



Kallikrein 12 downregulation reduces AGS gastric cancer cell proliferation and migration

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ABSTRACT. Abnormal expression of the kallikrein (KLK) family of serine proteases closely correlates with onset, progression, and prognosis of endocrine gland-related malignant tumors. The aim of this study was to evaluate how downregulation of *KLK12* influenced cell cycle and proliferation of the AGS gastric cancer cell line. *KLK12* was detected by western blot in GES-1 normal gastric epithelial and AGS cells. AGS cells were transfected with *KLK12* siRNA, a negative control siRNA, or subjected to a mock transfection, following which, we assessed mRNA and protein levels, cell proliferation, cell migration, and cell cycle progression. We found that *KLK12* levels were significantly higher in AGS cells than in GES-1 cells. Transfection of AGS cells with *KLK12* siRNA led to downregulation of *KLK12* mRNA and protein expression, reduced cell proliferation (0.47 ± 0.03 vs 0.92 ± 0.04 , $P < 0.01$), and lower cell counts ($3.92 \pm 0.25 \times 10^5$ vs $5.47 \pm 0.50 \times 10^5$, $P < 0.01$) with respect to the negative control. We observed that *KLK12* siRNA increased the number of AGS cells in G0/G1 and reduced those in S phase. Furthermore, downregulation of *KLK12* in AGS cells decreased

their ability to penetrate the membrane in a migration assay ($P < 0.05$). In conclusion, *KLK12* siRNA inhibited the proliferation and migration of AGS gastric cancer cells and caused their arrest in the G0/G1 phase of the cell cycle.

Key words: Kallikrein 12; Small interfering RNA; Cell proliferation; Cell cycle; Gastric cancer

INTRODUCTION

Human kallikreins (KLKs) form a polygenic family of serine proteases comprising 15 members. They are mainly present in tissues that produce or are regulated by steroid hormones (Tan et al., 2006; Angelopoulou and Karagiannis, 2015). Abnormal expression of KLKs such as *KLK12* closely correlates with onset, progression, and prognosis of endocrine gland-related malignant tumors, such as breast, prostate, and ovarian cancers (Lose et al., 2012; Lai et al., 2014). RNA interference, also known as post-transcriptional gene silencing, uses RNA to bind specifically to mRNA inside cells and thus block or decrease translation of the target gene. Few studies have hitherto targeted *KLK12* expression in this manner. Here, we evaluated the effect on growth after transiently transfecting gastric cancer cells with *KLK12* siRNA.

MATERIAL AND METHODS

Cell culture

Normal gastric epithelial (GES-1) and gastric cancer (AGS) cell lines were purchased from the China Center for Type Culture Collection (Wuhan, China) and cultivated in RPMI 1640 medium at 37°C in a 5% CO₂ atmosphere.

RNA interference

AGS cells were cultivated for 24 h on plates and divided into three groups: *KLK12* siRNA, negative control, and mock transfection control. Cells were incubated with 100 nM *KLK12* siRNA and liposomes (Dharmacon, Thermo Fisher Scientific, Waltham, MA, USA), control siRNA and liposomes, or RPMI 1640 medium at 37°C for 8 h. Subsequently, the medium was changed and cells were incubated for another 48 h. siRNA sequences were as follows: *KLK12*, sense strand 5'-AAACAGUGACAGCCACGUATT-3', anti-sense strand 5'-UACGUGGCUGUCACUGUUUGG-3'; negative control, sense strand 5'-UUCUCCGAACGUGUCACGUTT-3', anti-sense strand 5'-ACGUGACACGUUCGGAGAATT-3'. siRNAs were synthesized by Jiangsu Sainty Biotechnology (Jiangsu, China).

RNA extraction, reverse transcription, and real-time fluorescence quantitative PCR (q-PCR)

Total RNA was extracted using the Trizol method (Gibco BRL, Thermo Fisher Scientific), quality was assessed by formaldehyde electrophoresis, and the RNA was stored at -20°C. For reverse transcription, 5 µL RNA was denatured at 70°C for 5 min, followed by

addition of 10 μ L 5X reverse transcription buffer, 2 μ L dNTPs, 0.5 μ g random primers, and 300 U M-MLV reverse transcriptase (Promega, Madison, WI, USA). The reaction proceeded at 42°C for 60 min and was terminated by increasing the temperature to 70°C for 10 min. The PCR mix (20 μ L) contained 10 μ L 2X SYBR Green (TOYOBO, Osaka, Japan), 0.4 μ L 10 pmol each of sense and anti-sense strands, 2 μ L cDNA, and deionized water. Primer sequences were as follows: *KLK12*, sense strand 5'-GCCTCAACCTCTCCATCGTC-3', anti-sense strand 5'-CTTGAAGGACTCCCCACAC-3'; β -actin, sense strand 5'-CTGAGCAGATCATGAAGAC-3', anti-sense strand 5'-CTTGGTGGACGCATCCTGAG-3'. Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). β -actin was used as internal control. Each sample was loaded in triplicate. Reaction conditions were as follows: 95°C for 1 min; 95°C for 15 s and 60°C for 1 min, for a total of 40 cycles. The fluorescence generated during gene amplification was detected in real-time (7500 FAST Real-time PCR System, Thermo Fisher). Relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Yin et al., 2015).

Western blotting

Total protein was extracted using a kit (Life Technologies, Thermo Fisher Scientific). Protein extracts (400 μ g) were separated by polyacrylamide gel electrophoresis, electrotransferred onto a membrane, and blocked overnight at 4°C. Next, the membrane was incubated with mouse anti-human *KLK12* antibody (1:750; Abcam, Cambridge, UK) at 25°C for 2 h, with secondary antibody (1:2000, Santa Cruz) at room temperature for 1 h, and finally developed by electrochemiluminescence (ThermoFisher, MD, USA).

Cell proliferation assay

Exponentially growing AGS cells were seeded on 96-well plates (5 x 10³/well). Each condition was assayed in ten replicate wells. After transient transfection with *KLK12* or negative control siRNA, cells were cultured for 48 h and then incubated in the presence of tetrazolium dye (MTT; Beyotime Institute of Biotechnology, Haimen, China) for 4 h. Cells were resuspended in 150 μ L DMSO and shaken at low speed for 15 min. Absorbance at 490 nm was measured using an enzyme-linked immunosorbent assay reader.

Cell counting

AGS cells were seeded on 24-well plates at a density of 5 x 10⁴ cells/well. Each condition was assayed in triplicate. Cells were transfected with either *KLK12* or negative control siRNA, cultured for 48 h, and counted with a Coulter counter (Beckman Coulter, Brea, CA, USA).

Cell cycle analysis

AGS cells were seeded on 6-cm culture dishes (4 x 10⁵/mL). Twenty-four hours after inoculation, they were transiently transfected with either *KLK12* or negative control siRNA, cultured for another 48 h, washed with phosphate-buffered saline, fixed in 70% ethanol, and stained with propidium iodide solution containing 0.01% RNase (Beyotime Institute of Biotechnology) in the dark at 4°C for 30 min. Cell cycle progression was measured by flow cytometry (FACScan flow cytometer, Becton Dickinson, Mountain View, CA, USA).

Cell migration assay

The migratory properties of AGS cells were assessed using the CytoSelect 24-Well Cell Migration Assay Combo Kit (Cell Biolabs, San Diego, CA, USA). Cells were seeded on 24-well plates and incubated at room temperature for 10 min. After the density was adjusted to $1 \times 10^6/\text{mL}$, cells were resuspended in serum-free culture medium. Culture medium (500 μL) containing 10% fetal bovine serum was added to the lower chamber of 24-well plates and 300 μL cell suspension was added to the inside of each insert. Cells were cultured for 24 h in an incubator. At this point, non-migrating cells were removed with 2-3 damp cotton swabs. The remaining cells were transferred to sterile culture plates, stained with 400 μL 0.09% crystal violet solution, gently rinsed in water three times, air-dried, and observed under a microscope. Cells' migratory ability was assessed by counting the cells that penetrated the membrane insert in the upper, middle, lower, left, and right visual fields.

Statistical analysis

All data were analyzed by the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) and reported as means \pm SD. Multi-group comparisons were performed by one-way analysis of variance. Pairwise comparisons were conducted using the chi-square test. $P < 0.05$ was considered statistically significant.

RESULTS

KLK12 level in AGS and GES-1 cells

Western blotting showed that KLK12 was significantly higher in AGS than in GES-1 cells (Figure 1).



Figure 1. KLK12 protein expression in GES-1 and AGS cells.

KLK12 siRNA reduces KLK12 and KLK12 levels

qPCR showed that *KLK12* mRNA expression did not change significantly in negative and blank control groups; however, it decreased markedly in AGS cells transfected with *KLK12* siRNA ($P < 0.05$; Figure 2). The same cells produced significantly less KLK12 than the two control groups ($P < 0.05$), for which values were very similar (Figure 3).

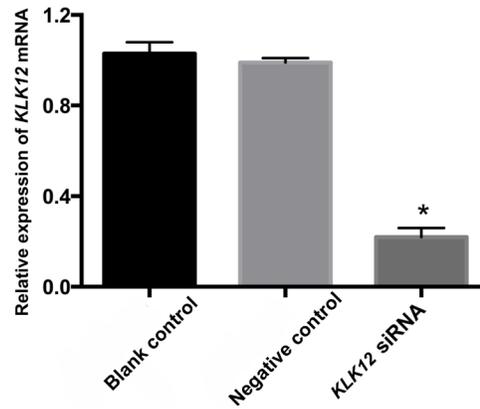


Figure 2. KLK12 mRNA expression in AGS cells transfected with *KLK12* siRNA. * $P < 0.05$ vs blank control and negative control groups.

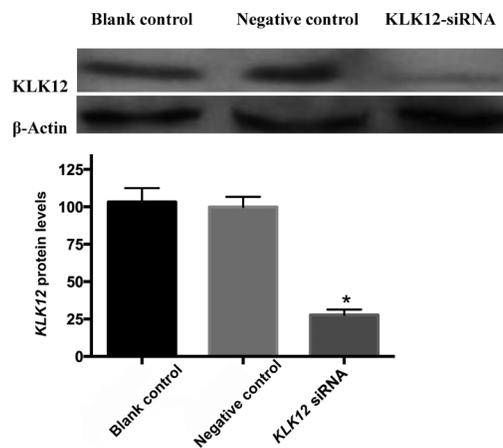


Figure 3. KLK12 protein expression in AGS cells transfected with *KLK12* siRNA. * $P < 0.05$ vs blank control and negative control groups.

Downregulation of *KLK12* retards AGS cell proliferation

Absorbance values from the MTT assay were 0.47 ± 0.03 and 0.92 ± 0.04 for the *KLK12* siRNA and negative control groups, respectively. Values were significantly different ($P < 0.01$). There were $(3.92 \pm 0.25) \times 10^5$ and $(5.47 \pm 0.50) \times 10^5$ cells in the *KLK12* siRNA and negative control groups, respectively, with the former displaying significantly slower proliferation ($P < 0.01$).

Effect of *KLK12* siRNA on cell cycle

Compared with the negative control group, significantly more *KLK12* siRNA-transfected cells were in G0/G1 and fewer were in S phase ($P < 0.05$; Figure 4). Cell cycle distribution was not significantly affected.

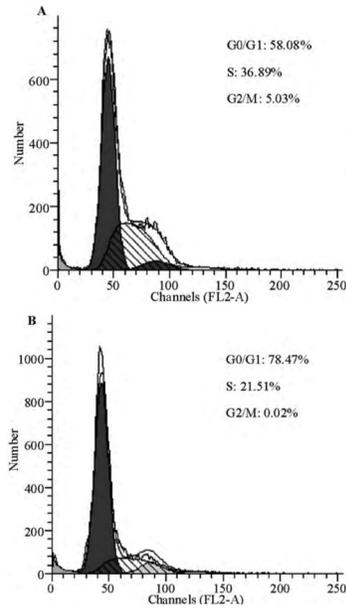


Figure 4. Effect of *KLK12* siRNA transfection on cell cycle. **A.** Negative control group; **B.** *KLK12* siRNA group.

Downregulation of *KLK12* impairs AGS cell migration

In the cell migration assay, membrane-penetrating AGS cells were stained blue. The number of cells penetrating the membrane in the blank (38.47 ± 3.22) and negative (41.14 ± 2.12) control groups was significantly higher than that in the *KLK12* siRNA group (17.87 ± 2.31) ($F = 644.059$, $P = 0.000$; Figure 5). Therefore, we concluded that *KLK12* siRNA significantly weakened *in vitro* migratory ability.

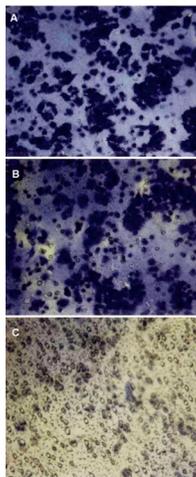


Figure 5. Effect of *KLK12* siRNA transfection on cell migration (200X magnification). **A.** Blank control group; **B.** negative control group; **C.** *KLK12* siRNA group.

DISCUSSION

Human KLK genes, located on chromosome 19q13.3-13.4, include 15 closely linked members that do not interfere with other genes. KLK1-KLK15 represent the largest gene cluster of proteases in the human genome (Sukumar et al., 2014; Kalinska et al., 2016). KLKs are abnormally expressed in prostate, ovarian, breast, and testicular cancers, indicating that they play important roles in the onset and progression of endocrine tumors. Moreover, KLKs have been recently detected in tumors of the digestive system. Nagahara et al. (2005) have reported that *KLK6* was highly expressed in gastric cancer tissues from 66 patients. Downregulation of *KLK6* by siRNA weakened proliferative capacity, cell cycle progression, and invasiveness of the MKN28 gastric cancer cell line. Feng et al. (2006) examined samples from 63 colorectal and 36 gastric cancer patients by RT-PCR, western blotting, and immunohistochemical staining. They observed that the transcriptional and translational levels of *KLK10* in tumor tissues significantly exceeded those of normal ones. In addition, *KLK10* overexpression in HCT116, HT29, CoLo205, LoVo, and AGS cell lines indicates a close link between KLK10 and the onset of tumors in the digestive system. Moreover, Huang et al. (2007) reported that *KLK10* overexpression in patients with gastric cancer indicated poor prognosis. We have previously found that *KLK12* expression was higher in gastric cancer tissues compared to that in normal gastric mucosae, especially in poorly differentiated adenocarcinomas (Zhao et al., 2012). Taken together, these findings suggest that blocking *KLK12* expression in gastric cancer cells may inhibit their growth and progression, representing a promising target for future therapy.

As a novel gene disruption technique, exogenous and endogenous RNA interference can specifically and efficiently suppress gene expression at the post-transcriptional level. Within a short time, it has been used in various applications as a potent gene therapy tool (Xiao and Deng, 2009; Lorenzer et al., 2015). Here, *KLK12* expression in AGS cells was significantly downregulated by RNA interference. As evidenced by the MTT assay and cell cycle analysis, downregulation of *KLK12* expression significantly inhibited proliferation by arresting cells in G0/G1 phase. During normal cell growth there is a slight risk of the DNA becoming damaged. If left unrepaired, such DNA may substantially destabilize the genome, resulting in cells becoming cancerous or directly participating in tumor onset and progression (Zhou and Elledge, 2000; Bohr et al., 2007; Hoeijmakers, 2007).

Having previously investigated the role of *KLK12* in MKN and SGC gastric cancer cell lines (Zhao et al., 2012), here we turned our attention to AGS cells. Although we observed that *KLK12* had a similar effect on cell proliferation and migration in all three cell lines, AGS cells displayed a lower degree of differentiation in the S phase (Ran et al., 2004). This is of relevance considering that *Helicobacter pylori* can easily adhere to and invade human gastric epithelial cells. It has been shown that infection with low-density *H. pylori* modified proliferation and apoptosis of AGS cells (Ito et al., 2008).

A decrease in KLK12 levels significantly inhibited migration of AGS cells. Hence, *KLK12* may promote the growth of gastric cancers, probably by transcriptionally regulating essential genes related to cell proliferation and cell cycle progression, as well as by affecting cellular behavior. Further studies on the downstream regulatory mechanisms of *KLK12* would be of great significance.

Conflicts of interest

The authors declare no conflict of interest.

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