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ORIGINAL RESEARCH

CLEC4D as a Novel Prognostic Marker Boosts the Proliferation and Migration of Gastric Cancer via the NF-κB/AKT Signaling Pathway

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Purpose: The functions of C-type lectin domain family 4 member D (CLEC4D), one member of the C-type lectin/C-type lectin-like domain superfamily, in immunity have been well described, but its roles in cancer biology remain largely unknown.

Patients and Methods: This study aims to explore the role of CLEC4D in gastric cancer (GC). Bioinformatics preliminarily analyzed the expression of CLEC4D in gastric cancer. Immunohistochemical staining was used to detect the expression level and clinical pathological characteristics of CLEC4D in gastric cancer. The biological function of CLEC4D in gastric cancer cell lines was verified through in vitro and in vivo experiments.

Results: In this study, CLEC4D expression was found to be markedly increased in gastric cancer (GC) tissues compared with matched normal gastric tissues, and high CLEC4D expression independently predicted unfavorable overall survival in patients with GC. Knockdown of CLEC4D markedly inhibited GC cell proliferation and migration. Mechanistically, CLEC4D knockdown deactivated the Akt and NF-κB signaling pathways in GC cells.

Conclusion: Together, these results demonstrate that aberrantly increased CLEC4D expression promotes cancer phenotypes via the Akt and NF-κB signaling pathways in GC cells.

Keywords: CLEC4D, gastric cancer, NF-κB signaling pathways, migration

Introduction

The numbers of gastric cancer (GC) cases and related deaths have declined gradually over the past 5 decades, GC remains a global health burden, as it is the fifth most frequently diagnosed malignancy and the fourth leading cause of cancer-related deaths around the world.[1] Complex host genetic and environmental factors interact with each other to contribute to the initiation and progression of GC, and most GC cases are diagnosed at advanced stages, at which point there are few effective treatment options to significantly improve patient outcomes.[2] Thus, a deeper understanding of the molecular mechanisms underlying disease progression is paramount to identifying novel biomarkers and understanding whether they can be targeted in GC treatment.

The immune surveillance of cells is guided by a large number of highly conserved pattern recognition receptors (PRRs).[3] These PRRs can recognize a wide range of ligands (carbohydrates, lipids, proteins, and glycoproteins).[4] PRRs

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play an important role in the occurrence and development of cancer. They participate in regulating immune response, inflammatory response, and anti-tumor response by recognizing abnormal signals and changes inside and outside the cell, which is of great significance for the immune surveillance and treatment of cancer.[5]

C-type lectin receptors (CLRs) are family of PRRs widely expressed in mammals, mainly involved in antigen recognition and signal transduction during the immune response.[6] The defining characteristic of CLRs is the presence of one or more C-type lectin domains (CTLDs) and the requirement for Ca^{2+} ions to achieve efficient binding to carbohydrate ligands.[7] CLRs are structurally diverse, including single-chain and multichain types, as well as soluble and membrane-bound types.[8] Membrane-bound CTLs are single-channel transmembrane proteins, which are further classified into type I (extracellular N-terminus) and type II (intracellular N-terminus) CTLs based on their topology.[9]

CLEC4D (also known as CLEC4D, CD303, or BDCA-2) belongs to the Dectin-2 subfamily of the C-type lectin receptor family.[10–12] It is a type II transmembrane glycoprotein and consists of a cytoplasmic domain, transmembrane domain, stem domain and extracellular carbohydrate recognition domain.[10–12] The distinct structural characteristic is that CLEC4D does not have a signal motif in the cytoplasmic domain, which suggests that CLEC4D might not relay intracellular signals in the same way as its four other family members.[10,11,13] In addition, the extracellular carbohydrate recognition domain of CLEC4D is atypical since this domain does not contain a conservative triple motif that is critical for recognizing Ca^{2+}-dependent carbohydrates.[12–15] CLEC4D was found to be expressed in multiple myeloid cells, such as neutrophils, monocytes, and dendritic cells;[10–12] thus, the defensive function of CLEC4D against bacterial pathogens has been extensively studied.[6,16] Currently, most research attention is focused on the ability of C-type lectins to play a role in innate and adaptive antimicrobial immune responses, but these proteins are increasingly recognized as playing an important role in cancer. Numerous studies have noted the expression of CLRs in both immune cells and tumor cells, highlighting their diverse roles in the progression of cancer.[17–20] Recently, the functions of CLEC4D have been uncovered in numerous types of cancer.[21–23] The weakened bactericidal ability of macrophages with CLEC4D gene deficiency leads to an increase in the load of Candida albicans, which triggers macrophage glycolysis and promotes the production of IL-22 by innate lymphocytes ILC3. IL-22 induces STAT3 phosphorylation in intestinal epithelial cells, ultimately promoting the development of colorectal tumors.[21] A study on the gene expression profile of peripheral blood mononuclear cell samples in patients with pancreatic ductal carcinoma showed significant upregulation of CLEC4D, suggesting its potential value as a diagnostic marker for pancreatic ductal carcinoma.[22] CLEC4s are associated with immune infiltration in hepatocellular carcinoma, providing a basis for finding new immunotherapeutic targets for HCC.[23] CLEC4D may mediate an increased risk of lung cancer by modulating the epigenetic clock (a biological indicator of organismal aging), indicating that CLEC4D could serve as a potential drug target for the treatment of lung cancer.[24] However, the function and overall mechanisms of CLEC4D in different cancer settings, in particular GC, remain incompletely defined.

In this study, we compared CLEC4D expression in GC and adjacent normal tissues and found that CLEC4D levels were markedly increased in GC samples relative to normal samples. More importantly, increased CLEC4D expression was indicative of poor patient outcomes. Depletion of endogenous CLEC4D expression significantly attenuated the aggressive phenotypes of GC cells by downregulating the Akt and NF-κB signaling pathways. Together, our results revealed that CLEC4D promotes cancer phenotypes in GC cells.

Materials and Methods

Gastric Cancer Dataset Analysis

The gene expression data and clinical information for gastric cancer were downloaded from the TCGA database (http://tcga-data.nci.nih.gov/tcga/). There were 375 gastric cancer tissues and 32 corresponding normal tissues. The expression value of FPKM was normalized to transcripts per kilobase million (TPM) for further analysis. Patients were divided into high-CLEC4D (n=246) and low-CLEC4D (n=104) expression groups according to the optimal cutoff value determined by the R package "Survminer". The CERES score in genome-wide CRISPR screening results of gastric cancer cell lines, for assessment of gene necessity, was acquired from the DepMap Database (https://depmap.org/portal/download/). Gene set enrichment analysis (GSEA) was performed using the R package clusterProfiler. An adjusted P value <0.05 was

considered to indicate significant enrichment of gene sets. R software (version 3.3.2) was used to conduct all bioinformatics and statistical analyses.

Patient Specimens and Immunohistochemistry (IHC)

Tumor and matched normal tissues were collected from 119 patients with GC who underwent curative gastrectomy between January 2004 and September 2007 at Tianjin Medical University Cancer Hospital (Tianjin, China), Xijing Hospital of Air Force Medical University (Xi'an, China), and Renji Hospital of Shanghai Jiao Tong University School of Medicine (Shanghai, China). None of these patients received neoadjuvant therapy before surgery. All studies involving human subjects were conducted after obtaining informed consent from the patients, and all experiments using these samples were approved by the Institutional Research Ethics Committee of Tianjin Medical University Cancer Institute and Hospital (Tianjin, China).

Rabbit anti-CLEC4D antibody (1:500) (ab175021, Abcam) was used for IHC staining. The detailed IHC procedures and IHC scoring were performed as described in our previous study.[25] The positive staining area was scored as 1–4, and the average staining intensity of tumor cells was scored as 0–3. The staining score of CLEC4D was then calculated as the positive areas multiplied by the average staining intensity, with a range between 0 and 12. Intensity scores between 0 and 4 were considered to indicate low expression of CLEC4D, whereas scores between 5 and 12 were considered to indicate high expression of CLEC4D. The 119 patients were categorized into a CLEC4D-high group (n=101) and a CLEC4D-low group (n=18). KM survival curves were calculated using GraphPad Prism 8.0.

Cell Lines and Cell Culture

The human GC cell line NCI-N87 and human embryonic kidney 293T cells were purchased from American Type Culture Collection (ATCC, USA). HGC-27 cells were obtained from MeisenCTCC Co., Ltd. (Zhejiang, China). The GC cell lines (NCI-N87 and HGC-27) were cultured in RPMI 1640 medium containing 10% FBS (Newzerum, New Zealand) and 1% antibiotics (penicillin and streptomycin). 293T cells were cultured in DMEM with 10% FBS and 1% antibiotics (penicillin and streptomycin). All cells were maintained in a humidified 5% CO_2 incubator at 37 °C. The identities of NCI-N87 and HGC-27 cells were confirmed through STR (Short Tandem Repeat) profiling, a method that analyzes the STR sequences of specific gene loci in the cell line. By generating unique DNA fingerprints, this technology validates the authenticity and uniqueness of the cell lines. For all cell types, cells with no more than 20 continuous passages were used in this study.

Knockdown and Overexpression in Gastric Cancer Cells

Endogenous *CLEC4D* expression was depleted via lentiviruses encoding short hairpin RNA (shRNA) targeting this gene. Lentiviral production and transduction were performed as described previously.[25] CLEC4D shRNA was incorporated into the pSIH-H1-puro vector. CLEC4D and scrambled control shRNA lentiviruses were packaged in 293T cells and then introduced into HGC-27 and NCI-N87 cells, respectively. The packaging plasmids were pMD2.G (Plasmid #12259, Addgene) and psPAX2 (Plasmid #12260, Addgene). These transduced cells were selected in puromycin-containing RPMI 1640 medium, and stable cell populations were established to examine the effect of CLEC4D knockdown on the aggressive phenotypes of GC cells. The CLEC4D shRNA targeting sequence was 5'-CCTTCGGTTATTGCTGTAGTT-3'.

The intact CLEC4D ORF was cloned into pLVX-IRES-puro vector. Empty pLVX-IRES-puro vectors were used as corresponding negative controls.

RT–qPCR

Total RNA was extracted from the cultured cells via RNAiso plus (Takara Bio, Shiga, Japan). cDNA was generated using the GoScript™ Reverse Transcription Kit (Promega). CLEC4D mRNA levels were measured by using TB Green Premix Ex TaqTM II (Takara Bio) on a QuantStudio 5 real-time PCR system (Applied Biosystems, Foster City, CA, USA). GAPDH was used for data normalization. The $2^{-\Delta\Delta Ct}$ method was used to determine the mRNA level of *CLEC4D*. The qPCR primer sequences of *CLEC4D* were 5'-GTAAGAGAGGCACAGGAGTGCA-3' and 5'-GGAAGGCTCTCCAGTCAATAGG-3'. The qPCR primer sequences for *GAPDH* were 5'-TGCACCACCAACTGCTTAGC-3' and 5'-GGCATGGACTGTGGTCATGAG-3'.

Immunoblotting

Whole-cell lysates were prepared by using lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate, pH=7.4) containing protease and phosphatase inhibitors (MCE, USA). A Pierce™ BCA protein assay kit (Thermo Fisher Scientific) was used to detect the protein concentrations. Immunoblotting was conducted as described previously (14). An Amersham Imager 600 System (GE) was used to obtain images. Protein ladder was purchased from Thermo Scientific (#26616).

The primary antibodies used for immunoblotting were as follows: anti-CLEC4D (1:1000) (ab175021, Abcam), anti-β-actin (1:1000) (GTX109639, GeneTex), anti-phospho-p65 (Ser536) (1:1000) (93H1, CST), and anti-phospho-Akt (Thr308) (1:1000) (D25E6, CST).

Detection of Cellular Proliferation in vitro

Cell Counting Kit-8 (CCK-8) assays and colony formation assays were used to examine the proliferation of GC cells in vitro. For the CCK-8 assay, HGC-27 and NCI-N87 cells were seeded in a 96-well plate at a density of 1000 cells/well and 2000 cells/well, respectively. Then, absorbance at a wavelength of 450 nm was measured at the indicated time points. For the colony formation assay, 1000 cells/well NCI-N87 cells and 500 cells/well HGC-27 cells were seeded into six-well plates. After approximately 12 days of culture, the cells were fixed using 4% paraformaldehyde for 15 min and stained with 0.1% crystal violet for 10 min at room temperature. Then, visible colonies with no less than 50 cells were photographed and counted.

The method used to evaluate GC cell proliferation in vitro was described in our previous study.[26]

Transwell Assay

HGC-27 cells from the control and CLEC4D-depleted groups were trypsinized and resuspended in serum-free RPMI 1640 medium. Then, 40,000 cells were seeded onto the upper chambers with or without precoated Matrigel®, and the lower chambers were filled with RPMI 1640 medium containing 20% FBS. After incubation for 24 hr, the cells were fixed in methanol for 10 minutes at room temperature and stained with 0.1% crystal violet solution. The migrated cells were captured at four random sites and quantified.

The method used to evaluate GC cell migration and invasion in vitro was described in our previous study.[26]

Animal Experiment

Control and CLEC4D-depleted NCI-N87 cells (2×10^6 cells) in the same volume of PBS were injected subcutaneously into the flanks of female BALB/c nude mice (4 weeks old). Beginning on the seventh day after inoculation, the sizes of the tumor masses were measured using calibration every two days. On the 23rd day after the injection, the mice were sacrificed to obtain the tumor masses, and their weights were measured. The tumor volume was calculated as follows: $V = \text{length} \times \text{width}^2 \times 0.5$. All animal assays were approved by the Institutional Animal Care and Research Advisory Committee of Tianjin Medical University (Tianjin, China) and were performed in accordance with Guidelines for the welfare and use of animals in cancer research. This study was conducted following the Animal Research: Reporting of in vivo Experiments (ARRIVE) guidelines and conformed with the Declaration of Helsinki.

Statistical Analyses

Descriptive statistics are presented as mean ± standard deviation (SD). Comparisons between two groups were made using a two-tailed *t*-test, and the proportion of Annexin V–positive apoptotic cells was determined using the χ^2 test. Analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., RRID: SCR002798). $P < 0.05$ was considered statistically significant. Statistical differences are marked by asterisks as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; and ns, not significant.

Results

Aberrant Upregulation of CLEC4D in GC Indicates a Poor Patient Prognosis

To explore the function of CLEC4D, we first analyzed CLEC4D mRNA levels in GC (n = 375) and normal (n = 32) tissue samples in The Cancer Genome Atlas (TCGA) database; the results showed that CLEC4D mRNA levels were significantly higher in GC tissues than in their normal counterparts (Figure 1a). Additionally, approximately 73% of patients with high CLEC4D expression in the TCGA cohort were diagnosed with Grade 3 disease, whereas approximately 54% of patients with low CLEC4D levels were diagnosed with Grade 3 disease (Figure 1b). We then used IHC to examine CLEC4D levels in GC specimens from our institution (n = 119), and the results showed that GC samples exhibited markedly higher CLEC4D protein levels than matched normal tissues ($P < 0.001$) (Figure 1c and d). Further analysis demonstrated that patients with high CLEC4D expression exhibited poorer survival than those with low expression in both the TCGA cohort ($P = 0.044$) and the in-house cohort ($P = 0.0358$) (Figure 1e and f). More importantly, multivariate regression analysis showed that high CLEC4D expression was an independent prognostic factor indicating unfavorable overall survival in patients ($P = 0.029$; Table 1). Together, these results revealed that CLEC4D is potentially associated with GC progression.

CLEC4D Knockdown Attenuates the Proliferation of GC Cells

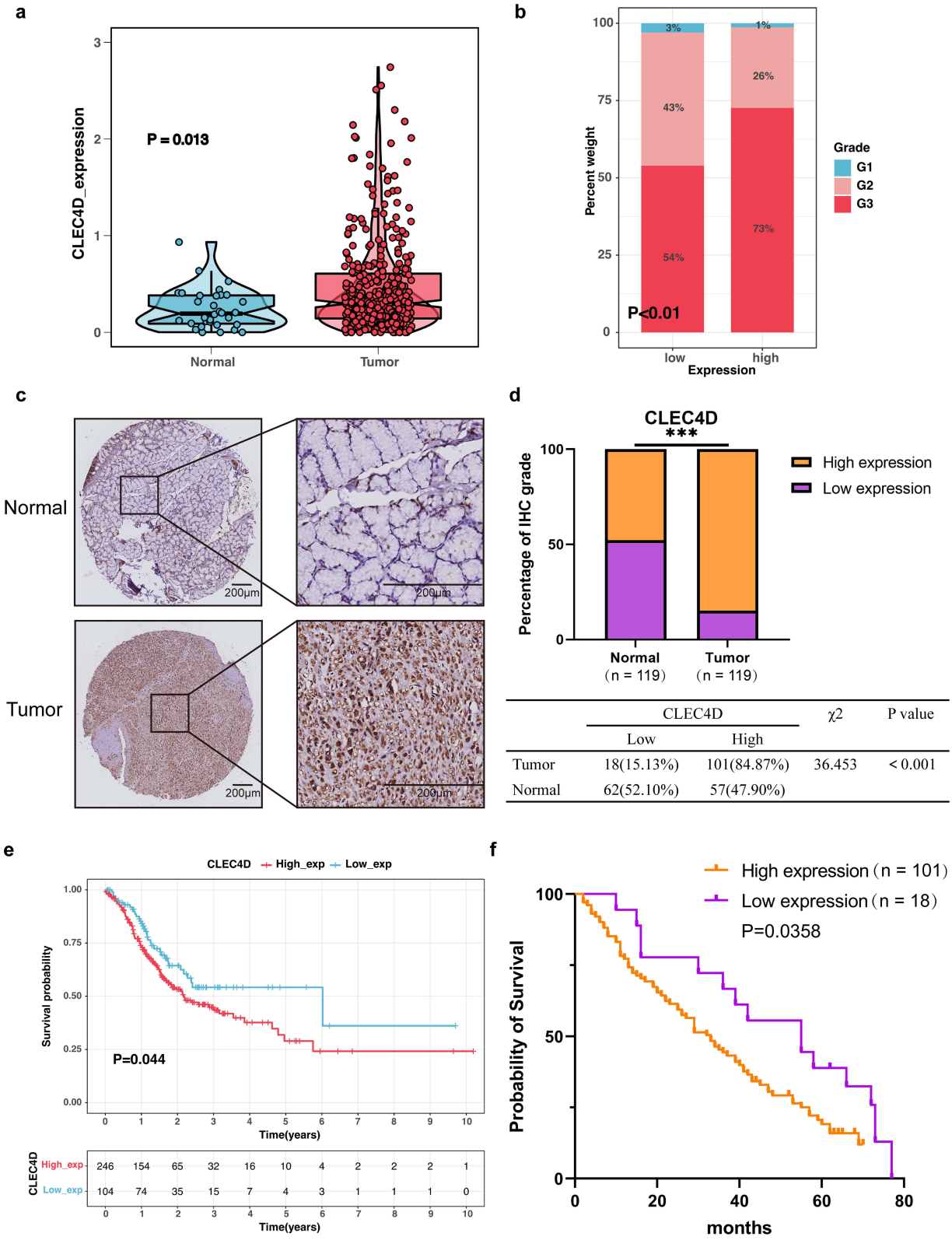
According to the results of immunohistochemistry, we confirmed that CLEC4D is predominantly localized to the cell membrane of GC cells (Figure S1a). To investigate the role of CLEC4D in GC biology, we first explored the necessity of the CLEC4D gene in gastric cancer cell lines within the large-scale CRISPR–Cas9 screening database. Most common gastric cancer cell lines presented with a negative CERES score, suggesting that knocking out CLEC4D could inhibit the proliferation and decrease the viability of gastric cancer cells (Figure 2a). Then, its expression was reduced in HGC-27 and NCI-N87 cells using lentivirus encoding a short hairpin RNA (shRNA) (Figure 2b). Flow cytometry was used to test the protein level of CLEC4D on the membrane in HGC-27 and NCI-N87 cells, confirming that the membrane expression of CLEC4D was markedly reduced in shC4D GC cells compared with shNC cells (Figure 2c). A CCK-8 assay was conducted to test whether CLEC4D knockdown affects the proliferation of GC cells. The results showed that CLEC4D knockdown significantly attenuated the propagation of HGC-27 and NCI-N87 cells (Figure 2d). Additionally, HGC-27/NCI-N87 cells with CLEC4D knockdown formed much fewer colonies than control cells (Figure 2e). Given that CLEC4D deficiency markedly inhibited the proliferation of GC cells in vitro, we then examined the effect of CLEC4D knockdown on growth and proliferation in vivo. We found that NCI-N87 cells with CLEC4D knockdown gave rise to tumor masses that were significantly smaller than those derived from the control cells within 23 days ($P < 0.01$) (Figure 2f). These results together demonstrate that CLEC4D knockdown markedly suppresses the proliferation of GC cells.

CLEC4D Deficiency Impairs the Migration and Invasion of GC Cells in vitro

In addition to its pro-proliferation effect, we used a Transwell assay to examine whether CLEC4D knockdown influenced migration in GC cells. As shown in Figure 3a, loss of CLEC4D markedly reduced the number of migratory HGC-27 cells ($P < 0.001$) (Figure 3a). Moreover, CLEC4D deficiency dramatically decreased the invasion capacity of HGC-27 cells ($P < 0.001$) (Figure 3b). Collectively, these data show that reduced CLEC4D expression could decrease the migration capacity of GC cells.

CLEC4D Knockdown Deactivates the Akt and NF-κB Signaling Pathways

To explore the molecular underpinnings of CLEC4D in GC cells, we first analyzed the potential implication of CLEC4D in key signaling pathways based on the TCGA GC cohort. We analyzed the correlation between CLEC4D expression and activated signaling pathways based on the TCGA GC cohort and found that the NF-κB signaling pathway was significantly correlated with CLEC4D expression (Figure 4a and b). The subsequent immunoblot assay validated that CLEC4D deficiency markedly decreased the amount of phospho-p65 (Ser536), indicating that knockdown of CLEC4D deactivated the NF-κB signaling pathway (Figure 4c). Given that CLEC4D could activate the Akt signaling pathway in



	CLEC4D		χ2	P value
	Low	High		
Tumor	18(15.13%)	101(84.87%)	36.453	< 0.001
Normal	62(52.10%)	57(47.90%)		

Figure 1 Increased CLEC4D expression indicates the poor prognosis in patients with GC. (**a**) Analysis of TCGA GC cohort showed that CLEC4D expression in GC tissues was markedly higher than normal tissues (P = 0.013). (**b**) Significantly more GC patients with higher CLEC4D expression were diagnosed at stage of Grade 3, compared with GC patients with lower CLEC4D expression (P < 0.01). (**c** and **d**). IHC showed that CLEC4D staining was stronger in GC tissues than in normal gastric mucosa. The representative images of one pair of tissues are presented here (**c**). Scale bars, 200 μm. Statistical analysis demonstrated that 101 out of 119 patients had high CLEC4D expression while 18 out of 119 patients had low CLEC4D expression (P < 0.001) (**d**). (**e**) Analysis of TCGA GC cohort showed that patients with higher CLEC4D expression had poorer prognosis (P = 0.044). Optimal cut-off analysis was determined in R using package "Survminer". (**f**) In-house GC samples showed that the overall survival of patients with high CLEC4D expression was also significantly worse (n=119) (P = 0.0358).

Table 1 Univariate and Multivariate Cox Proportional Hazards Regression Analysis with Respect to Overall Survival

Predictor	Univariate Analysis		Multivariate Analysis	
	HR(95% CI)	P	HR(95% CI)	P
Age, years				
≥65 vs <65	1.181(0.783–1.781)	0.428		
Gender				
Female vs Male	1.369(0.888–2.112)	0.155		
Size, cm				
>4cm vs ≤4cm	1.776(1.140–2.768)	0.011	1.653(1.056–2.587)	0.028
pT stage[a]				
pT3-4 vs pT1-2	0.970(0.548–1.718)	0.917		
pN stage[a]				
pN+ vs pN0	1.910(1.139–3.203)	0.014	1.768(1.048–2.983)	0.033
Tumor location				
Middle 1/3 vs Upper 1/3	1.192(0.562–2.530)	0.647		
Lower 1/3 vs Upper 1/3	0.742(0.417–1.318)	0.308		
More than 2/3 stomach vs Upper 1/3	1.123(0.583–2.164)	0.728		
Lauren type				
Diffuse vs Intestinal	1.296(0.790–2.125)	0.304		
Expression of CLEC4D				
High vs Low	1.899(1.026–3.515)	0.041	2.001(1.074–3.728)	0.029

Notes: [a]*pT* and *pN stage* is according to the 8th edition of the American Joint Committee on Cancer (AJCC) Staging System.

a previous report[27] and the cytostatic effect of CLEC4D knockdown in GC cells, we further examined the level of phospho-Akt (Thr308) in our model. Likewise, the phospho-Akt (Thr308) level was decreased in GC cells with CLEC4D knockdown vs control cells (Figure 4c). Consistent with the findings in GC cells, the Akt signaling pathway was also significantly enriched in samples with high CLEC4D expression in the TCGA GC cohort (Figure 4d). These results together suggest that CLEC4D promotes malignant phenotypes in GC cells by activating the Akt and NF-κB signaling pathways.

Overexpression of CLEC4D Promotes the Malignant Progression of Gastric Cancer

To further investigate the role of CLEC4D in gastric cancer progression, we conducted lentivirus infection to enhance the expression of CLEC4D in AGS cells. Immunoblot assays were performed to confirm the efficiency of overexpression (Figure S2a). CCK-8 assays demonstrated that high expression of CLEC4D significantly enhanced the proliferation of AGS cells compared to the control group (P<0.05) (Figure S2b). Moreover, CLEC4D overexpressing AGS cells formed more colonies compared to the control cells (P<0.05) (Figure S2c). AGS cells with CLEC4D overexpression exhibited stronger invasion and migration capabilities (P<0.05) (Figure S2e). Furthermore, high expression of CLEC4D significantly increased the expression of phosphorylated-p65 (Ser536) and phosphorylated Akt (Thr308) in AGS cells (Figure S2d).

Discussion

CLEC4D, which is predominantly expressed in myeloid cells, is a C-type lectin receptor (CLR), and these receptors function as pattern recognition receptors (PRRs). Therefore, its roles in inflammatory responses against fungal infections are evident. Under conditions of various microbial stimuli, CLEC4D is strongly upregulated increased and recognizes trehalose dimycolate, which is the fungal core component, in mice and humans.[28] Research has revealed that CLEC4D plays a crucial role in host defense against fungal infections by recognizing the mannose-rich surface structure of *Candida albicans* hyphae and inducing NF-κB-mediated inflammatory responses through spleen tyrosine kinase (Syk) signaling. CLEC4D and CLEC6A form a heterodimeric complex, enabling innate immune cells to highly detect *Candida*

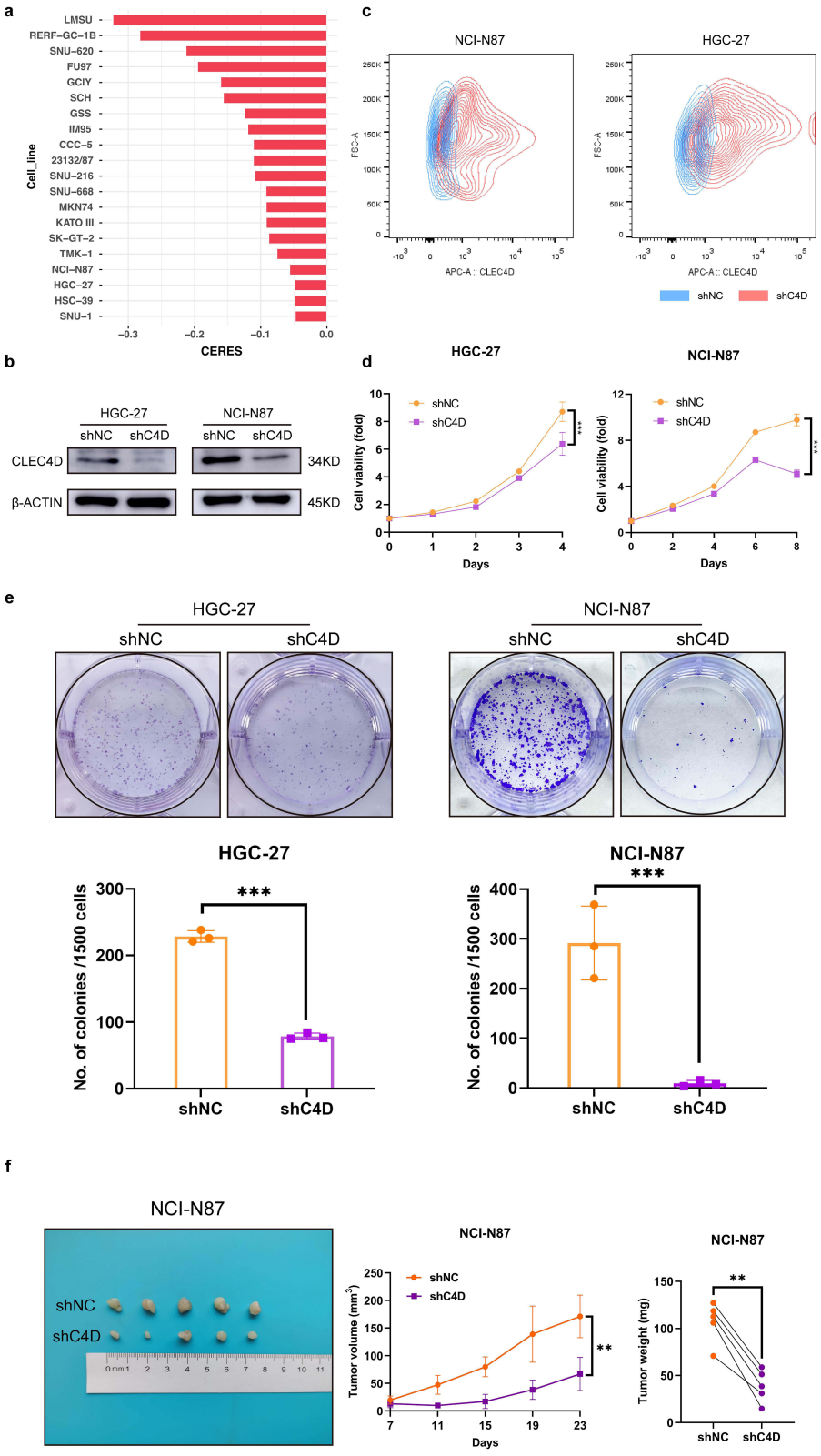


Figure 2 CLEC4D knockdown inhibits the proliferation in GC cells. (**a**) Analysis of DepMap Database (https://depmap.org/portal/download/) suggested that knockout of CLEC4D could attenuate the proliferation in a panel of GC cell lines. (**b**) We depleted the endogenous CLEC4D expression in HGC-27 and NCI-N87 cells using shRNA. Immunoblot assays were used to verify the knockdown efficiency. (**c**) Membrane expression of CLEC4D of GC cells was determined using flow cytometry. (**d**) CCK-8 assays showed that CLEC4D knockdown compared with control attenuated the propagation in HGC-27 and NCI-N87 cells. (**e**) HGC-27 and NCI-N87 cells with CLEC4D deficiency gave rise to far less colonies, compared with control cells did ($P < 0.001$). (**f**) Tumor xenografts model demonstrated that the growth of NCI-N87 cells was remarkably inhibited by CLEC4D knockdown in vivo. $^{**}P < 0.01$, $^{***}P < 0.001$.

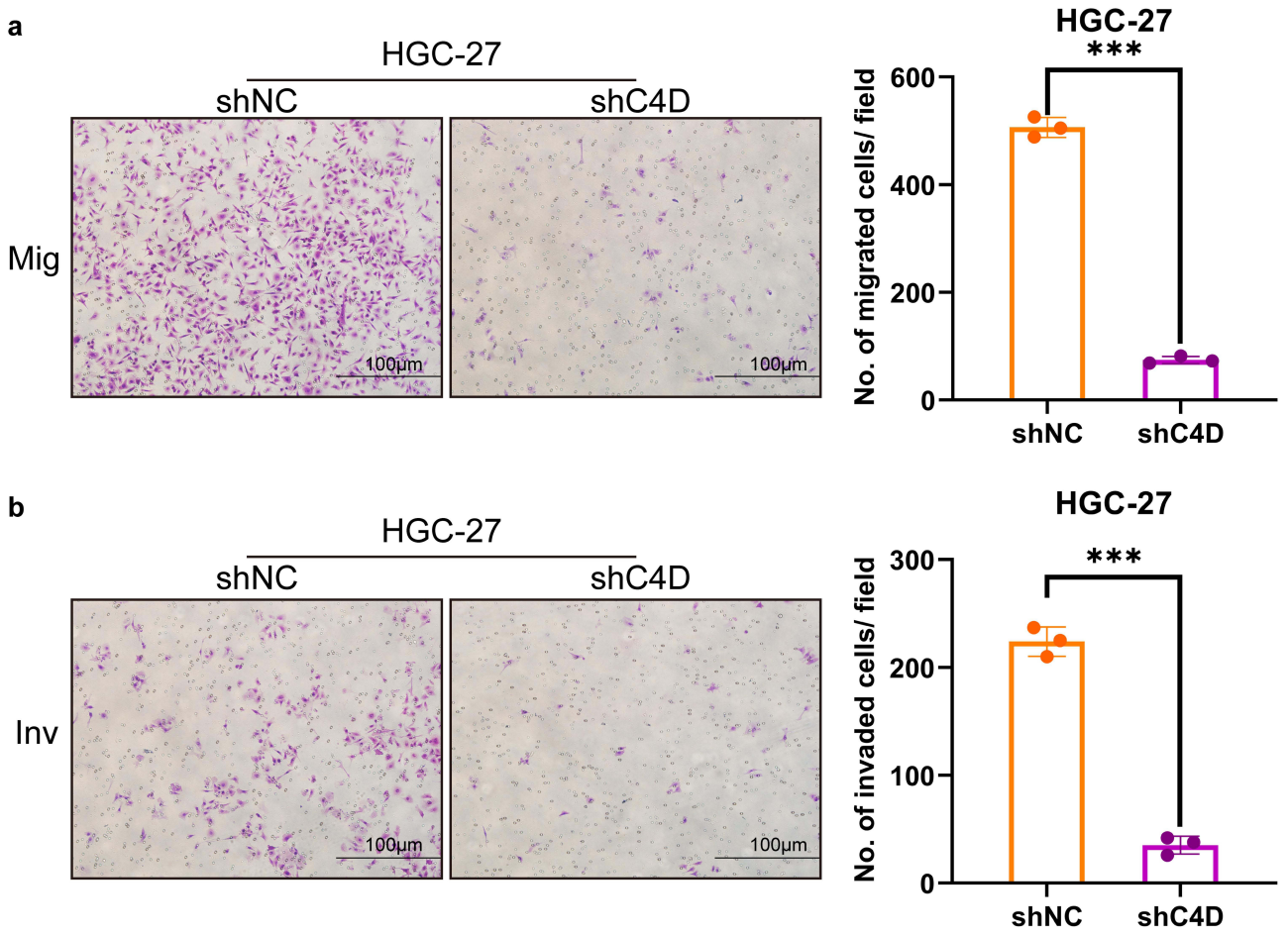


Figure 3 CLEC4D knockdown mitigates the migration and invasion in GC cells in vitro. (**a** and **b**) HGC-27 cells expressing CLEC4D shRNA (shC4D) displayed weaker migratory (**a**) and invasive (**b**) capacity relative to these cells expressing control shRNA (shNC). Scale bars, 100 μm. ***$P < 0.001$.

albicans infection and activate the NF-κB-dependent inflammatory response. CLEC4D can form homodimers to combat fungal infections, but it mainly forms a heterodimer called PPR with its protein family member CLEC6A. This heterodimer exhibits a higher affinity for binding to the main component, alpha-mannan, on the surface of *Candida albicans* hyphae compared to their respective homodimers. This endows host cells with heightened sensitivity to fungal infections.[13] CLEC4D is crucial in anti-mycobacterial immunity. The absence of CLEC4D specifically affects the binding between Mycobacterium and leukocytes, but it does not impact the phagocytic activity of leukocytes. It facilitates the identification of non-opsonic mycobacteria by pulmonary leukocytes, playing a vital role in the immune response against pulmonary tuberculosis. Loss of CLEC4D results in intensified inflammation and increased bacterial burden, accompanied by changes in IL-10 levels. CLEC4D-/- mice with IL-10 deficiency exhibit more severe pathology and survival.[16] Nevertheless, the presence of IL-10 levels offers protection to the majority of the infected CLEC4D-/- mice, mitigating the detrimental effects of lethal pathology, even in the face of elevated inflammation and bacterial burden.[16] Additionally, Clec4d-/- mice are more susceptible to dextran sodium sulfate (DSS)-induced colitis compared with wild-type mice. In Clec4d deficient mice treated with DSS, there is a significant increase in the specific fungal burden of the tropical commensal fungus C. Tropicalis in the intestine. The opportunistic pathogen C. tropicalis specifically induces NF-κB activation and cytokine production through the Clec4d signaling pathway. Antifungal treatment can effectively protect these mice from colitis invasion.[29]

The effects of CLEC4D in cancer biology have begun to be appreciated in recent years. As CLEC4D maintains tissue homeostasis, it is rational to suppose that this protein is probably implicated in regulating immunological responses in the microenvironment. In colorectal cancer, a blood-based 7-gene biomarker panel containing CLEC4D was reported to be

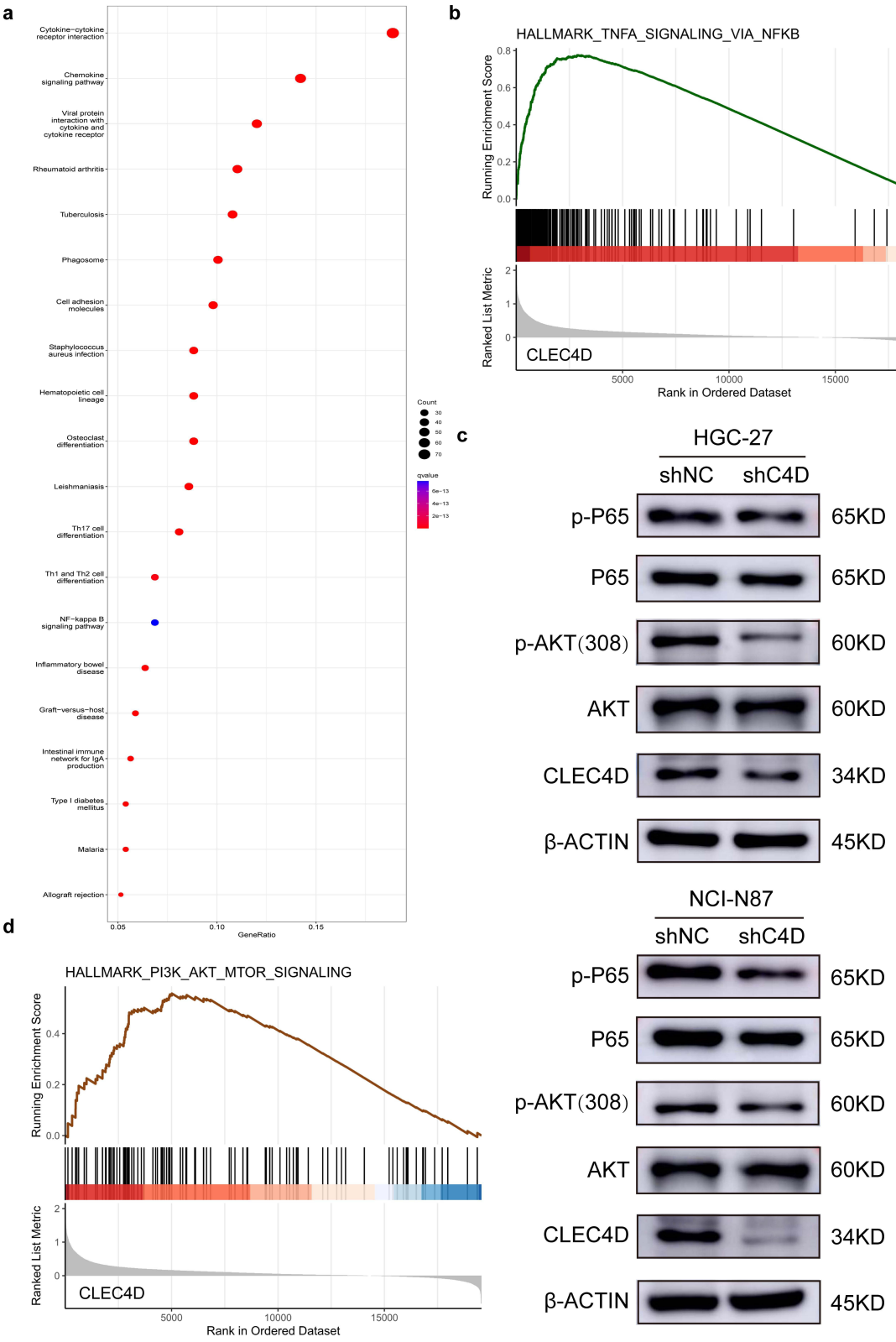


Figure 4 CELC4D depletion reduces Akt and NF-κB signaling pathway. (**a**) The KEGG enrichment analysis based on TCGA GC cohort showed 20 signaling pathways significantly associated with CLEC4D expression. (**b**) GESA analysis of TCGA GC cohort showed that high CELC4D expression positively correlated with NF-κB signaling pathways. (**c**) Immunoblot assays were conducted to show that reduced CELC4D level markedly decreased the expression of phospho-p65 (Ser536) and phospho-Akt (Thr308) in HGC-27 and NCI-N87 cells. (**d**) GESA analysis of TCGA GC cohort showed that high CELC4D expression positively correlated with Akt signaling pathways.

capable of improving clinical decision-making.[30] Additionally, CLEC4D expression in the microenvironment of hepatocellular cancer decreased as the tumor progressed.[13] More intriguingly, one recent report showed that Clec4d knockdown markedly mitigated the efficiency of anti-PD-1 treatment primarily because of its maintenance of myeloid cells' immunosuppressive function in mice.[31] Unlike previous investigations, we turned our attention to the expression and function of CLEC4D in GC cells themselves rather than in the GC microenvironment, since we found that CLEC4D mRNA and protein levels were significantly increased in GC tissues and several GC cell lines. Patients with high expression levels of CLEC4D show lower survival rates compared to those with low expression levels. Multivariate regression analysis revealed that high CLEC4D expression acts as an independent prognostic factor, predicting an unfavorable overall survival outcome for patients. These findings imply that CLEC4D may have a significant impact on the advancement of GC. Subsequently, the potential mechanisms by which CLEC4D exerted its effects on the proliferation and invasion of GC cells were investigated. The silencing of CLEC4D attenuates the proliferation and invasive abilities of gastric cancer cells, a phenomenon that has been confirmed through in vitro and in vivo experiments. Both CLEC4D and CLEC4E are capable of recognizing trehalose 6,6'-dimycolate (TDM) and inducing innate immune responses. Interestingly, stimulation of CLEC4D by TDM also leads to the activation of CLEC4E. It has been reported that CLEC4E is highly expressed in tumor-associated macrophages (TAMs), and specific silencing of CLEC4E effectively blocks the CLEC4E/Syk/NF-κB signaling pathway, thereby inhibiting TAM-driven cancer progression. In the context of innate immune response, the expression of CLEC4E is dependent on NF-κB activation mediated by CLEC4D.[32,33] CLEC4D induced the NF-κB signaling pathway by activating Syk and triggering the immune response.[32] Syk is a protein tyrosine kinase that couples activated immune receptors for downstream signal transduction activities to mediate various cellular reactions, including cell proliferation, differentiation, and phagocytosis.[34] In addition, Syk activates the Akt signaling pathway by binding to the promoter region of AKT1, thereby promoting cell proliferation.[35] CLEC4D inhibits the nuclear translocation of FOXO1 through the Syk-AKT1 signaling axis.[27] In our study, CLEC4D knockdown attenuated the proliferation and invasion of GC cells by reducing phospho-p65 (Ser536) and phospho-Akt (Thr308) levels. On one hand, CLEC4D may induce the NF-κB signaling pathway by activating Syk, to mediate the proliferation and invasion of cancer cells. On the other hand, changes in AKT phosphorylation suggest that CLEC4D may promote the progression of cancer cells through the AKT signaling pathway. The relationship between these two mechanisms is complex and interrelated. These preliminary results demonstrated that CLEC4D is probably expressed and functions in malignant cells, suggesting that this CLR might contribute to the neoplastic process in two different but intertwined manners.

There are some apparent limitations to this study. First, preliminary data on the clinical significance of CLEC4D expression and its biological functions have been reported in this manuscript; however, there is no detailed explanation of these phenotypes. Second, we did not investigate how CLEC4D knockdown deactivated the Akt and NF-κB signaling pathways. Although a previous study showed that Mincle, another member of the same family as CLEC4D, sustained tumor-associated macrophages via the Syk/NF-κB signaling axis, which provides a hint of how CLEC4D might relay intracellular signals,[33] more studies are required to delineate the molecular mechanisms.

Conclusion

In conclusion, we found that CLEC4D is overexpressed in GC and could be an independent indicator of poor prognosis in patients. Moreover, our results shed light on the oncogenic role of CLEC4D in GC, as it facilitates the malignant proliferation and progression of GC by activating the NF-κB and AKT pathways. CLEC4D may serve as a significant biomarker with potential diagnostic and therapeutic value for patients with gastric cancer, underscoring the importance of further exploration into its function.

Abbreviations

CLEC4D, C-type lectin domain family 4 member D; GC, gastric cancer; CLRs, C-type lectin receptors; PRRs, pattern recognition receptors; GSEA, Gene set enrichment analysis; shRNA, short hairpin RNA; Syk, spleen tyrosine kinase; DSS, dextran sodium sulfate; TDM, trehalose 6,6'-dimycolate; TAMs, tumor-associated macrophages.

Ethical Approval and Consent to Participate

All animal assays were approved by the Institutional Animal Care and Research Advisory Committee of Tianjin Medical University (Tianjin, China) and performed in accordance with relevant guidelines and regulations (AE-2023029) and the Guidelines for the welfare and use of animals in cancer research. Great efforts were made to minimize the number and pain of animals. This study was conducted following the Animal Research: Reporting of in vivo Experiments (ARRIVE) guidelines and conformed with the Declaration of Helsinki.

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Disclosure

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

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