthermore, patients with clinical psychological distress have significantly shortened survival time.[7] The impact of chronic stress on tumorigenesis in humans has also become increasingly concerning.[8] Although the molecular pathways of chronic stress have not been completely delineated, observations to date indicate a need for novel therapeutic paradigms that integrate a bio-behavioral perspective.

Many studies have explored the relationship between chronic stress and cancer.[9,10] Stress acts on the hypothalamus-pituitary-adrenal axis and the sympathetic nervous system, leading to abnormal hormone release[11] and elevated levels of stress-related catecholamines.[12] Levels of catecholamines are increased in individuals who experience chronic stress and are responsible for sympathetic nervous system effects on cardiac, respiratory, vascular, and other organ systems.[13] The concentrations of serum catecholamine and epinephrine in chronic unpredictable mild stress (CUMS) group were demonstrably higher than those in the control group in our previous study.[14] Furthermore, we found that the adrenoceptor beta 2 (ADRB2) agonist, isoproterenol, could induce chemoresistance in GC cells.[15] Senescent non-malignant cells exhibit a secretory profile under stress conditions, which may lead to cancer progression and chemoresistance.[16] In mice, chronic stress increased adiposity and serum corticosterone levels.[17]

Adrenoceptor beta 2 (β2AR, the protein name is ADRB2) is a prototypical G protein-coupled receptor.[18] Behavioral stress can enhance tumor angiogenesis and promote malignant cell growth by activating ADRB2 through the cAMP-protein kinase A signaling pathway.[19] Previous studies have shown that ADRB2 is involved in hepatocellular carcinoma,[20] prostate cancer,[21] and pancreatic cancer.[22]

The semaphorin and plexin family of ligand and receptor proteins are large super-protein family, which mediate axonal guidance in the developing nervous system. In recent years, some members of the semaphorins have been also shown to have diverse and important functions in tumor progression.[23] Previous studies have shown that PlexinA1 is highly expressed in glioblastoma, hepatocellular carcinoma, and prostate tumor.[24–27] Our previous research work has demonstrated that Plexin-A1 serves as a main receptor of semaphorin family and contributes to progression of GC.[28] To follow up our previous research work, we performed the present study, aiming to investigate the effects of chronic stress on GC progression and the underlying mechanism involving the β2AR/PlexinA1 pathway *in vivo*.

Materials and methods

PlexinA1 in TCGA data

The correlation of PlexinA1 (PLXNA1) gene expression with the clinical data of GC patients was analyzed using The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/).

PlexinA1 in KM-plotter and gastric cancer data

Clinical data of GC in the Kaplan–Meier (KM)-plotter database (https://kmplot.com/analysis/index.php?p=service) were examined to determine whether PlexinA1 was associated with the prognosis of GC patients.

Patients

Patients with pathologically diagnosed GC, who were hospitalized at the Affiliated Hospital of Chengde Medical University (Chengde, Hebei Province, China) between December 2020 and December 2022, were enrolled. The chronic stress states of patients were evaluated using the Self-Rating Anxiety Scale (SAS) and Self-Rating Depression Scale (SDS),[29,30] which includes 20 criteria each. The sum of the scores of each of the 20 items was multiplied by 1.25 to obtain the final stress score. The higher the scores of SAS and SDS, the higher the level of chronic stress.[31] Inclusion criteria included: (1) the age of patients ranged from 30 to 86 years old; (2) the pathological type of GC was gastric adenocarcinoma; (3) patients had no history of antipsychotic therapy; (4) patients had no communication or comprehension impairment regarding the study; and (5) patients signed the informed consent. Exclusion criteria included: (1) patients refused to participate in this study or had impaired communication; (2) patients had severe psychiatric disorders; and (3) patients with survival time less than three months. Tumor tissues and adjacent normal tissues were collected from the GC patients included in this study.

Animals

BALB/c nude mice (male, 4–5 weeks old, 36 mice) were obtained from the Beijing Huafukang Biotechnology (Beijing, China) and housed within the individually ventilated caging systems at Chengde Medical

Laboratory Animal Center. The mice were acclimated to the housing for 1 week prior to being included in the experiments; thus, the mice were 5–6 weeks old when they were used for the experiments. For the mice used in the orthotopic xenograft studies, they were given 1 week to physically heal from the surgery; thus, they were 6–7 weeks old at the time of exposure to the stressors. The stressors were given for 3 weeks. The behavioral assessments of animals were performed by video recording.

Establishment of chronic stress model

Chronic stress was induced by CUMS, which is currently the most widely used, reliable, and effective method for inducing chronic stress in rodents.[32] The protocol to apply CUMS to the mice is shown in Table 1. Each mouse was randomly subjected to one test stressor per day, and the same stressor was not applied consecutively. Control mice did not receive any stress and were fed normally. All experimental mice were weighed every three days. In order to improve the reproducibility of CUMS model,[33] we have taken the following measures: (1) To prevent the effects on mice from experiencing sleep deprivation for a long time, we minimized the experimental time and conducted all behavioral tests from 9:00 AM to 15:00 PM. (2) Since CUMS is effective only if the protocol is implemented in the daytime, all stressors are performed during the daytime, except for the light/dark cycle inversion experiment. (3) Because the organism's response to the action of nonspecific stress factors is dependent on the degree of familiarity with the hands of the experimenter and the main manipulations, the stressors given during the experiments are performed by the same person.

Cell culture

MKN45, a human GC cell line, was obtained from Mingzhoubio (Ningbo, China) and cultured with Roswell Park Memorial Institute-1640 medium containing 10% fetal bovine serum (Gibco, St. Louis, MO, USA) in an incubator at 37 °C with 5% CO_2.

Antibodies

The antibodies against Adrenoceptor beta 2 (ADRB2) (BIOSS, bs-21452R), PlexinA1 (BIOSS, bs-2692R), E-cadherin (Cell Signaling, 24E10), N-cadherin (Huabio, ET1607–37), alpha-smooth muscle actin (α-SMA) (Huabio, ET1607–43), Ki67 (Abcam, ab66155), GAPDH (Bioworld, AA54151), and Protein A/G (Santa, sc-2003) were used in Western blotting, co-immunoprecipitation (CO-IP), and immunohistochemistry (IHC) as described below.

Western blotting and co-immunoprecipitation

Proteins of tissues were extracted after lysis with Radio Immunoprecipitation Assay Lysis (RIPA) buffer (Thermo Scientific, Rockford, IL, USA), containing proteases and phosphatase inhibitors. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membrane was incubated with the corresponding primary antibodies at 4 °C overnight followed by incubation with the horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (ABclonal). The signal was detected using the ChemiDoc™ XRS+ Systems (BIO-RAD), and the imaging analysis software was used for quantitative analysis.

For CO-IP, the tissues were cut into small pieces and homogenized in 1 mL of pre-cooled RIPA buffer in a 1.5-mL tube at 4 °C for 10 min. The tissue homogenates were centrifuged at 10,000 g at 4 °C for 10 min, and the supernatants were collected as protein lysates. Approximately 300 ug protein was mixed with 1 μg primary antibody and incubated for 2 h at room temperature. Then, 20 μl of Protein A/G beads was added and

Table 1
CUMS protocol.

Stressor	Description
Water deprivation	Water deprivation for 24 h (h)
Food deprivation	Food deprivation for 24 h
Cage interchangeability	Mice were moved from the original cage into another empty cage for 24 h
Padding with wet sawdust	Water (125 mL) was distributed evenly over the padding sawdust for 24 h
Sawdust removal	All sawdust was removed for 24 h
Light/dark cycle	Light/dark cycle inversion for 24 h
Tail pinch	Paperclips pinched for 5 min
Cold stimulation	Mice were kept in a 4 ℃ environment for 5 min

CUMS, chronic unpredictable mild stress.

incubated at room temperature for 1.5 h. The samples were centrifuged at 2000 g for 5 min and the supernatants were removed. The beads were washed with RIPA buffer for four times. Finally, 40 μl 1×loading buffer was added to the beads. The samples were boiled for 10 min and loaded for sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis.

Immunohistochemistry

The paraffin-embedded tissues were cut into 4-μm sections. After deparaffination and hydration, antigen retrieval was performed by boiling in a sodium citrate solution. Then, 3% H_2O_2 was used to eliminate endogenous peroxidase activities. After that, incubation with primary antibodies was performed overnight at 4 °C followed by incubation with the secondary antibodies. Color developments were conducted with diaminobenzidine. The tissue sections were then stained with hematoxylin, dehydrated, mounted, and observed under a light microscope.

The staining intensity of each protein was scored 0 (negative), 1 (weak), 2 (moderate), or 3 (strong), and the degree of staining was assessed according to the positive staining percentage and scored as follows: 0 (0%), 1 (1%–25%), 2 (26%–50%), 3 (51%–75%), or 4 (76%–100%). The combination of the staining intensity score and the staining degree score was defined as the final staining score (0–12) of each protein. All scores were evaluated by two experienced pathologists in a blinded manner.

Animal grouping and treatment

In total, 36 mice were randomly divided into the following groups: control, CUMS, ICI118,551, CUMS +ICI118,551, 6-hydroxydopamine (6-OHDA), and CUMS+6-OHDA (n = 6 per group). β2AR antagonist ICI118,551 (ICI, 0.2 mg/kg/day, Cayman) and neurotoxin 6-OHDA (150 mg/kg/week, Cayman) were injected intraperitoneally. ICI118,551 was dissolved in phosphate buffer saline, and 6-OHDA was dissolved in normal saline containing 0.2% Vitamin C.

Mouse behavior assessment

The chronic stress behavior of mice was measured using the tail suspension test (TST)[34] and light/dark box (LDB).[35] The mice were transferred to the behavioral testing room for 30 min before the experiment to acclimate to the test room. In the TST, mice were suspended by taping at approximately 1 cm from the tip of the tail. The evaluation was performed for 6 min, and the duration of immobility was measured. In the LDB, light–dark box consisted of two compartments. One compartment was fully opaque, while the other was lit from the compartment ceiling by a 320 lux bulb. A small opening in the partition wall allowed free passage between the light and dark compartments. Mice were individually placed in the dark side of the light–dark box and were allowed to move freely for 10 min. Meanwhile, a video camera recorded their activities and analyzed using an automated tracking system (SuperMaze video tracking software, XinRuan Information Technology, Shanghai, China). All test chambers were cleaned with 75% ethanol before and after each test to avoid any olfactive cues. The observers were blinded to the treatment of the mice.

Mouse model of xenograft tumor

MKN45 cell suspension (2×10^8 cells in 1 mL of phosphate buffer saline) was injected subcutaneously into the nude mice to prepare subcutaneous tumors. The subcutaneous tumors were collected and the tumor tissue pieces with a volume of 1 mm^3 were prepared. Tissue pieces were implanted under the serous gastric membranes with an improved sewing method.[36] Briefly, mice were put under general anesthesia. Laparotomy was performed to expose the stomach. The gastric serosa was opened and the tumor tissue pieces were implanted beneath the gastric serosal layer. Mice were given buprenorphine through subcutaneous injection at a dose of 0.1 mg/kg at the time of surgery, and they were given an additional dose 6–8 h after the surgery for analgesia.

RNA-Seq analysis

Total RNA of xenograft tumors (six mice for each group) was extracted with Trizol (cat#15596018, Thermofisher) following the manufacturer's procedures. Next, cDNA was generated through reverse transcription using SuperScriptTM II Reverse Transcriptase (1896649, Invitrogen). Libraries were generated by polymerase chain reaction amplification, purified by AmPure XP magnetic beads (Beckman Coulter), and quantified using a Qubit 2.0 fluorometer (Thermo Fisher Scientific). After the heat-labile UDG enzyme (NEB, m0280) treatment of the U-labeled second-stranded DNAs, the ligated products are amplified with polymerase chain reaction. Next, we used Illumina NovaseqTM 6000 (LC Bio Technology Co, Ltd Hangzhou, China) to perform paired-end sequencing according to standard procedures (PE150). The RNA libraries were sequenced on the illumina NovaseqTM 6000 platform by LC Bio Technology CO, Ltd (Hangzhou, China). Bioinformatic analysis was

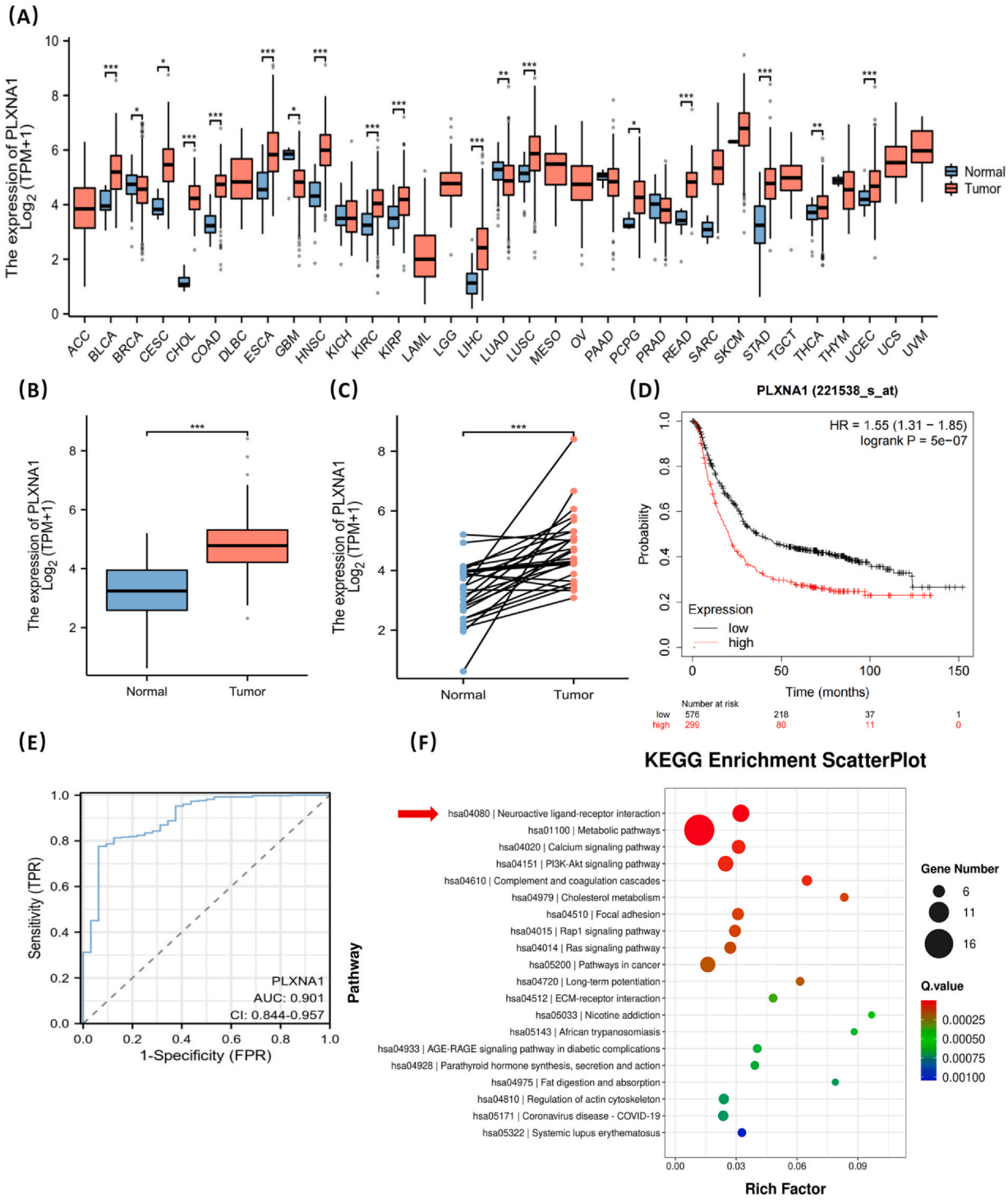

Fig. 1 Expression and prognostic value of PlexinA1 in gastric cancer. (a) Expression of PlexinA1 in pan-carcinoma in The Cancer Genome Atlas (TCGA) database. (b) Analysis of TCGA database shows the expression of PlexinA1 mRNA in unpaired gastric cancer tumor tissues. (c) Analysis of TCGA database shows the expression of PlexinA1 mRNA in paired gastric cancer tumor tissues. (d) PlexinA1 and overall survival in individuals with gastric cancer in the Kaplan–Meier-plotter database. (e) Receiver operating characteristic curve was used to evaluate the values of PlexinA1 expression in diagnosis of gastric cancer. (f) Gene set enrichment analysis (KEGG pathway analysis) of transcripts preferentially enriched neuroactive ligand-receptor interaction in the chronic unpredictable mild stress group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 2
Correlation of PlexinA1 with clinicopathological parameters in patients with gastric cancer in TCGA database.

Characteristics	Low expression of PLXNA1	High expression of PLXNA1	*P* value
n	187	188	
Age, n (%)			0.798
≤ 65	83 (22.4%)	81 (21.8%)	
> 65	102 (27.5%)	105 (28.3%)	
Pathologic T stage, n (%)			0.036
T1	12 (3.3%)	7 (1.9%)	
T2	47 (12.8%)	33 (9%)	
T3	82 (22.3%)	86 (23.4%)	
T4	39 (10.6%)	61 (16.6%)	
Pathologic N stage, n (%)			0.295
N0	60 (16.8%)	51 (14.3%)	
N1	49 (13.7%)	48 (13.4%)	
N2	34 (9.5%)	41 (11.5%)	
N3	30 (8.4%)	44 (12.3%)	
Pathologic M stage, n (%)			0.543
M0	166 (46.8%)	164 (46.2%)	
M1	11 (3.1%)	14 (3.9%)	
Pathologic stage, n (%)			0.142
Stage I	30 (8.5%)	23 (6.5%)	
Stage II	59 (16.8%)	52 (14.8%)	
Stage III	63 (17.9%)	87 (24.7%)	
Stage IV	16 (4.5%)	22 (6.2%)	
Gender, n (%)			0.639
Female	69 (18.4%)	65 (17.3%)	
Male	118 (31.5%)	123 (32.8%)	

TCGA, The Cancer Genome Atlas.

performed using the OmicStudio tools at https://www.omicstudio.cn/tool.

Statistical analyses

Data were analyzed using SPSS 19.0. Data are presented as mean ± standard error of means unless otherwise stated. The Student's t-test or one-way analysis of variance was used to compare continuous variables. The chi-square test was used to compare categorical variables. Pearson's correlation and Spearman's correlation tests were used to analyze the correlation between the two variables.

Results

Expression and prognostic value of PlexinA1 in gastric cancer

Analysis of TCGA data revealed that PlexinA1 expression was upregulated in 15 tumor types compared with the corresponding normal tissues, including bladder urothelial carcinoma, cervical squamous cell carcinoma and endocervical adenocarcinoma, cholangiocarcinoma, colon adenocarcinoma, esophageal carcinoma, head and neck squamous cell carcinoma, kidney renal clear cell carcinoma, kidney renal papillary cell carcinoma, liver hepatocellular carcinoma, lung squamous cell carcinoma, pheochromocytoma and paraganglioma, rectum adenocarcinoma, stomach adenocarcinoma, thyroid carcinoma, and uterine corpus endometrial carcinoma (Figure 1(a)). Analysis of TCGA database showed that PlexinA1 mRNA expression levels were higher in GC tissues than in unpaired (Figure 1(b)) or paired adjacent normal gastric samples (Figure 1(c)). KM-plotter database showed that the patients with high levels of PlexinA1 expression had worse overall survival outcomes than those with low levels of PlexinA1 expression (Figure 1(d)).

Based on the median level of PlexinA1 expression, GC patients were divided into two groups, namely high and low-PlexinA1 expression groups, to assess the relationship between PlexinA1 expression and the clinicopathological aspects of GC patients. PlexinA1 expression was found to be significantly linked with the tumor (T) stage (Table 2) ($P < 0.05$).

The receiver operating characteristic (ROC) curve was used to evaluate the diagnostic values of PlexinA1 and ADRB2 expression. The areas under the ROC curve were 0.901 for PlexinA1 in diagnosis of GC (Figure 1(e)). Of note, gene set enrichment analysis (KEGG pathway analysis) showed that genes are mainly enriched in neuroactive ligand-receptor interaction in the CUMS group (Figure 1(f)). The collective findings indicate that high expression of PlexinA1 is associated with a poor prognosis in patients with GC.

Chronic stress is associated with high expression of ADRB2 and PlexinA1 in GC

We next analyzed the expression levels of plexin-A1 with or without chronic stress in gastric samples from human patients. A total of 36 GC patients were recruited in this study, and their preoperative psychological state was evaluated with SAS and SDS. Among these patients, patients with chronic stress accounted for 33% (Figure 2(a)). According to the SAS and SDS scores, GC patients were divided into a chronic stress group and a group without chronic stress. The expression levels of ADRB2 and PlexinA1 in the collected tissue samples were measured with IHC. The expression levels of ADRB2 and PlexinA1 were higher in the chronic stress group than in the non-chronic stress group (Figure 2(b)), and their levels were higher in GC

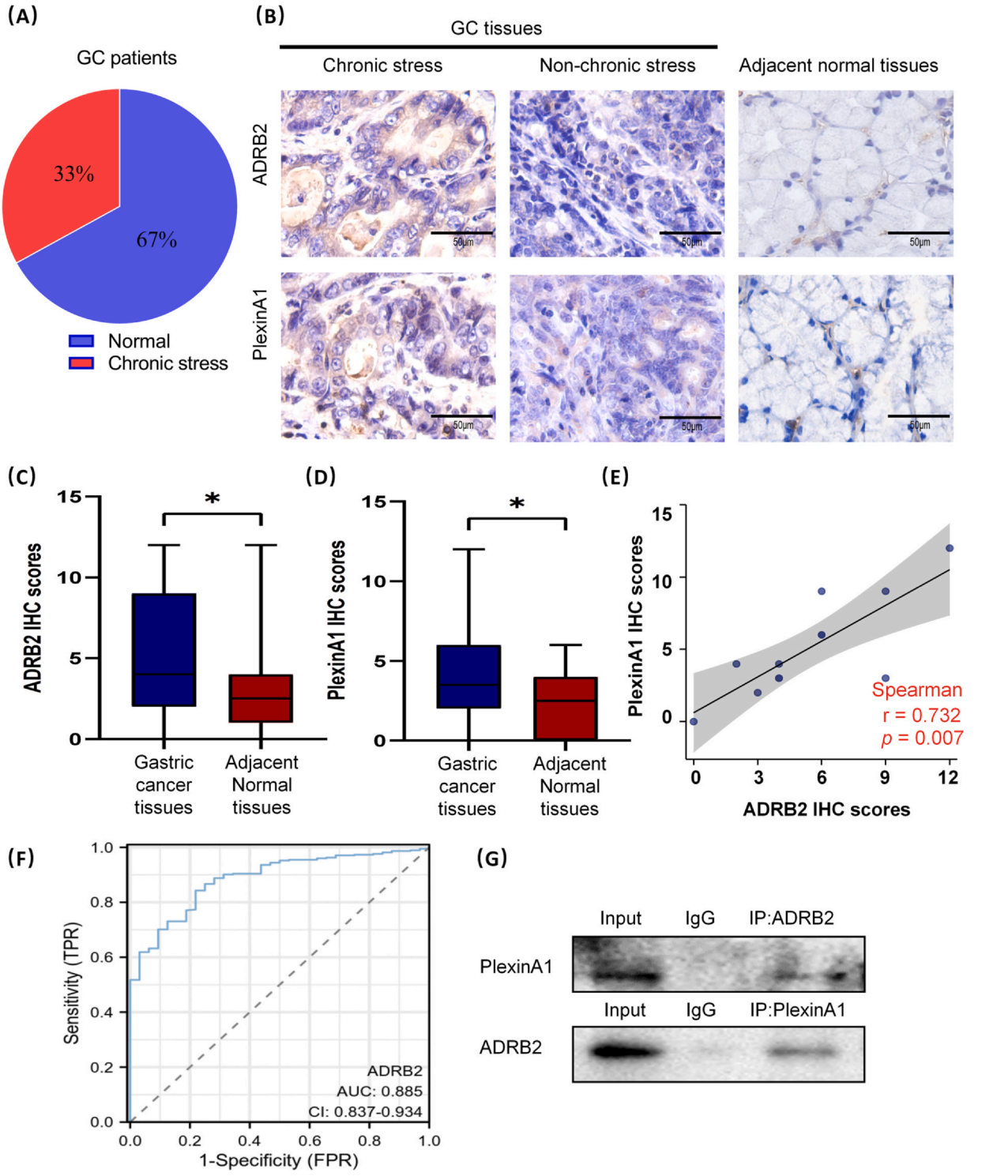

Fig. 2 The results were from 36 clinical patients. (a) Proportion of patients with chronic stress. (b) Immunohistochemistry results of ADRB2 and PlexinA1 in clinical tissue samples (40× magnification). (c-d) Histograms of ADRB2 and PlexinA1 expression levels. (e) The relationship between ADRB2 and PlexinA1. (f) Receiver operating characteristic curve was used to evaluate the values of ADRB2 expression in diagnosis of gastric cancer. (g) Co-immunoprecipitation assays were performed with clinical patient tissues. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. ADRB2, adrenoceptor beta 2; GC, gastric cancer.

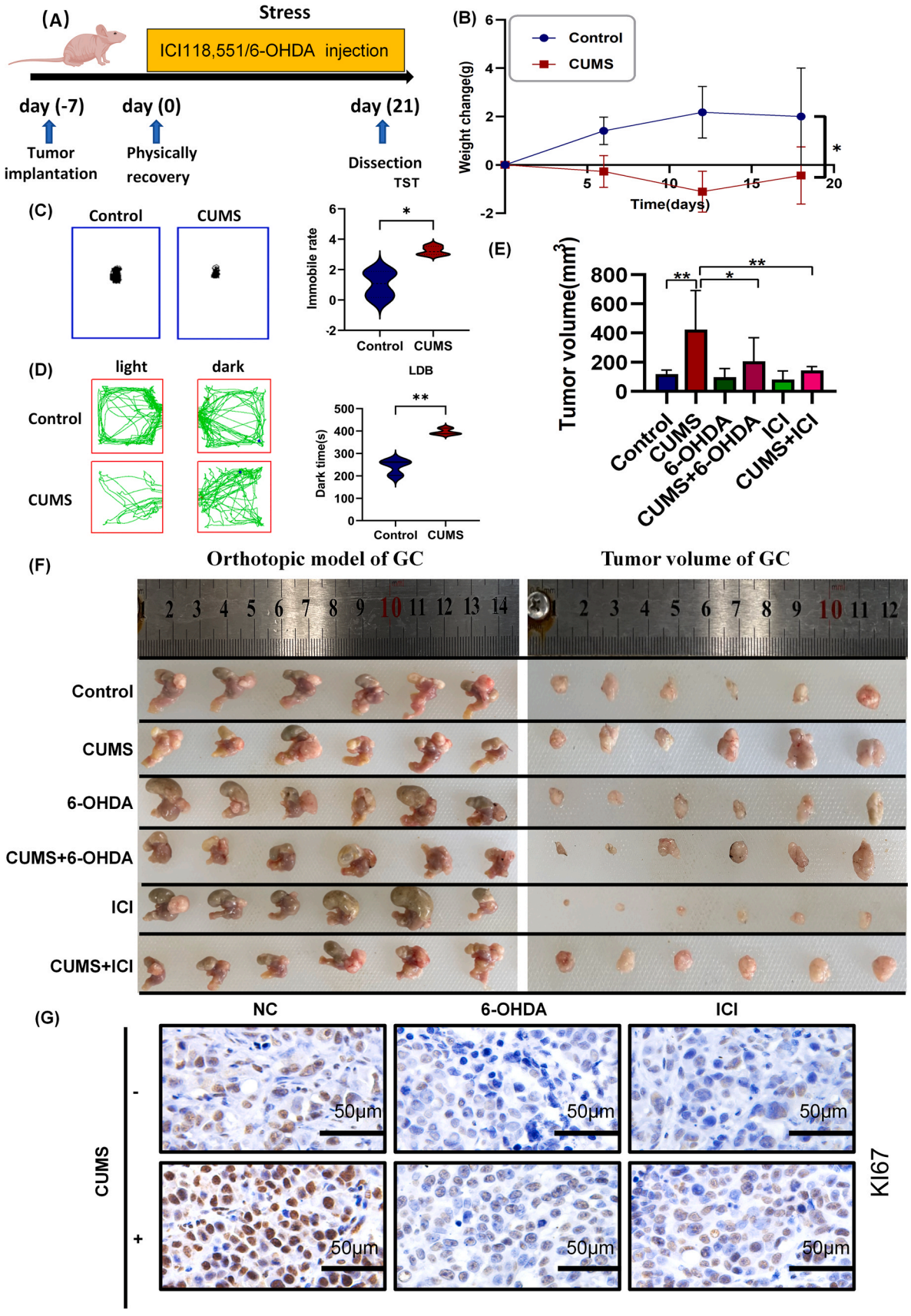
(A)
Stress
ICI118,551/6-OHDA injection
day (-7)
Tumor implantation
day (0)
Physically recovery
day (21)
Dissection
TST
(B)
Control
CUMS
Weight change(g)
Time(days)
(C)
Control
CUMS
Immobile rate
LDB
(D)
light
dark
Control
CUMS
Dark time(s)
(E)
Tumor volume(mm3)
Control
CUMS
6-OHDA
CUMS+6-OHDA
ICI
CUMS+ICI
(F)
Orthotopic model of GC
Tumor volume of GC
Control
CUMS
6-OHDA
CUMS+6-OHDA
ICI
CUMS+ICI
(G)
NC
6-OHDA
ICI
CUMS
KI67
50μm

Fig. 3 Morphological and behavioral results in mice. (a) Diagram of mouse experimental procedures. (b) Weight changes after CUMS was applied. (c) Behavioral results of animals in the tail suspension test, representative traces (left) and the immobile rate (right). (d) Behavioral results of animals in the LDB, representative traces (left) and the time spent in the dark box of mice (right). (e) Tumor sizes in different groups. (f) Orthotopic model of gastric cancer (left) and tumor volume of gastric cancer (right). (g) Ki67 immunohistochemistry results in different groups (40× magnification). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. CUMS, chronic unpredictable mild stress; GC, gastric cancer; LDB, light/dark box; 6-OHDA, 6-hydroxydopamine.

tissues than adjacent normal tissues (Figure 2(c) and (d)). Meanwhile, ADRB2 and PlexinA1 in GC tissues from GC patients with chronic stress were positively correlated ($r = 0.732$, $P < 0.01$) (Figure 2(e)). The areas under the ROC curve were 0.885 for ADRB2 in diagnosis of GC (Figure 2(f)).

Our previous data have reported that, in GC cell models, PlexinA1 might be a potential target for β2AR to regulate the malignant progression of GC.[37] Furthermore, the downstream targets of β2AR need further research *in vivo*. Co-IP assays were performed with proteins isolated from the GC tissues and binding between PlexinA1 and ADRB2 proteins was found (Figure 2(g)). This reveals that PlexinA1 may also be a potential target for the treatment of chronic stress GC patients.

Chronic stress promoted GC progression in vivo

To further explore the role of chronic stress in GC progression, we used the CUMS mouse model. It has been known that chronic stress promotes the release of stress-hormone primarily via the hypothalamus-pituitary-adrenal axis, and the adrenergic receptors preferentially bind to epinephrine. Epinephrine can promote the development and stemness of cancer cells via interaction with β2AR.[38] To examine the relationship of chronic stress, epinephrine, ADRB2, and PlexinA1 with GC progression, we divided BALB/c nude mice into control group, CUMS group, 6-OHDA group, ICI118,551 group, CUMS+6-OHDA group, and CUMS+ICI118,551 group. Figure 3(a) shows the schematic timeline of the experimental procedures. The mice were inoculated with orthotopic transplantation tumor. After a week of physically healing, CUMS was used to construct the chronic stress model. After 3 weeks, the mice were tested for behavioral changes. We measured the changes in the body weight of mice every 3 days and found that CUMS induced weight loss in mice in early days. The body weight of the stressed mice was significantly decreased over the time course compared to the control group (Figure 3(b)). To investigate the CUMS-induced anxiety-like or depression-like behaviors in mice, we performed the TST and LDB experiments. As shown in Figure 3(c) and (d), mice in CUMS group exhibited chronic stress-like behaviors, as evidenced by increased immobility time in the TST and longer time spent in the dark in the LDB compared with the control group, indicating that the chronic stress mouse model was successfully constructed.

We constructed an orthotopic mouse model of GC via gastric surgery by implanting tumor pieces under the gastric serosa in mice with or without CUMS. Meanwhile, tumor growth was also assessed. We found that weekly intraperitoneal injections of 6-OHDA and daily intraperitoneal injections of ICI118,551 for 3 weeks could eliminate chronic stress-induced GC progression after tumor transplantation. Compared with the control group, the tumor growth was faster in the CUMS group, and the groups of 6-OHDA and ICI118,551 had no significant difference from the control group (Figure 3(e)). However, compared with the CUMS group, the tumor volume was decreased in the CUMS+6-OHDA and CUMS+ICI118,551 groups (Figure 3(e) and (f)). There was a higher level of Ki67 expression in the CUMS group than the control group, while Ki67 levels were lower in the CUMS+6-OHDA and CUMS+ICI118,551 groups compared to the CUMS group (Figure 3(g)).

Western blot analysis showed that, compared with the control group, the protein expression levels of ADRB2 and PlexinA1 were higher in CUMS group. The 6-OHDA and ICI118,551 groups showed no significant difference from the control group. Compared with the CUMS group, the protein expression levels of ADRB2 and PlexinA1 were decreased in the CUMS+6-OHDA and CUMS+ICI118,551 groups (Figure 4(a)-(f)). The results from the IHC analysis were consistent with the Western blot analysis (Figure 4(g)).

Chronic stress promotes the epithelial-to-mesenchymal transition (EMT) of GC via epinephrine-β2AR/PlexinA1 pathway

We found that, compared with the control group, the protein expression level of E-cadherin (epithelial marker) was lower in the CUMS group, whereas the protein expression levels of N-cadherin and α-SMA (mesenchymal markers) were higher in the CUMS group (Figure 5(a)-(d)). Compared with the CUMS group, the protein expression level of E-cadherin increased in groups of CUMS+6-OHDA and CUMS +ICI118,551, whereas the treatment of 6-OHDA and ICI118,551 reversed this increase of N-cadherin and α-

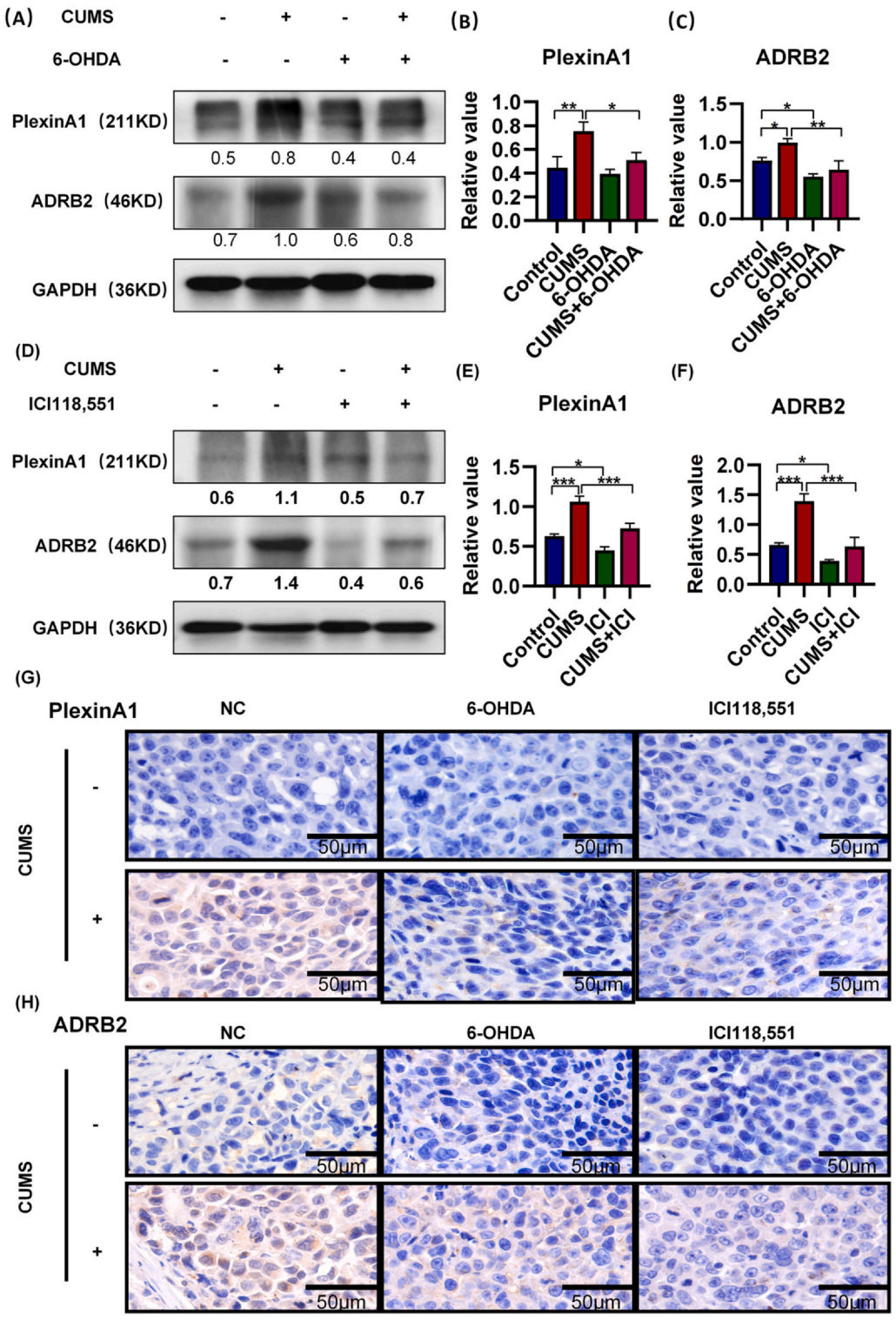

Fig. 4 Western blot and immunohistochemistry (IHC) analyses of ADRB2 and PlexinA1. (a)-(f). Western blot analysis and quantification of ADRB2 and PlexinA1 expression levels in different groups. (g) PlexinA1 IHC results in different groups (40× magnification). (h) ADRB2 IHC results in different groups (40× magnification). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. ADRB2, adrenoceptor beta 2; CUMS, chronic unpredictable mild stress; 6-OHDA, 6-hydroxydopamine.

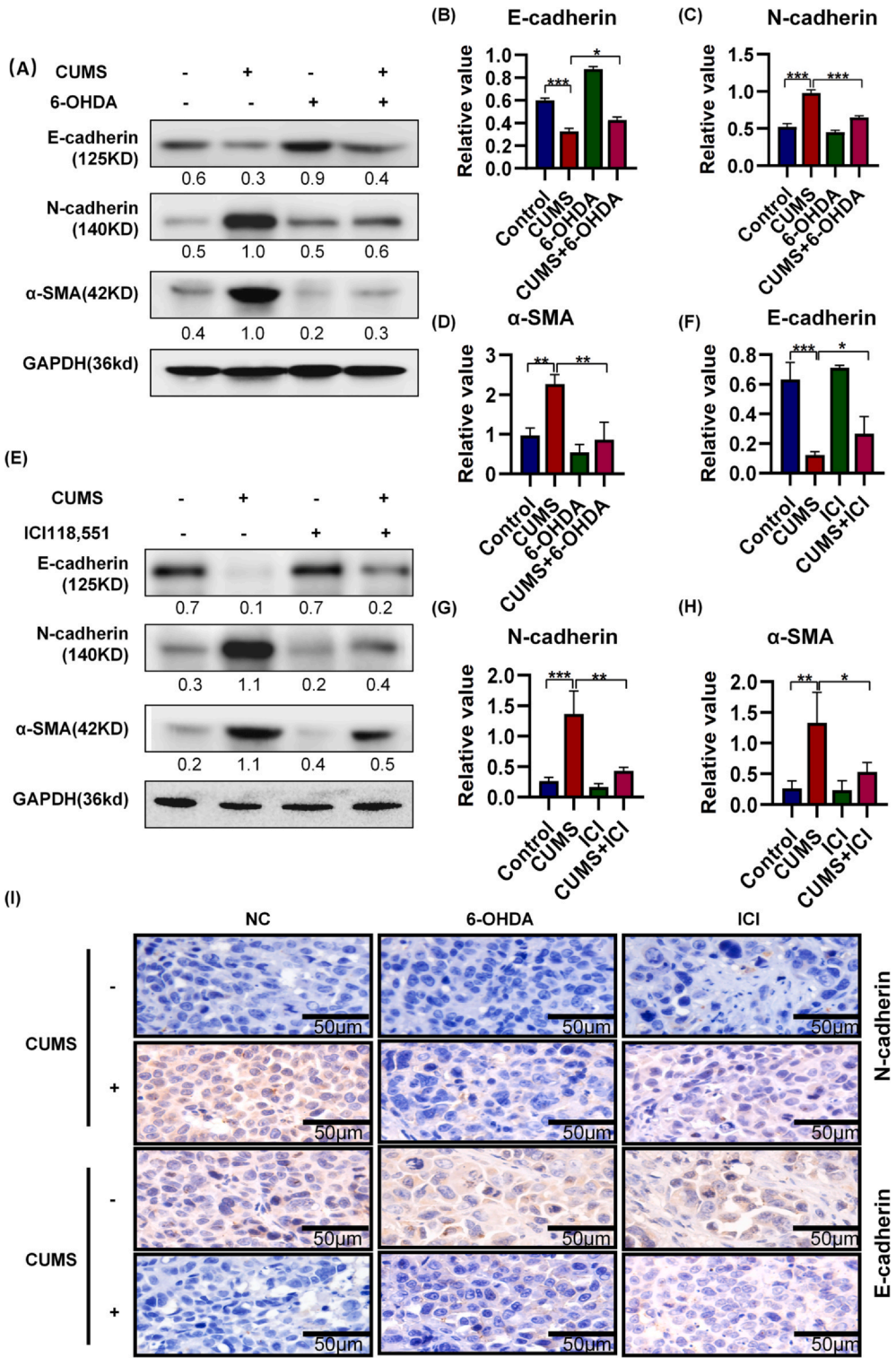

Fig. 5 Western blot analysis and immunohistochemistry of epithelial-to-mesenchymal transition-related markers. (a)-(h) Western blot analysis and quantification of N-cadherin, E-cadherin, alpha-smooth muscle actin expression levels in different groups. (i) Immunohistochemistry results of N-cadherin and E-cadherin in different groups (40× magnification). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. α-SMA, alpha-smooth muscle actin; CUMS, chronic unpredictable mild stress; 6-OHDA, 6-hydroxydopamine.

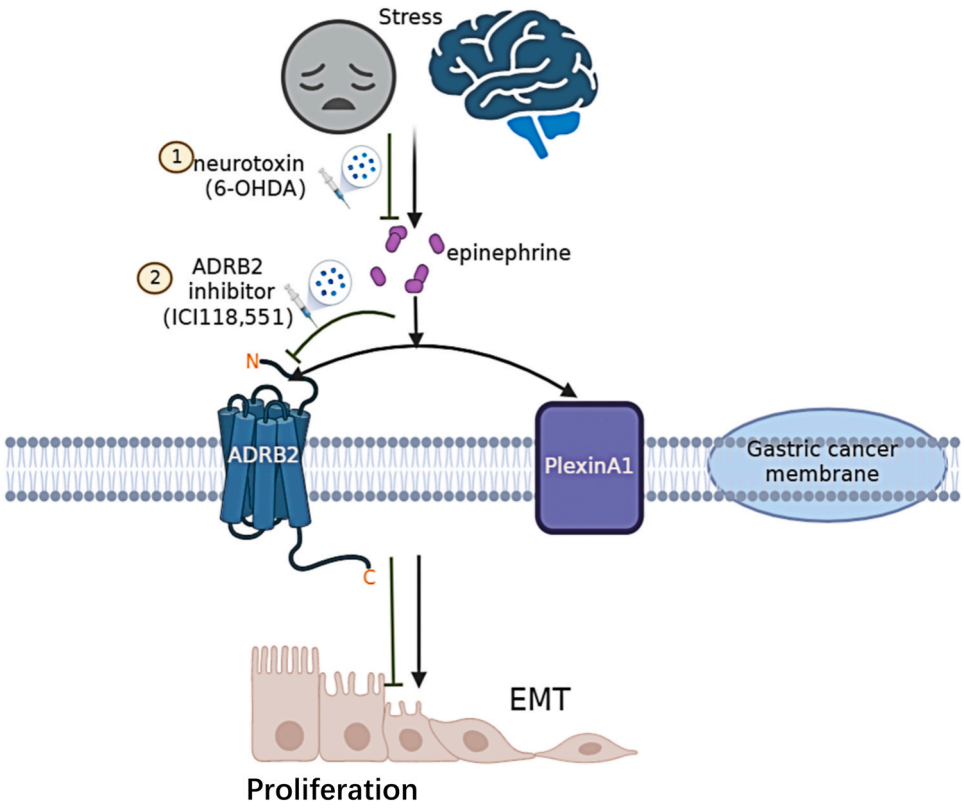

Fig. 6 Schematic diagram of the mechanism: chronic stress promotes gastric cancer proliferation and EMT through neuroendocrine system, while neurotoxin or β2AR inhibitor reverses these effects. EMT, epithelial-to-mesenchymal transition; 6-OHDA, 6-hydroxydopamine.

SMA protein expression levels (Figure 5(a)-(h)). Meanwhile, the IHC analysis obtained consistent results on the expression levels of N-cadherin and E-cadherin (Figure 5(i)).

Discussion

In recent years, β-blockers have been shown to block many of the deleterious effects of stress. However, large population-based case-control studies have not confirmed alterations in risk for invasive breast carcinoma with β-blocker use.[39] This suggests that there may be other important factors involved in the process of chronic stress-promoting tumor progression. In this study, we revealed the presence of a chronic stress-activated adrenoceptor beta 2/PlexinA1 signaling pathway, which promoted cell proliferation and EMT of GC. Chronic stress is considered an independent risk factor for GC poor prognosis.[40,41] Chronic stress is a state affected by psychological, social, and medical factors. To elucidate the effect of chronic stress on tumor growth of GC, we analyzed database and samples from clinical patients and performed animal studies. We found that chronic stress-induced epinephrine could promote GC proliferation and EMT through β2AR/PlexinA1 signaling pathway. We found that blocking epinephrine or β2AR abrogated the expression of PlexinA1 and decreased chronic stress-induced GC proliferation and EMT *in vivo*.

By analyzing the TCGA database and KM-Plotter, we found that the expression of PlexinA1 in GC tumor tissues was higher than that in gastric normal tissues, and the higher the expression level of PlexinA1, the worse the overall survival prognosis of patients. By analyzing patient information in the TCGA database, PlexinA1 expression was found to be linked with the T stage. We found that chronic stress accounted for about 33% of the included GC patients based on the SDS and SAS scale scores. We found that the expression levels of ADRB2 and PlexinA1 in GC tissues were increased compared with adjacent tissues. In the GC tissues, the expression levels of ADRB2 and PlexinA1 in the tissues of chronic stressed patients were higher than those in the non-chronic stress group. At the same time, Spearman correlation analysis found that ADRB2 and PlexinA1 protein expression were positively correlated in the GC tissues of patients with chronic stress.

Further, we found that ADRB2 protein bounds to PlexinA1 protein in the GC tissues. We speculate that, since both ADRB2 and PlexinA1 are transmembrane cell surface receptors, they may bind each other on the cytoplasmic membrane. However, we do not know which domain of each protein mediates the binding or whether they bind via cytosolic proteins, nor do we know if the binding is stimulated upon activation of the receptors. These questions may be addressed in future studies. Nevertheless, our results imply that ADRB2 and PlexinA1 may play an important role in chronic stress-induced progression of GC (Figure 6).

To examine the effect of chronic stress on GC progression, we used the CUMS model that utilizes multiple, discontinuous, and repetitive stress conditions, overcoming the influence of animals on the habituation of a single stressor.[42] The CUMS model was validated by weight weighing, TST, and LDB. Indeed, we found that GC tumor growth was increased in the CUMS group compared with the control group, suggesting that chronic stress promotes GC progression. Either ICI118,551 or 6-OHDA could reduce the GC tumor growth in the CUMS group, suggesting that blocking β2AR or sympathetic nerve signals to the GC tumors could inhibit GC tumor growth. To understand the underlying mechanism, we checked the expression of ADRB2, PlexinA1, and EMT markers. We found that both ICI118,551 and 6-OHDA treatments decreased the expression levels of ADRB2 and PlexinA1. Further, E-cadherin levels were decreased in the CUMS group compared with the control group, whereas N-cadherin and α-SMA levels were increased in the CUMS group compared with the control group, suggesting that CUMS enhanced EMT of the GC tumors. However, either ICI118,551 or 6-OHDA treatment could partially reverse the EMT phenotype, suggesting that blocking β2AR or sympathetic nerve signals to the GC tumors could act through reversal of EMT to inhibit GC progression.

Our study has some limitations: (1) The chronic stress state of animals includes complex psychological changes, which can lead to changes of behaviors, hormone levels, and immune system. For the successful inoculation of orthotopic transplanted tumors, nude mice were selected as animal models in this paper, which led to the lack of comprehensive immune system involvement; additional models with allograft mouse tumors would be needed to validate the findings; (2) one cell line was used, which should be validated with multiple cell line; and (3) as mentioned above, animal models of CUMS are highly sensitive to even the slightest changes and are influenced by various environmental and experimental factors. Reproducing the behavioral and physiological effects of CUMS has proven challenging for researchers. However, despite these difficulties, CUMS remains the best animal model for studying chronic stress. To enhance reproducibility, it is crucial to standardize the CUMS procedure and minimize the impact of influencing factors. This will enable scientists from different laboratories to obtain more consistent and reliable results.

Conclusions

Our findings suggest that chronic stress plays an important role in GC progression, and there is a potential for blocking the epinephrine-β2AR/PlexinA1 pathway in the treatment of GC. Further studies are warranted to establish blockade targets of the epinephrine-β2AR/PlexinA1 pathway as a potential therapy for improving the outcomes of GC patients with chronic stress.

Institutional Review Board Statement

The patients' consents for participation were approved by the institutional review board of Chengde Medical University (Ethics#2020003). Written informed consent was obtained from all patients who participated in this study. All animal experimental procedures adhered to the ethical guidelines and were approved by the Institutional Animal Care and Use Committee of Chengde Medical University (Ethics#2020013).

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Data availability statement Data will be made available on request. chengdie data (Original data) (Mendeley Data)

Declarations of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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