ESTABLISHMENT OF BONE METASTASIS MODEL OF BREAST CANCER AND EFFECT OF KP-10 ON MIGRATION AND OSTEOCLAST DIFFERENTIATION OF BREAST CANCER CELL LINE BT474

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ABSTRACT

Objective: *To analyze the effect of Kp-10 on the migration and osteoclast differentiation of breast cancer cell line BT474.*

Methods*: Sixteen clean grade female rats were selected. After anesthesia, the solution prepared by breast cancer BT474 cells was injected between the second and third ribs of mice to establish the bone metastasis model of breast cancer. BT474 cells were stimulated with different concentrations of Kp-10 to observe the number of cell migration. Bone marrow mononuclear cells were isolated and cultured from the femur and tibia. Osteoclast differentiation was induced by breast cancer conditioned medium in vitro. The number of osteoclasts was detected by trap staining. RT-PCR was used to detect the effect of KP-10 on the expression of coordinated genes in bone metastasis of breast cancer.*

Results: *After treated with 0.01, 0.05 and 0.1nm concentrations of Kp-10, the migration number of BT474 cells increased gradually with the increase of Kp-10 concentration, which was significantly higher than that of the control group without Kp-10 (P<0.05). The migration number of BT474 cells reached the peak at the concentration of 0.05 nm. The number and area of osteoclasts were observed and counted by trap staining under a microscope. The results showed that the area of osteoclasts was significantly higher than that of the control group (0nm). With the increase of Kp-10 concentration, the number of osteoclasts increased significantly (P<0.05). When the concentration of Kp-10 was 0.05nm, the promoting effect of KP-10 on osteoclast differentiation was the most obvious. Compared with the control group (0nm), the expression of IL-11, CTGF, OPN, MMP1 and RANKL in the treatment of 0, 0.05, 0.1, 0.5 and 1nm concentrations of Kp-10 significantly increased the expression of IL-11, CTGF, OPN, MMP1, RANKL in the control group (0 nm) (P<0.05).*

Conclusion: *Kp-10 can significantly promote the migration of breast cancer cells BT474 and osteoclast differentiation induced by breast cancer cells, and the related mechanism may be achieved by promoting the expression of breast cancer synergistic genes.*

Keywords: *Breast cancer, bone metastasis, Kp-10; BT474 cells, migration, osteoclast differentiation.*

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Introduction

Breast cancer is one of the most common malignant tumors in women. It is a malignant tumor occurring in the glandular epithelial tissue of the breast, and its incidence ranks first among female malignant tumors, and has been increasing year by year in recent years[(1)]. The etiology and pathogenesis of breast cancer are still not completely clear. Studies have found that women with breast high-risk factors are prone to breast cancer, including family history, early menarche, late menopause, late childbearing without breastfeeding, and other factors. Mammary gland is not an important organ to maintain human life activities, but due to the loss of normal cell characteristics of breast cancer cells, once they fall off, they will spread throughout the body with blood or lymph, forming metastasis and endangering life[(2-3)]. The 5-year survival rate of early breast cancer can reach 98%, and the survival rate of patients is significantly reduced to 10% if cell metastasis occurs. The formation and metastasis of

breast cancer are the result of multi-gene and multi-factor effects as well as other malignant tumors. Bone, lung, brain and liver are common metastatic sites. Bone pain, bone injury and other related events caused by bone metastasis have seriously affected the quality of life of patients[4]. KISS1 gene was discovered as a tumor suppressor gene in malignant melanoma cells, containing a peptide chain of 145 amino acids, among which Kisspeptins-10 (KP-10) was the shortest and most active polypeptide[5].

In this study, by establishing a bone metastasis model of breast cancer, Kp-10 was applied to breast cancer cell BT474 to analyze the effect on cell migration and osteoclast differentiation.

Methods

Experimental reagents and instruments

Human breast cancer BT474 cells were purchased from Shanghai Guandao Bioengineering Co., LTD; MEM medium was purchased from Wuhan Yipu Biotechnology Co., LTD; Fetal bovine serum was purchased from Wuhan Punosai Life Technology Co., LTD; Kp-10 was purchased from Albicin (Shanghai) Biotechnology Co., LTD; Recombinant mouse RANKL and M-CSF cytokines were purchased from Shanghai Kemin Biotechnology Co., LTD; TRAP staining kit was purchased from Beijing Baolaibo Technology Co., LTD; PCR kit was purchased from Shanghai Caiyou Industrial Co., LTD; BCA protein quantitative kit was purchased from Shenyang Wanshi Biotechnology Co., LTD; Sodium dodecyl sulfate was purchased from Shanghai Baoman Biotechnology Co., LTD; Trizol lysate was purchased from Guangzhou Dongsheng Biotechnology Co., LTD.

Fluorescence real-time quantitative PCR instrument was purchased from Shanghai Tusen Vision Technology Co., LTD; Normal temperature centrifuge purchased from Shanghai Shiwei Experimental Instrument Technology Co., LTD; Fluorescence microscope was purchased from Guangzhou Website Scientific Instrument Co., LTD; Pipette and pipette gun were purchased from Shanghai Yanyi Biological Technology Co., LTD; Centrifugal tube purchased from Bunsen (Tianjin) Health Technology Co., LTD; Fluorescence scanning film imager was purchased from Shanghai Yuyan Scientific Instrument Co., LTD; Electrophoresis apparatus and membrane transfer apparatus were purchased from Beijing Yiaobai Technology and Trade Co., LTD; The water bath was purchased from Jiangsu Jirui Biotechnology Co., LTD; The carbon dioxide incubator was purchased from Esco (Shanghai) Trading Co., LTD.

BT474 cell culture

BT474 cells of breast cancer were cultured with DMEM medium mixture, cells were counted with cell counting plate, cell suspension (containing 2×10^7 cells) was extracted and placed into EP tube, stood at room temperature for 30min, centrifuged at 12000rpm for 15min, supernatant was discarded, and washed with PBS buffer for 3 times.

Establishment of mouse breast cancer model and culture of bone marrow monocytes: Female BALB/c mice for Guangzhou for bo biological technology co., LTD., 8 weeks, the design of breast cancer cells left ventricle injection model, after anesthesia to solution of the preparation of breast cancer BT474 cells injected into mice high-centralized between ribs, after 10 min in live imaging observation, cell cycle and the whole body blood system, shows the left ventricle injection is successful, Bone metastasis was examined by imaging and X-ray in vivo, as shown in Figure 1. The femur and tibia of mice were removed and placed in sterile Petri dishes. The femur and tibia were cut open, respectively, and the bone marrow cavity was washed with serum-free α-MEM medium containing antibiotics.

The bone marrow cells were taken and placed in a sterile centrifuge tube. After the cell suspension was transferred to petri dish, 5ng/ml M-CSF was added overnight. After 1~2 days, 80~90% of the cells adhered to the wall, relatively pure bone marrow macrophages could be obtained.

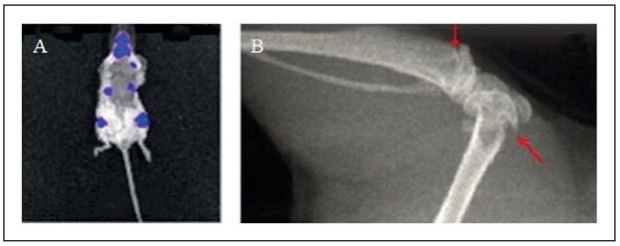

Figure 1: In vivo imaging (A) and X-ray examination of A mouse animal showing bone metastases.

Detection method

• Transwell cell migration experiment: BT474 cells were starved in serum-free medium for 12h, and digested by trypsin. After centrifugation, the cells were washed with PBS buffer, and BT474 cells were suspended in DMEM medium again with a concentration of 0.8 million /mL after dilution. Cells were placed in the migration chamber, 100μ L of cell

suspension was added into the Transwell chamber, 600 μL DMEM medium was added into the lower chamber, and KP-10 with different concentrations (0, 0.01, 0.05, 0.1nM) were added into the lower chamber and incubated at 37°C for 6-8h. The cells were fixed with 4% paraformaldehyde for 10min. The unmigrated cells were inserted with cotton swabs. After PBS was clear, the cells were stained with 0.1% crystal violet for 5min.Imageproplus6.0 software counted the migrated cells.

• TRAP staining was used to observe osteoclast differentiation:BT474 cells were inoculated into a 6cm Petri plate, and different concentrations of KP-10 (0, 0.01, 0.05, 0.1nM) were added, respectively. Mouse monocytes were inoculated into a 96-well plate, and 10ng/mL M-CSF and 50ng/ mL RANKL were added to induce osteoclast differentiation. After the control group cells were differentiated into osteoclasts, 4% paraformaldehyde was added and fixed for 20min. After PBS was clear, triton-X100 was used to permeate for 5min and incubated at room temperature for 20min. TRAR staining solution was added to incubate for 1h.

• Real-time quantitative PCR analysis of bone metastasis coordination gene expression in breast cancer:BT474 cells were inoculated into 6-well plates, different concentrations of KP-10 (0, 0.05, 0.1, 0.5, 1nM) were added, Trizol 1mL/well was added, total RNA was extracted, the extracted RNA was measured, the required sample quantity was calculated, and cRNA was obtained by reverse transcription using the reverse transcription kit, and the reaction system was configured. The gene fragment was amplified by RT qPCR kit.

Reaction conditions: pre-denaturation at 95°C for 4min, 95°C for 30s, 60°C for 30s, 372 °C for 30s, 35 cycles of reaction, gene expression by $2^{-\triangle\triangle CT}$ formula.

Gene: connective tissue growth factor (CTGF), Connective tissue growth factor, Osteopontin, interleukin-11OPN, Matrix metalloproteinases-1 (MMP-1), Nuclear factor Kappa B receptor activator ligand (RANKL).

Statistical methods

SPSS21.0 software package was used for analysis of data in this study, and measurement data were represented by ($\bar{x}\pm s$).

Comparison of p2-p2 data was tested by T, and comparison of data between multiple groups was performed by ANOVA. P<0.05 was considered to be statistically significant.

Results

Effect of KP-10 on migration ability of BT474 in breast cancer cells

BT474 cells were treated with 0.01, 0.05 and 0.1nM KP-10, and then fixed and stained for 6-8 h. The cells penetrating the multi-compartment membrane were shown in Figure 2.

The number of cell migration increased gradually with the increase of KP-10 concentration, which was significantly higher than that of the control group without KP-10 (0nM), and the difference was statistically significant (P<0.05), and the number of cell migration reached the peak at 0.05nM. As shown in Figure 3.

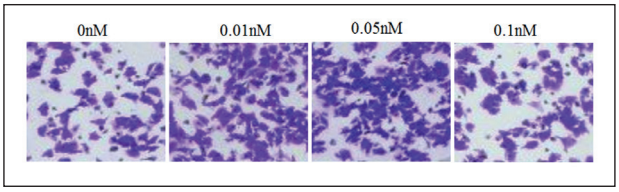

Figure 2: Observation of different KP-10 concentration groups through the compartment membrane cells.

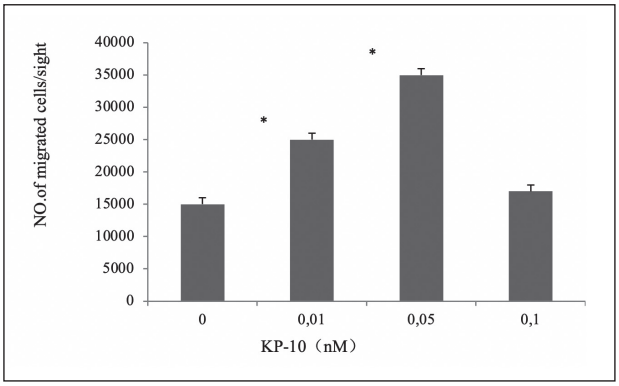

Figure 3: Comparison of migration numbers of BT474 cells in breast cancer groups with different KP-10 concentrations.

*Note: Compared with control group (0nM) *P<0.05.*

Effect of KP-10 on osteoclast differentiation of mouse bone marrow monocytes induced by conditioned medium for breast cancer

After the addition of 0.01, 0.05 and 0.1nM KP-10 conditioned medium, TRAP staining was performed, and the number and area of osteoclasts formed were observed and counted under the microscope. The results showed that the area occupied by osteoclasts was significantly higher than that of the control group (0nM), as shown in Figure 4. With the increase of KP-10 concentration, the number of osteoclasts increased significantly. Compared with the control group (0nM), the difference was statistically significant (P<0.05),

kP-10 promoted osteoclast differentiation most obviously when the concentration was 0.05nM. As shown in Figure 5.

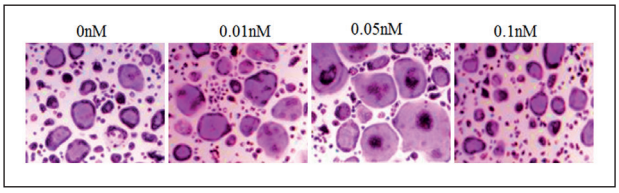

Figure 4: Effect of KP-10 on the area occupied by mouse bone marrow monocyte osteoclasts induced by conditioned medium for breast cancer.

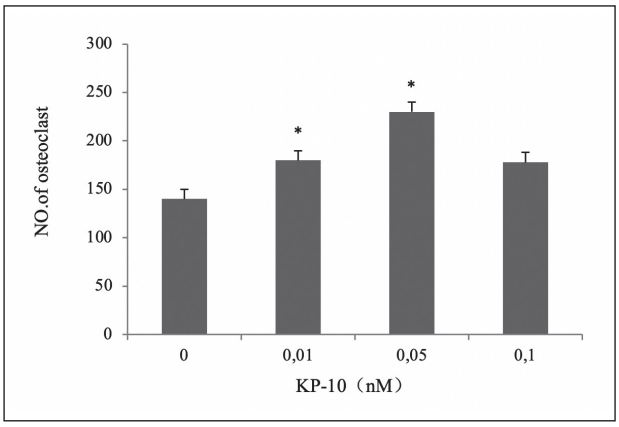

Figure 5: Effect of KP-10 on the number of bone marrow monocyte osteoclasts induced by conditioned medium for breast cancer in mice.
*Note: Compared with control group (0nM) *P<0.05.*

Effect of KP-10 on bone metastasis coordination gene expression in breast cancer

After KP-10 treatment at the concentration of 0, 0.05, 0.1, 0.5 and 1nM, the expression of il-11, CTGF, OPN, MMP1 and RANKL in breast cancer bone metastasis coordination genes was significantly higher than that in the control group (P<0.05), the expression of IL-11 and CTGF reached the peak when the concentration of KP-10 was 0.05nM, but the effect of increasing the concentration on OPN, MMP1 and RANKL was not obvious. See Table 1.

Kp-10	IL-11 mRNA	CTGF mRNA	OPN mRNA	MMP1 mRNA	RANKL mRNA
0 nM	1.02±0.03	1.02±0.13	1.01±0.02	1.00±0.02	1.00±0.01
0.05 nM	5.21±0.78*	1.78±0.16*	3.58±0.47*	6.01±0.74*	11.02±0.32*
0.1 nM	1.11±0.08	1.32±0.11	0.98±0.06	1.13±0.05	2.50±0.75
0.5 nM	1.13±0.09	1.50±0.08	0.62±0.04	0.99±0.13	1.52±0.41
1 nM	1.12±0.05	1.01±0.07	0.33±0.02	0.72±0.08	0.72±0.13

Table 1: Effect of KP-10 on bone metastasis coordination gene expression in breast cancer.
*Note: Compared with control group (0nM) *P<0.05.*

Discussion

Breast cancer is the most common female malignant tumor with malignant biological behavior characteristics, it has many local recurrence and distant metastasis, and bone is the most easy to shift. Cancer cells can through local infiltrating into the circulatory system, blood vessels and tissue around the tumor cell falls off release in the blood circulation, then through the endothelial cells from blood vessels. Osteolytic bone injury is caused by deposition and proliferation in bone, and the occurrence of this process depends on the interaction between breast cancer cells and local bone microenvironment, resulting in the destruction of bone structure and function[6-7].

Bone metastasis of breast cancer is mainly osteolytic type, which often causes a series of bone related events such as intractable bone pain, pathological fracture, and nerve compression symptoms, bringing great pain to patients and improving the mortality of patients. Breast cancer bone metastases is an extremely complex multi-step process, and the bone metastases of bone cells and breast cancer are associated, in this process, the balance between osteoblasts and osteoclasts are destroyed, and activation of osteoclasts, continuously to dissolve osseous lesion as the main performance, its bone dissolve and bone structure damage is caused by increased osteoclast activity[8-9]. Bone contains a variety of growth factors in the process of bone resorption and remodeling are activated and release, if in breast cancer cells to bone, they can take advantage of the unique bone microenvironment and osteoblast and osteoclast exchange of biological information, lead to dissolve bony or osseous changes, appeared fractured bone destruction and pain, and eventually death[10-11].

KISS1 gene, as a member of the tumor suppressor gene family, has attracted clinical attention. It is expressed at different levels in brain, placenta, pancreas, liver, and other tissues, and is lost in a variety of tumor tissues with metastasis[12-13]. Studies have found that KISS1, as a tumor suppressor gene, exists in a variety of cells, and the expression product KP of KISS1 gene is reduced in a variety of malignant tumors. KISS1 gene plays an important role in initiating adolescent development, regulating energy balance and regulating animal biological rhythms[14-15]. In this study, by analyzing the effect of KP-10 interference on the migration of BT474 cells in breast cancer, it was found that the

number of cell migration increased gradually with the increase of kp-10 concentration after KP-10 treatment, which was significantly higher than that of the control group without KP-10 (0nM $P<0.05$), the number of cell migration reached the peak at the concentration of 0.05nM, suggesting that KP-10 may play a promoting role in the development of breast cancer. Osteoclast plays an important role in bone metabolism. In this study, TRAP staining showed that the area and count of osteoclast in groups with different concentrations of KP-10 were significantly higher than those in the control group. When the concentration was 0.05nM, KP-10 had the most obvious promoting effect on osteoclast differentiation. These results suggest that KP-10 can promote osteoclast differentiation and accelerate the process of bone destruction caused by breast cancer. After KP-10 treatment at the concentration of 0, 0.05, 0.1, 0.5 and 1nM, the expression of IL-11, CTGF, OPN, MMP1 and RANKL in breast cancer bone metastasis coordination genes was significantly higher than that in the control group ($P<0.05$), suggesting that KP-10 may improve the metastasis activity of breast cancer cells by promoting the expression of breast cancer cogene.

In conclusion, KP-10 can significantly promote the migration of BREAST cancer cell BT474 and osteoclast differentiation induced by breast cancer cells, and the related mechanism may be realized by promoting the expression of breast cancer cogenes.

References

1) Hu K F, Kong X Y, Zhong M C, et al. Brucine inhibits bone metastasis of breast cancer cells by suppressing Jagged1/Notch1 signaling pathways[J]. Chinese Journal of Integrative Medicine, 2017, 23(2): 110-116.
2) Jiang P, Gao W, Ma T, et al. CD137 promotes bone metastasis of breast cancer by enhancing the migration and osteoclast differentiation of monocytes/macrophages[J]. Theranostics, 2019, 9(10): 2950-2966.
3) Ke X , Lin W , Li X , et al. Synergistic dual-modified liposome improves targeting and therapeutic efficacy of bone metastasis from breast cancer[J]. Drug Delivery, 2017, 24(1): 1680-1689.
4) Tstutomu Kawaguchi, Li Yan, Qianya Qi. Abstract 4436: Novel microRNA signature score to predict bone metastasis and prognosis of breast cancer[J]. Cancer Research, 2017, 77(13 Supplement): 4436-4436.
5) Allawi F S, Daghestani M H, Daghestani M H, et al. Rs4889 polymorphism in KISS1 gene, its effect on polycystic ovary syndrome development and anthropometric and hormonal parameters in Saudi women[J]. Journal of Biomedical ence, 2018, 25(1): 50-56.
6) Hiraga T, Ninomiya T. Establishment and characterization of a C57BL/6 mouse model of bone metastasis of breast cancer[J]. Journal of Bone and Mineral Metabolism, 2019, 37(2): 235-242.
7) Bellanger, Aurélie, Donini C F , Vendrell J A , et al. The critical role of the ZNF217 oncogene in promoting breast cancer metastasis to the bone[J]. Journal of Pathology, 2017, 242(1): 73-89.
8) Hara T, Iwadate M, Tachibana K, et al. Metastasis of breast cancer cells to the bone, lung, and lymph nodes promotes resistance to ionizing radiation[J]. Strahlentherapie Und Onkologie, 2017, 193(10): 848-852.
9) Matthew M C, Shengmei F, Wei C, et al. Osteoclast proton pump regulator Atp6v1c1 enhances breast cancer growth by activating the mTORC1 pathway and bone metastasis by increasing V-ATPase activity[J]. Oncotarget, 2017, 8(29): 47675-47690.
10) Johnson R W, Suva L J . Hallmarks of Bone Metastasis[J]. Calcified Tissue International, 2018, 102(2): 141-151.
11) Guo S, Zhu W, Yin Z, et al. Proanthocyanidins attenuate breast cancer-induced bone metastasis by inhibiting If-3/c-jun activation[J]. 2019, 30(10): 998-1005.
12) Shiori M, Marimo S, Naoko I, et al. Neonatal Estrogen Causes Irreversible Male Infertility via Specific Suppressive Action on Hypothalamic Kiss1 Neurons[J]. Endocrinology, 2019, 12(5):1223-1233.
13) Hirofumi O, Hayato A, Hajime K, et al. Kiss1 hexadecapeptide directly regulates gonadotropin-releasing hormone 1 in the scombroid fish, chub mackerel.[J]. Biology of Reproduction, 2017, 10(2): 376-388.
14) Yamasaki M, Kuwahara A, Iwasa T, et al. Development-related changes in the expression of the ovarian Kiss1 and Kiss1r genes and their sensitivity to HCG in prepubertal female rats[J]. Journal of Reproduction and Development, 2017, 63(4): 409-414.
15) Pazarci P, Kaplan H, Alptekin D, et al. The effects of daylight exposure on melatonin levels, Kiss1 expression, and melanoma formation in mice[J]. Croatian Medical Journal, 2020, 61(1): 55-61.

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