miR-548d-3p inhibits the invasion and migration of gastric cancer cells by targeting GKN1

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Abstract
Background: The aim of this study was to explore the function and mechanism of GKN1 in gastric cancer (GC) progression.

Methods: Firstly, we used GEO2R to perform differential gene analysis on GSE26942 and GSE79973 and constructed the protein–protein interaction network of differential genes by STRING. Next, the cytoHubba, Mcode plugins, and GEPIA were used to obtain our follow-up research object GKN1. Then, the function of GKN1 in GC was verified by scratch and transwell assay in GC cells. We further analyzed the genes related to GKN1 through LinkedOmics, and exported top 100 genes positively or negatively correlated with GKN1. Meanwhile, Metascape was performed on these genes. Finally, we analyzed the miRNAs that bind to GKN1 through miRDB and verified the correlation between miR-548d-3p and GKN1 using dual-fluorescence and quantitative PCR experiments.

Results: Bioinformatics analysis showed that there were 52 differential genes on GSE26942 and GSE79973. In addition, the results of functional assays indicated that overexpressed GKN1 can inhibit GC cell migration and invasion, while GKN1 knockdown demonstrated the opposite effect. Additionally, Metascape analysis results showed that the 3′-UTR region of mRNA is rich in AU sequences, based on which we infer that mRNA may be regulated by miRNA. Dual-fluorescence and quantitative PCR assays clarified that miR-548d-3p may be one of the target miRNAs of GKN1, which was up-regulated in GC tissues.

Conclusions: In summary, we clarified that miR-548d-3p regulates GKN1 to participate in GC cell migration and invasion, and provides a possible target for the prognostic diagnosis and treatment of GC.

KEYWORDS
gastric cancer, GKN1, invasion, migration, miR-548d-3p
1  |  INTRODUCTION

As one of the most common types of gastrointestinal cancer, gastric cancer (GC) has the fifth highest incidence rate and the third-highest mortality rate among all malignant tumors globally. Nowadays, surgery is still the only cure for gastric carcinoma in situ. However, since GC usually remains undiagnosed until advanced stage, accompanied by metastasis to lymph nodes or distant organs, surgical resection is usually not an option. So far, no effective therapy has been found for metastatic GC. For this reason, the discovery of new metastasis marker genes and the exploration of the metastatic dissemination mechanism are of great significance for improving the therapeutic efficacy and prognosis of GC patients.

Gastrokeine-1 (GKN1), a small protease-resistant protein containing 185 amino acid residues, is a stomach-specific protein secreted by gastric mucus-producing cells. GKN1 mRNA and its proteins are mainly highly expressed in normal gastric epithelial cells and can also be expressed in oral mucosa, epithelial metaplasia tissues (such as Barrett’s esophagus), placenta, ovarian tumor, and smooth muscle tissues. Researches showed that GKN1 might be a product of the cell growth process, and that increased GKN1 expression in gastric mucosal cells could protect gastric mucosa through the regeneration of gastric mucosa, promote the repair of injured mucosa, and inhibit tumor progression. Meanwhile, other studies suggested that the amino-terminal hydrophobic region and the BRICHOS domain of GKN1 are possibly the main functional domains suppressing tumor activity, and that any of its change or deletion at the gene level could result in a loss of function of GKN1 proteins. Oien K A et al. discovered through Western blotting that GKN1 is specifically highly expressed in normal gastric mucosal tissues, but not expressed in GC, suggesting that the expression absence of GKN1 can be associated with the occurrence of GC. These results indicated that GKN1 can be a diagnostic marker of GC and plays an important role in the development and progression of tumor. Nevertheless, the specific mechanism of GKN1 in GC remains elusive.

MiRNA (miRNA) is an endogenous noncoding single-stranded DNA molecule, which binds to the 3′-UTR of target mRNA to block the translation of relevant target DNA regulating pathological and physiological processes, thereby achieving the goal of negative regulation of gene expression. Research revealed that miR-548d-3p is up-regulated in GC and targets RSK4 to inhibit the apoptosis of GC cells and enhance the proliferation, migration, and invasion of GC cells. In osteosarcoma cells, miR-548d-3p exerts an inhibitory effect on tumor by down-regulating KRAS expression. In addition, one study found that miR-548d-3p regulates the proliferation and apoptosis of breast cancer cells by acting on TP53BP2. All of these studies pointed out that miR-548-3p has an important role in the proliferation and pathological processes of tumor.

In this study, we verified the regulatory effect of GKN1 on the migration and invasion of GC cells with a series of in vitro experiments. Besides, through database prediction and dual-luciferase reporter assays, we determined that miR-548d-3p is one of the miRNAs targeting GKN1, and that miR-548d-3p promotes the migration and invasion of GC cells by targeting GKN1.

2  |  MATERIALS AND METHODS

2.1  |  Microarray data analysis

Gene expression omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) is an international public database repository mainly used for storing high-throughput microarray and next-generation sequencing functional genomics data sets. In this study, we downloaded gene expression profiles GSE26942 and GSE79973 from GEO, and conducted a differential gene analysis through GEO2R with the screening conditions of |logFC| > 2 and p < 0.05. Next, the analysis results were visualized in the form of volcano maps using SangerBox, and the intersection between differentially expressed genes (DEGs) was obtained with a Venn diagram.

2.2  |  PPI analysis

The STRING (http://string-db.org/) database was used for PPI network construction and hub gene identification. Then, the PPI network was visualized using the Cytoscape (www.cytoscape.org) software—an open-source bioinformatics software program for visualizing molecular interaction networks. With the Hubba plug-in in Cytoscape, we obtained the top 10 hub genes by the calculation of degrees.

2.3  |  Correlation analysis

LinkedOmics (http://www.linkedomics.org/login.php) is a database accessible to the public, containing 32 cancer types from the Cancer Genome Atlas (TCGA) and the multi-omics and clinical data of 11,158 patients. It offers a unique platform for biologists and clinicians to access, analyze, and compare multi-omics data within and across tumor types. In this study, we utilized the LinkedOmics database to identify GKN1-related genes and conduct a correlation analysis of GKN1 gene expression. Subsequently, we carried out Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of the target gene.

2.4  |  Functional enrichment analysis

By importing the top 100 genes positively or negatively correlated with GKN1 expression into Metascape (https://metascape.org/gp/index.html#/main/step1) and clicking Expression Analysis, we performed GO and KEGG analyses, including biological process, molecular function, and cell component, as well as KEGG pathway enrichment analysis, to determine the biological function of relevant genes, with the screening criteria being p < 0.05.
2.5 | Cell culture

The MNK45 and SGC-7901 cell lines were purchased from the Cell Bank of Type Culture Collection, Chinese Academy of Sciences. Cell cultures were prepared and maintained in accordance with standard cell culture processes, and all mediums were supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin/streptomycin (HyClone). All cells were cultured in incubators containing 5% CO2 at 37°C, with medium replaced every 2 days.

2.6 | Cell transfection

Negative control (NC), pcmv3, pcmv3-GKN1-FLAG, and si-GKN1 were purchased from Shanghai GenePharma Co., Ltd. MNK45 and SGC-7901 cells were seeded in a 6-well plate, respectively, and the above-mentioned siRNAs or plasmids were transfected into to cells using Lipofectamine 2000 (Invitrogen). 48h later, cells were collected in preparation for the following measurement. The transfection efficiency was evaluated through RT-qPCR.

2.7 | RT-qPCR assays

The mRNA expression level was determined through RT-qPCR. According to the manufacturer’s instructions, the TRIzol reagent (Thermo) was used to extract total RNA from cells. Next, TaqMan RT-PCR kit (Applied Biosystems) was adopted for reverse transcription of total RNA to cDNA. Then, SYBR green (Toyobo) was utilized for RT-qPCR in Applied Biosystems 7300 (Thermo Fisher Scientific, Inc.) to detect the GKN1 level. With GAPDH as the endogenous control for GKN1 expression, the \(2^{-\Delta\Delta Ct}\) method was adopted for quantitative comparison. See Table 1 for the primers used.

2.8 | Scratch wound-healing assays

Scratch wound-healing assays were adopted to assess the migratory ability of cells. MNK45 and SGC-7901 cells were seeded in a 6-well plate. 16–24h later, the cells were transfected for 4–6 h, and scratch wound-healing assays were performed with a pipette

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**Table 1** Sequence information of primers

| Primer          | Sequence 5′-3′                      |
|-----------------|------------------------------------|
| qPCR-GAPDH-forward | AATGGTGAAGTGGTGTA                 |
| qPCR-GAPDH-reverse | CGTGATGGAGTCATACTGGA               |
| qPCR-GKN1-forward | CAAACTGCTGGAAGTGGCC               |
| qPCR-GKN1-reverse | GAGTCTGGTGCCGCAAAAGC             |
| GKN1 WT-forward | CCGCTGAGTCCAATGCTTTGCTGGACT       |
| GKN1 WT-reverse | ATAAAAGTGGGCCCACCGTGCCTC CACAGAAGG |

**Figure 1** Differentially expressed mRNAs in GSE26942 and GSE79973 were identified. (A) 61 differentially expressed mRNAs were screened in GSE26942, of which 22 ascended and 49 descended. (B) 792 differentially expressed mRNAs were screened in GSE79973, of which 189 ascended and 603 descended. (C) By Venn diagram, 52 overlapping mRNAs were obtained in DEGs of GSE26942 and GSE79973. DEGs, differentially expressed genes
tip. After washing the cells with PBS, medium containing 2% blood serum was added to culture cells. A microscope was used to observe and measure the initial distance (0 h) and the distance 24 and 48 h after scratching (40x).

### 2.9 | Transwell assays

Transwell assays were utilized to assess the in vitro migration and invasion ability of cells.

To assess migratory ability with transwell assays, cells were transferred for 24 h and digested, followed by resuspension of MNK45 and SGC-7901 cells using serum-free RPMI-1640. 1 x 10^5 cells were seeded to the upper chamber (BD Biosciences), and RPMI-1640 medium containing 10% fetal bovine serum was added to the bottom chamber. 48 h later, migrant cells were fixed with 4% paraformaldehyde solution and stained with 10% crystal violet solution at room temperature. Five random visual fields were selected for photographing (100x) with a light microscope, and the Images software was used for quantitation.

To assess invasion ability with transwell assays, MNK45 and SGC-7901 cells were digested with trypsin, collected, and resuspended in serum-free medium, with concentration adjusted to 1 x 10^6 cells/ml. After being diluted at 1:5 using RPMI 1640 medium, Matrigel (Corning) was used to coat the upper chamber of transwell and allowed to dry at room temperature. The following steps are the same as above. Eventually, the number of cells stained with crystal violet under microscope was the invasion cell count.

### 2.10 | Exploration of miRNAs bound to GKN1

The MiRDB database^{24} (http://mircode.org/index.php) was used to look for miRNA targeting GKN1 3’UTR, and the dbDEMC database (https://www.picb.ac.cn/dbDEMC/index.html)^{25} was deployed to find out miRNA expression level in different tumors.
2.11 | Dual-luciferase reporter assays

Dual-luciferase reporter assays were performed using the dual-luciferase reporter assay kit (Promega, Madison, WI, USA). Psicheck2-GKN1-WT or psicheck2-GKN1-MUT and miR-548d-3p mimic or NC mimic were transfected to HEK-293 T cells, and cells were lysed 24 h later. Then, luciferase and renilla cell substrates were added for measurement. Each experiment was repeated 3 times.

2.12 | Statistical analysis

All data were analyzed with the GraphPad Prism 8.0 software (San Diego), and data were expressed as mean value ± SE. T test was conducted to determine whether there is a significant difference between 2 groups, while differences between multiple groups were compared using one-way or two-way ANOVA. Bonferroni was adopted as a post hoc test, with *p < 0.05, **p < 0.01 and ***p < 0.001.

3 | RESULTS

3.1 | Identified the differentially expressed mRNAs in GSE26942 and GSE79973

The GSE26942 data set mainly includes 205 GC tissues and 12 paraneoplastic tissues. Through differential analysis, we obtained 61 differentially expressed mRNAs in GC, among which 22 were up-regulated and 49 down-regulated (Figure 1A). GSE79973 includes 10 GC tissues and paraneoplastic tissues, and 792 differentially expressed mRNAs were obtained after differential analysis, including 603 up-regulated and 189 down-regulated ones in GC (Figure 1B). Differentially expressed mRNAs were visualized in the form of volcano maps. Through Venn diagram, we obtained 52 differentially genes overlapping in GSE26942 and GSE79973 (Figure 1C).

3.2 | PPI network construction and key gene screening

To further explore the role of differentially expressed genes in GC, we predicted protein interactions with the Cytoscape software. The STRING software was used to construct a PPI network, which revealed the direct or indirect interactions between different proteins (Figure 2A). The cytoHubba plug-in of Cytoscape was utilized to analyze hub genes with the degree calculation method, and the top 10 key genes were obtained (Figure 2B), with GKN1 ranking first. Module analysis was conducted using the MCODE plug-in, and 3 modules were obtained. The seed genes of module 1, module 2, and module 3 were GSTA1, TFF1, and GKN2 respectively (Figure 2C).

3.3 | Key gene expression analysis

We further analyzed hub genes and seed genes with the GEPIA database, and the results showed decreased expression of GKN1, GKN2, and GSTA1 in GC. However, the relative folds of GKN1 and GKN2 expression in GC as compared to the control group displayed greater difference than TFF1. Considering that GKN1 and GSKN2 belonged to the same family, and that GKN1 ranked first in the results of hub gene screening, we selected GKN1 for subsequent research (Figure 3).

FIGURE 3 Expression of GKN1, GKN2, ALDHA1, and TFF1 in GC tissues. The expression of GKN1, GKN2, ALDHA1, and TFF1 in GC tissues was analyzed by GEPIA database, and all the above genes are highly expressed compared with normal tissues in STAD. GC, gastric carcinoma
We evaluated the role of GKN1 in GC in vitro and verified the transfection efficiency of siRNA GKN1 and pcmv3-GKN1 in GC cells (MNK45 and SGC-7901) through RT-qPCR (Figure S1). The invasion and migratory ability of cells were measured with scratch wound-healing assays and transwell assays. In scratch wound-healing assays, the overexpression of GKN1 in MNK45 (Figure 4A) and SGC-7901 (Figure 4B) cell lines exhibited lower migration rate than the negative control group. Compared with the NC group, interference with the expression of GKN1 promoted the transverse migration of MNK45 (Figure 4A) and SGC-7901, indicating that GKN1 can inhibit the transverse migratory ability of GC cells. Furthermore, in transwell migration and invasion assays, GKN1 overexpression displayed weaker migratory and invasion ability than the NC group in MNK45 (Figure 4C) and SGC-7901 (Figure 4D) cells lines, while the interference with GKN1 expression yielded opposite results compared with the NC group, indicating that GKN1 can inhibit the migration and invasion of GC cells.

3.5 | The correlation analysis of significant genes related to GKN1

Genes positively or negatively correlated with GKN1 in GC were selected according to LinkedOmics (Figure 5A). Through Pearson's test, we assessed the correlation between GKN1 and differentially
expressed genes in GC. The heat map displayed the top 100 genes of marked positive correlation (Figure 5B) or negative correlation (Figure 5C) with GKN1 in GC.

3.6 | Functional enrichment analysis

Functional enrichment analysis of the top 100 genes of the closest positive or negative correlation with GKN1 was performed using Metascape. The results showed that these genes were mainly enriched in digestion, gastric acid secretion, hormone metabolic process, positive regulation of reactive oxygen species biosynthetic process, aspartic-type endopeptidase activity, and, the most noteworthy of all, mRNA 3′-UTR AU-rich region binding. These results suggest that GKN1 could be regulated by miRNA. Thus, subsequently, we will study the miRNAs regulating GKN1 expression in GC (Figure 6).

3.7 | GKN1 is target of miR-548d-3p

It is known to all that microRNA (miRNA) binds to the target mRNA by identifying specific sites to regulate the post-transcriptional level. Through the miRDB database, we analyzed the miRNAs binding to GKN1 and obtained 17 miRNAs (hsa-miR-325; hsa-miR-205-3p; hsa-miR-892c-5p; hsa-miR-6832-5p; hsa-miR-1245b-3p; hsa-miR-548d-3p; hsa-miR-548z; hsa-miR-548bb-3p; hsa-miR-548h-3p; hsa-miR-548ac; hsa-miR-3942-3p; hsa-miR-510-3p; hsa-miR-4803; hsa-miR-4738-3p; hsa-miR-580-5p; hsa-miR-3923; and hsa-miR-3123). TCGA was adopted to analyze the expression of 17 miRNAs in different tumors, and the results demonstrated differential expressions of 17 miRNAs in multiple tumors including GC, among which hsa-miR-548d-3p exhibited relatively high expression in GC (Figure 7A,B). As the only miRNA up-regulated in GC, miR-548d-3p was selected for further study. In addition, in dual-luciferase report assays, reduced level of luciferase activity was noticed in cells co-transfected with psicheck2-GKN1-WT 3′UTR and miR-548d-3p mimic, while no obvious difference in luciferase activity was observed between the psicheck2-GKN1-MUT group and the NC mimic group, suggesting that GKN1 is a candidate target of miR-548d-3p (Figure 7C). Additionally, RT-qPCR results showed that miR-548d-3p mimic can down-regulate GKN1 expression in MNK45 and SGC-7901 cells, while miR-548d-3p inhibitor can up-regulate GKN1 expression in MNK45 and SGC-7901 cells (Figure 7E,F).

4 | DISCUSSION

Gastric cancer (GC) is one of the most common malignant tumors in China with high mortality rate. Due to the atypical symptoms of GC, early diagnosis is relatively difficult, and most GC patients are diagnosed at either intermediate or advanced stage. At present, surgery is the primary means of treatment for GC. On account of metastasis and relapse, the 5-year survival rate of GC is only 30–50%. Nevertheless, the specific molecular mechanism for the metastasis and relapse of GC is not fully understood. Therefore, finding...
(A) Differential Expression Profile in cancer vs normal

(B) hsa-miR-548d-3p with 372 cancer and 32 normal samples in STAD

(C) GKN1 WT: 5’...AAUAUGGGCUCCAGUGGUUUUUU...

miR-548d-3P: 3’...CGUUUUCUUUGACACAAAAAC...

GKN1 MUT: 5’...AAUAUGGGCUCCAGUGGUUUUUU...

(D) Fluorescence Ratio

(E) The Expression of GKN1

(F) The Expression of GKN1
new promising target genes is an urgent task for exploring effective treatment methods for GC.

Located on chromosome 2p13 with 6 exons, GKN1 is also known as AMP-18 (antrum mucosal protein-18), CA11, foveolin, or TFIZ2. Studies have revealed marked down-regulation of GKN1 in epithelial cells of gastric mucosa infected with Helicobacter pylori and GC cells, suggesting that GKN1 can be a suppressor gene of GC. A recent study demonstrated that GKN1 inhibited the activation of gastrin-induced NF-κB signaling pathway, thereby inhibiting the proliferation of GC cells; the study of Yoon J et al. indicated that CKN1 could reduce the production of active oxygen, inhibit the PI3K/Akt pathway, and inactivate NF-κB-dependent matrix metal-binding protein, thus inhibiting EMT and GC cell migration. According to our research data, the expression of GKN1 in GC was obviously higher than in paraneoplastic normal tissues. Furthermore, GKN1 was involved in the migration and invasion of GC cells as a tumor promoter. All data were consistent with previous studies.

Through dual-luciferase report assay, we also verified that miR-548d-3p can directly target GKN1 in GC cells. Previous researches indicated that, as a target of miR-548d-3p, RSK4 also participated in the progression of GC cells. miR-548d-3p is a renowned miRNA promoting or inhibiting cancer in different tumor types. The abnormal expression of miR-548d-3p is considered to play an inhibitory role in the growth of trypsin. It was reported that miR-548d-3p inhibited the growth of osteosarcoma by inducing KRAS expression, and, by binding to SOCS5/SOCS6, miR-548d-3p can also regulate the JAK–STAT pathway to promote cell migration, meanwhile inhibiting the apoptosis of cells. Thus, the different roles of miR-548d-3p in various tumors reflected the diversity and complexity of its functions. In GC, our results suggested that miR-548d-3p promoted the invasion and migration of GC cells, which was consistent with previous reports.

In this study, we found marked down-regulation of GKN1 in GC, and its inhibitory role in the migration and invasion of GC. It is worth noting that GKN1 is a proven target of miR-548d-3p, and miR-548d-3p targets GKN1 to regulate the migration and invasion of MNK4S and SGC-7901 cells. Furthermore, the identification of the specific targeting of GKN1 by miR-548d-3p offers novel insights into its mechanism of action in cancer development. Nevertheless, this paper is inadequate due to a lack of clinical trials to verify the relationship between miR-548d-3p and GKN1, which is our future research direction.

5 | CONCLUSION

We identified the miRNA targeting GKN1-miR-548d-3p and studied the function of GKN1 in gastric cancer. According to the results of our study, miR-548d-3p accelerated the proliferation of GC cells and tumorigenesis by reducing the expression of GKN1.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.
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