



# KISS1 protein expression is associated with worse prognosis in osteosarcoma patients: a long-term follow-up study

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**Background:** A long-term follow-up study was used to analyze the relationship between KISS1/GPR54 protein expression and the clinical prognosis of osteosarcoma (OS) patients.

**Methods:** Forty-four paraffin-embedded OS samples conserved during the period from 2005 to 2009 were studied. Information about gender, age, pathological type, surgical stage, and clinical outcome was collected from the medical records.

**Results:** This study included 25 males (56.81%) and 19 females (43.19%) with a median age at diagnosis of 18 years (range, 12–74 years). The follow-up duration ranged from 97 to 162 (121.36±15.46) months. The overall survival and metastatic rates were 43.18% and 65.91% respectively. For KISS1, the rate of distal metastasis and mortality in positive expression patients were 85.00% (17/20) and 75.00% (15/20) respectively, and 50.00% (12/24) and 41.67% (10/24) in negative expression patients respectively. In terms of survival time, KISS1 positive and negative patients were 55.35±14.29 and 97.46±13.06 months, respectively.  $P < 0.05$  was considered statistically significant in all three of the above aspects. For GPR54, the rate of metastasis and mortality of positive patients were 66.67% (24/36) and 61.11% (22/36), respectively, and 62.50% (5/8) and 37.50% (3/8) in negative expression patients. The survival time of GPR54 positive and negative expression patients were 73.97±11.81 and 92.75±15.42 months, respectively; however,  $P > 0.05$  in these three aspects.

**Conclusions:** Expression of KISS1 protein correlated with higher distal metastasis and shorter survival time in OS patients after an average of 10.11 years of follow-up. GPR54 protein did not affect the prognosis when expressed alone.

**Keywords:** Osteosarcoma (OS); KISS1; GPR54; clinical prognosis; long-term follow-up

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## Introduction

Osteosarcoma (OS) is a common primary malignant bone tumor occurring in adolescence, and mostly affects males (1). In recent years, several clinical applications, including neo-adjuvant chemotherapy combined with surgery and other comprehensive treatment, have significantly improved its

clinical prognosis. However, the 5-year survival rate is still hovering around 50–70% (2) due to early lung metastasis and inflicts a grievous toll on the community and families.

KISS1 gene was found to be cDNA clone fragment (3), located on the long arm of chromosome 1 (1q321q34) (4), which generates a biologically active secretory peptide

named kisspeptin-54 (5,6). GPR54 was discovered (7) to be a receptor protein, consisting of seven transmembrane (TM) domains, belonging to the orphan G protein receptor family. In 2001, researchers found human GPR54 homologs, known as AXOR12 (8) or hOT7T175 (5), composed of 398 amino acids. Kisspeptin-54 can specifically bind with receptor GPR54 and perform its biological role (6).

Research on the metastasis mechanism and clinical prognosis in OS have been intensively active of late. However, long-term follow-up reports are rare. This study was conducted for an average of 10.11 years of follow-up and aimed to analyze the relationship between KISS1 and GPR54 protein expression and clinical prognosis in OS patients.

## Methods

### *Samples and data collection*

Forty-four paraffin-embedded OS samples and 15 osteochondroma samples were provided by the Pathology Department of the First Affiliated Hospital of Fujian Medical University. The OS samples were conserved during the period from 2005 to 2009 and included 25 males (56.81%) and 19 females (43.19%) with a median age at diagnosis of 18 years (range, 12–74 years; mean age 23.68 years). All patients had been given a period of treatment of chemotherapy (combined treatment of doxorubicin, cyclophosphamide, and ifosfamide) before surgery. Information about gender, age, pathological type, surgical stage, and clinical outcome was collected from the medical record.

The relationship between the expression of KISS1 or GPR54 protein and the clinical prognosis of OS patients were reported in our previous study (9,10) with short-term (average less than 2 years) follow-up results. With a regular annual follow-up, the average follow-up time was 10.11 years (121.36±15.46 months; range, 97 to 162 months) lasting until June 2018. The rates of lung metastasis and mortality have changed, and we here report the most recent follow-up results.

### *Immunohistochemistry*

The immunohistochemical staining of paraffin specimens from patients was used to detect the expression of KISS1 and GPR54 protein. Serial 2.5 µm slices were cut from formalin-fixed and paraffin-embedded OS specimens. First,

sections were dewaxed in xylene and rehydrated in a graded series of alcohols before being washed by distilled water. Then, the sections were boiled with high power in the microwave oven to a boiling point, and then continuously boiled at medium-to-low power for 15 minutes with EDTA (0.05 mol/L Tris, 0.001 mol/L EDTA, and pH 9.0) to retrieve antigens. After boiling, sections were washed 3 times with PBS and then treated with 3% H<sub>2</sub>O<sub>2</sub> for blocking unspecific protein-binding sites for 15 minutes. Second, the slides were incubated with a monoclonal mouse anti-KISS1 antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-GPR54 antibody (1:700, Phoenix Pharmaceuticals, Burlingame, CA, USA) at 37 °C for 1 hour. Next, slides were incubated with Polymer Helper and Poly Peroxidase-anti-Mouse IgG (GBICO, USA) for 25 minutes. Third, 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 and GPR54 antigen. Finally, the sections were counterstained with hematoxylin and mounted with coverslips after standard dehydration. Pictures were captured with a BX-51 microscope (Olympus, Japan) by image processing software DP-70 (Olympus, Japan), and have been published with the results of the short-term follow-up studies (9,10).

### *Statistical analyses*

All statistical analyses were conducted by SPSS21 (SPSS Inc., Chicago, IL, USA). The distribution of data was assessed for normality using the Kolmogorov-Smirnov test. Continuous variables were presented as the mean ± standard error of the mean (SEM) or median with range. For normally distributed data, Student's *t*-test was used to compare the differences between 2 independent groups. Otherwise, the Mann-Whitney U test was used. Chi-square test was performed to compare differences of rates among different groups. Survival curves were drawn by the Kaplan-Meier method and compared by the log-rank test. Statistical significance was assumed when P<0.05.

## Results

### *Overall clinical prognosis*

After more than an average of 10 years of follow-up, the follow-up durations (121.36±15.46 months) ranged from

**Table 1** Comparison of the KISS1/GPR54 positive and negative patients with OS

| OS patients             | KISS1 (+) (n=20),<br>n (%) | KISS1 (-) (n=24),<br>n (%) | P value       | GPR54 (+) (n=36),<br>n (%) | GPR54 (-) (n=8),<br>n (%) | P value       |
|-------------------------|----------------------------|----------------------------|---------------|----------------------------|---------------------------|---------------|
| Gender                  |                            |                            |               |                            |                           |               |
| Male                    | 12 (60.00)                 | 13 (54.17)                 | 0.70          | 21 (58.33)                 | 4 (50.00)                 | 0.97          |
| Female                  | 8 (40.00)                  | 11 (45.83)                 |               | 15 (41.67)                 | 4 (50.00)                 |               |
| Age (mean ± SEM) (year) | 24.50±2.68                 | 23.00±2.85                 | 0.44          | 23.69±2.32                 | 23.63±2.83                | 0.39          |
| Locations               |                            |                            |               |                            |                           |               |
| Femur                   | 9 (45.00)                  | 11 (45.83)                 | 0.89          | 16 (44.44)                 | 4 (50.00)                 | 0.34          |
| Tibia                   | 7 (35.00)                  | 7 (29.17)                  |               | 13 (36.11)                 | 1 (12.50)                 |               |
| Other                   | 4 (20.00)                  | 6 (25.00)                  |               | 7 (19.44)                  | 3 (37.50)                 |               |
| Pathological type       |                            |                            |               |                            |                           |               |
| Telangiectatic OS       | 1 (5.00)                   | 2 (8.33)                   | 0.27          | 2 (5.56)                   | 1 (12.50)                 | 0.63          |
| Small cell OS           | 2 (10.00)                  | 0 (0.00)                   |               | 2 (5.56)                   | 0 (0.00)                  |               |
| Other                   | 17 (85.00)                 | 22 (91.67)                 |               | 32 (88.89)                 | 7 (85.50)                 |               |
| Surgery stages          |                            |                            |               |                            |                           |               |
| IIA                     | 2 (10.00)                  | 5 (20.83)                  | 0.57          | 4 (11.11)                  | 3 (37.50)                 | 0.19          |
| IIB                     | 18 (90.00)                 | 19 (79.17)                 |               | 32 (88.89)                 | 5 (62.50)                 |               |
| Metastasis              |                            |                            |               |                            |                           |               |
| Present                 | 17 (85.00)                 | 12 (50.00)                 | $\chi^2=5.95$ | 24 (66.67)                 | 5 (62.50)                 | $\chi^2=0.00$ |
| Absent                  | 3 (15.00)                  | 12 (50.00)                 | 0.01          | 12 (33.33)                 | 3 (37.50)                 | 1.00          |
| Mortality               |                            |                            |               |                            |                           |               |
| Death                   | 15 (75.00)                 | 10 (41.67)                 | $\chi^2=4.94$ | 22 (61.11)                 | 3 (37.50)                 | $\chi^2=0.68$ |
| Survival                | 5 (25.00)                  | 14 (58.33)                 | 0.03          | 14 (38.89)                 | 5 (62.50)                 | 0.41          |
| Survival time (months)  | 55.35±14.29                | 97.46±13.06                | 0.01          | 73.97±11.81                | 92.75±15.42               | 0.18          |

SEM, standard error of the mean; OS, osteosarcoma.

97 to 162 months, 19 patients were alive, 25 patients died, and the median survival time was 35 months (mean ± SEM 63.05±7.88 months). The overall survival rate was 43.18% (19/44), and the metastatic rate was 65.91% (29/44) in the course of the disease. These data points were 54.55% (24/44) and 61.36% (27/44) respectively, at the early follow-up. Cases of metastasis included 26 lung metastases, 1 bone metastasis, 1 axillary lymph node metastasis, and 1 retroperitoneal metastasis. Furthermore, there was no statistical difference in the expression of KISS1/GPR54 protein in terms of gender, age, pathological subtype, and surgical stage (Table 1).

### *KISS1 expression and clinical prognosis analysis*

Among patients with KISS1 protein positive expression, the distal metastasis rate was 85% (17/20), the mortality rate was 75% (15/20), the median survival time was 18 months, and the mean survival time was 55.35±14.29 months. As for the KISS1 protein negative patients, the rate of distal metastases and mortality was 50% (12/24) and 41.67% (10/24), and the mean survival time was 97.46±13.06 months. There was significant difference in the mortality rate (Figure 1), metastasis rate (Figure 1), and mean survival time (Figure 2) ( $P<0.05$ ), suggesting that positive expression

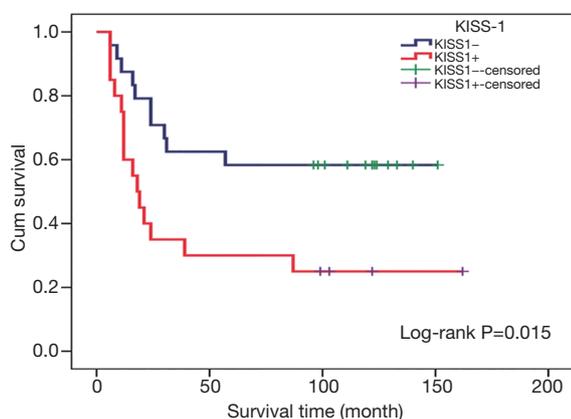
| A         | Survival |        | P value | C         | GPR54 (+) |        | GPR54 (-) |   | *P value |
|-----------|----------|--------|---------|-----------|-----------|--------|-----------|---|----------|
|           | Present  | Absent |         |           | Present   | Absent |           |   |          |
| KISS1 (+) | 5        | 14     | 0.026   | KISS1 (+) | 16        | 3      | 1         | 0 | 1.000    |
| KISS1 (-) | 15       | 10     |         | KISS1 (-) | 8         | 9      | 4         | 3 |          |
| GPR54 (+) | 14       | 22     | 0.409   | #P value  | 0.018     |        | 1.000     |   |          |
| GPR54 (-) | 5        | 3      |         |           |           |        |           |   |          |

| B         | Present  |       | P value | D         | GPR54 (+) |       | GPR54 (-) |   | *P value |
|-----------|----------|-------|---------|-----------|-----------|-------|-----------|---|----------|
|           | Survival | Death |         |           | Survival  | Death |           |   |          |
| KISS1 (+) | 17       | 3     | 0.015   | KISS1 (+) | 5         | 14    | 0         | 1 | 1.000    |
| KISS1 (-) | 12       | 12    |         | KISS1 (-) | 9         | 8     | 5         | 2 |          |
| GPR54 (+) | 24       | 12    | 1.000   | #P value  | 0.171     |       | 0.375     |   |          |
| GPR54 (-) | 5        | 3     |         |           |           |       |           |   |          |

\*Compare the rate of mortality or distal metastasis between GPR54 (+) and GPR54 (-).  
 #Compare the rate of mortality or distal metastasis between KISS1 (+) and KISS1 (-).

**Figure 1** The relationship between KISS1/GPR54 expression and clinical prognosis: (A,B) there had significant difference between KISS1 expression and mortality rate or metastases rate, but not between GPR54 expression and mortality rate or metastases rate; (C) KISS1 proteins expression was positively correlated with the metastases in patients with GPR54 proteins expression, but not in patients with GPR54 proteins absent; (D) KISS1 proteins expression did not affect the survival rate both in patients with GPR54 proteins expression and absent.



**Figure 2** Kaplan-Meier test for survival time (month): survival time curve was illustrated according to the immunohistochemistry result of KISS1. There was a trend (P=0.015) toward a shorter survival time in patients with KISS1 protein positive expression.

of KISS1 protein correlated with more mortality and metastasis, and shorter survival time in OS patients, which is consistent with the results of our early follow-up (9).

**GPR54 expression and clinical prognosis analysis**

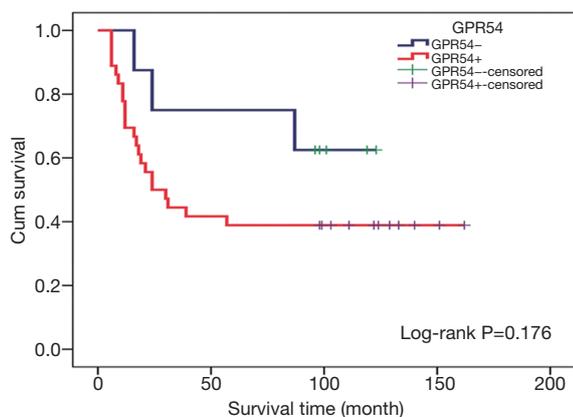
In GPR54 protein positive expression patients, the metastatic rate was 66.67% (24/36), the mortality rate was 61.11% (22/36), the median survival time was 24 months,

and the mean survival time was 73.97±11.81 months. In GPR54 protein negative expression patients, the metastasis rate was 62.5% (5/8), the mortality rate was 37.5% (3/8), and the mean survival time was 92.75±15.42 months. There was no significant difference in mortality rate (Figure 1), metastasis rate (Figure 1), or mean survival time (Figure 3) (P>0.05), which is inconsistent with the results of our early follow-up (10).

Furthermore, there were 19 cases with KISS1 and GPR54 proteins, both positive, in which 16 cases were distal metastases, and 14 ended in death. For those 7 cases with both KISS1 and GPR54 proteins negative, 4 cases had distal metastases, and 2 cases ended in death. The analysis showed that there was a significant statistical difference in distal metastases (Figure 1) rather than mortality (Figure 1), indicating that both KISS1 and GPR54 positive expression was negatively correlated with the metastasis rate of patients with OS.

**Discussion**

The important role of the KISS1/GPR54 system in human tumors has been demonstrated, but the specific effects are still controversial. Some reports have shown that KISS1/GPR54 genes inhibit tumor occurrence or transfer. For instance, it has been reported that B16-BL6 melanoma cell morphology was changed significantly after the transfection



**Figure 3** Kaplan-Meier test for survival time (month): survival time curve was illustrated according to the Immunohistochemistry result of GPR54. No significant different of survival time existed between patients with GPR54 protein positive and negative expression.

of the GPR54 gene, and the ability of invasion and metastasis markedly decreased (11). The lack of KISS1 and GPR54 gene was found to be associated with the occurrence of lymph node metastasis tumors in esophageal cancer (12). Another study (13) reported that KISS-1 expression was decreased in pancreatic cancer tissue, but GPR54 (mRNA) expression increased. Zohrabian *et al.* (14) discovered that KISS1 gene expression decreased in lung adenocarcinoma tissue. Furthermore, it was reported that KISS1 expression in uveal melanoma, patients with low KISS1 expression were associated with a higher risk of metastatic disease (15). By down-regulating  $G\alpha_q$ -p63RhoGEF-RhoA signaling pathways, Cho *et al.* (16) found that the KISS1 receptor GPR54 gene could delay the development of breast cancer. Recently, Yin *et al.* (17) reported that KISS1 inhibited the proliferation of OS *in vitro* by accelerating the processes of apoptosis and autophagy in the cells.

Conversely, the high expression of KISS1/GPR54 showed poor prognosis in several other kinds of malignant tumors. In 2003, it was found that the KISS1/GPR54 gene was highly expressed in all advanced liver cancer tissues (18). Results reported by Gao *et al.* (19) also showed that the expression level of KISS1 was higher in tumor tissues than benign lesions and normal tissues in the ovarian epithelial tumor. The positive expression rate of KISS1 was also found to be higher in the development of colorectal cancer tumors than normal tissue (20). In addition, Kostakis *et al.* (21) reported a contradictory phenomenon: the expression of KISS1 was

much higher in normal colonic mucosa than in malignant colonic mucosa and higher in more advanced tumors than early tumor tissue. Moreover, among malignant tissues, KISS1 expression was higher in larger tumors (>4 cm) than in smaller ones ( $\leq 4$  cm), higher in stages III and IV than in stages I and II, and higher in patients with lymph node metastases.

According to the previous studies, the KISS1/GPR54 gene may participate in the occurrence and development process in different tumors through different molecular mechanisms, as an inhibitor in some tumors and a promoter in others. The complex molecular mechanism of KISS1/GPR54 gene in tumors remains to be further studied. Here, we investigated the prognosis of OS patients with KISS1 or GPR54 alone or together.

Among the 36 patients with GPR54 protein positive expression, the KISS1 protein was positively expressed in 19 patients, 16 of whom had distant metastasis, 14 of these patients who occurred distant metastasis resulting in death. For the other 17 patients with KISS1 protein negative expression, distant metastasis occurred in 8 patients, all of whom died. This indicates that the rate of mortality and distal metastasis were both significantly higher in the patients with both KISS1 and GPR54 protein positive expression. On the other hand, among the 20 patients with positive KISS1 protein expression, the only one with negative GPR54 protein expression had lung metastasis and died, which suggests that the KISS1 positive expression was associated with clinical outcomes, but not the expression of GPR54 protein. In contrast, KISS1 protein expression was positively correlated with metastasis in patients with GPR54 protein expression, but not in patients with GPR54 protein absent. What the above indicates is that, as an endogenous receptor of KISS1, GPR54 protein affected the prognosis of patients with OS by integrating with KISS1 protein, and KISS1 protein affected the prognosis perhaps through the GPR54 signaling pathway. The results of the long-term follow-up were distinct from the short-term follow-up results we reported 7 years ago (10).

Some limitations exist in this study. First, this is a retrospective evaluation of a limited number of patients. Second, immunohistochemical staining was the only validated experiment method in this study, but our previous experiments had proven that KISS/GPR54 protein expression in OS cell lines (U-2 OS, Saos-2, MG-63) and KISS protein expression were related with aggressive ability in cell lines. Despite these limitations, this study is noteworthy since we are first to report a long-term clinical

prognosis analysis concerning the predictive effect of KISS1/GPR54 protein expression in OS patients.

## Conclusions

In summary, after more than an average of 10.11 years of follow-up, we found that KISS1 protein expression significantly increased in OS patients who had distal metastases, with a negative correlation to survival time and survival rate; these findings are consistent with the results of our earlier follow-up. GPR54 protein affects the prognosis of patients with OS due to being integrated with the KISS1 protein but does not affect the prognosis when expressed alone, an observation is inconsistent with the results of early follow-up. The molecular mechanism of the KISS/GPR54 protein system in tumors is very complex, and thus, further study is needed.

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## Footnote

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2019.08.21>). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All studies were approved by the ethics committee of Fuzhou Second Hospital Affiliated to Xiamen University and the First Affiliated Hospital of Fujian Medical University (FUM-2010-012). Individual informed consent was waived.

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