RESEARCH COMMUNICATION

KISS1 Expression in Osteosarcoma: High in Chinese Clinical Cases, but Lower in Cell Lines

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Abstract

Purpose: Osteosarcoma is the most common primary bone malignancy with a notorious feature of high metastasis. KISS1 has been identified as a putative human metastasis suppressor gene in numerous types of cancer. This study was aimed to evaluate the relationship between expression of KISS1 and invasion ability in osteosarcoma cell lines, and the relationships between KISS1 expression levels and prognosis of clincial cases. Methods: Expression levels of KISS1 in 3 types of osteosarcoma cell lines (MG-63, Saos-2 and U-2 OS) and a normal osteoblast cell line (hF-OB 1.19) were examined using semi-quantitative RT-PCR and immunochemistry staining. Transwell assays were used to detect the cell invasion ability. The osteosarcoma cell lines and specimen sections of osteosarcoma together with control were immuno-stained with KISS1 antibody. The relationship between the clinical data and the expression of KISS1 was evaluated. Results: KISS1 mRNA expression was moderate in U-2 OS, weak in Saos-2 and lost in MG-63. Transwell assays displayed a gradually increased aggressive phenomenon in osteosarcoma cell lines U-2 OS, Saos-2 and MG-63. However, a contrary conclusion was obtained with clinical specimen, a higher positive rate of KISS1 expression being displayed in osteosarcoma patients, especially in metastastic cases, compared to the benign osteochondroma patients. Furthermore, significant earlier distant metastasis was observed in KISS1 positive than negative cases. Conclusion: KISS1 expression levels were found to be decreased with the increasing aggressive ability in osteosarcoma cell lines. However, expression of KISS1 positively correlated with metastasis in osteosarcoma patients.

Keywords: Osteosarcoma - KISS1 - metastasis - prognosis

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Introduction

Osteosarcoma is the most common primary bone malignancy, which is the very frequently appeared in adolescents (Weber, 2005). Almost 80% of osteosarcomas happened at the rapidly growing sites of longitudinal bones, such as distal femur, proximal tibia and proximal humerus. One of notorious features of this tumor is its high metastasis. Despite a standard combination treatment of chemotherapy and surgery in modern years, the 5-year survival rate of osteosarcomas is still about 60% in the age group under 25 years (Mirabello et al., 2009), and distant metastasis is the key fetal cause, especially lung metastasis. Therefore, studies about anti-metastasis of osteosarcomas are worthwhile to put effort in.

KISS1 has been identified as a putative cancer metastasis suppressor in numerous investigations. Using subtractive hybridization and differential display technology, Lee et al. initially discovered that KISS1 gene expressed in a non-metastatic melanoma cell line neo6/C8161.1, however absent in metastatic melanoma cell line C8161 (Lee et al., 1996), that indicated it may be a metastasis suppressor in melanoma cell lines. Further investigations revealed KISS1 gene located in human chromosome1 (1q32) (West et al., 1998), encodes a number of peptides (kp-54, kp-14, kp-13, kp-10) named kisspeptin (Ohtaki et al., 2001), which are abundant expressed in the syncytiotrophoblast of placenta (Muir et al., 2001).

Subsequently, KISS1 was widely studied in different human tumors, and the true effect of KISS1 is still under determination. Numerous clinical reports demonstrated that a loss or reduction of KISS1 expression in different human cancers inversely correlate with tumor progression, metastasis and survival. For instance, loss or insufficient expression of KISS1 were revealed in melanoma (Shirasaki et al., 2001), esophageal squamous cell carcinoma (Ikeguchi et al., 2004), pancreatic cancer cells (Masui et al., 2004), uveal melanoma (Martins et al., 2008), bladder cancer (Sanchez-Carbayo et al., 2003b) and gastric carcinoma (Dhar et al,. 2004) associated with advanced progressions and poor prognosis. However, overexpression of KISS1 are paradoxically observed in hepatocellular carcinoma (Ikeguchi et al., 2003) and human breast cancer (Martin et al., 2005), positively correlated to their metastasis and progression.

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Furthermore, in non-small cell lung (Karapanagiotou et al., 2011), KISS1 shows a lack of direct involvement of metastin in the diagnosis and metastatic potential.

Despite the high metastasis nature of osteosarcoma and numerous studies of other metastasis suppressor genes' function on it, the study about the correlation of KISS1 and osteosarcoma is still a brilliant new topic for us. In a former study, Marta et al. (Sanchez-Carbayo et al., 2003a) detected that KISS1 gene existed in osteosarcoma cell line U-2 OS by using the cDNA microarray technology. However, no available investigation, to our best known, is about the expression of KISS1 in osteosarcoma cases or in other osteosarcoma cell lines, furthermore, the relationship between KISS1 expression and clinic outcome or cell lines invasion abilities.

Materials and Methods

Cell culture

The cell lines, including human osteosarcoma cell lines Saos-2, MG-63 and U-2 OS, and human osteoblast cell line hF-OB1.19, were purchased from the Chinese Academy of Sciences (Shanghai, China) repository and were used within 10 passages. Saos-2 cells were propagated in a-MEM, U-2 OS and MG-63 cells in DMEM, and hF-OB1.19 cells in DMEM/F-12 (1:1). The complete growth media included 10% fetal calf serum in three of the osteosarcoma cell lines and 15% in hF-OB1.19, were added 100U/ml penicillin and 100 μg/ml streptomycin. All of the cells were incubated in at 37°C in a humid atmosphere of 5% CO2/95% air.

Samples and data collection

The study of human osteosarcoma tissue was approved by the Ethics Committee of the First Affiliated Hospital of Fujian Medical University. 44 formalin-fixed and paraffin-embedded osteosarcoma samples and 15 osteochondroma sample were provided by Pathology Department of the First Affiliated Hospital of Fujian Medical University. The osteosarcoma samples were conserved during the period from 2005 to 2009, including 25 male (56.81%) and 19 female (43.19%) with a median age at diagnosis of 18 years (range 12-74 years, mean age 23.68 years). By the end of April 2011, the follow-up durations ranged from 6 to 76 months (median 17.5 months, mean±SD 23.11±15.81 months), 20 cases were died (survival time range 6-38 months, median 13 months, mean 15.05±8.24 months) and 24 cases have been lived since from 9-76 months (median 33 months, mean 29.83±14.79 months). All osteosarcoma patients had been given a period treatment of chemotherapy (combinating treatment of ADM, CP and IFO) before surgery, except for 4 cases found lung metastasis when osteosarcoma had been diagnosed. Information of gender, age, pathological types, surgical stages and clinical outcome were collected from the medical records (Table 1).

Immunocytochemistry

Firstly, cells were grown to approximately 80% confluence on slides, fixed in 95% alcohol for 30 minutes, immersed in 0.1% TritonX-100 for 20 minutes, and then treated with 3% hydrogen peroxide (3% H2O2) for blocking unspecific protein binding sites for 15 minutes. Secondly, the slides were incubated with a monoclonal mouse anti-KISS1 antibody (1:50, Santa Cruz, USA) at 37°C for 1 hour. Then slides were incubated with Polymer Helper and Poly Peroxidase-anti-Mouse IgG (GBICO, USA) for 25 minutes respectively. Thirdly, the peroxidase activity was visualized through a color reaction with DAB (3, 3'-diaminobenzidine, Maixin Biotechnology, Fuzhou, China). Brown and yellow colors indicated positive results for KISS1 antigen. Finally, the sections were counterstained with hematoxylin and mounted with cover slips after standard dehydration. Pictures were captured from microscope BX-51 (Olympus, Japan) by image processing software DP-70 (Olympus, Japan).

Table 1. Comparison of the Patients with Osteosarcoma Who Had Positive Immunohistochemistry Staining of KISS1 and Those Negative

Osteosarcoma Patients	KISS1 (+) (n=20) Number (%)	KISS1 (-)(n=24) Number (%)	P- value
Gender			
Male	12 (60.00)	13(54.17)	P =0.70
Female	8 (40.00)	11(45.83)	
Age			
≤ 24	12 (60.00)	17(70.83)	P =0.96
> 24	8 (40.00)	7 (29.17)	
Locations			
Femur	9 (45.00)	11 (45.83)	P=0.89
Tibia	7 (35.00)	7 (29.17)	
Other	4 (20.00)	6 (25.00)	
Pathological Type			
Telangiectatic Os	1 (5.00)	2 (8.33)	P=0.27
Small Cell Os	2 (10.00)	0(0.00)	
Other	17 (8500)	22 (91.67)	
Surgery Stages			
IIA	2 (10.00)	5 (20.83)	P =0.33
IIB	18 (90.00)	19 (79.17)	
Metastasis[a]			
Present	17 (85.00)	10 (41.67)	χ^2=7.698[b]
Absent	3 (15.00)	14 (58.33)	P = 0.006
Mortality[c]			
Death	13 (65.00)	7 (29.17)	
Survival	7 (35.00)	17 (70.83)	

[a]Metastasis survival time ranger from 0-63 months (median 11 months, and mean± SD, 14±13.71 months); [b]χ2 was compared by the log-rank test; [c]Owing to insufficient follow-up time, the mortality rate was not calculated

RNA preparation and semiquantitative RT-PCR amplification

Semiquantitative reverse transcriptase (RT-PCR) was used to detect the expression of KISS1gene in normal osteoblast cell line hF-OB1.19 and human osteosarcoma cell lines Saos-2, MG-63 and U-2 OS respectively. Placenta tissues, as a positive control, were provided by a healthy pregnant volunteer, according to the institutional ethical guidelines. At first, total RNA was isolated from the cultured cells using TRIZOL (Invitrogen, USA) and quantized by u.v.260/280nm to an absorption ratio of >1.6. Total RNA (2μL) was reverse transcribed to cDNA in a final volume of 20 ml using MuLvRT (Invotrogen, USA) following the manufacturer's instructions. Then,

Table 2. The Immunohistochemistry Result of Osteosarcoma Cases and Osteochondroma Cases

Cases	KISS1		P-Value
	Positive	Negative	
Osteosarcoma	20	24	P=0.03
Osteochondroma	2	13	

cDNA was used as the template for PCR. All PCRs were performed by the ABI Prism 7700 Sequence Detection System (Perkin–Elmer Applied Biosystems), under the conditions recommended by the manufacturer. The thermal cycling conditions comprised 1 cycle of 5 min at 94°C, 28 cycles of 45 sec at 94°C , another 28 cycles of 30 sec at 59°C or 58°C (KISS1 and β-actin respectively), of 30 sec at 72°C, and finally 1 cycle of 7 mins at 72°C for termination. Thirdly, the PCR products were resolved on 1.2% low melting agarose gels. Forward (F) and reverse (R) primes were as follows: KISS1 (F) 5'-GACCTGCCTCTTCTCACCAA-3', KISS1 (R) 5'-CAGTAGCAGCTGGCTTCCTC3'. Amplification of the β-actin fragment was used for RT–PCR normalization.

Invasion assay

24-well cell culture chambers containing inserts with 8-μm pores (Transwell, Corning, USA) and coated with 70μl 20% matrigel (ECM gel, BD, Germany) were utilized to examine the invasion of osteosarcoma cell lines. Approximately 4×105 cells were implanted in the upper chamber of every insert. After 20h of incubation, cells on the upper surface of inserts were wiped off by a cotton swab. Cells had invaded to the lower surface were fixed with 95% ethanol, stained with hematoxylin and eosin, and counted in 10 different fields at 200× magnification.

Immunohistochemistry

Serial 2.5 μm slices were obtained from formalin-fixed and paraffin-embedded osteosarcoma specimens. Firstly, sections were dewaxed in xylene, rehydrated in alcohols respectively before being washed by distilled water. Then the sections were boiled with high power in the microwave oven to boiling point, and then continuously boiled at Med-Low power for 15 minutes with EDTA (0.05 mol/L Tris, 0.001 mol/L EDTA, and pH 9.0) to retrieve antigen of KISS1 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After boiling, cooled sections were washed 3 times with PBS, and then treated with 3% H_2O_2 for blocking unspecific protein binding sites for15 minutes. The remaining steps were as same as the immunocytochemistry, with placenta as a positive control.

Statistical analyses

All statistical analyses were conducted by SPSS16.0 (SPSS Inc, Chicago, IL, USA). Continuous variables are presented as the mean ± standard error or as the median and range. ANVOVA was used to analyze the mRNA expression levels and invasion ability. Nonparametric test and χ^2 tests were performed for clinical data. Survival curves were drawn by the Kaplan-Meier method, and compared by the log-rank test. Statistical significance was assumed when $P < 0.05$.

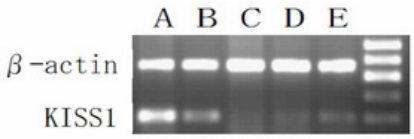

Figure 1. Results of Semiquantitative RT-PCR. A: Placenta Tissue, B: hF-OB 1.19, C: MG-63, D: Saos-2, E: U-2 OS

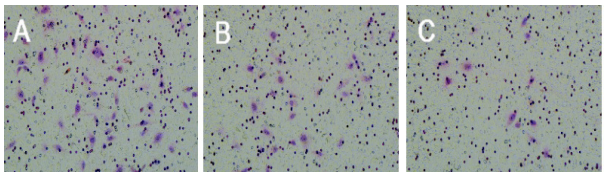

Figure 3. Result of Cells Invasion Assay. A: MG-63, B: Saos-2, C: U2-OS (200× magnification)

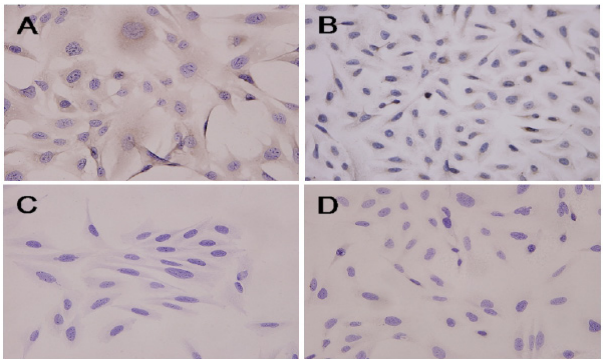

Figure 2. Results of KISS1 Immuncytochemistry Staining. A: hF-OB 1.19, B: U-2 OS, C: Saos-2, D: MG-63 (400× magnification)

Results

mRNA expression of KISS1 in osteosarcoma cell lines

hF-OB1.19 cell line (mean±SD, 0.838±0.054) depicted stronger expression of KISS1 mRNA than all of the three osteosarcoma cell lines. And in osteosarcoma cell lines, KISS1 mRNA levels (standardized use β-actin) were moderate in U-2 OS cell line (mean±SD 0.720±0.043), weak in Saos-2 cell line (mean±SD 0.629±0.031), almost lost in MG-63 cells. Placenta tissue was used for positive control. The experiments showed significant statically difference between hF-OB1.19 and U-2 OS ($P < 0.001$), U-2 OS and Saos-2 ($P < 0.001$) cell lines respectively (Figure 1).

KISS1 expression in three osteosarcoma cell lines

A monoclonal mouse anti-KISS1 antibody was used to examine the expression of the protein production of KISS1 in normal osteoblast cell line hF-OB1.19 (Figure 2A) and osteosarcoma cell lines (Figure 2B-D). hF-OB1.19, the normal osteoblast cell also showed a stronger expression in result of immunocytochemsitry, comparatively to KISS1 in U-2 OS, Saos-2 and MG-63 cell lines. KISS1 in U-2 OS, Saos-2 and MG-63 cell lines continuously showed moderate, weak and lost expression respectively.

The invasion ability of three osteosarcoma cell lines

Osteosarcoma cells were cultured in 24-well chambers for 24h, after stained with hematoxylin, 10 different fields (200×magnification) were counted to test the numbers

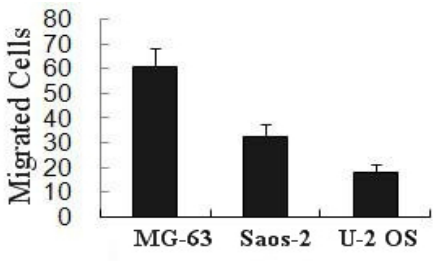

Figure 4. Statistic of the Migrated Cells. Significant statically differences (P < 0.005) between each other

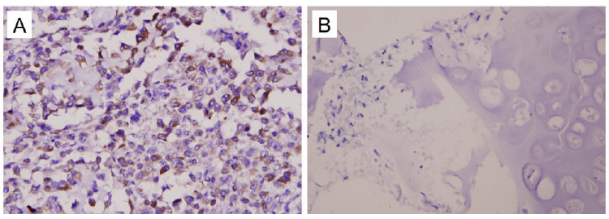

Figure 5. Results of KISS1 Immunohistochemistry Staining. A:A positive staining of KISS1 in osteosarcoma patients, B: A negative staining in benign osteochondroma patients (200× magnification)

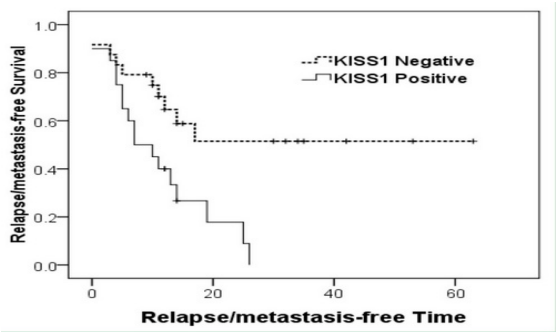

Figure 6. Result of Kaplan-Meier Test for Relapse/ Metastasis-free survival. Relapse/metastasis-free survival curve was illustrated according to the Immunohistochemistry result of KISS1. There was a trend (p=0.006) toward increased earlier metastasis in patients with negative result of KISS1. Full line indicated the KISS1 positive group. Dotted line indicated the KISS1 negative group

of migrated cells. The three types of osteosarcoma cells displayed an increasing migrating potential: U-2 OS (mean ± SD, 18.1±3.21), Saos-2 (mean ± SD, 32.4±5.36) and MG-63 (mean ± SD, 61±7.15). There were also significant statically differences (P < 0.005) between each other (Figure 3, Figure 4).

The KISS1 expression in osteosarcoma clinical case by immunochemistry staining and correlation with the distance metastasis

The percentage of KISS1 positive patients in osteosarcoma patients (45.45%) was higher than that in the benign osteochondroma patients (13.33%) (Table 2, Figure 5). Osteosarcoma patients were divided into positive (20 cases 45.45%) and negative (24 cases 54.55%) groups according to the results of immunohistochemistry by a cut off level of 50%. Especially, relapse/metastasis osteosarcoma patients showed a significantly higher KISS1 positive rate of 62.96%, compared to all non-metastatic patients (17.65%) in this research. In additional, we investigated the relationship between KISS1 expression and the relapse/metastasis-free survival (ranger from 0-63 months, median 11 months, and mean± SD, 14±13.71 months). The 20 KISS1 positive patients (median 8.5 months, mean± SD, 9.85±7.13 months) had relatively earlier distant metastasis compared to the 24 KISS1 negative patients (median 12 months, mean± SD, 18.62±16.34 months). The statistics difference was significant confirmed by the Kaplan-Meier Test (P=0.006) (Figure 6). Furthermore, the expression levels of KISS1 were not associated with gender, age, pathological subtype and surgical stages.

Discussion

This is the first study to investigate the expression of KISS1 in human osteosarcoma cell lines and tissues. In the study of the cell lines, MG-63 cells, which lack of KISS1 gene expression, showed a significantly stronger invasive capability than Saos-2 and U-2 OS which had a weak or moderate expression of KISS1 gene. Each of the three osteosarcoma cell lines showed statistically significant beyond the osteoblast cell line hf-OB1.19. Moreover, the statistic difference between the KISS1 expression of Saos-2 and U-2 OS was significant, that Saos-2 had a relatively stronger invasion capability than U-2 OS. These comparisons suggested that a lower expression of KISS1 gene might associate with a stronger invasive capability. Our in vitro results showed the role of KISS1 as metastasis suppressor in osteosarcoma cell lines same as the formal studies.

However, after investigating the clinical information of all the osteosarcoma patients, we obtained a result on the contrary of the conclusion we have gotten from in vitro experiments. According to our experimental results, we proposed that patients with positive expression of KISS1 occupied a higher proportion in osteosarcoma patients (45.45%), compared to that in the osteochondroma control samples (13.33%). And, the KISS1 positive group presented earlier distant metastasis than the negative group. The comparisons indicated that a higher expression of KISS1 could associate with a poor prognosis and progression in osteosarcoma cases. According to these, we suggested that KISS1, as a metastasis related gene, was still correlated to metastasis process of osteosarcoma, despite the presenting higher expression rate.

Interestingly, we found two other independent studies in breast cancer, which together contributed a consistent result with our discovery during the research. In 1997, Lee et al. (Lee and Welch, 1997) reported that KISS1 functions as a metastasis suppressor gene in human breast cancer line MDA-MB-435. This result was conflicted by the research of Martin et al. in 2005, who suggested that KISS1expression was increased in human breast cancer, particularly in patients with aggressive tumors and with mortality, and over-expression of KISS1 in breast cancer cell line MDA-MB-231 result in more aggressive phenotype (Martin et al., 2005). Further study was done by Marot et al. in 2007, who concluded that the conflicting results from Martin and Lee were caused by ER-α (Marot et al., 2007). These researches indicated KISS1 may be affected by other factors, and casts totally contradict

effects between in vitro and in clinical specimen, which was consistent to our study.

For explaining why expression of KISS1 associated with a poor prognosis and progression in some malignant tumors, we found that there were generally three suggestive opinions present. The first, Martin et al. (Martin et al. 2005)proposed that the placenta is also an invasive tissue, and the behavior of invading trophoblasts is similar to the invasion of cancer cells. In their research, over-expression of KISS1 in breast cancer cells associate with more aggressive phenotype. Therefore, they supposed the similar overexpression of KISS1 in breast cancer and in placenta may largely due to the similar invasive nature of the two tissues, which was contradicted to the published data about KISS1 gene. The second, Katagiri et al. (2009) explained that the high level of plasma KISS1 may be secreted from the adjacent normal tissue as a self-defense mechanism reaction in pancreatic cancer patients. However, why this self-defense mechanism reaction has not been seen in other human tumors, it was still an unclear hypothesis. The third, Ikeguchi et al. (2003) postulated that increased estrogen levels might cause the elevated expression of KISS1 in hepatocellular carcinoma, because patients with HCC generally suffer from liver cirrhosis and prone to a hyperestrogenic state.

Hence, According to Ikeguchi et al. (2003), we proposed that the imbalance of steroid hormone may also be one of the reasons for the high expression of KISS1 in osteosarcoma. Osteosarcoma, which prefers boys (Qureshi et al. 2010), were considered as (sex steroid) hormone-dependent tumors (Svoboda, Hamilton and Thalhammer, 2010). It was well known that osteosarcoma is most frequently appeared in a period of puberty, when steroid hormone, such as testosterone (Biro et al., 1995, Legro et al., 2000) and estradiol (Ikegami et al., 2001), changed sharply and prone to disorder in adolescents. This may suggest that disorder of steroid hormone might be an undetermined factor to disturb the process of osteosarcoma. In Additionally, Fohr et al. (2000) and Dohi et al. (2008) found that the proliferation of osteosarcoma cell line MG-63 was significantly increased by estrogen, and significantly suppressed by blockers for ER and AR (androgen receptors) respectively. From above, we considered KISS1 expressed in osteosarcoma not only related to the level of estrogen, but also might be affected by various steroid hormone synchronously. However, the regulation mechanisms of KISS1 in osteosarcoma need further researches.

To our knowledge, this is the first study to investigate the expression of KISS1 in human osteosarcoma tissues and cell lines. The experimental results showed totally different results: expression of KISS1 gene was decreased with the increasing invasive ability in osteosarcoma cell lines in vitro, but elevated KISS1 was observed with early distant metastasis in osteosarcoma patients.

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