In vivo and *in vitro* inhibition of human gastric cancer progress by upregulating Kank1 gene

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Abstract. Recent research has found that Kank1 gene, as one of the important members of the Kank gene family, plays an important role in the occurrence and development of malignant tumors. As a brand new cancer suppressor gene, its expression is significantly downregulated or missing in various kinds of malignant tumors. However, no report on the specific role of Kank1 in the genesis and development of gastric cancer is available yet. In the present study, we found significantly lower expression of Kank1 gene in human gastric cancer cells. When Kank1 gene was upregulated, it was found that the proliferation of gastric cancer cells was obviously inhibited, cell apoptosis occurred and caspase family expression changed with mainly activation of caspase-3 and caspase-9 as well as the apparent imbalance in Bcl-2/Bax ratio. We also found that upregulating Kank1 gene may result in the inhibition of the ability of the invasion and metastasis of tumor cells and the significant change in the expression level of MMP-7 of MMP family, which were confirmed by the *in vivo* nude mouse experiment, as upregulating Kank1 gene expression may result in decline in the nude mouse tumor formation rate and the significant reduction in the transplanted tumor size. The results above indicate that upregulating the Kank1 gene can inhibit the progress of gastric cancer *in vivo* and *in vitro* and its mechanism is closely relevant to apoptosis and the tumor invasion and metastasis.

Introduction

Cancer is an incurable disease, which not only brings enormous burden to the countries, especially the developing countries, but also affects the economic development to a certain degree (1). As per the latest researches, the morbidity and morality of cancer has been increasing year after year, and gastric cancer is one of main causes of death among all types of cancers, ranking the 3rd in China (2). Gastric cancer in the advanced stage tends to metastasize, and although the cancer is treated by surgery, radiotherapy or chemotherapy or other means (3), the results are unsatisfactory as the survival time of the patients after operation is short and relapse is common. There is increased research on the molecular targeted treatment of gastric cancer, and it has become more important to explore the molecular mechanism of the occurrence and development of gastric cancer (4,5). Based on the above, our research group, by referring to the literature, discovered Kankl (6), as one possible ideal and reliable molecular target for future molecular targeted treatment, providing new significance to the clinical understanding of the biological behavior of gastric cancer.

In 2002, the Japanese scholars cloned the candidate tumor suppressor gene Kank1 (7). Kankl plays the role of inhibiting the occurrence and development of tumors to a certain extent (8). The low expression of Kankl has been found in kidney (9), brain glioma (10), cervical (11), bladder (12), liver (13,14), pancreatic cancer (15) and various tumor tissues including lung cancer (16,17). It mainly exists in the cytoplasm, adjusts the actin polymerization in the cytoskeleton and participates in the cell motility (18). It also forms a compound with β-catenin to shuttle among the nucleoplasm to regulate the development of many malignant tumors. It has been found in some research that downregulation of Kankl gene may participate in the genesis and development of bone tumor subcellular distribution of β-catenin, playing a key role in the genesis and via activating JAK2-Stat (19) tumor-promoting signaling pathway. The mechanism of the tumor genesis and development is complicated and varied, while the missing or mutation of cancer suppressor gene has significant impact on the tumor genesis (20). We have found low expression of Kankl in gastric cancer cells. It was found after upregulating Kankl gene that the proliferation of tumor cells was significantly inhibited, and with apoptosis. At the same time, upregulation of Kankl gene can lead to the increase of Bax expression and decrease of Bcl-2 expression, and the expression of caspase family is changed, mainly with caspase-3 and caspase-9 activation. In addition, after the upregulation of Kankl gene, the ability of tumor cell invasion and metastasis was reduced. *In vivo* experiments showed upregulation of Kank1 may lead to lower tumorigenicity rate and significant reduction in tumor size. In summary, we conclude that the upregulation of Kankl gene can inhibit gastric cancer development *in vivo*, and the mechanism is closely related to apoptosis and tumor invasion and metastasis.

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Materials and methods

Cell strain. Human gastric cancer cell strain SGC-7901, MKN-49P cells and normal human gastric epithelial cell line GES-1 and RGM-1 cells were cultured in the RPMI-1640 culture medium containing 10% fetal bovine serum (FBS) in the incubator containing 5% CO_2 at 37˚C of saturated humidity. Both the culture medium RPMI-1640 and FBS were from Gibco (Carlsbad, CA, USA).

Antibodies and reagents. Kank1, Bax, Bcl-2 and MMP-7 antibodies were all from Cell Signaling Technology Inc. (Danvers, MA, USA). siRNA and plasmid were synthesized by Shanghai GenePharma, Co., Ltd. (Shanghai, China). Caspase-3 Assay kit and caspase-9 Assay kit were from (Abcam, Cambridge, UK). RT-PCR kit was obtained from (Takara Bio, Shiga, Japan). PI was from Sigma-Aldrich (St. Louis, MO, USA) and Annexin V-FITC apoptosis assays kit was from BD Biosciences (San Jose, CA, USA).

Cell culture. Human gastric cancer cell strain SGC-7901 and MKN-49P and normal gastric mucosa epithelial cell strains GES-1 and RGM-1 were cultured in the RPMI-1640 culture medium containing 10% fetal calf serum (FCS), 100 μg/ml penicillin and 100 μg/ml streptomycin, and then were cultured in the incubator containing 5% CO_2 at 37˚C.

Cell transfection. Human gastric cancer cells in logarithmic growth phase (SGC-7901 and MKN-49P) were collected by trypsin, and SGC-7901 and MKN-49P cells were plated at the density of $6x10^5$/ml in 6-well plates for culture. Transfection was carried out when the cells grew to >75% confluence. Negative oligonucleotide was used as control. The culture medium was replaced without serum and antibiotics, add respectively the mixed solution of siRNA and plasmid with Lipofectamine 2000, adjusted to a predetermined concentration by referring to the transfection reagent instructions. We used an RT-PCR kit for PCR analysis.

RNA extraction and RT-PCR analysis. RNA cells were collected and extracted for purity and integrity analysis before the reverse transcription. After calculating the concentration of RNA, we used the RT-PCR Kit (Takara) for RT-PCR reaction with steps according to the instructions. Kank1 and β-actin gene primers were designed and synthesized by Invitrogen. The forward primer of Kank1 gene was 5'-CTTGA CACAGTATTTTCACGCTTTTG-3' and the reverse primer, 5'-AAGTAAATGTGACACGGTAAAAAGG-3'; β-actin gene forward primer, 5'-CTGGGACGACATGGAGAAAA-3' and reverse primer, 5'-AAGGAAGGCTG GAAGAGTGC-3'. PCR reaction system 25 μl at the following reaction conditions: 94˚C for 2 min, 94˚C degeneration for 30 sec, 60˚C annealing for 30 sec, 72˚C extension for 30 sec, total 31 cycles. The PCR product was electrophoresed with 1.5% agarose gel, then scanned and analyzed with gel imaging system.

Cell viability. Cells were collected with trypsin and inoculated into 96-well plates, with 5 double wells in each group. For the transfection group, complete culture solution was used after 24 h and 20 μl MTT solution was added 48 h later (0.5 mg/ml)/well. After the cells were incubated for 4 h at 37˚C, the supernatant was removed and 150 μl of dimethyl sulfoxide (DMSO) was added to each well for 10 min oscillation until the complete dissolution of the purple crystal. The optical density value of the 490 nm wavelength of each well was measured by the microplate reader.

Caspase viability. Human gastric cancer cells after transfection, human gastric cancer cells without transfection and blank plasmid transfected cells were collected to determine the activity of caspase-3 and caspase-9 according to the caspase activity analysis kit. The fluorescence spectrophotometer was used at an excitation wavelength of 460 nm.

Analysis of apoptosis with flow cytometry. We digested and collected the cells of all groups for PBS cell suspension and pipetting preparation into single cell suspension. According to Annexin V-FITC cell apoptosis analysis kit steps, 10 μl of Annexin V and 5 μl of PI staining were added, respectively for staining in the dark at room temperature for 15 min and flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) was used to detect cell apoptosis, and each experiment was repeated 3 times.

Cell invasion and migration ability analysis. The cells of all groups were cultured in 6-well Transwell culture plate of BD, the upper chamber of 8 μm membrane was covered with Matrigel diluted at 1:6 and each culture well was evenly laid with ~$2x10^6$ cells to culture for 36 h. The cells that had gone through the membrane were fixed to the bottom of the culture well with formalin and stain with Crystal violet. The number of cells of 10 fields (x200) was counted for each well.

Western blot assay. Human gastric cancer cells and their stably transfected cell strain were collected, washed with PBS twice, and 1 ml protein lysate was added (Sigma-Aldrich) containing 10 μl protease inhibitor for cell lysis. The concentration of protein was measured by BSA method. Equal amount of protein was added to each well for loading and separated with 10% SDS-PAGE gel, and then the protein was transferred to PVDF membrane with semi-dry method and 5% skim milk powder was used for sealing at 4˚C overnight. TBST solution was diluted and the first antibody added at 37˚C with the first antibody dilution ratio (phosphorylated PI3K antibody 1:500, phosphorylated-Akt antibody 1:1000, MMP-7 antibody 1:800). The membrane was washed with TBST 3 times, second antibody incubation at 37˚C for 1 h, and TBST solution shaking and washing 4 times. ECL luminescence reagent was added, with X exposure imaging, scanning strip, and the gray analysis and β-actin were used as the reference standard.

Inoculation in nude mice. The animal experiment program has been approved by the Ethics Committee of Zhengzhou City People's Hospital. The nude mice were purchased from the Animal Experimental Center of Henan Medical University, and there were 20 male mice of 6-8 weeks, weighing ~20 g. The mice were randomly divided into 2 groups with 10 mice in each group. The human gastric cancer cells were divided into 2 groups: the constructed Kank1 gene stable expression

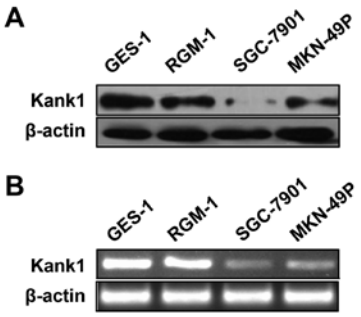

Figure 1. The expression level of Kank1 gene and protein in gastric cancer cells. (A) Kank1 protein expression level in gastric cancer cells. (B) The expression level of Kank1 mRNA in gastric cancer cells. Each individual experiment sample was repeated 3 times.

of MKN-49P cells were divided into a group, and non-transfection group, and they were, respectively, planted in 2 groups of nude mice via subcutaneous injection; the impact of Kank1 on the transplanted tumor formation ability of nude mice was observed. The effect of upregulating Kank1 gene on the growth of transplanted tumor in nude mice was also observed and the tumor growth was measured 21 and 28 days. The nude mice were sacrificed 4 weeks later with cervical spine method, and the subcutaneous transplantation tumor tissue under sterile condition was cut-off for indicator analysis.

Statistical analysis. All the experimental data were analyzed with SPSS 18.0 statistical software. T-test and variance analysis were used. $P<0.05$ was considered to indicate the significant statistical difference.

Results

Kank1 gene expression is low in human gastric cancer cells. We used RT-PCR and western blot assay to determine the expression of Kank1 mRNA and protein in human gastric cancer cells (SGC-7901 and MKN-49P cells) and normal human gastric epithelial cell line GES-1 cells and RGM-1 cells. The results showed that, as compared with normal human gastric mucosal epithelial cell lines, the expression of Kank1 mRNA and protein in SGC-7901 and MKN-49P cells also decreased. These results indicated that both protein and gene of Kank1 were expressed in gastric cancer at low levels (Fig. 1).

Kank1 gene inhibits growth of human gastric cancer cells. In order to further confirm the effect of Kank1 gene on the growth of human gastric cancer cells, we used transfection experiments, respectively for the transfection of siKank1 and successfully constructed and transfected Kank1 plasmid, upregulated the expression level of Kank1 gene in human gastric cancer cells and analyzed using the MTT method. We found that as compared with the control group and transfection negative oligonucleotide control group, the proliferation ability of SGC-7901 and MKN-49P cells declined significantly after the upregulation of the expression of Kank1 and when silencing Kank1, compared with the control group and transfection negative oligonucleotide control group, cell proliferation rate increased significantly which suggests that

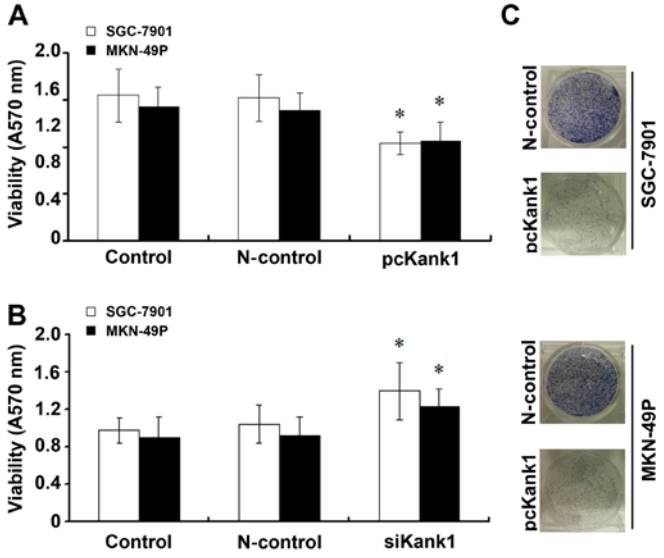

Figure 2. Impact of Kank1 gene on growth of human gastric cancer cells. (A) Cell proliferation after Kank1 overexpression in SGC-7901 cells and MKN-49P cells was measured using MTT assays. (B) Cell proliferation after Kank1 knockdown in SGC-7901 cells and MKN-49P cells was measured using MTT assays. (C) The results of colony formation assays that were conducted after Kank1 overexpression in SGC-7901 cells and MKN-49P cells. *$P<0.05$. Data from three independent experiments.

Kank1 gene can inhibit the growth of human gastric cancer cells (Fig. 2).

Upregulation of Kank1 gene induces apoptosis of human gastric cancer cells. In order to investigate whether the Kank1 gene is associated with apoptosis of human gastric cancer cells, we increased the expression level of Kank1 gene, and used Annexin V-FITC/PI double labeling method to determine the cell apoptosis rate. We found that after the expression of Kank1 was upregulated, the apoptosis rate of SGC-7901 and MKN-49P cells increased significantly as compared with the control group (Fig. 3A). The changes of expression level of Bel-2 and Bax proteins were determined with western blot assay (Fig. 3B). In addition, we used caspase activity assay kit to upregulate Kank1 gene expression level and found viability enhancement of caspase-3 and caspase-9 (Fig. 3C). These results indicate that upregulation of Kank1 gene can lead to apoptosis of human gastric cancer cells.

Upregulation of Kank1 gene induces apoptosis of human gastric cancer cells by inhibiting PI3K/Akt pathway. In order to investigate the molecular mechanism of apoptosis of human gastric cancer cells induced by of upregulating Kank1 gene, we referred to the relevant literature and found that Kank1 gene may induce apoptosis of human gastric cancer cells by inhibiting the PI3K/Akt pathway. We used western blot assay to determine the changes in the expression level of PI3K/Akt pathway proteins PI3K and Akt, and we tested the changes in their phosphorylation level at the same time. We found reduction in the expression level of PI3K protein after the upregulation of Kank1 gene. Moreover, phosphorylation of Akt protein also decreased after the upregulation of the Kank1 gene (Fig. 4). Our results indicated that upregulation of Kank1

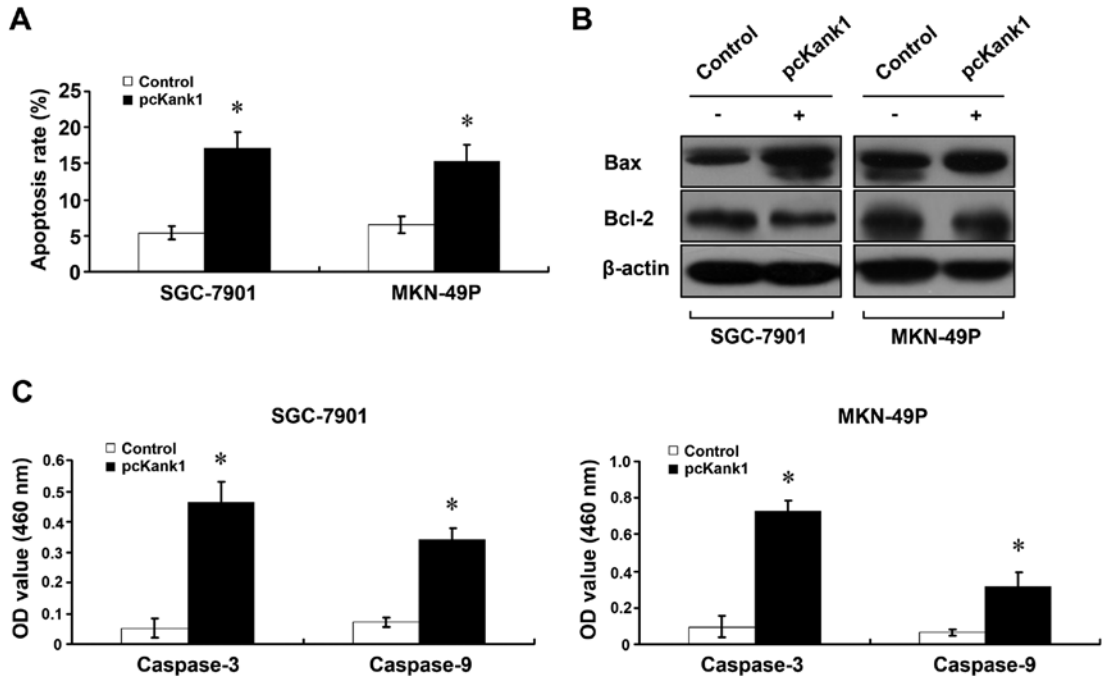

Figure 3. Upregulation of Kank1 gene results in apoptosis of human gastric cancer cells. (A) Annexin V-FITC/PI double labeling flow cytometric analysis on the apoptosis rate. (B) Western blot assay analysis of Bel-2 and Bax protein expression level. (C) Viability determination of caspase-3 and caspase-9. Compared with the control group; *P<0.05. Data from three independent experiments.

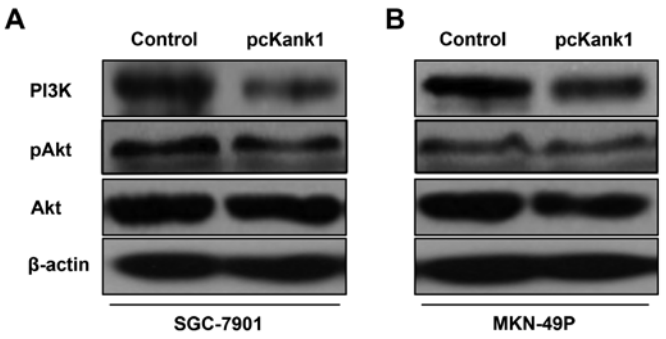

Figure 4. Upregulation of Kank1 gene induces apoptosis of human gastric cancer cells by inhibiting the PI3K/Akt pathway. (A) PI3K/Akt proteins expression level after upregulating Kank1 gene in SGC-7901 cells was analyzed by measuring protein levels by western blotting. (B) PI3K/Akt protein expression level after upregulating Kank1 gene in MKN-49P cells was analyzed by measuring protein levels by western blotting. As compared with the control group; *P<0.05. Results are representative of three independent experiments.

gene can induce apoptosis of human gastric cancer cells by inhibiting the PI3K/Akt signaling pathway.

Upregulation of Kank1 gene inhibits invasion and metastasis of gastric cancer cells. We have also found that upregulation of the Kank1 gene can inhibit human gastric cancer cell invasion and metastasis. We analyzed the change in the invasion and migration ability of gastric cancer cells via cell invasion and metastasis test. We have found that invasion and metastasis ability of the human gastric cancer cells (SGC-7901 and MKN-49P cells) with upregulated Kank1 gene, both declined significantly as compared with the control group of oligonucleotides (Fig. 5A). We have found by western blot assay that the expression level of MMP-7 protein in human gastric cancer cells declined by upregulation of Kank1 gene (Fig. 5B). Our results indicated that upregulation of Kank1 gene can inhibit the invasion and metastasis of human gastric cancer cells and downregulate the expression of MMP-7 protein.

Effects of upregulation of Kank1 gene on the growth of transplanted tumor in nude mice. We found that at any time, the tumor volume of the group with upregulated Kank1 gene were significantly lower than that of the control group (Fig. 6A). At the same time, the weight of tumor harvested at surgery in the group of upregulated Kank1 gene was significantly lower than the control group (Fig. 6B). These results strongly suggest that upregulating Kank1 gene can significantly inhibit the progress of gastric cancer *in vivo*.

Discussion

At present, gastric cancer is a major cause of cancer death in the world, although early detection and standard treatment approaches have improved, gastric cancer mortality rates have remained high (21). As is known, the complicated pathogenesis of malignant tumor is closely related to the missing or mutation of tumor suppressor genes or the overexpression of cancer gene and some signaling pathways. The research explores the occurrence and development of gastric cancer gene from the perspective of tumor suppressor gene deletion or mutation. Kankl is one of the key members of Kank family gene (22). Kankl gene is kocated in human chromosome 9p24.3 containing 12 exons with the total length of ~27.7 kp (5). Kankl protein consists of three parts, in which ankyrin repeat domain and coiled coil domain are the functional domains where Kankl protein and other proteins combine for their biological

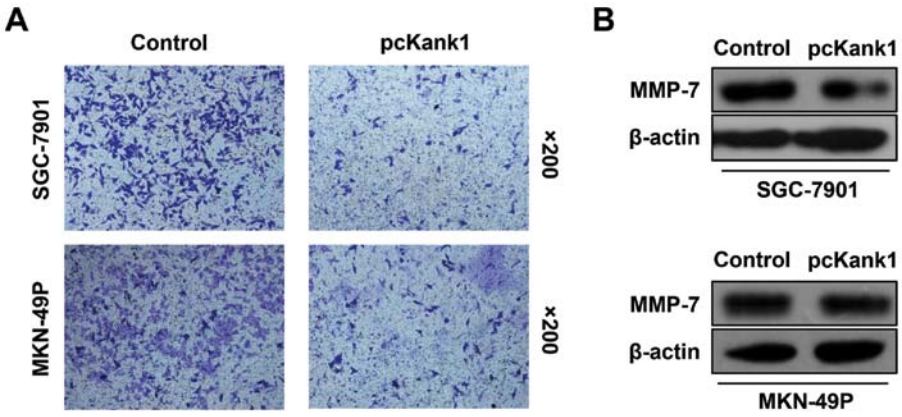

Figure 5. Upregulating Kank1 gene inhibits the invasion and metastasis of human gastric cancer cells. (A) Cell invasion assay determination of invasion of human gastric cancer cells after upregulation of Kank1 gene. (B) Western blot analysis of MMP-7 protein expression level; *P<0.05. Each independent experimental sample was repeated 3 times.

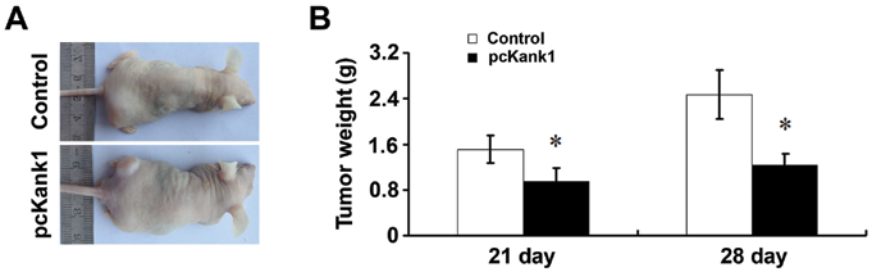

Figure 6. Upregulation of Kank1 gene can inhibit the growth of gastric cancer in nude mice. (A) Tumor growth volume measured at 28 days after inoculation. (B) Tumor tissue weight at 21 and 28 days after inoculation. As compared with the control group, *P<0.05.

roles (6,10). Kank1 is mainly distributed in the cytoplasm, and associated with cell motility. In the development, invasion and metastasis of a variety of diseases including malignant tumors, Kank1 often forms a compound with β-catenin to shuttle in the nucleoplasm to adjust the distribution of β-catenin in the nucleus and strengthen the transcription of β-catenin to affect the occurrence and development of tumor to a certain extent (23).

As a candidate tumor suppressor gene, Kank1 gene is lowly expressed in a variety of malignant tumors, such as kidney (24) and liver cancer (14). No report on the research of gastric cancer in this aspect is available yet. The incidence and mortality of gastric cancer is ranking top three in malignant tumors, but its pathogenesis is still unclear. Therefore, it is imperative to explore the effect of Kank1 gene on tumor development and metastasis of gastric cancer. We found through research that the Kank1 gene and protein expression in human gastric cancer cells are low as compared with normal gastric cells. Thus, we can draw the conclusion that there is a certain close relationship between Kank1 gene and gastric cancer development process, and the Kank1 gene may well be a potential therapeutic target for gastric cancer. In order to further elucidate the relationship between Kank1 gene and the existence and development of gastric cancer as well as its detailed mechanism of action, we used transfection and other methods to upregulate Kank1 gene expression in human gastric cancer cells, and observed the biological changes of human gastric cancer cell with Kank1 gene overexpression through a series of experiments. We found that upregulating the expression of Kank1 can inhibit the proliferation of human gastric cancer cells and promote cell apoptosis. Therefore, we believe that the low expression of Kank1 in gastric cancer promotes the proliferation of cancer cells to a certain extent.

The mechanism of how Kank1 gene regulates the process of gastric cancer cell proliferation is still unknown. In order to clarify this key issue, we found that Kank1 gene and PI3K/AKT signaling pathway are related (25). To a certain extent, PI3K/AKT signaling pathway regulates the proliferation, differentiation, migration and infiltration or other functions of gastric cancer cells (26). When we upregulated the expression level of Kank1 gene in human gastric cancer cells, we found that the phosphorylation expression level of PI3K protein and AKT protein was significantly inhibited. Therefore, from the cell proliferation signaling pathway we found that Kank1 could inhibit the proliferation of tumor cells and promote cell apoptosis by inhibiting PI3K/AKT signaling pathway.

The mitochondrial pathway and death receptor pathway of apoptosis is the classical pathway of apoptosis of various tumor cells (27). Bcl-2 and Bax play a key role in mitochondrial apoptotic pathway (28), the expression of Bcl-2 in a variety of tumors, including gastric cancer, is high while Bax is the opposite (29). The knowledge on the mechanism of how Kank1 gene regulates apoptosis of human gastric cancer is still very limited. Thus, after upregulating Kank1 gene expression in human gastric cancer cells, we found via

western blot assay and RT-PCR, the shift of Bax and Bcl-2, the decline in the expression level of Bax, and Bax translocation from the cytosol to the mitochondrial membrane, which could change the permeability of the mitochondrial membrane, promote the release of cytochrome *c* from mitochondria into cytoplasm, then start the apoptosis cascade, eventually leading to cell apoptosis. Thus, we speculated that the Kank1 gene promoted apoptosis in gastric cancer cells through regulating Bel-2 family of anti/pro-apoptotic proteins (Bcl-2 and Bax), we also demonstrated that the phase of Kank1 gene and mitochondrial pathway leading to apoptosis of tumor cells. As known, caspase family activation and cascade amplification is a necessary condition for cell apoptosis regardless whether apoptosis is regulated externally or internally (30). We upregulated Kank1 gene in human gastric cancer cells and found that the expression level of pro-caspase-9 and pro-caspase-3 both decreased significantly. In conclusion, we found that the Kank1 gene can promote apoptosis of human gastric cancer cells by regulating the change of mitochondrial membrane potential, and activating mitochondria to release apoptosis enzyme activation factor to activate caspases.

Tumor invasion and metastasis is one of the important causes of cancer death, as it is found that ~90% of the tumor patients died of metastatic tumor (31). We found that overexpression of the Kank1 gene can significantly inhibit the invasion and metastasis of human gastric cancer cells. MMP-7, as an important member of the MMP family, on the one hand, its physiological function can degrade certain proteins in the extracellular matrix, such as elastic protein and fibronectin (32), on the other hand, it can activate protease activity, and promote the release of the growth factor (33). MMP-7 promotes the invasion and metastasis ability of various tumors, and the latest research has found that MMP-7 is used as a biomarker to evaluate the proliferation, differentiation and metastasis of gastric cancer (34,35). We found that the upregulation of Kank1 gene can reduce the expression of MMP-7 in gastric cancer. These results indicated that upregulation of Kank1 gene can inhibit the invasion and metastasis of human gastric cancer cells through inhibition of MMP-7 expression in gastric cancer.

In conclusion, we found in the present study that the reduction of Kank1 gene expression in gastric cancer, and the upregulation of Kank1 gene can inhibit the PI3K/AKT signaling pathway, and acts on the mitochondria pathway to regulate Bcl-2/Bax to further induce the inhibition of the proliferation of human gastric cancer cells leading to apoptosis. Kank1 gene in the future may become a potential therapeutic target and provides theoretical basis for clinical treatment of gastric cancer.

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