The metastasis suppressor gene KISS-1 regulates osteosarcoma apoptosis and autophagy processes

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Abstract. The expression of the metastasis suppressor gene KISS-1 in osteosarcoma cells during apoptosis and autophagy was evaluated. MG-63 osteosarcoma cells were transfected with either KISS-1 overexpression or KISS-1 knockdown expression vector *in vitro*, and compared with cell lines transfected with empty vector. After 12, 24, 48 and 72 h of cell culture, the cell proliferation was examined. The MTT method was used to detect apoptosis by flow cytometry, and the mRNA levels of apoptosis and autophagy markers caspase-3, Bcl-2, Bax, LC3 and Beclin1 were assessed by RT-PCR. Our results showed that cells in the control and low expression group kept proliferating during the cell culture period of 72 h, while the cells in the overexpression group progressively decreased in number. Also, the proliferation rate of the low expression group was significantly higher than that of the control group. The relative mRNA expression levels of caspase-3 and Bax mRNA in the control and low expression group showed no change (the expression was lowest in the low expression group). Moreover, the mRNA level of Bcl-2 increased in both cell groups. The mRNA expression levels of caspase-3 and Bax in the overexpression group were increased, and the level of Bcl-2 was reduced significantly. At the same time, the relative expression level of LC3 and Beclin1 mRNA in the control and low expression groups remained the same, and that of the overexpression group increased. The mRNA levels of LC3 and Beclin1 in the overexpression group were the highest, and that of the low expression group the lowest. The differences were statistically significant ($P<0.05$). Based on these results, we showed that KISS-1 inhibited the proliferation of osteosarcoma *in vitro*, probably by accelerating the processes of apoptosis and autophagy in the cells.

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Introduction

Osteosarcoma is one of the most common primary bone cancers among teenagers, it is prone to metastasis in its early stages, and its five-year survival rate is only 20-65% (1). Research shows that the occurrence and metastasis of osteosarcoma are closely related with the abnormal expression of the KISS-1/G protein-coupled receptor 54 (GPR54) gene system (2). The metastasis suppressor gene KISS-1 acts as a tumor suppressor gene in many tumors, such as in thyroid, pancreatic, bladder, ovarian and stomach cancer (3,4), while it acts as a growth promoter in breast and liver cancer (5). In osteosarcoma cells cultured *in vitro*, the expression of the KISS-1 gene is negatively correlated to the cell proliferation and invasion (6), moreover, after KISS-1 transfection and overexpression, the proliferation and invasion of human osteosarcoma MG-63 and U-2OS cells decline. However, clinical studies have found that the patients with high expression of KISS-1 and GPR54 suffer a higher tumor metastasis rate and mortality (7,8). This study was designed with those findings in mind, we used KISS-1 overexpression vector or siRNA to either highly express or knockdown expression of KISS-1 in the MG-63 osteosarcoma cell line *in vitro*, in order to analyze the mechanism of proliferation, apoptosis and autophagy of the tumor cells.

Materials and methods

Cell culture. MG-63 human osteosarcoma cells were brought from Shanghai Biotechnology Cell Experimental Center (Shanghai, China), and were cultured in the Dulbeco's modified Eagle's medium (DMEM) nutrient fluid, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and streptomycin of 100 μg/ml final concentration (Beyotime Institute of Biotechnology, Jiangsu, China). The cells were placed in an incubator at 37˚C, with 5% CO_2 and saturated humidity. Cells were left to grow to confluency of more than 80%, then adherent cells were digested with 0.25% trypsin and subcultured on a new flask. Every 2-3 days cells were subcultured, and maintained under the same conditions until ready for experiments. Cells for transfection experiments were selected when in the logarithmic phase.

Group division. We induced overexpression and knockdown expression of KISS-1 mRNA in cells of the osteosarcoma

Table I. Sequences of the primers.

	Forward primers	Reverse primers
Caspase-3	5'-GCCGTAGCAGCACGTAAATAA-3'	5'-GTGCAGGGTCCGAGGT-3'
Bcl-2	5'-ACTACTTCTCCCGCCGCTAC-3'	5'-GAAATCAAACAGAGGCCGCATG-3'
Bax	5'-CCAGCACTTCACGCATCAG-3'	5'-GCTGTCTAGCCAGAGTTTCAC-3'
LC3	5'-CTCGAGCTATGCCGTCCGAGAAGACCTTCA-3'	5'-GAATTCTTACACAGCCATTGCTGTCCCGAA-3'
Beclin1	5'-CTGAGGAGCAGTGGACAAAGG-3'	5'-GGAAGAGGGAAAGGACAGCAT-3'
Inter reference GAPDH	5'-TGCTTCACCACCTTCTTGA-3'	5'-TCACCATCTTCCAGGAGC-3'

cell line MG-63 *in vitro*, and used the same type of cells as control by transfecting them with empty vector (GenePharma, Shanghai, China). The cDNA of KISS-1 from the osteosarcoma cells was amplified by RT-PCR, and the pcDNA3.1-vector (GenePharma) was used to make constructs for eukaryotic expression using standard molecular cloning techniques. Tranfections of the MG-63 cells with KISS-1 expression vector were done using Lipofectamine (both from GenePharma), and screened by G418 (1 mg/ml). MG-63 cells with stable high expression of KISS-1 were subcultured. Additionally, a number of small interfering RNAs (siRNAs) were designed to knock down the KISS-1 expression in the MG-63 cells. The most effective siRNA segment giving the best silencing of KISS-1 expression was used in a eukaryotic expression vector pSUPER-KISS-1-siRNA, and transfected using Lipofectamine (both from GenePharma) into the cells (screened by G418 at 1 mg/ml). The MG-63 cells with stable low expression of KISS-1 were subcultured and used in subsequent experiments. The control cell line was made by transfecting the empty pcDNA3.1-vector into MG-63 cells. KISS-1 expression was verified by RT-PCR method in all the resulting cell lines.

Research methods. After 12, 24, 48 and 72 h of transfections, the cell proliferation was examined for each cuture flask with the method of MTT. Apoptosis was detected by flow cytometry, the mRNA levels of caspase-3, Bcl-2 and Bax (apoptosis markers) as well as the mRNA levels of LC3 and Beclin1 were detected by RT-PCR.

MTT method. Cells were collected from the culture flasks when confluence reached 85% using trypsin digestion; 2,000 x g of the cell culture medium containing resuspended cells were centrifuged for 15 min, the cell pellet was saved and the supernatant discarded. Phosphate-buffered saline (PBS) was used to resuspend the pellet to a final concentration of $1x10^6$ cells/ml; 100 μl of the resuspended cells were placed on each well of a 96-well plate. The cells were incubated at 37˚C with 5% CO_2 and saturated humidity for recovery and until attached. Then, 40 μl of MTT solution was added to each well. The cells were further cultured in the incubator for 4 h. In the following steps, the supernatants were removed, and 150 μl of DMSO were added to each well, shaking for 10 min. Finally, the absorbance (optical density, OD) at 490 nm wavelength was read in a microplate reader. The reference wavelength was 630 nm. The process was repeated three times to average the results.

Flow cytometry. The cells were collected and washed in PBS. The binding buffer in the apoptosis assays kit (ZSGB-BIO, Beijing, China) was used to make a cell suspension, with the cell density adjusted to $1x10^6$/ml; 100 μl of cell suspension were mixed with 5 μl of Annexin V-FITC, and allowed to react for 5 min at room temperature in the dark. Then, 10 μl of propidium iodide was added, the cells were left to react for 15 min at room temperature in the dark. Finally, 400 μl of 1X binding buffer were added, before FACSCalibur flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) within 1 h to detect the cell apoptosis.

RT-PCR method. The traditional TRIzol method (KeyGene Biotech, Nanjing, China) was used to extract RNA, and its concentration and purity were detected with a UV spectrophotometer. cDNA was synthesized using a Sensiscript RT kit. Primers were synthesized by Takara Bio, Inc. (Otsu, Japan), the sequences are shown in Table I.

The reaction system consisted of 2.5 μl 5X buffer + 1.5 μl $MgCl_2$ + 0.5 μl dNTP + 1 μl GAP-43 with sense and antisense primers + 0.3 μl Taq enzyme + 2 μl cDNA template and water to 25 μl. The reaction conditions were 95˚C for 5 min, 95˚C for 30 sec, 62˚C for 30 sec, 72˚C for 30 sec, and repeating the cycle 35 times, with a final at 72˚C for 10 min extension. The dissociation curve was obtained and the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of mRNA.

Statistical analysis. SPSS 20.0 software (IBM, Armonk, NY, USA) was adopted for statistical analyses. All quantitative data were expressed as mean ± standard deviation (mean ± SD). The one-way analysis of variance (ANOVA) method was used for comparison among groups. The LSD-t method was used for comparison between two groups, and the repeated measure ANOVA was used for comparisons within one group. The differences were considered statistically significant at $P<0.05$.

Results

Comparison of the cell proliferation rate. The cell proliferation rates were evaluated for 72 h. The rates kept increasing for cells in the control and low expression groups, and decreased in the overexpression group. The rates in the low expression group were apparently higher than those in the control group. The differences had statistical significance ($P<0.05$) (Table II).

Table II. Comparison of cell proliferation rate.

Groups	12 h	24 h	48 h	72 h	F-value	P-value
Control	0.64±0.15	0.82±0.23	1.13±0.34	1.35±0.36	6.532	0.002
Overexpression	0.52±0.12	0.46±0.13	0.38±0.09	0.33±0.06	6.421	0.006
Low expression	0.68±0.14	0.91±0.18	1.22±0.23	1.46±0.29	6.958	<0.001
F-value	3.625	4.548	6.123	8.624		
P-value	0.025	0.019	0.009	<0.001		

Table III. Comparison of cell apoptosis rate (%).

Groups	12 h	24 h	48 h	72 h	F-value	P-value
Control	15.6±4.5	15.9±4.7	16.3±3.8	16.7±4.2	1.236	0.285
Overexpression	24.6±6.6	27.8±6.9	35.4±8.2	41.6±8.5	5.628	0.018
Low expression	8.7±2.2	9.2±2.3	9.0±2.4	9.4±2.1	0.629	0.342
F-value	5.627	6.328	6.854	7.523		
P-value	0.013	0.004	<0.001	<0.001		

Table IV. Comparison of the relative expression levels of apoptotic mRNA molecules.

Groups	Control group	Overexpression group	Low expression group	F-value	P-value
Caspase-3					
12 h	0.2341±0.0639	0.3256±0.1124	0.1532±0.0527	3.625	0.032
24 h	0.2143±0.0527	0.3864±0.1325	0.1426±0.0634	4.123	0.028
48 h	0.2056±0.0684	0.4235±0.1527	0.1352±0.0727	4.432	0.023
72 h	0.2135±0.0729	0.4659±0.1632	0.1428±0.0834	4.865	0.017
Bcl-2					
12 h	0.5426±0.1532	0.5213±0.1827	0.6214±0.1532	3.527	0.033
24 h	0.5893±0.1426	0.4629±0.2134	0.6638±0.1627	4.326	0.025
48 h	0.6124±0.1827	0.4123±0.1965	0.7214±0.1824	4.932	0.015
72 h	0.6569±0.1932	0.3526±0.1538	0.7528±0.2131	5.327	0.010
Bax					
12 h	0.2135±0.0628	0.4257±0.1824	0.1023±0.0624	4.102	0.029
24 h	0.2262±0.0762	0.4826±0.2031	0.1124±0.0537	4.426	0.022
48 h	0.2351±0.0839	0.5328±0.2152	0.0968±0.0162	5.127	0.014
72 h	0.2158±0.0528	0.5925±0.2236	0.1325±0.0539	5.329	0.009

Comparison of cell apoptosis rates. During the first 72 h after transfection, the cell apoptosis rates of the control and low expression groups did not change significantly, but that in the overexpression group increased. The rate of the overexpression group was significantly higher than that of the control group, and the low expression group had the lowest rates. The differences amongst groups had statistical significance (P<0.05) (Table III).

Comparison of the relative mRNA expression levels of apoptotic molecules. With time, the relative expression levels of caspase-3 and Bax mRNAs in the control and low expression groups remained the same, while the level of Bcl-2 increased. By contrast, in the overexpression group, the relative expression levels of caspase-3 and Bax mRNAs increased, and the level of Bcl-2 decreased. The levels of caspase-3 and Bax in the overexpression group were higher than those in the control group, with the low expression group showing the lowest level. The level of Bcl-2 in the overexpression group was lower than that in the control group, and the low expression group had the highest level. The differences had statistical significance (P<0.05) (Table IV).

Comparison of the relative expression levels of mRNAs of autophagy markers. With time, the relative expression levels of LC3 and Beclin1 mRNAs in the control and low expression

Table V. Comparison of the relative expression levels of autophagy mRNA molecules.

Groups	Control group	Overexpression group	Low expression group	F-value	P-value
LC3					
12 h	0.1629±0.0862	0.3341±0.1321	0.0968±0.0132	4.236	0.033
24 h	0.1724±0.0764	0.3935±0.1247	0.1023±0.0235	4.827	0.028
48 h	0.1638±0.0912	0.4452±0.1635	0.0864±0.0321	5.324	0.020
72 h	0.1725±0.0834	0.5124±0.1824	0.0985±0.0245	5.629	0.014
Beclin1					
12 h	0.1123±0.0737	0.2659±0.1302	0.0762±0.0326	4.123	0.035
24 h	0.1052±0.0629	0.3124±0.1242	0.0635±0.0257	4.632	0.030
48 h	0.1324±0.0538	0.3762±0.1124	0.0824±0.0241	5.127	0.024
72 h	0.1262±0.0624	0.4213±0.1028	0.0823±0.0169	5.532	0.016

group remained virtually unchange, while the level in the overexpression group increased. The expression in the overexpression group was higher than that of the control group, and the low expression group had the lowest levels. The differences had statistical significance ($P<0.05$) (Table V).

Discussion

KISS-1/GRP54 gene is mainly expressed in placental tissues, but can be also expressed in the central nervous system, testis, ovary, pancreas and small intestine (9). Kisspeptin is the translation product of KISS-1, it acts as the endogenous ligand of GPR54. The interaction between kisspeptin and GPR54 can activate the receptor, causing a cascade of effects through signaling pathways within the cells, which includes activation of phospholipase, calcium influx and regulation of collagenase activity, activating PI to release arachidonic acid, activating the MAPKs network and the extracellular signal-regulated protein kinases 1 and 2 (ERK1/ERK2) (10). The same interaction, however, can also inhibit the chemotactic activity of CXCR4, which plays an important role in promoting tumor metastasis (11). In addition, KISS-1 in cells can promote the release of LH, and mediates the positive and negative feedback regulation of estrogen (12). Kisspeptin is related with the formation of the placenta and the invasion by trophoblast cells (13), a process similar to the invasion of a tumor.

Our results showed how the cell proliferation rates of the control and low expression group kept increasing with time, while that of the overexpression group was decreasing. Also, the rate of the low expression group was higher than that of the control group, and the overexpression group had the lowest rate. Based on this, it appears that KISS-1 can play a role as anti-oncogene. Along with the replication of transfected plasmid in the overexpression group, the levels of KISS-1 in that group were high, and the tumor proliferation activity was inhibited. By contrast, the low levels of expression of KISS-1 in the low expression group, lead to faster malignant proliferation. KISS-1 also displayed a role in apoptosis of the osteosarcoma cells. The cells with lower levels of expression (control and low expression groups) showed no difference in apoptosis rate with time, while cells overexpressing KISS-1 showed increasing apoptosis rates. The rate of apoptosis was highest in the KISS-1 overexpression group and lowest in the KISS-1 low expression group. KISS-1 in normal osteosarcoma cells had a relatively weak effect on promoting apoptosis. Caspase-3 is the 'key molecule' for apoptosis of the eukaryotic cells, drastically determining the level of apoptosis (14). Endogenetic-mitochondrion signaling apoptosis pathways play an important role in the apoptosis of tumor cells. The proteins, Bcl-2 and Bax have functions as anti-apoptosis and pro-apoptosis molecules, respectively. Their mutual antagonism influences the direction of apoptosis (15). In our study, the relative expression levels of caspase-3 and Bax mRNA in control group and low expression group remained relatively unchanged, and the level of Bcl-2 increased, while in the overexpression group, the relative expression levels of caspase-3 and Bax mRNA increased, and the level of Bcl-2 decreased. Accordingly, the cells in the control and low expression group had low apoptosis activity, while the overexpression group cells displayed a high rate of apoptosis. Autophagy is different from apoptosis, and is characterized by typical autophagosomes formed inside the cells. Beclin1 is considered necessary for autophagy in the early stages. A complex formed with class III PI3K stimulates the formation of autophagosomes, Beclin1 guides related proteins to target the autophagosomes (16). LC3 is closely related to Beclin1 and is used as an autophagy marker in cells (17). During the time of cell culture, the relative expression levels of LC3 and Beclin1 mRNAs in the control and low expression groups had no change, but the level increased in the overexpression group, indicating that in this group the autophagy process was probably accelerated.

Based in our results, we conclude that KISS-1 can inhibit the proliferation of osteosarcoma *in vitro* by accelerating the processes of apoptosis and autophagy. KISS-1 is expected to become a new drug development target in the search for an effective treatment for osteosarcoma.

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