

Original Article

EHD3 promotes gastric cancer progression via Wnt/ β -catenin/EMT pathway and associates with clinical prognosis and immune infiltration

Jing Yu, Yunmeng Yan, Chunlan Hua, Hairong Song

Department of Clinical Laboratory, Key Clinical Laboratory of Henan Province, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China

Received May 12, 2023; Accepted August 21, 2023; Epub September 15, 2023; Published September 30, 2023

Abstract: Gastric cancer (GC) shows high levels of heterogeneity and predicts a poor prognosis. The expressions of EHD3 are found to be misregulated in a number of tumors. However, the clinical significance and potential function of EHD3 expression in GC patients remain unknown. In this study, we found that EHD3 expression was distinctly increased in GC specimens and cell lines in both TCGA datasets and our cohort. High levels of EHD3 expression were linked to worse outcomes for patients with GC in clinical tests. Nomogram based on multivariate assays displayed good predictive accuracy for GC patients, as evidenced by C-indices and calibration graphs. Low levels of EHD3 mRNA were discovered in GC tissues due to EHD3 methylation's negative regulation of EHD3. In addition, EHD3 was observed to be related to several immune cells and might play a role in successful immunotherapy. Functionally, it was verified that knockdown of EHD3 remarkably suppressed the proliferation, migration and invasion of GC cells in vitro and in vivo. Results of Western blot confirmed that knockdown of EHD3 suppressed the expressions of β -catenin, MMP-9, and N-cadherin, while promoting the expression of E-cadherin. Overall, this research identified a novel GC-related gene EHD3 which might be a novel prognostic biomarker involved in tumor microenvironment. EHD3 promoted the proliferation and metastasis of GC cells through influencing the Wnt/ β -catenin/EMT signaling pathway, suggesting it as a novel treatment target for GC patients.

Keywords: EHD3, gastric cancer, biomarker, tumor microenvironment, Wnt/ β -catenin/EMT signaling, metastasis

Introduction

Gastric cancer (GC) is an exceptionally aggressive and deadly type of cancer, and its rising incidence and mortality rates make it a significant global health issue [1]. In the United States alone, the latest cancer statistics (2019) reported 17,230 new cases of GC and 11,140 deaths caused by this disease [2]. Despite the development of various therapeutic strategies, patients with advanced GC continue to face an extremely poor prognosis, largely due to the high risk of metastasis and recurrence [3, 4]. One of the main challenges in treating GC is the lack of effective therapeutic options for advanced stages of the disease. Additionally, the molecular mechanisms underlying the development, invasion, and metastasis of GC are still not fully understood. Consequently, identifying novel metastasis-related genes and

elucidating their underlying mechanisms are crucial steps towards developing more effective anti-cancer treatments [5]. To date, numerous studies have been conducted to identify potential metastasis-related genes, and their findings have underscored the significance of various cellular pathways, including cell adhesion, migration, and invasion [6]. However, more research is necessary to adequately explore the complex network of molecular interactions involved in GC metastasis.

EHD3 [Eps15 homology (EH) domain-containing protein 3] is a member of the Eps15-homology domain (EHD) family of proteins, which are involved in regulating endocytic membrane trafficking in cells [7]. Endothelial cells, which border the interior surface of blood arteries and play an important part in the modulation of vascular homeostasis, are the cells that predomi-

EHD3 promotes gastric cancer progression

nantly produce the protein that is encoded by the EHD3 gene [8]. The EHD3 protein has been demonstrated to play a role in multiple cellular processes, encompassing endocytosis, intracellular trafficking, and signaling. Specifically, it interacts with a variety of proteins engaged in membrane transport, including clathrin, caveolin, and the SNARE complex [9, 10]. Studies have suggested that mutations in the EHD3 gene may be associated with several human diseases. For example, EHD3 is involved in regulating blood pressure, and mutations in the gene may contribute to hypertension [11]. Other studies have revealed that EHD3 participates in regulation of glucose metabolism, and mutations in the gene may lead to the development of type 2 diabetes [12]. Previously, some studies have investigated the function of the EHD3 gene in several cancers, including glioblastoma and colorectal cancer, amongst others [13-15]. However, the expression and function of EHD3 in GC have not been reported.

Our research aimed to carry out an in-depth investigation into the significance of EHD3 in GC by making use of the RNA sequencing data acquired from TCGA datasets, in addition to the bioinformatics and statistical techniques. Furthermore, the functions of EHD3 in the developments of GC were carefully examined by conducting experiments both *in vitro* and *in vivo* to test our hypotheses.

Materials and methods

Tissue samples

GC specimens and corresponding adjacent non-tumor tissues were procured from 14 patients who had undergone curative resection between 2021 and 2022 at the First Affiliated Hospital of Zhengzhou University. Prior to the surgery, none of the patients received any form of chemotherapy or radiotherapy. Pathological confirmation was performed to validate the collected samples, which were subsequently preserved in liquid nitrogen to facilitate total RNA extraction. The present study was carried out in accordance with the ethical standards laid down by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University, and all participating patients provided informed written consent.

Cell lines, culture conditions and cell transfection

The human GC cell lines SGC-7901, AGS, MGC-803 and BGC-823, as well as the human normal stomach epithelium cell line GES-1 were derived from the renowned National Infrastructure of Cell Line Resource in Beijing, China. After that, these cell lines were grown in either RPMI 1640 or DMEM. The culture medium was placed in an incubator set to 37 degrees Celsius with 5% carbon dioxide. For efficient cell transfection, the cutting-edge Lipofectamine 3000 method was employed, strictly following the guidelines provided by the manufacturer (Invitrogen, USA). During this procedure, EHD3 shRNA sequences were inserted into PLVX-shRNA vectors (sh-EHD3-#1, sh-EHD3-#2) painstakingly by the illustrious Genetong Biological organization (Xiamen, Fujian, China) in order to achieve the desired results. Further, sh-EHD3-#1, sh-EHD3-#2, or a control lentivirus vector was individually co-transfected with pVSVG, pRRE, and pREV plasmids into GC cells precisely. The cutting-edge technology of Lipofectamine 2000 chemicals was utilized when these cells reached a confluent state of 70%.

RNA isolation and RT-qPCR

Total RNA was meticulously isolated from the specimens with the aid of the highly reliable TRIzol reagent (Takara). Subsequently, RNA samples were meticulously quantified. RNA samples with an OD value of 260/280 were displayed in a manner that conformed to the highest possible standards using a reagent specifically designed for this purpose. For precise quantification of relative transcript abundance, RT-qPCR was meticulously conducted, employing a LightCycler480 System (Roche Ltd.) instrument and the SYBR Green PCR kit. GAPDH transcripts were deployed as internal controls to ensure the highest levels of accuracy in the quantification of relative transcript abundance. To achieve the most robust and reliable analysis, all data were meticulously examined with the $2^{-\Delta\Delta Ct}$ methods, which have been proven to be highly effective in ensuring the highest levels of accuracy and consistency in molecular analysis. RT-PCR for EHD3 was carried out with the forward primer 5'-TCAGGAACTCAACGCC-TTG-3' and reverse primer 5'-CTCCAGCACAGGGTTAGGTAG-3'. GAPDH forward and reverse

EHD3 promotes gastric cancer progression

primers contained 5'-GGAGCGAGATCCCTCCA-AAAT-3' and 5'-GGCTGTTGTCATACTTCTCATGG-3', respectively.

Cell counting kit-8 (CCK-8) assay

96-well plates were seeded with MGC-803 and SGC-7901 cells at a density of 2×10^3 cells per well, followed by overnight incubation. The cells in each well were then treated with 10 μ l of CCK-8 solution (Dojindo, Kumamoto, Japan) for a period of two hours. The optical density (OD) values of these cells were further measured using a microplate reader at a wavelength of 450 nm. The same procedure was repeated for three consecutive days to determine the OD values.

Colony formation assay

The cells in the logarithmic growth phase were treated with 0.25% trypsin to enable digestion. They were then dissociated using a plastic pipette and suspended in RPMI 1640 medium. The cells were seeded into a six-well plate at a density of 1×10^3 cells and incubated for one week. Afterwards, the cells were labeled with 0.1% crystal violet for 30 minutes and then preserved with 4% paraformaldehyde for 15 minutes. The colony formation capacity was evaluated relying on a microscope.

EdU assay

After being transfected, the GC cells were distributed at a density of 1×10^4 cells per well into 96-well dishes and then treated with the BeyoClick™ EdU Cell Proliferation Kit (Beyotime, Shanghai, China). DAPI solution was added to stain the cell nuclei. Finally, the cells were observed for EdU-positive cells using a fluorescence microscope (Olympus, Tokyo, Japan).

Transwell assay

To assess the invasive abilities of MGC-803 and SGC-7901 cells, Transwell assays were performed. Specifically, 2×10^5 cells of each type were placed into the upper chambers of Transwell plates which were pre-coated with diluted Matrigel® (1:5; BD Biosciences), and 500 μ l medium containing 10% FBS was added in the lower chambers. The cells on the upper membrane were gently brushed off after 48

hours of incubation at 37 degrees Celsius, and the broken cells through the membrane were stained with 0.1% crystal violet for 10 minutes while the plates were maintained at room temperature. Finally, the number of cells that had infiltrated the bottom chamber was tallied in five arbitrarily selected microscopic fields. It was hypothesized that the amount of tumor cells entering the bottom chamber was indicative of their capacity to migrate or invade.

Wound-healing assay

Various batches of transfected GC cells were placed into 6-well culture plates and allowed to grow until reaching a confluency of approximately 80%. Subsequently, the cell monolayer was intentionally damaged through the use of a 200 μ l pipette tip. The migratory behavior of the cells was assessed at both 0 and 24 hours by virtue of an Olympus microscope.

TUNEL staining

After being cultivated for a full 24 hours, the cells were then given two washes in PBS, and then fixated with 4% paraformaldehyde for 15 minutes, followed by permeation with 0.25% Triton-X 100 for 20 minutes. The TUNEL analyses were implemented in compliance with the manufacturer's guidelines provided by Roche. In a nutshell, the cells were first treated with a cocktail of terminal deoxynucleotidyl transferase reaction for 45 minutes at 37 degrees Celsius. Both procedures happened at the same time. Final nuclear staining was performed using either hematoxylin or methyl green.

Western bolt analysis

The Radio Immunoprecipitation Assay (RIPA) lysate solution was utilized in order to obtain the complete protein from GC cells. With twenty milligrams of total protein running through an SDS-PAGE gel at a concentration of 10%, the resulting proteins were transferred to the nitrocellulose membrane. The membranes were blocked in 5% nonfat milk at room temperature for 1 h, followed by incubating with specific primary antibodies against EHD2 (ab154784), N-cadherin (ab76057), E-cadherin (ab15148), MMP-9 (ab38898), β -catenin (ab32572) and β -actin (ab124964) overnight at 4°C. After that, the membranes were incubated with a secondary antibody that was conjugated with horse-

EHD3 promotes gastric cancer progression

radish peroxidase. A package for ECL Western blotting was adopted to identify the protein band. β -actin was considered as the loading control for this experiment.

Animal treatment

Pathogen-free housing was provided for female BALB/c mice that were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. They ranged in age from 4-6 weeks and weighed between 18 and 20 grams. A randomized experiment was conducted on ten mice, with each group consisting of five mice. The rodents in each group were offered different treatments. The sample size was determined considering statistical power and ethical guidelines. The inclusion and exclusion criteria of animals were independently established in accordance with the guidelines provided by AAA-LAC. Both MGC-803 and MGC-803-shRNA cells were inserted into the subcutaneous tissue of rodents in each group using a volume of 100 μ L of PBS. All animal experiments were conducted in strict accordance with the Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Zhengzhou University.

TCGA database

The dataset applied for RNA-sequencing contained 375 GC samples and 32 normal stomach tissues, each with corresponding clinical information downloaded from the TCGA datasets.

Analysis of DEGs between EHD3-high and -low expression GC groups

The unpaired Student's t-test included in the DESeq2 (3.8) software was used to identify and confirm genes with different levels of expression in individuals with EHD3-high and EHD3-low. These genes are referred to as differentially expressed genes, or DEGs. A heat map and volcano graphs were employed to provide comprehensive graphical representation of all DEGs.

Function enrichment analysis

DEGs were subjected to enrichment analysis of biological processes in Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes

(KEGG), and disease ontology (DO) using the clusterProfiler R and DOSE packages in order to predict their underlying functions [16]. The most important functional words distinguishing GC and normal categories were determined by GSEA. $P < 0.05$ was considered statistically significant. If the p -value for the enriched gene set was less than 0.05, with a false detection rate less than 0.025, the gene set was therefore deemed significantly enriched.

The construction of nomogram and calibration curves

The "rms" package in the R software was adopted to generate both a nomogram and calibration plots. The nomogram allowed to assess the level of agreement between the predicted and actual probabilities, while the calibration plots served as an essential role in forecasting survival rates for 1, 3, and 5 years.

Association between EHD3 and immune cell type fractions

The relative fractions of 22 subtypes of infiltrating immune cells (TIICs) in TCGA STAD dataset were estimated with the aid of the CIBERSORT algorithm (<https://cibersort.stanford.edu/>), using normalized gene expression data. The LM22 gene signature was used. Only patients with CIBERSORT $P < 0.05$ were included for further analysis, with the sum of immune cell type estimates equaled 1 for each sample. The proportions of TIICs between low and high expression groups were calculated for further comparison. A statistically significant result was determined at $P < 0.05$. Besides, the impact of EHD3 on TILs was assessed by evaluating the relationship between EHD3 and immune checkpoints in different groups. Further, potential responses to immune checkpoint inhibitors (ICIs) were predicted by the Tumor Immune Dysfunction and Exclusion (TIDE) algorithm.

Statistical analysis

Statistical analysis was conducted using Prism 7.0 (GraphPad software, CA, USA) and R version 3.5.2. The differences between two groups of EHD3 were analyzed through the Student's t-test. ROC analysis was employed to validate the diagnostic value of EHD3 for GC patients. The impact of EHD3 expressions on the overall survival (OS) and progression-free survival (PFS) of GC patients was assessed through

EHD3 promotes gastric cancer progression

Kaplan-Meier analysis. Cox models were employed to conduct univariate and multivariate analyses. A *p*-value of less than 0.05 was considered statistically significant.

Results

High expression of EHD3 in GC and its association with clinical variables

Initially, pan-cancer analyses revealed that EHD3 expression was dysregulated in various types of tumors, and its expression trend varied between different tumors (**Figure 1A**). Importantly, a distinct increase in EHD3 expressions was found in GC specimens compared to non-tumor specimens (**Figure 1B**). To validate this finding, RT-PCR analysis was accordingly conducted, which showed consistent results with the TCGA dataset (**Figure 1C**). ROC analysis further confirmed that high EHD3 expression had a strong diagnostic potential for GC, with an AUC value of 0.8036 (95% CI: 0.6372 to 0.9700), indicating its ability to distinguish GC specimens from non-tumor specimens (**Figure 1D**). Moreover, through investigating the associations between EHD3 expressions and clinical variables in GC patients, it was observed that EHD3 expressions significantly increased in GC patients with advanced clinical stages (**Figure 1E** and **1F**). Nevertheless, no distinct association between EHD3 expression and grade, T stage, M stage, and N stage (**Figure 1E** and **1F**) was detected. Additionally, a heat map was drawn to present the differences in the distribution of clinical characteristics between patients whose EHD3 expression was high or low (**Figure 1G**). Notably, all GC patients were divided into a high-expression group (n=187) and a low-expression group (n=188) based on the median expression level of EHD3.

The prognostic value of EHD3 expression in GC patients

To investigate the prognostic significance of EHD3 expression in GC patients, Kaplan-Meier analysis was conducted using TCGA datasets, finding that patients with high EHD3 expression exhibited a shorter overall survival (OS) than those with low EHD3 expression (*P*=0.021, **Figure 2A**; *P*=0.004, **Figure 2B**). Time-dependent ROC analysis revealed diagnostic accuracy of EHD3 expression (**Figure 2C**). Additionally, univariate analysis manifested that age, clinical

stage and EHD3 expression were related to clinical outcomes of GC patients (**Figure 2D**). Furthermore, multivariate analysis confirmed that EHD3 expression (HR=1.330, 95% CI: 1.061-1.668, *P*=0.014) was an independent prognostic factor for OS (**Figure 2E**). It is obvious that this independent prognostic risk factor could provide clinicians with a quantitative method to predict the likelihood of overall survival at 1, 3, and 5 years for GC patients (**Figure 2F** and **2G**).

The association between EHD3 expression and DNA methylation in GC

Initially, an analysis of the methylation level of EHD3 was completed, with **Figure 3A** displaying a clear distribution of 13 CpG sites. Furthermore, a moderate inverse correlation between EHD3 expression and DNA methylation was detected (**Figure 3B**). To pinpoint the CpG sites most strongly linked to EHD3 mRNA expression, Pearson correlation analysis was performed accordingly. Our findings indicated that methylation of cg00981472, cg01163837, cg05882522, cg06773122, cg08251399, cg13795465, cg15355118, cg18444347, cg24743639, cg25202298, cg25428398, cg25840208, and cg2723003 was significantly and negatively associated with EHD3 expression (**Figure 3C**).

Correlation of EHD3 with the proportion of TICs

The immune cell landscape was explored with the help of the CIBERSORT approach, revealing the makeup of immune cells in GC samples (**Figure 4A**) and the interrelationships between immune cell types (**Figure 4B**). Furthermore, a total of ten types of tumor-infiltrating immune cells (TICs) related to EHD3 expression were identified (**Figure 4C-E**). Among these, six types of TICs displayed a positive correlation with EHD3 expressions, including Tregs, naïve B cells, resting CD4 memory T cells, monocytes, memory B cells, and resting mast cells. In contrast, four types of TICs exhibited a negative correlation with EHD3 expression, namely CD8 T cells, resting NK cells, M1 macrophages, and activated CD4 memory T cells. These findings further supported the notion that EHD3 levels impacted the immune activity within the tumor microenvironment.

EHD3 promotes gastric cancer progression

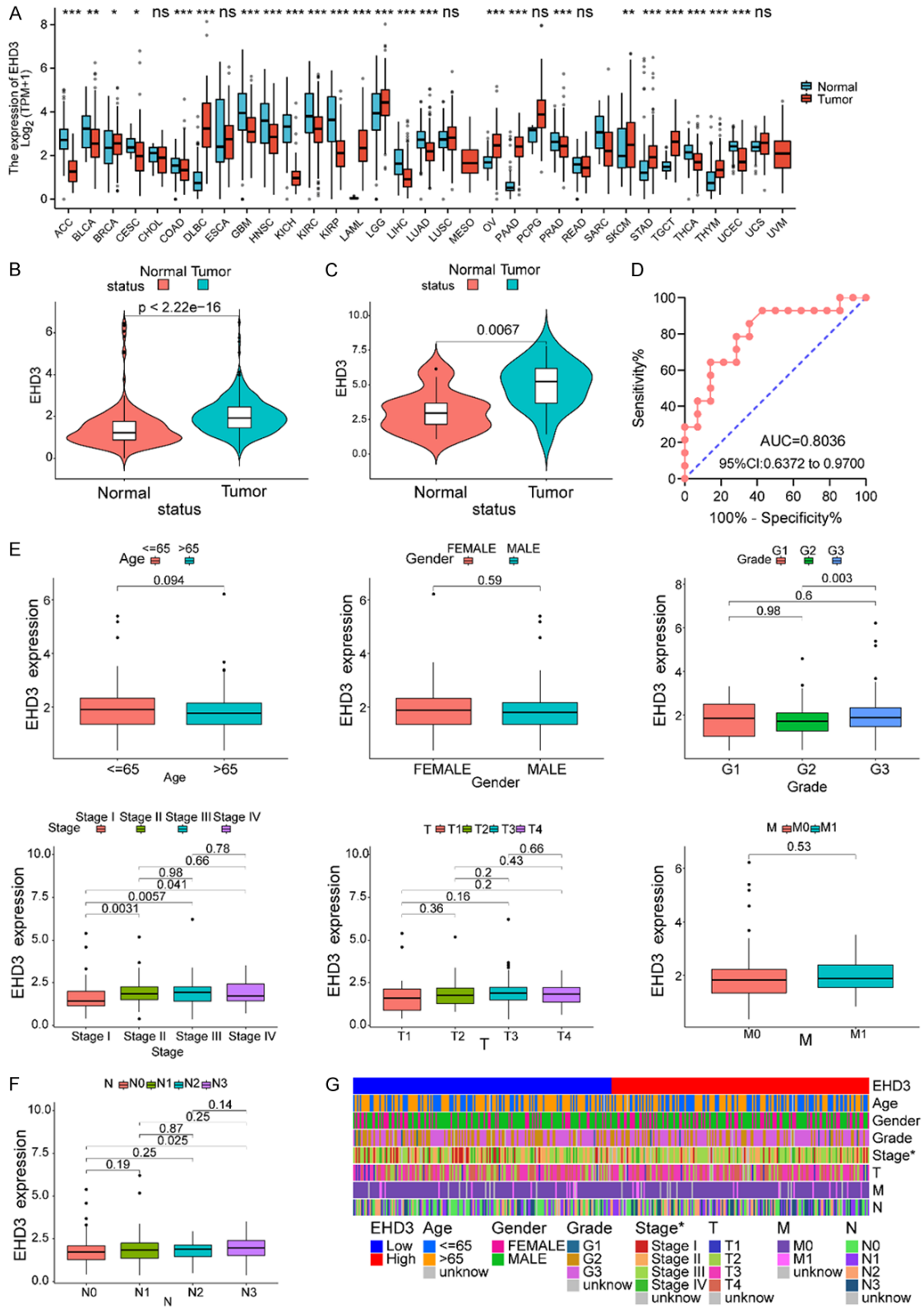


Figure 1. EHD3 expression was upregulated in GC and predicted an advanced clinical stage. A. Pan-cancer analysis of EHD3 in the TCGA and GTEx datasets. B. The expressions of EHD3 in GC specimens and non-tumor specimens in the TCGA and GTEx database. C. RT-PCR for the expression of EHD3 in our cohort. D. ROC assays were applied to examine the diagnostic value of EHD3 expression of GC. E and F. Association between the expression of EHD3 and clinical characteristics in GC patients. G. The association between EHD3 expression and different clinical factors were shown using Heat map. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

EHD3 promotes gastric cancer progression

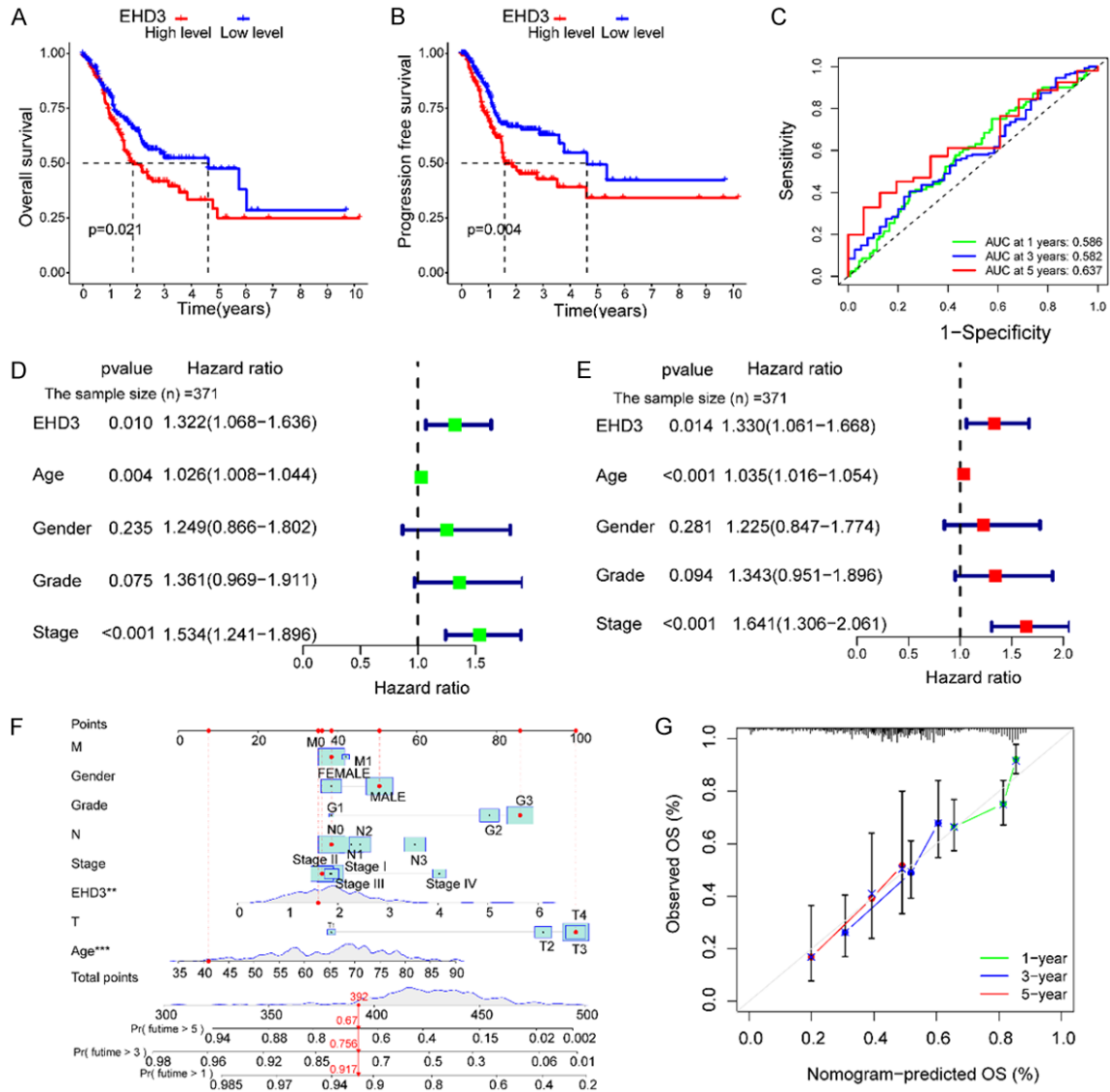


Figure 2. The prognostic values of EHD3 expressions in GC patients. A and B. Kaplan-Meier curve of EHD3 expression for OS and PFS of GC patients. C. ROC curves drawn using TCGA data. D and E. Univariate Cox analysis and Multivariate assays of the risk factors related with survival of GC. F. Construction of nomogram by the EHD3 expressions and clinical factors. G. The calibration plot. *P<0.05, **P<0.01, ***P<0.001.

Prediction of EHD3 expression in GC for sensitivity to immunotherapy

As immune cell function is regulated by various immune checkpoints, the relationship between EHD3 and different immune checkpoints was further explored. The results indicated a close association between EHD3 expression levels and multiple immune checkpoints (**Figure 5A**). Moreover, a negative correlation was observed between EHD3 expressions and tumor mutation burden (**Figure 5B**). The IPS algorithm was employed for the prediction of immune responses. According to the

data from the Cancer Immunome Atlas (TCIA), IPS scores were calculated based on the expression of PD-1 and CTLA4. Samples with high IPS scores (the top 25%) were considered as PD-1 or CTLA4 positive. All patients were further divided into four groups on the basis of PD1 and CTLA4 expression levels. It was revealed that in the PD1_negative_CTLA4_negative, PD1_positive_CTLA4_negative, and PD1_negative_CTLA4_positive groups, patients with low EHD3 expressions displayed higher scores of IPS (**Figure 5C-F**). The above results suggested that EHD3 might play a crucial role in regulating immune checkpoints and

EHD3 promotes gastric cancer progression

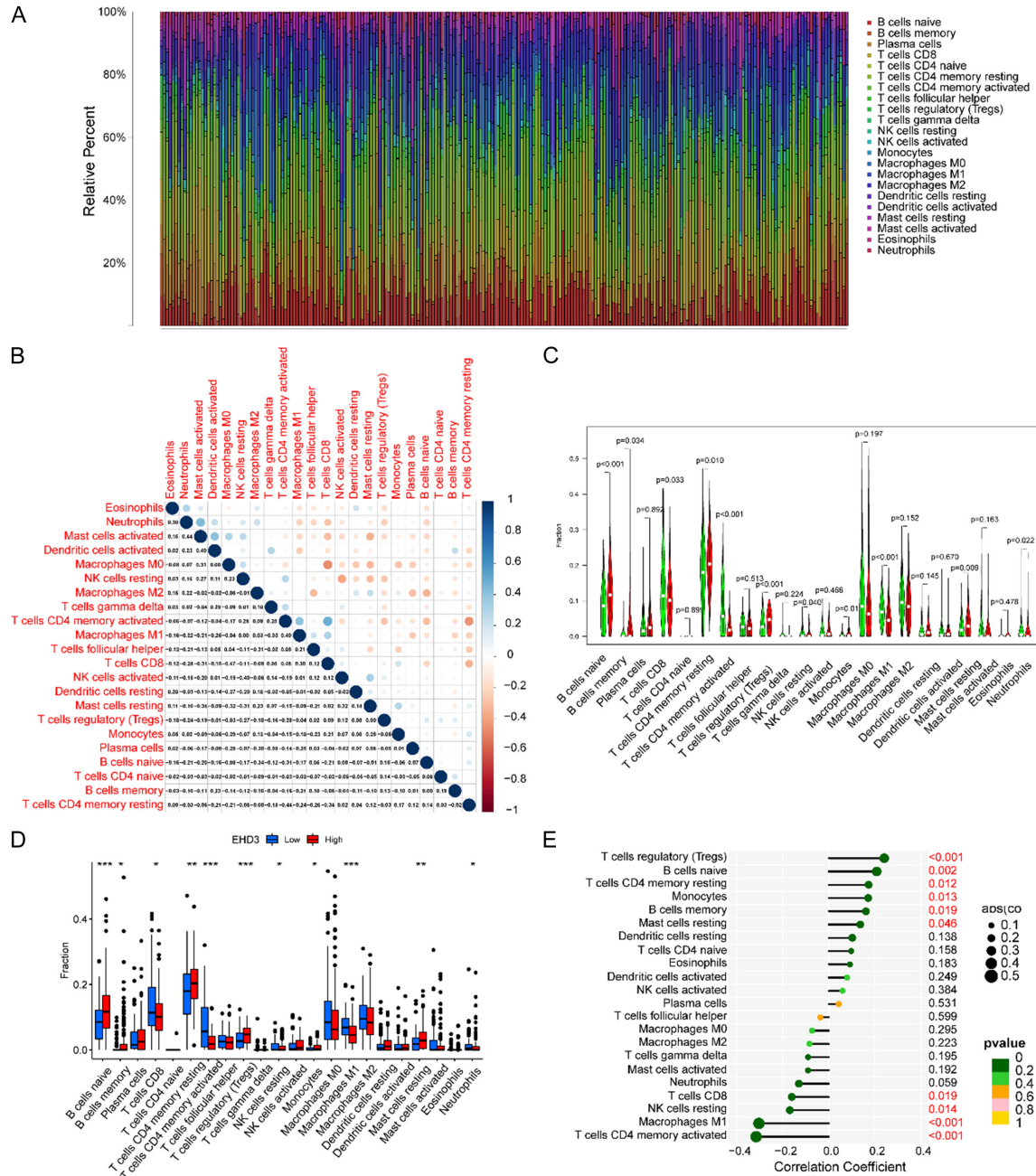


Figure 4. Correlations of TICs proportion with EHD3 expressions. A. Distribution of 21 different TIC types in GC tumor tissues as shown by a bar diagram. B. A heatmap depicting the relationships between twenty-one different TIC types. C and D. Differentiation of 21 distinct types of immune cells was compared between GC tumor tissues with low and high EHD3 expression using a violin plot, with the median EHD3 expression level serving as 100%. E. Correlation between EHD3 expressions and infiltrating immune cells in GC. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

gested that 226 DEGs were mainly associated with lymphoblastic leukemia, brain disease, myopathy, muscle tissue disease and muscular disease (Figure 6B). GO assays revealed that EHD3-associated DEGs were primarily involved in muscle system process, muscle contraction, regulation of membrane potential, neuronal cell

body, contractile fiber, myofibril, activator activity of signaling receptors, receptor ligand activity and hormone activity (Figure 6C). KEGG analysis suggested that 226 DEGs were principally enriched in neuroactive ligand-receptor interaction (Figure 6D). Through carrying out GSEA, it was found that CALCIUM_SIGNALI-

EHD3 promotes gastric cancer progression

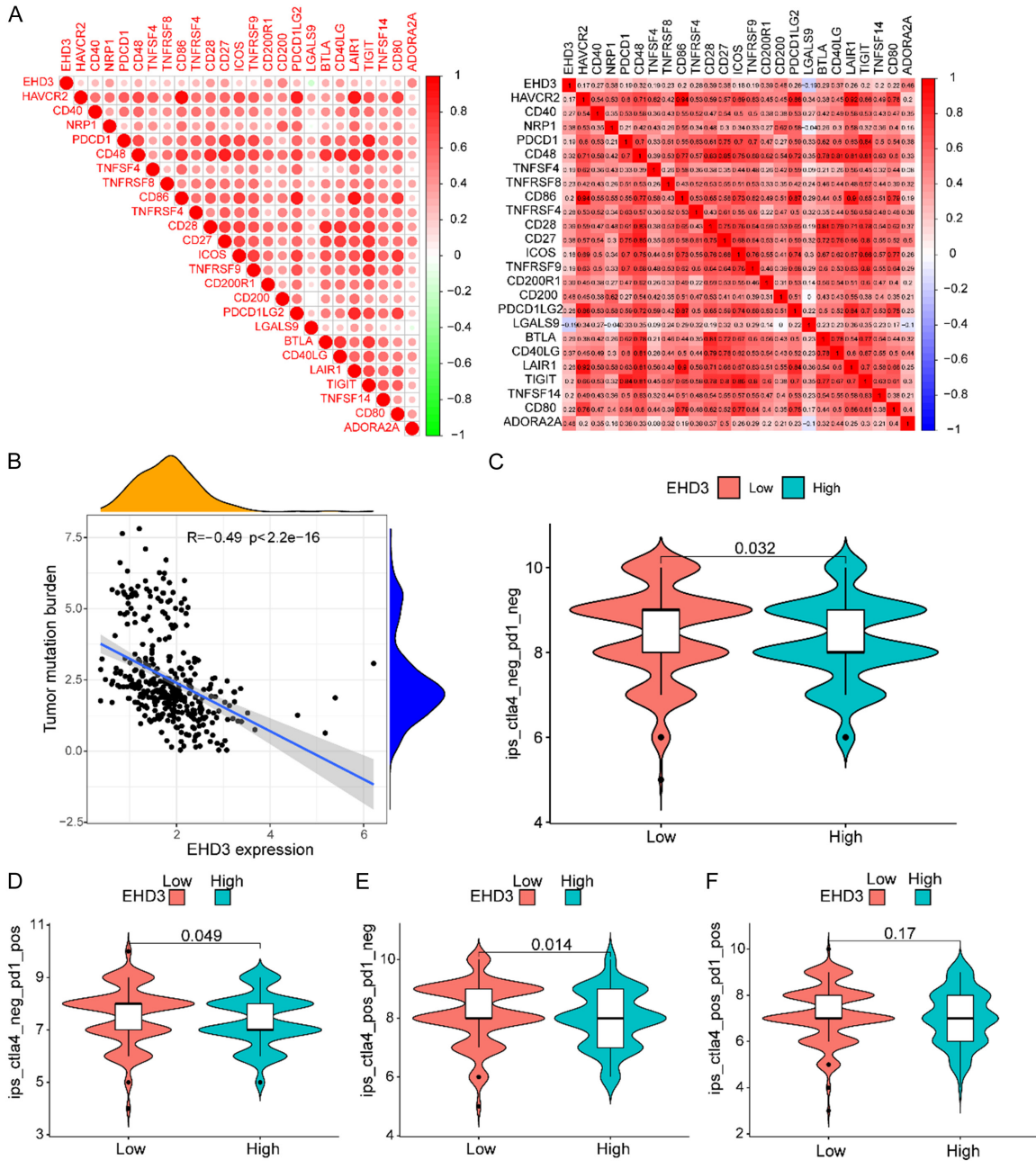


Figure 5. Prediction of EHD3 expressions in GC for sensitivity to immunotherapy. A. A close association between EHD3 expression levels and multiple immune checkpoints. B. A negative correlation between EHD3 expression and tumor mutation burden. C-F. Differences in IPS between EHD3 groups with high and low transcript levels when CTLA-4 or/and PD1 is positive. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

NG_PATHWAY, CELL_ADHESION_MOLECULES_CAMS, FOCAL_ADHESION and NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION were dynamically correlated with high EHD3 expression, while OLFACTORY_TRANSDUCTION was significantly enriched in the low EHD3 expression group (Figure 6E). It could be concluded from the above results that EHD3 might play a sig-

nificant role in various biological processes and diseases, including but not limited to muscle system processes, neuroactive ligand-receptor interactions, calcium signaling pathways, cell adhesion, and olfactory transduction. These findings provided important clues for gaining insight into the functions of EHD3 and its role in the onset and development of diseases.

EHD3 promotes gastric cancer progression

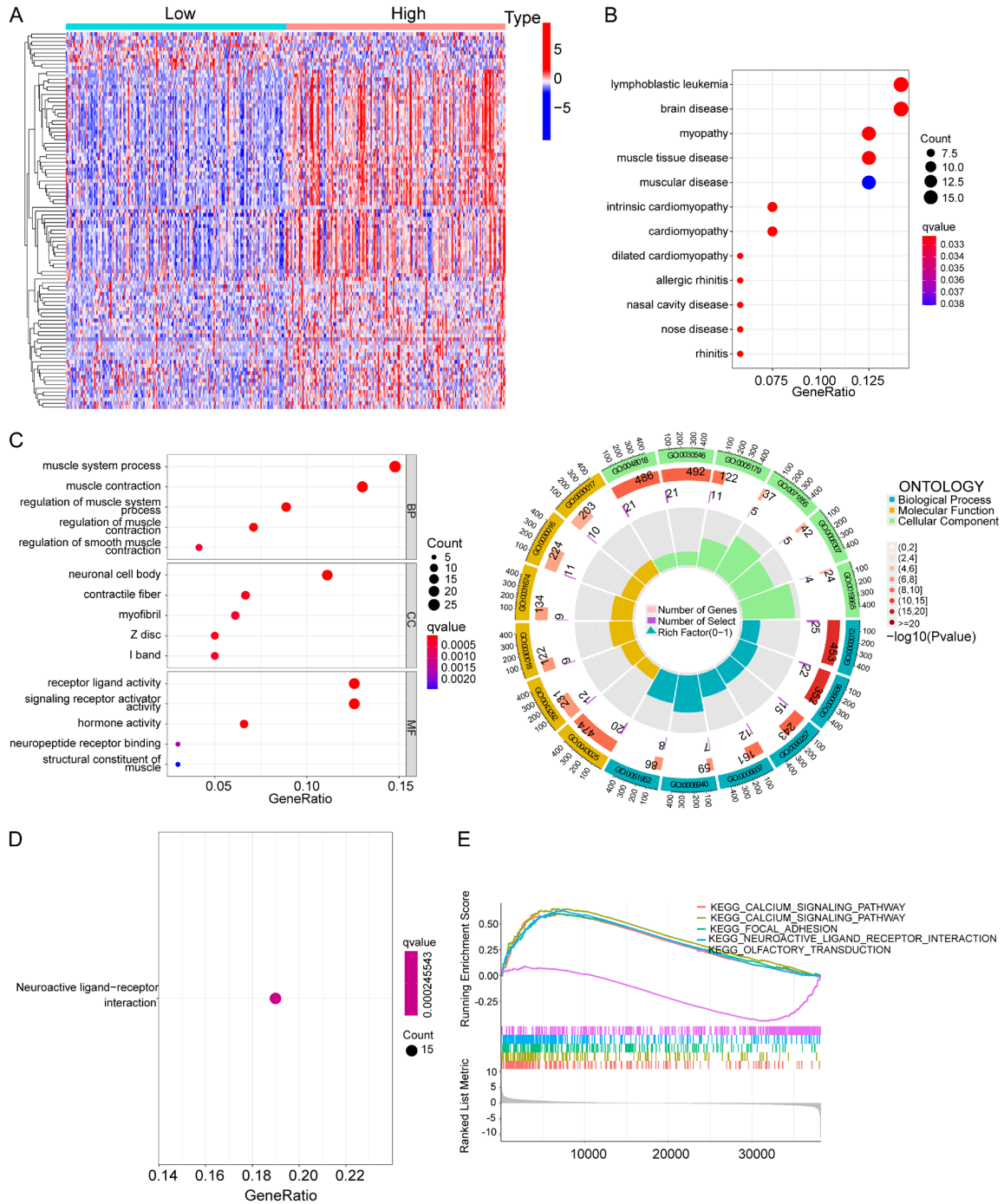


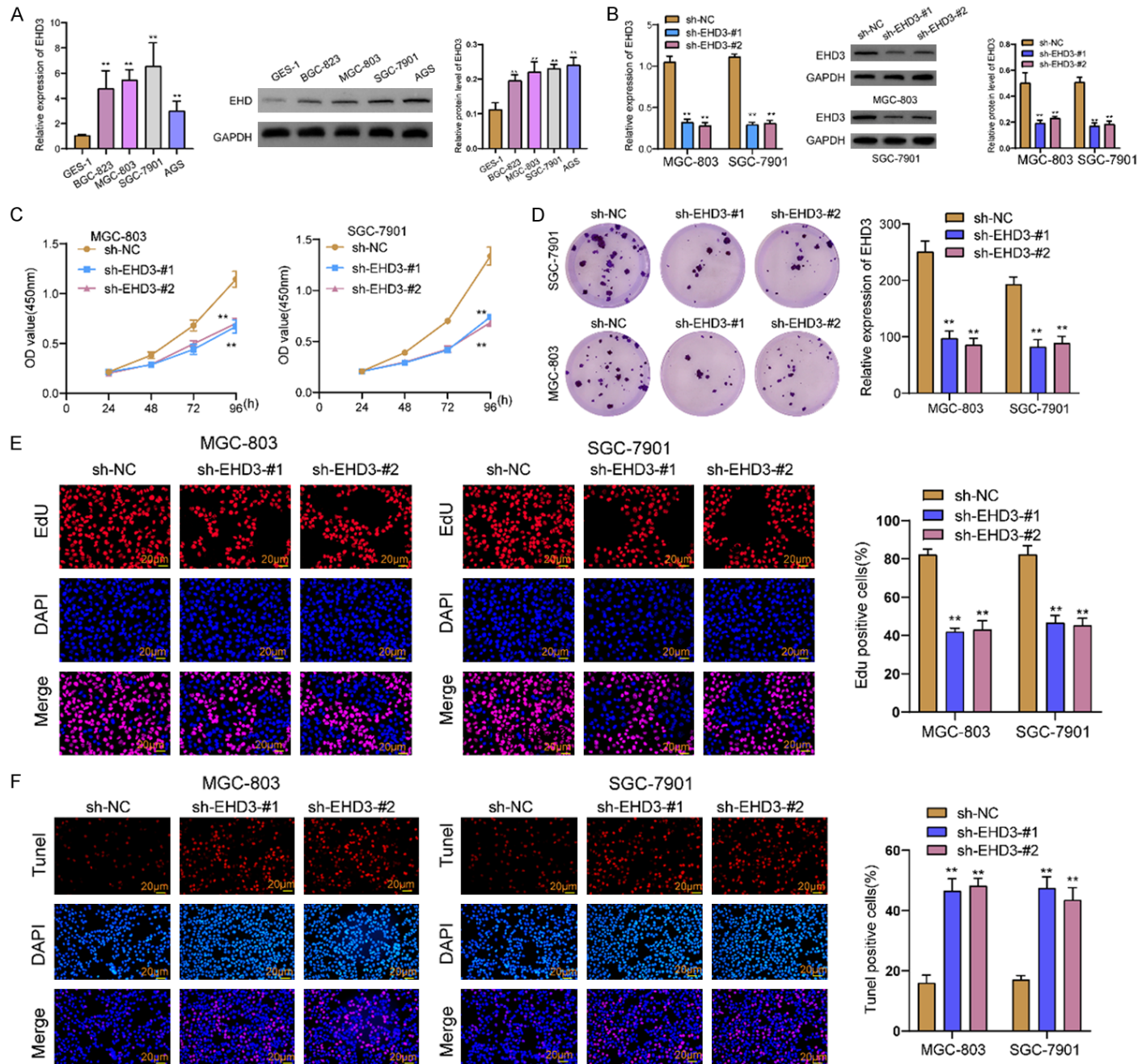
Figure 6. The function assays of EHD3-associated DEGs in GC. A. The representative DEGs associated with EHD3 were shown in Heat map. B. DO analysis. C. Bubble graph for GO enrichment. D. Barplot graph for KEGG pathways. E. Gene Set Enrichment Analysis. *P<0.05, **P<0.01, ***P<0.001.

Inhibition of EHD3 knockdown on the proliferation and metastasis of GC cells via Wnt/ β -catenin/EMT signaling pathway

The possible function of EHD3 in GC cells was investigated by analyzing its expression in a number of different GC cell lines. EHD3 mRNA

and protein levels were distinctly higher in four GC cell lines compared to GSE-1 cells (**Figure 7A**). Then, shRNA plasmids were used to knock-down EHD3 expression in MGC-803 and SGC-7901 cells, achieving an efficiency of over 65% (**Figure 7B**). The results of CCK-8 assays, EdU staining, and clonogenic assays demonstrated

EHD3 promotes gastric cancer progression



EHD3 promotes gastric cancer progression

Figure 7. EHD3 knockdown impaired the proliferation and promoted cell apoptosis. A. RT-PCR and western blot assays for the expression of EHD3 in four GC cells and GSE-1 cells. B. qPCR and western blot assays detected EHD3 levels in MGC-803 and SGC-7901 cells after transfection with EHD3 shRNAs. C. CCK-8 assays. D. Clonogenic assays. E. EdU assays. F. TUNEL assays. Scale bars: 20 μm . * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

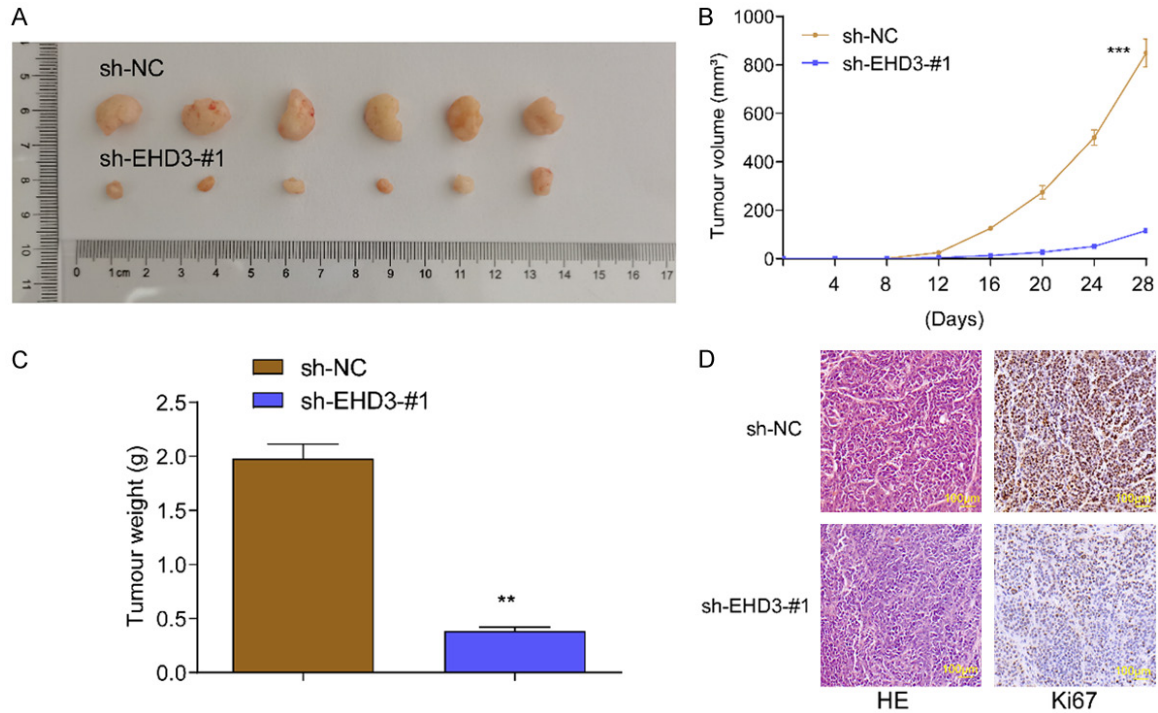


Figure 8. In vivo mice studies validated that EHD3 depletion suppressed tumor growth. A. Images of MGC-803 tumors and a size contrast to the original cells are presented. B. The tumor volume-time curves. C. The tumor weights. D. Relative Ki67 staining. Scale bars: 100 μm . * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

that EHD3 knockdown markedly suppressed the proliferation of MGC-803 and SGC-7901 cells (**Figure 7C-E**). Moreover, TUNEL assays revealed that EHD3 knockdown significantly increased GC cell apoptosis (**Figure 7F**). Subsequently, in vivo experiments were performed. The tumor volumes and weights in the EHD3-deficient group were remarkably smaller than those in the sh-control group, suggesting that repressing EHD3 expressions significantly inhibited tumor growth (**Figure 8A-C**). H&E and immunohistochemistry results showed that EHD3 knockdown suppressed the abnormal proliferation of GC cells (**Figure 8D**). In addition, the effects of inhibition of EHD3 on the migration and infiltration of GC cells were investigated. According to the results of the wound healing experiment, EHD3 knockdown substantially restrained the capacity of GC cells to migrate (**Figure 9A**). The transwell assays further revealed that MGC-803 and SGC-7901 cells represented lower invasive ability after EHD3 knock-

down (**Figure 9B**). Furthermore, the expressions of N-cadherin, E-cadherin, MMP-9 and β -catenin related to the Wnt/ β -catenin/EMT pathway were examined. It was observed that silence of EHD3 suppressed the expressions of N-cadherin, β -catenin and MMP-9, while promoting the expression of E-cadherin (**Figure 9C**).

Discussion

In recent years, there have been significant advances in the research of GC, particularly in understanding the underlying molecular mechanisms and identifying potential biomarkers for clinical diagnosis and treatment [17, 18]. One notable breakthrough is the advancement of precision medicine, which focuses on targeting specific mutations or pathways in tumor cells. This approach has yielded promising results in clinical trials, leading to improved survival rates for patients with advanced GC. However,

EHD3 promotes gastric cancer progression

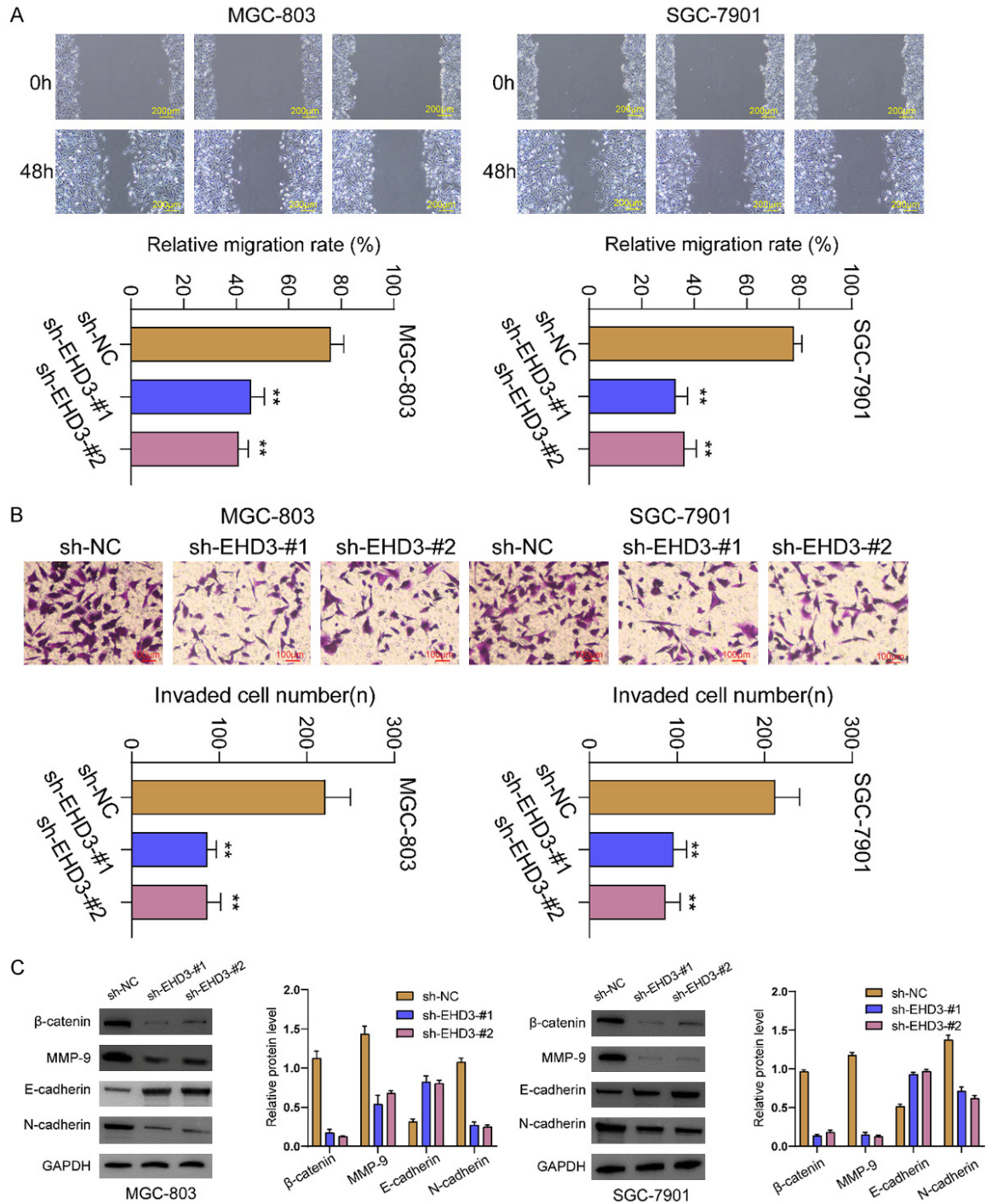


Figure 9. Knockdown of EHD3 suppressed the metastasis of GC cells via Wnt/ β -catenin/EMT signalling pathway. A. Cell migration of MGC-803 and SGC-7901 was evaluated by wound healing assays after transfection with EHD3 shRNAs. Scale bars: 200 μ m. B. Cell invasion of MGC-803 and SGC-7901 cells was assessed by transwell invasion assays. Scale bars: 100 μ m. C. The levels of N-cadherin, E-cadherin, MMP-9 and β -catenin following EHD3 shRNAs treated in MGC-803 and SGC-7901 cells were assessed through western blot. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

despite these achievements, the diagnosis of GC still remains a serious challenge. Currently, the most commonly used diagnostic markers

for GC include carbohydrate antigen 72-4, carbohydrate antigen 19-9 and carcinoembryonic antigen [19, 20], which are proteins produced

EHD3 promotes gastric cancer progression

by cancer cells and can be detected in the blood. While they are valuable in identifying patients with advanced GC, they lack the necessary specificity for early detection. Furthermore, these markers can also be elevated in other conditions, such as inflammation, liver disease, and pancreatitis, leading to false positives and unnecessary testing [21]. As a consequence, more sensitive biomarkers are needed to be further demonstrated. In this study, a novel GC-related gene EHD3 was identified which was highly expressed in GC specimens. High EHD3 expression was confirmed to predict an advanced clinical stage and poor prognosis. Our finding suggested EHD3 as a novel diagnostic and prognostic biomarker for GC patients.

In the meantime, research on DNA methylation in GC has attained significant progresses as well. Epigenetic transformation known as DNA methylation is an important component in the process of controlling how genes are expressed [22]. In cases of GC, abnormal DNA methylation patterns have been discovered, with hypermethylation of genes that suppress tumors and hypomethylation of genes that promote tumor growth being two of the more prevalent characteristics [23]. Studies have shown that DNA methylation profiles can be used as biomarkers for early detection, prognosis, and prediction of response to therapy in GC [24]. Additionally, DNA methylation-targeted therapies have presented promise in preclinical studies, indicating that they may be a potential therapeutic strategy for GC treatment. In the initial phase of this study, Pearson correlations were employed to examine the potential impact of EHD3 methylation status on the expression of EHD3 mRNA. A significant and robust negative association was determined between EHD3 methylation levels and EHD3 expression, particularly evident in GC tissues. Further, we proceeded to identify particular CpG sites in the EHD3 DNA promoter at which methylation was significantly correlated with the production of EHD3 mRNA. Surprisingly, virtually all of the CpG sites displayed substantial correlations with the production of EHD3 gene products. Overall, our findings indicated that methylation of EHD3 played a role in the negative regulation of EHD3.

Tumor microenvironment (TME) refers to non-cancerous cells and molecules surrounding a

tumor [25], which are involved in the initiation, progression, and metastasis of cancer. An increasingly large number of studies have focused on understanding the complexity of the TME and how it influences the behavior of cancer cells. One area of intense research has been the role of TME in GC. The TME in GC is characterized by a variety of cells, including endothelial cells, immune cell and cancer-associated fibroblasts [26, 27]. These cells interact with each other and with cancer cells to promote tumor growth and invasion. Recent studies have identified several key signaling pathways and molecules participating in TME-mediated GC progression [28, 29]. For example, the interaction between cancer cells and immune cells in the TME can modulate the expression of immune checkpoint molecules, such as PD-1 and PD-L1, which play a critical role in immune evasion by cancer cells. In addition, the TME can promote the activation of signaling pathways, such as the Wnt/ β -catenin pathway, which are involved in cancer cell proliferation and invasion [30]. In this research, it was observed that the expressions of EHD3 imposed a substantial effect on the infiltration of several immune cells. It is common knowledge that macrophages and CD8⁺ cells are essential cell subgroups responsible for the immune function of leukocytes against malignancies. At present, the immunophenoscore (IPS) is an algorithm that is commonly used to forecast the immune response. It was proved in this paper that GC patients with low expressions of EHD3 exhibited higher scores of IPS, suggesting sensitivity to immunotherapy. Our findings indicated EHD3 as a potential target for immunotherapy.

Given the above bioinformation analysis based on TCGA datasets, it was reasonable to suspect that EHD3 may be a carcinogenic gene. Accordingly, loss-of-function experiments were performed to explore the potential function of EHD3 in GC. The results confirmed that knock-down of EHD3 distinctly suppressed the proliferation, migration and invasion of GC cells. However, a previous study reported that EHD3 was lowly expressed in glioma and its overexpression increased cell growth both in vitro and in vivo, which was inconsistent with our findings, suggesting that EHD3 may exhibit different roles depending on the types of tumors. Finally, to explore the potential mechanisms

involved in EHD3 function on promoting GC progression, our attention focused on Wnt/ β -catenin/EMT signalling pathway. Recent studies have manifested that aberrant activation of this pathway contributes to tumor initiation, progression, and metastasis in GC [31, 32]. The dysregulation of β -catenin, a key downstream effector of the Wnt pathway, leads to the accumulation of β -catenin in the nucleus, exhibiting a regulatory effect on the expressions of functional genes involved in tumor growth and metastasis. Several studies have identified various molecular mechanisms, including mutations in Wnt pathway genes, epigenetic modifications, and miRNA dysregulation, which can activate or inhibit the Wnt/ β -catenin pathway in GC [33, 34]. Hence, the results suggested that knockdown of EHD3 distinctly suppressed the activity of Wnt/ β -catenin/EMT signaling pathway. However, specific mechanisms by which EHD3 regulated Wnt/ β -catenin/EMT pathway needed to be further studied.

Conclusions

Significant discoveries on the possibility of EHD3 as a novel biomarker for GC, its relationship with progression, poor mortality, and immune infiltration, as well as its function in supporting tumorigenesis through abnormal inflammation and immune response, were uncovered by this research. The oncogenic characteristics of EHD3 were also carefully validated, including promotion of the proliferation, migration, and invasion of GC cells through the Wnt/ β -catenin/EMT signaling pathway. These findings indicated that EHD3 may not only be able to provide a potential new pathway for investigating clinicopathological implications and molecular pathogenesis of GC when served as an effective predictor of treatment outcomes, but also may play a role in the treatment of other cancers.

Disclosure of conflict of interest

None.

Address correspondence to: Hairong Song, Department of Clinical Laboratory, Key Clinical Laboratory of Henan Province, The First Affiliated Hospital of Zhengzhou University, No. 1 Jianshe Road East, Zhengzhou, Henan, China. E-mail: song-hairong5320@163.com

References

- [1] Miller KD, Nogueira L, Devasia T, Mariotto AB, Yabroff KR, Jemal A, Kramer J and Siegel RL. Cancer treatment and survivorship statistics, 2022. *CA Cancer J Clin* 2022; 72: 409-436.
- [2] Xia C, Dong X, Li H, Cao M, Sun D, He S, Yang F, Yan X, Zhang S, Li N and Chen W. Cancer statistics in China and United States, 2022: profiles, trends, and determinants. *Chin Med J (Engl)* 2022; 135: 584-590.
- [3] Machlowska J, Baj J, Sitarz M, Maciejewski R and Sitarz R. Gastric cancer: epidemiology, risk factors, classification, genomic characteristics and treatment strategies. *Int J Mol Sci* 2020; 21: 4012.
- [4] Johnston FM and Beckman M. Updates on management of gastric cancer. *Curr Oncol Rep* 2019; 21: 67.
- [5] Hamilton JP and Meltzer SJ. A review of the genomics of gastric cancer. *Clin Gastroenterol Hepatol* 2006; 4: 416-425.
- [6] Seeneevassen L, Bessède E, Mégraud F, Lehours P, Dubus P and Varon C. Gastric cancer: advances in carcinogenesis research and new therapeutic strategies. *Int J Mol Sci* 2021; 22: 3418.
- [7] Lu Q, Insinna C, Ott C, Stauffer J, Pintado PA, Rahajeng J, Baxa U, Walia V, Cuenca A, Hwang YS, Daar IO, Lopes S, Lippincott-Schwartz J, Jackson PK, Caplan S and Westlake CJ. Early steps in primary cilium assembly require EHD1/EHD3-dependent ciliary vesicle formation. *Nat Cell Biol* 2015; 17: 531.
- [8] Jones T, Naslavsky N and Caplan S. Differential requirements for the Eps15 homology domain proteins EHD4 and EHD2 in the regulation of mammalian ciliogenesis. *Traffic* 2022; 23: 360-373.
- [9] Zhang L, Ding L, Li Y, Zhang F, Xu Y, Pan H, Wan X, Yan G, Yu F and Li R. EHD3 positively regulated by NR5A1 participates in testosterone synthesis via endocytosis. *Life Sci* 2021; 278: 119570.
- [10] Juszczak GR and Stankiewicz AM. Glucocorticoids, genes and brain function. *Prog Neuropsychopharmacol Biol Psychiatry* 2018; 82: 136-168.
- [11] Yang HQ, Jana K, Rindler MJ and Coetzee WA. The trafficking protein, EHD2, positively regulates cardiac sarcolemmal K(ATP) channel surface expression: role in cardioprotection. *FASEB J* 2018; 32: 1613-1625.
- [12] Imamura M, Takahashi A, Matsunami M, Horikoshi M, Iwata M, Araki SI, Toyoda M, Susarla G, Ahn J, Park KH, Kong J, Moon S, Sobrin L; International Diabetic Retinopathy and Genetics CONsortium (iDRAGON); Yamauchi T, Tobe K, Maegawa H, Kadowaki T and Maeda S. Ge-

EHD3 promotes gastric cancer progression

- nome-wide association studies identify two novel loci conferring susceptibility to diabetic retinopathy in Japanese patients with type 2 diabetes. *Hum Mol Genet* 2021; 30: 716-726.
- [13] Amessou M, Ebrahim AS, Dilly A, Joseph M, Tabolina M, Chukkapalli S, Meroueh L, Syed JT, Liddane A, Lang SL, Al-Katib A and Kandouz M. Spatio-temporal regulation of EGFR signaling by the Eps15 homology domain-containing protein 3 (EHD3). *Oncotarget* 2016; 7: 79203-79216.
- [14] Wang YH, Chang SC, Ansar M, Hung CS and Lin RK. Eps15 homology domain-containing protein 3 hypermethylation as a prognostic and predictive marker for colorectal cancer. *Bio-medicines* 2021; 9: 453.
- [15] Chukkapalli S, Amessou M, Dekhil H, Dilly AK, Liu Q, Bandyopadhyay S, Thomas RD, Bejna A, Batist G and Kandouz M. Ehd3, a regulator of vesicular trafficking, is silenced in gliomas and functions as a tumor suppressor by controlling cell cycle arrest and apoptosis. *Carcinogenesis* 2014; 35: 877-885.
- [16] Yu G, Wang LG, Han Y and He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* 2012; 16: 284-287.
- [17] Dunbier A and Guilford P. Hereditary diffuse gastric cancer. *Adv Cancer Res* 2001; 83: 55-65.
- [18] Ouyang J, Xie Z, Lei X, Tang G, Gan R and Yang X. Clinical crosstalk between microRNAs and gastric cancer (Review). *Int J Oncol* 2021; 58: 7.
- [19] Sexton RE, Al Hallak MN, Diab M and Azmi AS. Gastric cancer: a comprehensive review of current and future treatment strategies. *Cancer Metastasis Rev* 2020; 39: 1179-1203.
- [20] Necula L, Matei L, Dragu D, Neagu AI, Mambet C, Nedeianu S, Bleotu C, Diaconu CC and Chivu-Economescu M. Recent advances in gastric cancer early diagnosis. *World J Gastroenterol* 2019; 25: 2029-2044.
- [21] Matsuoka T and Yashiro M. Biomarkers of gastric cancer: current topics and future perspective. *World J Gastroenterol* 2018; 24: 2818-2832.
- [22] Moore LD, Le T and Fan G. DNA methylation and its basic function. *Neuropsychopharmacology* 2013; 38: 23-38.
- [23] Usui G, Matsusaka K, Mano Y, Urabe M, Funata S, Fukayama M, Ushiku T and Kaneda A. DNA methylation and genetic aberrations in gastric cancer. *Digestion* 2021; 102: 25-32.
- [24] Choi SJ, Jung SW, Huh S, Chung YS, Cho H and Kang H. Alteration of DNA methylation in gastric cancer with chemotherapy. *J Microbiol Biotechnol* 2017; 27: 1367-1378.
- [25] Xiao Y and Yu D. Tumor microenvironment as a therapeutic target in cancer. *Pharmacol Ther* 2021; 221: 107753.
- [26] Vitale I, Manic G, Coussens LM, Kroemer G and Galluzzi L. Macrophages and metabolism in the tumor microenvironment. *Cell Metab* 2019; 30: 36-50.
- [27] Wu T and Dai Y. Tumor microenvironment and therapeutic response. *Cancer Lett* 2017; 387: 61-68.
- [28] Pan Y, Yu Y, Wang X and Zhang T. Tumor-associated macrophages in tumor immunity. *Front Immunol* 2020; 11: 583084.
- [29] Kumar V, Ramnarayanan K, Sundar R, Padmanabhan N, Srivastava S, Koiwa M, Yasuda T, Koh V, Huang KK, Tay ST, Ho SWT, Tan ALK, Ishimoto T, Kim G, Shabbir A, Chen Q, Zhang B, Xu S, Lam KP, Lum HYJ, Teh M, Yong WP, So JBY and Tan P. Single-cell atlas of lineage states, tumor microenvironment, and subtype-specific expression programs in gastric cancer. *Cancer Discov* 2022; 12: 670-691.
- [30] Katoh M. Canonical and non-canonical WNT signaling in cancer stem cells and their niches: cellular heterogeneity, omics reprogramming, targeted therapy and tumor plasticity (Review). *Int J Oncol* 2017; 51: 1357-1369.
- [31] Guo Q, Xu J, Huang Z, Yao Q, Chen F, Liu H, Zhang Z and Lin J. ADMA mediates gastric cancer cell migration and invasion via Wnt/ β -catenin signaling pathway. *Clin Transl Oncol* 2021; 23: 325-334.
- [32] Katoh M. Multi-layered prevention and treatment of chronic inflammation, organ fibrosis and cancer associated with canonical WNT/ β -catenin signaling activation (Review). *Int J Mol Med* 2018; 42: 713-725.
- [33] Zhang M, Hu S, Min M, Ni Y, Lu Z, Sun X, Wu J, Liu B, Ying X and Liu Y. Dissecting transcriptional heterogeneity in primary gastric adenocarcinoma by single cell RNA sequencing. *Gut* 2021; 70: 464-475.
- [34] Ji L, Qian W, Gui L, Ji Z, Yin P, Lin GN, Wang Y, Ma B and Gao WQ. Blockade of β -catenin-induced CCL28 suppresses gastric cancer progression via inhibition of treg cell infiltration. *Cancer Res* 2020; 80: 2004-2016.