Role of Delta/Notch-like epidermal growth factor-related receptor in gastric cancer patients and cells and its clinical significance

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Gastric cancer (GC) is a common digestive system malignancy. The aim of this study was to explore the role of Delta/Notch-like epidermal growth factor-related receptor (DNER) in GC patients and cells. Gene expression omnibus data base public databases were used to analyze the DNER expression in GC patient. A total of 30 cases of GC and adjacent tissue samples were retrospectively obtained to analyze the DNER expression. MTT assay was conducted to measure the cell viability. The apoptosis rate of GC cells was determined by flow cytometry. The migration and invasion were detected by transwell assay. Real-time polymerase chain reaction and western blot were performed to measure the DNER expression. Bioinformatics tools exhibited that DNER expression is significantly upregulated in the GC, which was also found in GC tissues and cells. The high levels of DNER were closely related the tumor size, sex and lymph node metastasis. Additionally, the survival rate of patients with high DNER expression is decreased. Furthermore, knockdown of DNER inhibits the proliferation, migration and invasion, and induces the apoptosis rate of the GC cells. DNER was upregulated in GC and knockdown of DNER inhibits the growth and metastasis of DNER. DNER may be a potential prognostic biomarker and therapeutic target of GC patients.
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Introduction

Gastric cancer (GC) is a common digestive system malignancy. GC has the second highest incidence rate after lung cancer and the third highest mortality rate. It ranks third in GC-related deaths in China [1]. In China, the detection rate of early GC is relatively low, accounting for only about 20%. Most patients are in the advanced stage at the first diagnosis, and the 5-year overall survival rate of GC patients is less than 50% [2,3]. At present, the exact molecular mechanism of GC is still unclear, and clinical biomarkers for early diagnosis and prognosis are also lacking. In recent years, with the rapid development of high-throughput technology, it has greatly promoted the development and progress of disease genomics. Some studies suggested that the use of bioinformatics tools to explore the relationship between molecular changes and the prognosis of the disease can mine effective molecular markers to judge the prognosis of the disease [4,5]. It was demonstrated that many biochemical molecular markers are involved in the occurrence and development of tumors, and can be used for early screening of tumors [6]. Therefore, it is necessary to explore novel and specific diagnostic markers in the development of GC.

Delta/Notch-like epidermal growth factor-related receptor (DNER) is a transmembrane protein, which was discovered in various peripheral cells. DNER is a member of the noncanonical Notch ligand family and binds to the Notch1 receptor [7,8]. The DNER structure includes extracellular segment containing 10 tandem epidermal growth factor-like sequences and intracellular segment enriched with enzyme amino acids. The extracellular segment is similar to the delta extracellular segment of Notch, which can act as a ligand to bind to Notch and activate the corresponding signaling pathway [9]. The intracellular segment includes a tyrosine-rich region at the proximal membrane and a di-leucine-rich region at the carboxy-terminal. The endocytosis mediated by the carboxy-terminal di-leucine region is related to the polar distribution of DNER in central nerve cells. Phosphorylation of the tyrosine-enriched region will inhibit the endocytosis of DNER, which will cause the accumulation of DNER on the cell membrane and induce abnormal changes in nerve cells [10]. DNER was originally described as highly expressed in Purkinje neurons and participates in the development of cerebellar [11]. In recent researches, it has demonstrated that

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DNER is closely related to the growth and metastasis of cancer and stem cells [12,13]. However, to our knowledge, the study of DNER on GC development had not been reported.

Therefore, we demonstrated that DNER acts as an oncogene in GC and plays an important role in occurring and progression of GC via bioinformatics analysis and clinical characteristics of GC patients. In addition, this study also aimed to explore the effects of DNER on the growth and metastasis of the GC cells

Materials and methods

Patients samples

A total of 30 cases of GC and adjacent tissue samples were retrospectively obtained from the First Affiliated Hospital of Soochow University Hospital. The patients were recruited from June 2021 to March 2022. This study was approved by the ethics committee of First Affiliated Hospital of Soochow University Hospital.

Cell culture and transfection

The human GC cell lines (KATO III, NCI-N87, SNU-1 and HGC-27) and normal human gastric mucosal epithelial cell GES-1 were purchased from Cell Bank of Chinese Academy of Sciences. A total of 10% fetal bovine serum and 1% penicillin streptomycin were added to the Dulbecco's Modified Eagle's medium, and all cells were cultured at 37 °C, 5% CO_2.

Cells in the logic growth phase were transfected with siRNA targeting DNER (si-DNER 1#, si-DNER 2#) and controls (si-nc) (Genpharma, El Jadida, Maroc) with Lipofectamine 3000 reagent (Invitrogen, San Jose, California, USA) according to the instructions.

Real-time polymerase chain reaction

The total RNA was extracted with Trizol (Thermo Fisher Scientific, San Jose, California, USA) according to instructions. Thereafter, the total RNA was reverse-transcribed into cDNA by the reverse transcription kit (Takara, Tokyo, Japan). The primer sequence of DNER and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: F: 5′-GGGCAGCAGACCACTATGG-3′, R: 5′-CCAGGGTTGATGGCCTGAG-3′; GAPDH, F: 5′-AATGGACAACTGGTCGTGGAC-3′, R: 5′-CCCTCCAGGGGATCTGTTTG-3′. The PCR reaction conditions were as followed: 95 °C, 5 s; 45 cycles of 95 °C, 5 s; 60 °C, 1 min; 95 °C, 5 s; 60 °C, 1 min. The relative mRNA expression was calculated by $2^{-\Delta\Delta Ct}$ method. GAPDH was used as internal reference.

MTT assay

The cells in logarithmic phase were cultured according to 1×10^5 cells/well in 12 well culture plate. The cells were taken at 24, 48 and 72 h, respectively. Then the absorbance (optical density) at 490 nm was measured by a microplate reader (BioRad, Hercules, California, USA) after the cells were treated with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Beyotime, Shanghai, China) for 4 h.

Flow cytometry

The apoptosis rates of the MCF-7 and MDA-MB-468 cells were determined with Annexin V/Propidium Iodide (PI) Apoptosis Detection Kit (Beyotime). In brief, the cells were collected and stained with 5-μl Annexin V-APC and 5-μl PI in the dark for 10 min. Then the flow cytometer (BD, Franklin Lakes, New Jersey, USA) and Flow Jo V10 software (Tree Star Software, San Carlos, California, USA) were used to analyze the apoptotic cells.

Transwell assay

The transwell assay was used to analyze the invasion and migration ability of the MCF-7 and MDA-MB-468 cells. Invasion assay: 500-μl medium containing 10% fetal calf serum was added to the lower chamber, and 200-μl cell suspension with Matrigel-coated membrane was added to the upper chamber of the Transwell chamber. After 1 day, the cells in the upper chamber were carefully wiped off, and the cells at the bottom of the transwell membrane were fixed and stained with 4% polyformaldehyde and 0.5% crystal violet. The average number of cells in five randomly selected fields of view under the microscope represents the number of invaded cells. Migration assay: the difference is that 200-μl cell suspension is added to the upper chamber of the transwell chamber not precoated with Matrigel, and the rest procedure was the same as that of invasion assay.

Western blot

After 48 h of culture, the cells of each group were collected and the radio immuno precipitation assay lysis buffer (Beyotime) was used for protein extraction. Bicinchoninic acid assay protein concentration detection kit (Beyotime) was used to detect protein concentration. Then 20-μg protein and 4 μl 2 × SDS buffer were mixed evenly and denatured at 100 °C for 10 min. Next, the samples were separated by SDS-PAGE gel electrophoresis and then transferred to polyvinylidene fluoride membrane. Then the membranes were blocked with 5% skim milk for 1 h, and washed three times. After that, the first antibody (anti-DNER, 1:1000; anti-GAPDH. 1:1500; Abcam, Cambridge, Massachusetts, USA) was added and incubated overnight at 4 °C, Next day, the membranes were incubated with Horse Radish Peroxidase-labeled IgG antibody (1:5000, Abcam) at room temperature for 2 h. After washed for three times, the BeyoECL Plus ECL kit (Beyotime) was used to visualize the bands. GAPDH was used as an internal reference.

Immunohistochemistry

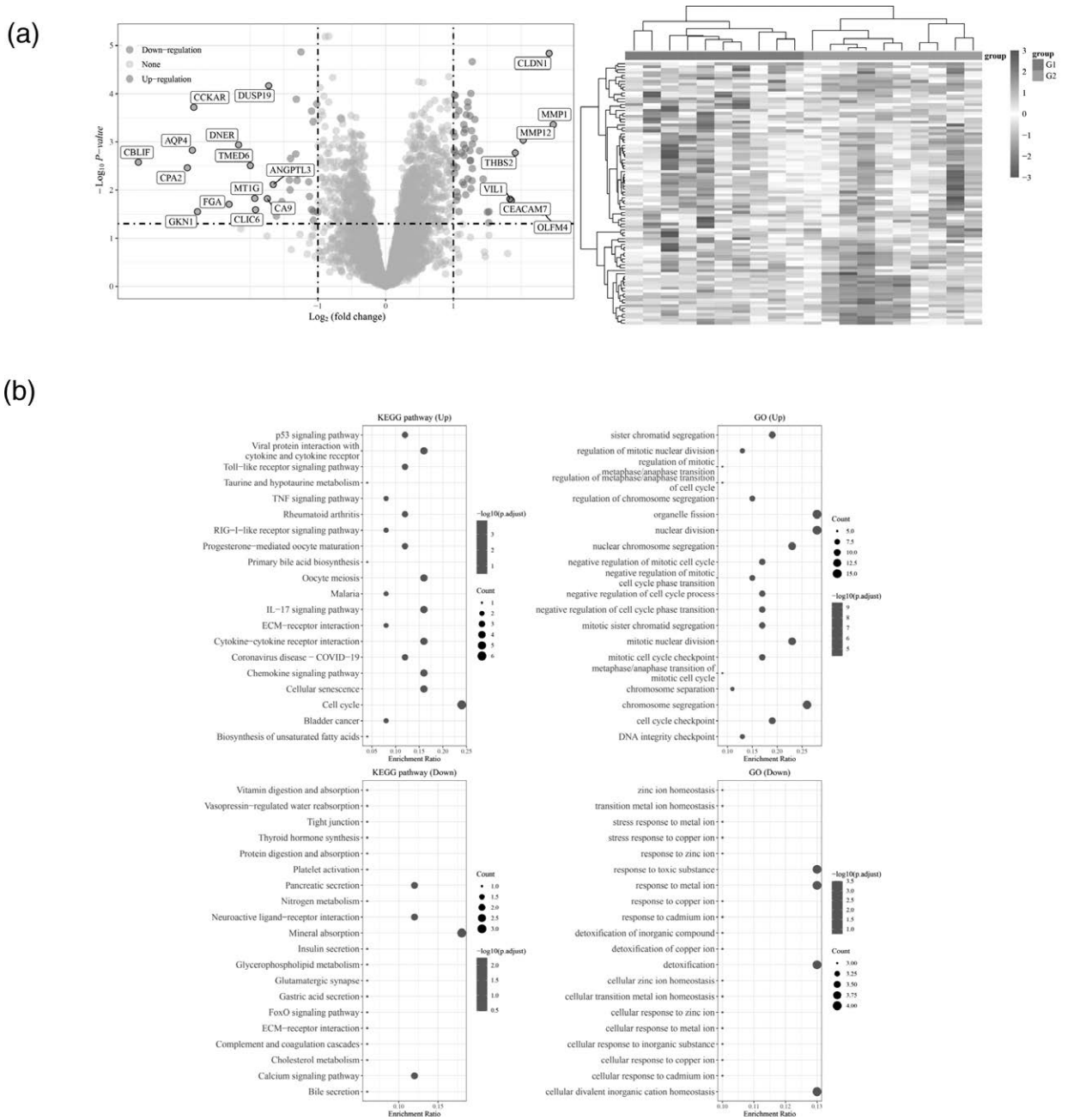
The tissue is made into paraffin sections with a thickness of about 4 μmol/l. Then the sections were proceed to deparaffinization, dehydration, antigen thermal retrieval and deionized water incubation. Next, the sections were incubated with DNER primary antibody overnight at 4 °C. Next day, the sections were washed twice with PBS

and incubated at 37 °C for 2 h and then sections were incubated with IgG polymer for 30 min. Finally, 100-μl DAB chromogenic reagent was added to the sections until brownish–yellow particles appear under the microscope to stop the color reaction.

Statistical and bioinformatics analysis

The data in the current study were expressed as mean ± SD. SPSS 20.0 (SPSS Inc., Chicago, Illinois, USA) software was utilized for all the statistical analysis of the study and analysis of variance was used for comparison among groups. Bioinformatics tools were performed to analyze box plot, principal component analysis results, heatmap, volcano map, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment pathways from the gene expression omnibus (GEO) database (http://www.ncbi.nih.gov/geo). Kaplan–Meier method was used to analyze the survival curves. $P < 0.05$ was considered a statistically significant difference.

Fig. 1



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Results

Bioinformatics analysis discovered of DNER expression in GC patient

Bioinformatics tools were performed to discover DNER in GC tissue and adjacent tissue of GEO dataset (GSE118897). These heatmap and volcano plot results showed the abnormally expressed genes (Fig. 1a). In addition, the GO and KEGG database analysis represented the top-20 significant up- and downregulated pathways of GC patients(Fig. 1b).

DNER expression was upregulated in the GC tissues

As shown in Fig. 2a and b, we found that the DNER expression was upregulated in the GC tissues compared with the adjacent tissue. Similarly, the results of immunohistochemistry also showed that the level of DNER in GC tissues is higher than that of the adjacent tissues (Fig. 2c). In addition, Kaplan–Meier survival curves showed that survival rate of patients with high DNER expression is lower compared with that of patients with low DNER expression (Fig. 2d).

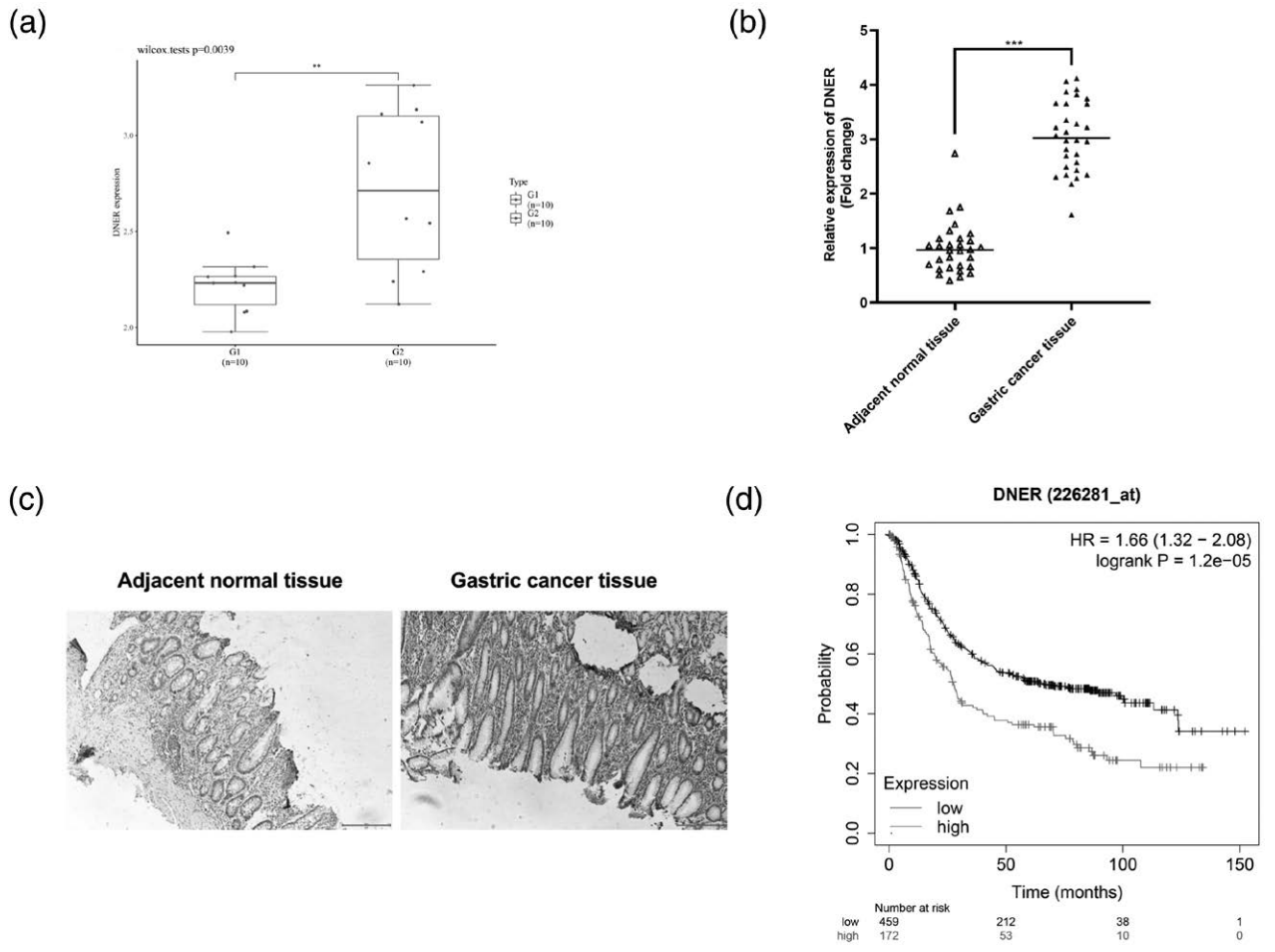
Clinical characteristics

A total of 30 GC patients were enrolled to measure the DNER expression in GC tissue and adjacent tissue. Further, the patients were characterized by clinical parameters such as age, sex, tumor size, lymph node metastasis and vascular invasion. We confirmed that DNER expression is positively correlated with tumor size, sex and lymph node metastasis (Table 1).

DNER expression was upregulated in the GC cells

Subsequently, we explored that DNER expression in the GC cells. We found that DNER expression is significantly upregulated in the KATO III, NCI-N87, SNU-1 and HGC-27 cells compared with the GES-1 cells. In addition, the expression of DNER was higher in HGC-27 and KATO III cells compared with other GC cells

Fig. 2



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(Fig. 3). Therefore, we selected HGC-27 and KATO III cells for further experiments.

Knockdown of DNER relieved the malignant behaviors of the GC cells.

Finally, we explored the effects of DNER knockdown on the cell viability, apoptosis, migration and invasion of the HGC-27 and KATO III cells. After si-DNER transfection, the DNER expression was significantly downregulated in mRNA and protein levels (Fig. 4a and b). In addition, after si-DNER transfection, the cell viability, migration and invasion abilities of the HGC-27 and KATO III cells were significantly suppressed, and the apoptosis rate was significantly promoted(Fig. 4c and d).

Table 1 **Clinical information of the patients**

	DNER low ($n = 15$)	DNER high ($n = 15$)	*P* value
Age			
<50	6	7	0.7125
≥50	9	8	
Sex			
Male	9	8	0.7125
Female	6	7	
Tumor size			
<3 cm	12	6	0.0253
≥3 cm	3	9	
Tumor differentiation			
Well-moderate	10	8	0.4561
Poor	5	7	
Tumor metastasis			
Yes	2	8	0.0201
No	13	7	

DNER, Delta/Notch-like epidermal growth factor-related receptor.

Discussion

The occurrence and development of GC is a complex process. Nowdays, endoscopy is still the main method of screening for early GC [14]. However, endoscopy is an invasive examination, most patients or healthy people are intolerant and afraid of endoscopy, which makes endoscopy temporarily not included in the routine screening for early GC [15]. At present, the clinical biomarkers for early screening of GC mainly include carbohydrate antigen 724 (CA724), carbohydrate antigen 19-9 (CA19-9) [16], carbohydrate antigen 242 (CA242) [17] and carcinoembryonic antigen [18]. However, due to environmental pollution, food safety, dietary structure and other factors, the previous serological markers can not meet the clinical needs. Therefore, researchers are constantly looking for more sensitive and specific molecular markers to improve the accuracy of early GC screening. In this study, we confirmed that DNER was significantly upregulated in GC through Bioinformatics tools, which also indicated that DNER may be a clinical biomarkers for the diagnosis of GC.

In order to further verify the role of DNER in GC, we analyzed the DNER expressions in GC tissues and adjacent tissues. DNER is a transmembrane protein with multidomain structure, which mediates cell–cell interaction through Notch ligand [19]. DNER was highly

Fig. 3

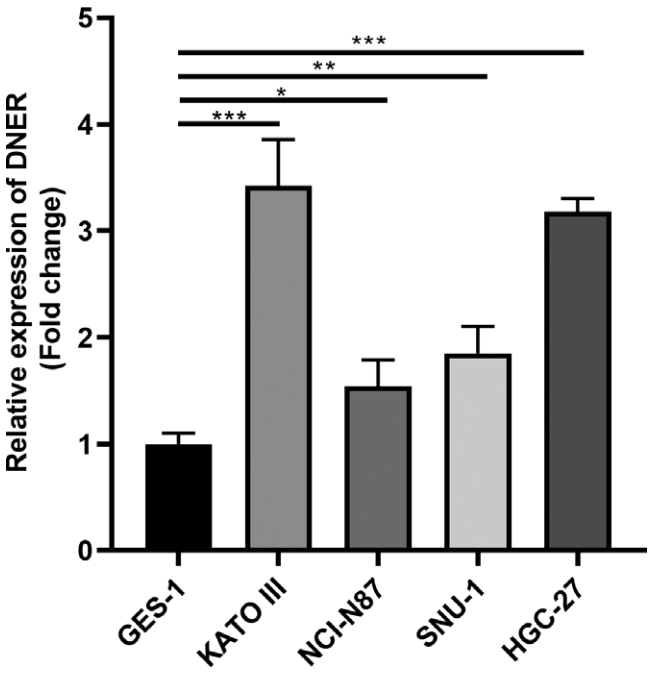

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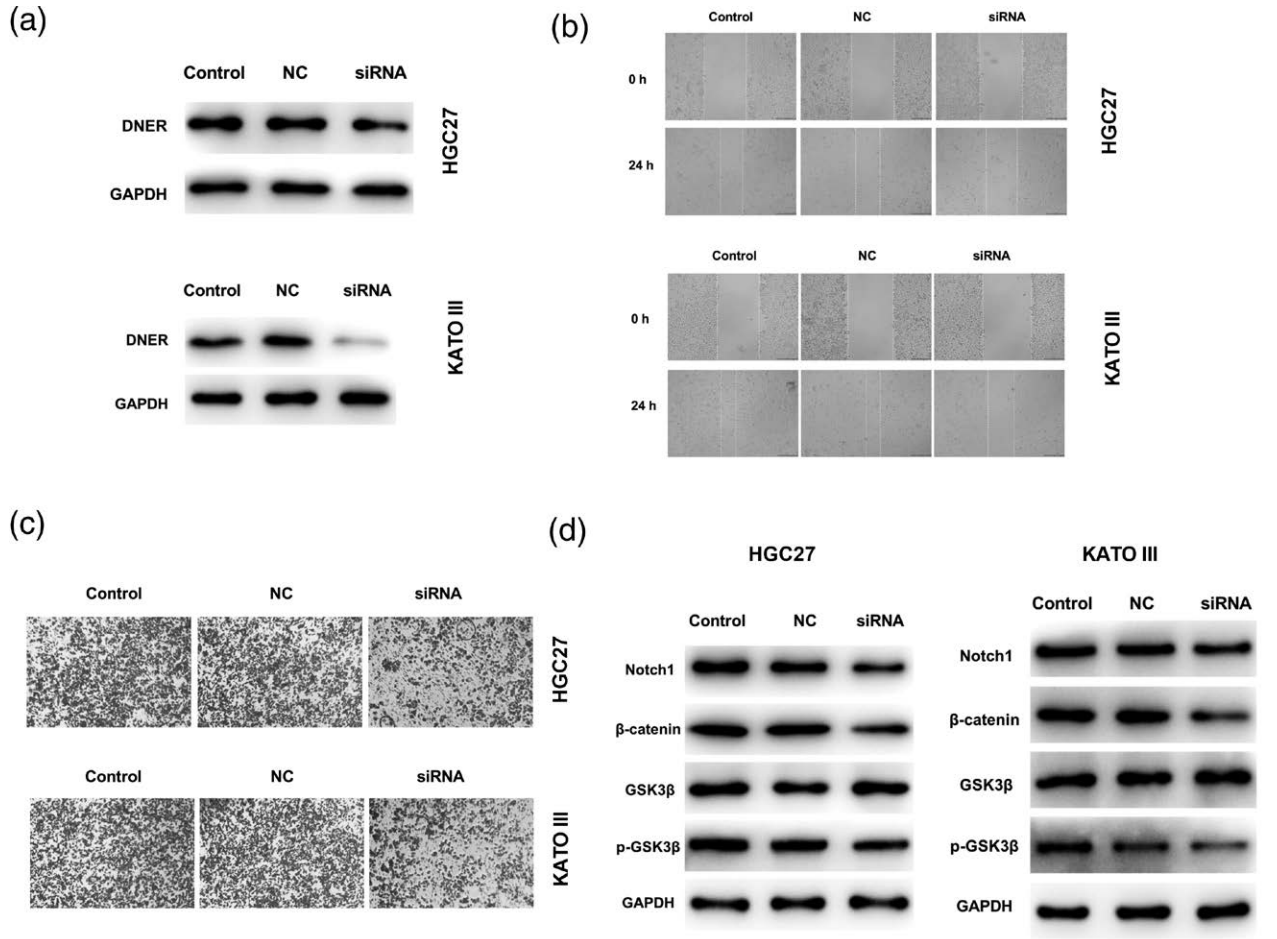
Fig. 4



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expressed in cerebellum, hippocampus neurons and Purkinje cells of glioblastoma cells. Some studies have shown that DNER, as an atypical Notch ligand, can be inhibited by histone deacetylase, thus inhibiting the growth and development of glial-derived neurospheres, producing tumor heterogeneity and inducing differentiation. It can be seen that DNER plays a negative role in neuroma [20]. However, other studies have come to the opposite conclusion, proving that DNER is highly expressed in several human cancer cell lines such as breast cells and prostate cancer cells at mRNA and protein levels. Silencing DNER in tumor cell xenotransplantation model can reduce cell transformation and carcinogenicity [21,22]. Therefore, DNER may play different roles in different tumor tissues. In this study, we found that DNER is upregulated in the GC tissues of GC patient, which was also consistent with the results of immunohistochemistry. Clinically, we found that DNER expression is positively correlated with tumor size, sex and lymph node metastasis, and high level of DNER is closely related to the poor prognosis of GC patients.

Finally, we selected human GC cell lines HGC-27 and KATO III to perform rescue experiments. The proliferation and metastasis of the tumor is the important factor resulting the poor prognosis of GC. A growing number of researches are devoted to explore novel molecular markers to regulate the malignant behavior of tumor cells and improve the cure rate of cancer. Tian *et al.* [23] suggested that downregulation of SERPINH1 expression inhibits the survival, invasion and migration of SGC-7901 cells. Chen *et al.* [24] demonstrated that THBS4 promotes the proliferation and metastasis of the MGC-803 and BGC-823 cells. In addition, Wang *et al* [21] showed that DNER induces epithelial-mesenchymal transition and suppressed the apoptosis rate of breast cancer cells, which illustrated that DNER functions as an oncogene in breast cancer. Similarly, Wang *et al.* [22] also confirmed that DNER promotes the occurrence and progression of prostate cancer. However, the role of DNER in GC cells

is still unclear. In the current study, we silenced DNER expression in GC cells by siRNA transfection. The results showed that knockdown of DNER inhibits the cell viability, migration and invasion, and induces the apoptosis of the GC cells. These results implied that the DNER expression is closely related to the malignant behavior of GC cells.

Taken together, our results suggest that the high expression of DNER is associated with poor prognosis of GC patients, and is positively correlated with tumor size, sex and lymph node metastasis of GC patients. In addition, DNER may play a role in the occurrence and development of GC by regulating proliferation, apoptosis, migration and invasion of GC cells. This study confirmed that DNER may function as a potential targeted molecular marker for the diagnosis and therapy of GC.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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