

Original Article

Inhibition of KiSS-1 on metastasis of nasopharyngeal carcinoma implant tumor in nude mice

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Abstract: Background: Nasopharyngeal carcinoma (NPC) is a kind of head-neck malignant neoplasm originated from the nasopharyngeal epithelium and is mainly prevalent in Southern China and Southeast Asia countries. KiSS-1 is an inhibitor of tumor metastasis in a range of cancers. Methods: We establish a cell substrain of SUNE-1-5-8F (NPC cell line from humans) that trnsfected with lentiviral vectors carried with KiSS-1 gene and were selected by puromycin. A transplantation tumor animal model in BALB/c-nu mice was successfully established with a substrain that stably overexpressed KiSS-1. Results: Our result showed that the size of transplantation tumor in the nude mice with KiSS-1 overexpression in transplantation tumor was not difference from the size of transplantation tumor in the controlled transplantation tumor mice. We detected metastatic tumor in lung but not in liver. Moreover, we also found that in the nude mice with KiSS-1 overexpression in transplantation tumor showed extremely fewer meta-static tumor in lung compared with the controlled transplantation tumor mice model. In conclusion, KiSS-1 may be beneficial for the inhibition of metastasis of human NPC. Conclusion: This study may throw light on the treatment of NPC and may help improve the prognosis of patients with NPC.

Keywords: KiSS-1, metastasis, nasopharyngeal carcinoma, implantation tumor, nude mice

Introduction

Incidence of nasopharyngeal carcinoma (NPC) varies between countries and regions [1, 2]. For instance, among the Caucasian population, the incidence is below 1 case per 100,000 individuals [3]. However, in some Asia regions, such as in Hong Kong and the Guangdong Province in Southern China, the incidence of NPC is 25 cases per 100,000 individuals [3] and ranks on the most common tumor among the head and neck cancers in Asia. For the atypical early symptoms of NPC, the major patients are initially diagnosed with advanced disease and resulted in a high rate of mortality [3]. It was reported that combination of chemotherapy and radiotherapy improves the curative effect in regionally-advance patients [4, 5]. However, the incidence of local recurrence and distant metastasis still remains a high level [6]. Therefore, more detailed mechanism of metastasis

and incidence and more effective systemic therapy should be explored.

KISS-1 gene was identified to inhibit metastasis of human malignant melanoma by Lee JH et al in 1996 [7] and located on chromosome 1q32. As indicated by Northern Blot, KiSS-1 expressed in multiple tissues such as in brain, lung, liver, heart and skeletal muscle and also expressed in a low degree in pancreas and kidney and in a high degree in placenta [8]. Kisseptin, encoded by *KISS-1*, consists of a number of peptides such as kisspeptin-10, kisspeptin-13, kisspeptin-14, kisspeptin-54 [9], are endogenous ligands to a G protein-coupled receptor, generally including hOT7T175 or AXOR12 or GPR54 [9]. In these years, some researchers tried to explore the mechanism of KiSS-1 in the inhibition of metastasis, including KiSS-1 obstacles the migration of breast cancer cells via restraining TNF α -induced NF- κ B pathway [10]. Over-

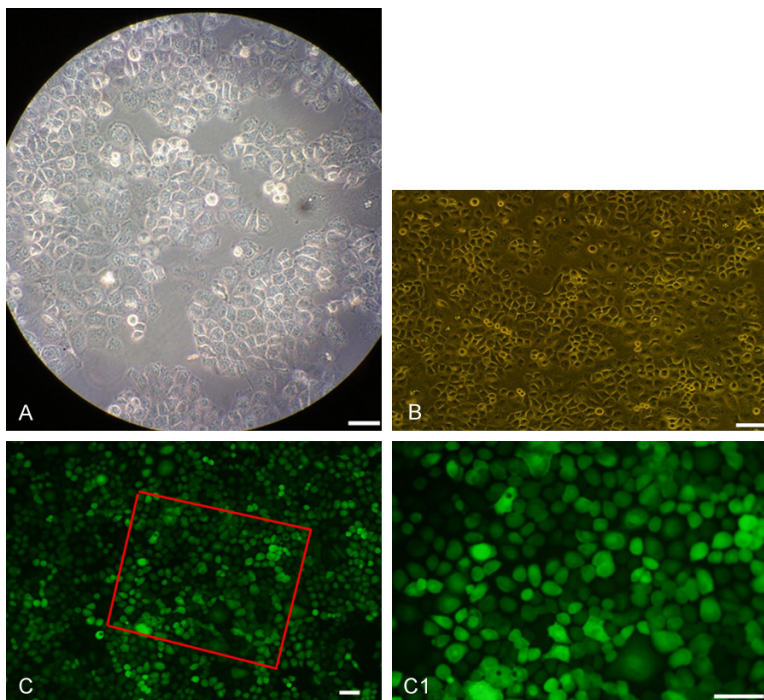


Figure 1. Observation of SUNE-1-5-8F cell line. (A) Observation of SUNE-1-5-8F cell under an ordinary optical microscopy. (B) Observation of transfected SUNE-1-5-8F cell line under an ordinary optical microscopy. (C) Observation of transfected SUNE-1-5-8F cell line under a fluorescence microscopy. (C1) Magnified from (C). Scale bar = 50 μm .

expression of KiSS-1 was also reported to suppress migration and invasion of the breast cancer cells [11] and decrease MMP-9 expression through inhibiting the activation of NF- κ B in fibrosarcoma cells [12]. Recently, a clinical retrospective study reported that low expression of kisspeptin in NPC was tightly related with clinical stage, patients with low level expression of kisspeptin indicated a poorer distant metastasis-free survival compared with patients with high expression of kisspeptin, thus, the authors suggested kisspeptin as a potential prognostic indicator for metastasis in NPC [13]. However, there is no evidence to show the role of KiSS-1 in the metastasis of NPC.

In this study, we transfected SUNE-1-5-8F cell line, which is a highly metastatic cell line of NPC and express KiSS-1 in a low level, to prepared two substrain of SUNE-1-5-8F, including the high expression of KiSS-1 substrain and the control substrain. We establish an experimental prototype of metastatic tumor in nude mice with the SUNE-1-5-8F cells and investigate the role of KiSS-1 in the inhibition of metastasis of NPC. Our study is valuable in exploring a new approach to NPC therapy.

Materials and methods

Reagents

RPMI 1640 culture medium, penicillin/streptomycin (P/S) and trypsin were commercially got from HyClone (Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Gibco BRL (Grand Island, NY, USA). Anti-KiSS-1 antibody was from Becton Dickinson (Franklin Lakes, NJ, USA). Primers of KiSS-1 and SYBR-GRE MIX were from Sangon Biotech (Shanghai, China) commercially. Lentivirus was constructed by GenePharma (Shanghai, China).

Cell culture

The human SUNE-1-5-8F cell line was obtained from SinobestBio (Shanghai, China). Cells were incubated in a culture flask or 12-well plate in a moist incubator of 5% CO_2 at 37°C. SUNE-1-5-8F cell was maintained in RPMI 1640 culture medium containing 10% FBS and 1% P/S. Subcultures were initiated when the cell density reached 80% with 0.25% trypsin and then washed twice with PBS.

Animals

BALB/c-nu mice were purchased from Sikerui Biotech (Nanjing, Jiangsu, China). A total of 40 female healthy nude mice (BALB/c-nu) aged of 4-6 weeks were used in this study and maintained under a regular 12 hour light/dark photoperiod (lights on from 7:00 to 19:00) with food and water available ad lib. They were raised in Specific Pathogen Free environment (SPF) and housed in an exclusive stainless steel cage in a room maintained at $25\pm 1^\circ\text{C}$ and $70\pm 4\%$ relative humidity. Feeding and treatment of animals were performed in accordance with guidelines provided in the *Guide for the Care and Use of Laboratory Animals*.

Cell transfection and selection of cell strain

SUNE-1-5-8F cells were seeded in the 6-well plates at a density of 10^6 /well, transfection

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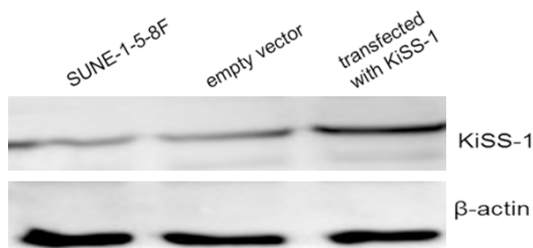


Figure 2. Determination of KiSS-1 gene expression by western blot. Three groups were detected, one group is SUNE-1-5-8F cell line, one group transfected with empty lentiviral vectors and one group is a sub-strain that transfected with KiSS-1.

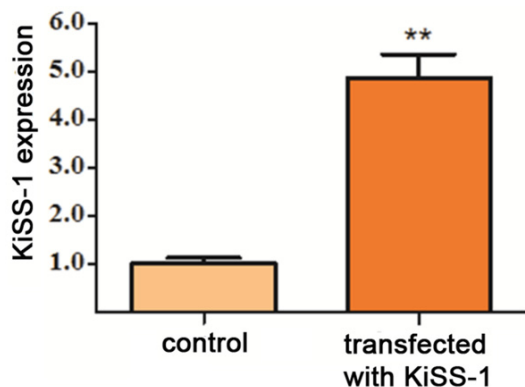


Figure 3. The mRNA Expression level of KiSS-1 gene. The mRNA expression level of KiSS-1 was detected in the substrain of SUNE-1-5-8F cell line that transfected with KiSS-1 and in the SUNE-1-5-8F cell line that not transfected. ** $P < 0.01$, Student's t-test was used.

was performed after 18-24 hours and at a density about 2×10^5 per well prior to treated with polybrene. Medium containing lentivirus was changed after incubated for 24 hours followed by maintaining culture for 3 days, after that, puromycin was used to select positive transfection cells.

Western blot

Following three washes in ice-cold PBS, RIPA lysis buffer (Beyotime Biotech, Haimen, Jiangsu, China) containing 1% phenylmethylsulphonyl fluoride (PMSF; Beyotime, Haimen, Jiangsu, China) and 3% phosphatase inhibitor cocktail tablets were added. Protein extracts (8 μ g or 10 μ g) were loaded to SDS-PAGE on 10% gels and then transferred electronically to polyvinylidene difluoride (PVDF) membranes (Beyotime Biotech, Haimen, Jiangsu, China) by elec-

tro-blotting. The membranes were blocked with 5% non-fat milk for 1 hour before incubation with primary antibody of KiSS-1 overnight at 4°C.

RT-PCR

Reverse transcription was performed using 2 μ g RNA per reaction system with a First Strand cDNA Synthesis Kit commercially from Thermo Scientific (Heidelberg, Germany). The real time PCR was performed with the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR Green master mix. The primer for KiSS-1 and β -actin were as follows: KiSS-1 forward: 5'-CCTCTGGACATTCACCCAGC-3', KiSS-1 reverse: 3'-GCTGCCAAGAAACCAGTGAG-5'; β -actin forward: 5'-CCTGGAGAAGAGCATATGAGC-3', β -actin reverse, 5'-ACAGGATCCATACCCAGG-3'. The amplification conditions were set as follows: 30 seconds at 95°C, and then 40 cycles of 5 seconds at 95°C, 30 seconds at 55°C, and 34 seconds at 72°C, in the next step, 40 seconds at 95°C. The reaction specificities were confirmed by analysis of melting curve. The relative expression level was determined by the differences in the CT values between the target mRNA and an internal reference (β -actin). The relative change of mRNA levels between the experimental group and control group was calculated using the $2^{-\Delta\Delta CT}$ method.

HE staining

Samples were removed from mice and fixed in 10% formalin solution. The next procedure was performed in accordance with guideline previously reported [14].

Immunohistochemistry

Mice were fixed with 4% paraformaldehyde solution (PH = 7.2). Tumors were dissected and postfixed in 4% paraformaldehyde overnight at 4°C. A 5 μ m section was deparaffinized in xylene and rehydrated through descending graded concentrations of ethanol. After antigen retrieval, nonspecific binding site was blocked with donkey serum albumin for 1 hour at room temperature. In the next, primary antibody was diluted with serum and incubated for 24-48 hours in 4°C. After washed with 0.01 MKPBS, secondary antibody diluted with 0.01 MKPBS was added and incubated for 2 hours in room

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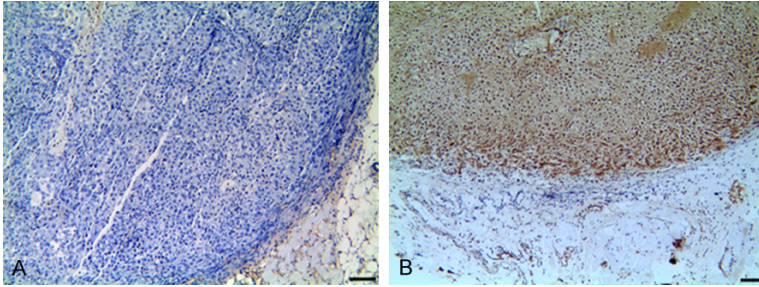


Figure 4. KiSS-1 expression in the transplantation tumor in the axilla detected by immunohistochemistry. A. Expression level of KiSS-1 in control group. B. Expression level of KiSS-1 in transfected group. Scale bar = 50 μm .

temperature followed by washed with 0.01 MKPBS for 3 times, 5 minutes each, next dyed with TMB Substrate.

Statistical analysis

All statistical analysis was performed using IBM SPSS statistics 20 software (SPSS Inc., Chicago, IL, USA). Data was calculated as a percentage obtained from at least 3 repeated experiments and showed as mean \pm standard deviation. Statistical differences between groups were analyzed by using approach of Student's t-test or chi-square test. $P < 0.05$ was defined as statistically significant.

Results

SUNE-1-5-8F cell line grew rapidly and passaged every two days (**Figure 1A**) as well as the transfected and puromycin-selected (2 $\mu\text{g}/\text{ml}$) SUNE-1-5-8F cell substrain (**Figure 1B**). Transfected SUNE-1-5-8F cell substrain was with green fluorescence under an excitation laser at a wavelength of 488 nm under a fluorescence microscopy (**Figure 1C, 1C1**) for the sake of reporter gene, which also suggested a stable substrain of SUNE-1-5-8F transfected with KiSS-1 carried by lentivirus.

After stable transfected SUNE-1-5-8F NPC lines were generated after puromycin selection, we detected the expression of KiSS-1 gene by western blot. According to our data, expression of KiSS-1 in SUNE-1-5-8F cell line that transfected with KiSS-1 gene is improved prominently compared with cell line that not transfected or transfected with empty vectors (**Figure 2**).

We also detected the expression level of KiSS-1 mRNA in each group by Real-time PCR. Our

result showed that KiSS-1 mRNA expression is prompted significantly in the substrain that transfected with KiSS-1 viral vectors (**Figure 3**, $P < 0.01$ Student's t-test was used).

In this study, we establish the nude-mouse transplanted tumor model with injection of 0.2 ml SUNE-1-5-8F cell substrain (at a concentration of 1×10^6) that transfected with

KiSS-1 or without in the axilla. We closely observed the development of the transplanted tumor until the mouse behaved low activity and displayed an emaciated physical condition. After the mouse was sacrificed, the transplantation tumor and liver as well as lung were removed. We detected the expression of KiSS-1 in the transplantation tumor in the axilla by immunohistochemistry and found that KiSS-1 gene expression is in a much higher level compared with the control group (**Figure 4**). Our study showed that the weight of the transplantation tumor not showed a significant difference between transfected with KiSS-1 group and the control group (**Figure 5**, $P > 0.05$ Student's t-test was used).

In our study, the removed lungs and livers were fixed in Bouin buffer and were observed after two days. The metastatic foci in the lung showed a white appearance. Meanwhile, we not observed any metastatic foci exactly in the removed livers. We observed and analyzed the morphology of the metastatic foci in the removed lungs, our result showed that many metastatic cell in the lung showed a clear nucleolus, with a larger cell nucleus and showed a number of irregular mitosis and arranged irregularly (**Figure 6**). In addition, we found that the number of metastatic foci in the lungs in the transfected group showed a decreased level compared with the control group (**Table 1**, $P < 0.05$ Chi-square test was used).

Discussion

NPC is prone to invades adjacent regions and metastasizes to regional lymph nodes and distant organs, most commonly to lung, is the main cause of treatment failure, and the inci-

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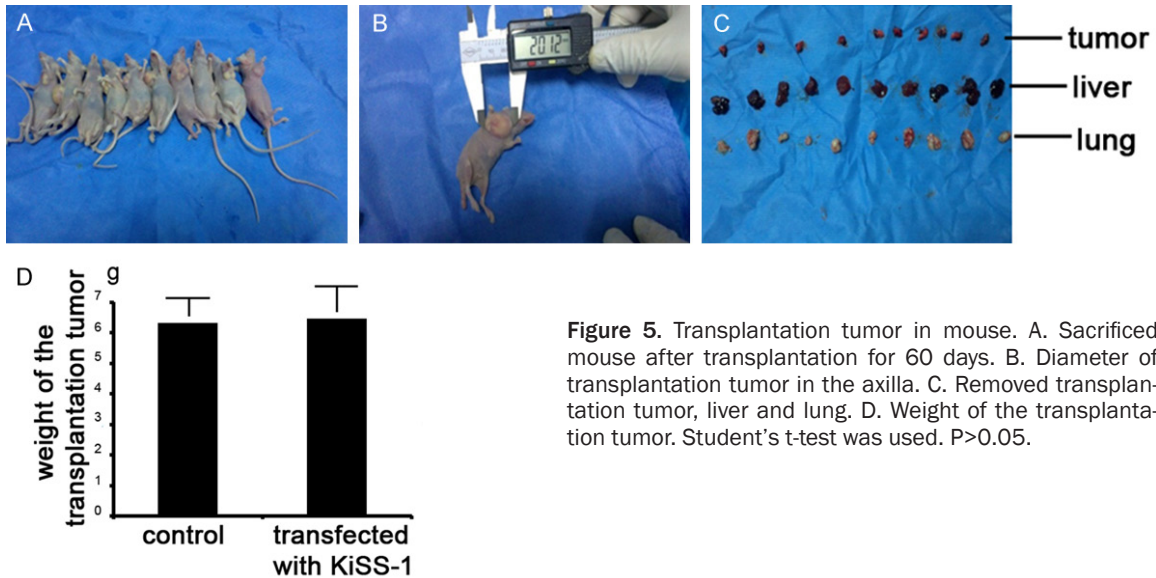


Figure 5. Transplantation tumor in mouse. A. Sacrificed mouse after transplantation for 60 days. B. Diameter of transplantation tumor in the axilla. C. Removed transplantation tumor, liver and lung. D. Weight of the transplantation tumor. Student's t-test was used. $P > 0.05$.

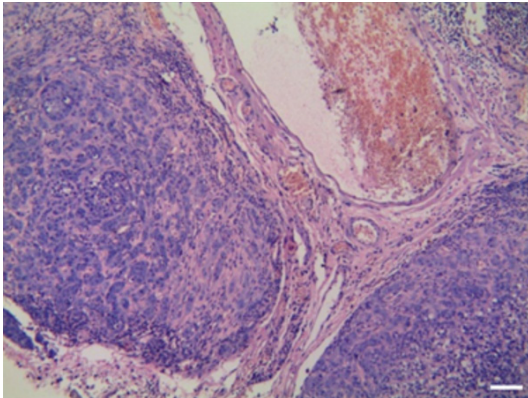


Figure 6. HE staining of the metastatic foci in the removed lungs. A number of metastatic cell in the lung and showed a clear nucleolus, with a larger cell nucleus and showed a number of irregular mitosis and arranged irregularly. Scale bar = 50 μm .

Table 1. Metastasis in lung

Group	Metastasis	No metastasis	Rate of metastasis
A	1	19	5%
B	8	12	40%

$P < 0.05$. Chi-square test was used.

dence of distant metastasis is more common in patients with advanced disease [15]. Radiotherapy is the primary treatment method for NPC. The outcome for patients with early-stage disease of NPC is usually satisfactory; however, the response of locoregionally advanced NPC to radiotherapy is unfavorable. In these

years, many researchers focus on the study of tumor metastasis suppressor gene.

In this study, we established a transplantation tumor of NPC in nude mice successfully with a substrain that overexpressed KiSS-1 and was verified by expression level of mRNA and protein. In previous study, human NPC was passaged directly in nude (thymusless) mice to generate a transplantation tumor. Thus, to our best knowledge, this was an improved approach to generate the transplantation tumor in nude mice and was able to study the specific cell substrain of NPC.

To date, several studies have shown that KiSS-1 is aberrantly low-expressed in several types of metastatic cancers [7, 16] suggesting its therapeutic potential in cancers. In our study, we transfected the SUNE-1-5-8F cell with lentiviral vectors which carried with KiSS-1 and generated a stable substrain with an overexpression of KiSS-1. Our data demonstrated that KiSS-1 not affect the size of transplantation tumor. Previous study reported that KiSS-1 overexpression inhibits anchorage-independent growth but not anchorage-dependent growth of prostate cancer cells [17]. That is to say, KiSS-1 inhibits growth of matrix-independent tumor but not of matrix-dependent tumor. Therefore, this previous study is consistent with our data. In addition, it was reported that KiSS-1 increased apoptosis in tumor tissues such as osteosarcoma [18] and normal tissues such as testicular [19]. An essential characteristic of

metastatic cancer cells is resistance to anoikis [20, 21]. This attribute allows the survival of metastatic cancer cells during systemic circulation and facilitates their metastasis to distant organs. Enhanced expression of KiSS-1 in prostate cancer cells also partially abated the cell's motility and invasiveness and increased chemosensitivity of prostate cancer cells [17]. Moreover, another study reported that in human colorectal cancer tissues, the mRNA expression level of Kiss-1 had a negative correlation with Dukes staging, TNM staging, reduction of Kiss-1 expression was also linked to poor prognosis for the patients [22, 23]. All these data suggested the beneficial role of KiSS-1 on resistance to tumorigenesis.

In conclusion, we set up a transplantation tumor of NPC in nude mice successfully with a substrain that overexpressed KiSS-1 in this study. We found that the tumor suppressor gene KiSS-1 was able to attenuate the metastasis of human NPC but not able to influence the size of the primary cancer of NPC. Our study may be helpful for exploring a new method to treat NPC in the future.

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Disclosure of conflict of interest

None.

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