Therapeutic Discovery

Prognostic Role of KiSS-1 and Possibility of Therapeutic Modality of Metastin, the Final Peptide of the KiSS-1 Gene, in Urothelial Carcinoma

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Abstract

The KiSS-1 gene has been reported to be a metastasis suppressor gene in human melanoma. The gene product was isolated from human placenta as the ligand of GPR54, a G protein–coupled receptor, and the C-terminally amidated peptide of 54 amino acids is called metastin. The binding of metastin to GPR54 has been shown to inhibit tumor metastasis in some tumor cells; however, its function remains unclear in urothelial carcinoma. We first evaluated KiSS-1 expression and GPR54 expression in 151 patients with upper urinary tract urothelial carcinoma to determine their prognostic significance. Next, we examined the role of metastin in the invasiveness and lung metastasis of MBT-2 variant (MBT-2V), which is a highly metastatic murine bladder cancer cell. Multivariate analysis revealed that KiSS-1 expression was an independent predictor of metastasis and overall survival. However, GPR54 expression was not selected. Hematogeneous metastasis had a significantly lower level of KiSS-1 expression compared with lymph node metastasis. Metastin treatment significantly reduced the invasiveness of MBT-2V cells and inhibited the DNA-binding activity of NF-κB by blocking its nuclear translocation, leading to a reduction in the expression and activity of matrix metalloproteinase-9. Metastin treatment dramatically prevented the occurrence of lung metastatic nodules (6.3 ± 2.3, n = 15) compared with controls (30.4 ± 5.1, n = 15; P < 0.01), as well as had survival benefit. KiSS-1 plays an important role in the prognosis of upper tract urothelial carcinoma and metastin may be an effective inhibitor of metastasis in urothelial carcinoma through its blockade of NF-κB function. Mol Cancer Ther; 11(4); 853–63. ©2012 AACR.

Introduction

Bladder cancer is the most common neoplasm occurring along the urothelium. On the contrary, upper tract urothelial carcinoma is very rare, accounting for approximately 5% of all urothelial carcinomas (1). The prognosis of urothelial carcinoma patients with locally advanced and/or positive lymph nodes is poor. Five-year survival rates for advanced bladder cancer were reported to be 18% to 29% in patients with positive lymph nodes, and 34% to 41% in those with pT3/4 disease (2). Meanwhile, 5-year survival rates for advanced upper tract urothelial carcinoma were reported to be 35% in patients with positive lymph nodes, and 12% to 54% in patients with pT3/4 disease (3). Systemic chemotherapy with a cisplatin-containing regimen is often proposed for patients with metastatic or locally advanced urothelial carcinoma. Cisplatin-based chemotherapy has a short-term therapeutic effect against metastatic urothelial carcinoma with a response rate of about 50%; however, longer survival after receiving systemic chemotherapy is low, with a 5-year survival rate of only 13% to 15% (4–6). Furthermore, cisplatin-based chemotherapy sometimes has severe side effects, and especially in upper tract urothelial carcinoma, dose reduction must be considered because of the loss of renal function that occurs after nephroureterectomy. Thus, a new therapeutic strategy for treating highly aggressive urothelial carcinoma is strongly warranted.

The KiSS-1 gene has been reported as a metastasis suppressor gene in human melanoma (7). The gene product kisspeptin was isolated from human placenta as the endogenous ligand of an orphan G protein–coupled receptor known as GPR54 (8). KiSS-1 encodes a 145–amino acid peptide, which is further processed to a C-terminally amidated peptide with 54 amino acids (metastin; ref. 9), 14-amino acid peptide (kisspeptin-14), 13-amino acid peptide (kisspeptin-13), and 10-amino acid peptide (kisspeptin-10; refs. 8, 10).

The roles of KiSS-1 expression and GPR54 expression in tumor progression and metastasis have been evaluated in some types of cancer. It was reported that KiSS-1 plays a tumor suppressor role in melanoma, thyroid cancer, esophageal cancer, gastric cancer, and bladder cancer.
(11–15). Meanwhile, the opposite has been reported in breast cancer and hepatocellular carcinoma (16–18). GPR54 was reported to be a tumor suppressor in esophageal cancer and ovarian cancer (13, 19), whereas a study in hepatocellular carcinoma showed the opposite result (17). Still, the clinical importance of the association between KiSS-1 expression and the biologic features of urothelial carcinoma. Sanchez-Carbayo and colleagues showed that low KiSS-1 expression was a significant risk factor for vascular invasion and was associated with poor survival in bladder tumor patients (15). However, there is no information of clinical significance about KiSS-1 expression and GPR54 expression in upper tract urothelial carcinoma.

Only one previous paper has evaluated the inhibitory effect of metasin in tumor metastasis in an in vivo model (9). Ohtaki and colleagues showed that metasin inhibited pulmonary metastasis in a melanoma animal model. After gene manipulation of the transfection of GPR54 into B16-BL6 melanoma cells, the mice were inoculated in the footpads with these cells and then administered metasin. The results showed that pulmonary metastases were significantly decreased by metasin treatment. No study has evaluated whether metasin has an inhibitory effect on tumor metastasis without any genetic manipulation in an in vivo model.

In this study, we evaluated (i) the association between the tumor characteristics of 151 cases of upper tract urothelial carcinoma and the expression of KiSS-1 and GPR54 by immunohistochemistry to determine the clinical role of KiSS-1 and GPR54 expression in tumor metastasis and survival in upper tract urothelial carcinoma. We also examined (ii) the inhibitory effect of metasin on the invasive ability of a highly metastatic murine bladder cancer cell line, MBT-2 variant (MBT-2V), which was established from a parent MBT-2 tumor with multiple lung metastases (20), and (iii) whether metasin could prevent lung metastasis and have survival benefit in a MBT-2V bladder cancer lung metastasis model without any genetic manipulations.

**Materials and Methods**

**Cell culture, agents, and animals**

MBT-2V cells were established and characterized by our laboratory researchers, Horinaga and colleagues (20). The MBT-2V cells are maintained in RPMI-1640 (Invitrogen) with 10% FBS at 37°C in a humidified 5% CO2 atmosphere and are always available in our laboratory. Metasin (C258H400N78O79) was generously provided by Takeda Pharmaceutical. Eight-week-old female C3H/HeN mice were purchased from Sankyo Laboratory Service. Mice were kept under standardized laboratory conditions with free access to food and water.

**Tissue samples**

A total of 151 patients who had been surgically treated for upper tract urothelial carcinoma at Keio University Hospital from 1987–2004 were enrolled in the study. These patients did not undergo any chemotherapy or radiation therapy before surgery. The median follow-up was 58 months (range: 4–247 months) and the mean patient age was 65.9 years (range: 41–89 years). Nephroureterectomy with a bladder cuff was the most common procedure (147 patients, 97.4%). A partial ureterectomy was done in 4 patients. Regional lymphadenectomy was generally carried out in patients with suspicious lymph nodes on preoperative axial imaging or with adenopathies detected during intraoperative examination. Extended lymphadenectomy was not routinely done. The specimens were fixed with 10% formalin and embedded in paraffin. All slides were reviewed by a genitourinary pathologist. Tumors were staged according to the American Joint Committee on Cancer–Union Internationale Contre le Cancer TNM classification. Tumor grading was assessed according to the 2004 WHO/International Society of Urologic Pathology consensus classification (21). Lymphovascular invasion (LVI) was defined as the presence of tumor cells within an endothelium-lined space without underlying muscular walls. The study was conducted subject to the guidelines of the Declaration of Helsinki and had no influence on the treatment of the patients. The Keio University Hospital ethical committee granted ethical approval for the study (ID: UMIN000003066).

**Immunohistochemistry**

A paraffin section (4-μm thick) of one representative paraffin block of the upper tract urothelial carcinoma patients and a cytopsin slide of MBT-2V cells were used. The paraffin sections were deparaffinized in xylene and dehydrated in a graded ethanol series and an antigen retrieval procedure was carried out by heating the slides in 10 mmol/L citrate buffer (pH 6.0) at 121°C for 10 minutes. The endogenous peroxidase activity was blocked by incubation with 1% hydrogen peroxide/methanol for 15 minutes. MBT-2V cells were centrifuged onto a glass microscope slide, air dried, and then fixed in acetone for 15 minutes.

To evaluate KiSS-1 expression, after incubation with 10% normal goat serum for upper tract urothelial carcinoma and 10% normal horse serum for MBT-2V, the sections were incubated at 4°C overnight with primary KiSS-1 antibody (for upper tract urothelial carcinoma: 1:35 dilution, Takeda Pharmaceutical, for MBT-2V: 1:1,000 dilution; Phoenix Pharmaceuticals). After washing with PBS, they were incubated with secondary antibodies for 30 minutes (for upper tract urothelial carcinoma: no dilution, antimouse ABC; Santa Cruz Biotechnology, for MBT-2V: no dilution, antirabbit ImmPRESS; Vector Laboratories). To evaluate GPR54 expression, after incubation with 10% normal horse serum, the sections were incubated at 4°C overnight with primary GPR54 antibody for upper tract urothelial carcinoma and MBT-2V (for upper tract urothelial carcinoma: 1:400 dilution, for MBT-2V: 1:1,000 dilution;
Real-time PCR

To evaluate the relative mRNA expression, total RNA of murine placental tissue and MBT-2V cells treated with metastin or medium alone for 24 hours was isolated using RNA iso (TAKARA BIO), and the quantity and quality were evaluated by spectrophotometry. Reverse transcription of RNA to cDNA was done using a PrimerScript 1st strand cDNA Synthesis Kit (TAKARA BIO). Quantitative gene expression was conducted for KiSS-1 (Mm00617576_m1), GPR54 (Mm00475046_m1), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Mm99999915_g1) with gene-specific probes (Applied Biosystems) using a TaqMan Universal Probe Master Mix and a StepOne Real-Time PCR System (Applied Biosystems). The KiSS-1, GPR54, and MMP-9 to 18S ribosomal RNA (Mm432636_d1) expression was conducted for KiSS-1 (Mm00617576_m1), GPR54 (Mm00442991_m1), and GAPDH (Mm00475046_m1), matrix metalloproteinase (MMP)-9 (Mm00442991_m1), and GAPDH (Mm00475046_m1), matrix metalloproteinase (MMP)-9 (Mm00442991_m1), and GAPDH (Mm00475046_m1) to 18S ribosomal RNA (Mm432636_d1) expression were calculated. In the total patient population, the mean percentage of KiSS-1 and GPR54 staining in the population of cancer cells was 25% and 85%. KiSS-1 staining in less than 25% and GPR54 staining in less than 85% were defined as low KiSS-1 expression and low GPR54 expression, respectively. Immunoreactivity for KiSS-1 and GPR54 was analyzed independently by 2 uropathologists. The associations between KiSS-1 or GPR54 and clinicopathologic features were assessed with the χ² test and Mann–Whitney U test. Metastasis-free survival and overall survival curves were constructed using the Kaplan–Meier method and were compared using the log-rank test. The prognostic factors assessed were age, gender, tumor grade, pathologic tumor stage (pT), LVI, tumor location, KiSS-1 expression, and GPR54 expression. The Cox proportional hazards model with stepwise forward selection was used to assess the prognostic indicators for survival, and P < 0.05 indicated statistical significance. These analyses were carried out with the STATA version 9.2 statistical software package (Stata Corporation).

Immuno histochemical evaluation in human samples

To evaluate the staining of KiSS-1 and GPR54, the number of cancer cells with positive staining in the cytoplasm in at least 3 representative fields (× 400 magnification) and the mean percentage of the positive cancer cells were calculated. In the total patient population, the mean percentage of KiSS-1 and GPR54 staining in the population of cancer cells was 25% and 85%. KiSS-1 staining in less than 25% and GPR54 staining in less than 85% were defined as low KiSS-1 expression and low GPR54 expression, respectively. Immunoreactivity for KiSS-1 and GPR54 was analyzed independently by 2 uropathologists. The associations between KiSS-1 or GPR54 and clinicopathologic features were assessed with the χ² test and Mann–Whitney U test. Metastasis-free survival and overall survival curves were constructed using the Kaplan–Meier method and were compared using the log-rank test. The prognostic factors assessed were age, gender, tumor grade, pathologic tumor stage (pT), LVI, tumor location, KiSS-1 expression, and GPR54 expression. The Cox proportional hazards model with stepwise forward selection was used to assess the prognostic indicators for survival, and P < 0.05 indicated statistical significance. These analyses were carried out with the STATA version 9.2 statistical software package (Stata Corporation).

Real-time PCR

Total RNA of murine placental tissue and MBT-2V cells treated with 10⁻⁶ mol/L of metastin or medium alone (control) for 24 hours was isolated using RNA iso (TAKARA BIO), and the quantity and quality were evaluated by spectrophotometry. Reverse transcription of RNA to cDNA was done using a PrimerScript 1st strand cDNA Synthesis Kit (TAKARA BIO). Quantitative gene expression was conducted for KiSS-1 (Mm00617576_m1), GPR54 (Mm00475046_m1), matrix metalloproteinase (MMP)-9 (Mm00442991_m1), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Mm99999915_g1) with gene-specific probes (Applied Biosystems) using a TaqMan Universal PCR Master Mix and a StepOne Real-Time PCR System (Applied Biosystems). The KiSS-1, GPR54, and MMP-9 to GAPDH mRNA ratios were calculated for each sample to evaluate the relative mRNA expression.

Cell proliferation assay

A water-soluble tetrazolium salt (WST-1) assay (Roche) was used to evaluate cell viability. Briefly, MBT-2V cells were seeded in 96-well plates at a density of 2 x 10⁴ cells per well and incubated for 24 hours. The cells were then washed twice with PBS and given fresh medium containing various concentrations of metastin (10⁻⁷, 10⁻⁶, and 10⁻⁵ mol/L). Cells treated with medium alone served as controls. After incubation for 24, 48, and 72 hours, 10 µL of WST-1 solution was added to each well and incubation continued for 30 minutes. Absorbance was measured with a Microplate reader (Bio-Rad) at a reference wavelength of 450 nm.

In vitro cell invasion assay

MBT-2V cell invasion assay was assessed by counting the number of cells that migrated through transwell inserts with a polyethylene terephthalate membrane (8-µm pore size) coated with a uniform layer of BD Matrigel Basement Membrane Matrix (BD Biosciences), according to the protocol recommended by the manufacturer. Briefly, 5 x 10⁴ MBT-2V cells treated with 10⁻⁷, 10⁻⁶, and 10⁻⁵ mol/L of metastin or medium alone (control) for 24 hours in 0.5 mL RPMI-1640 with 1% FBS were seeded in the upper chamber, whereas the lower chamber was loaded with 0.75 mL RPMI-1640 with 10% FBS. After 48 hours at 37°C in a well-humidified incubator, cells remaining inside the inserts were removed, and the cells that had invaded into the reverse side of the inserts were rinsed and counterstained using a Diff-Quik stain kit (Sysmex). The cells that had migrated through the membranes were quantified by determination of the number of cells in 3 randomly chosen visual fields at ×200 magnification.

Zymography

MBT-2V cells treated with 10⁻⁶ mol/L of metastin or medium alone (control) were incubated for 24 hours. MMP-9 enzyme activity was evaluated using a Gelatin Zymography Kit (Cosmo Bio). Briefly, 15 µL of each supernatant diluted with 15 µL of zymogram sample buffer and each mixture was subjected to electrophoresis. After electrophoresis, the gel was incubated at room temperature for 1 hour on a rotary shaker in a rinse buffer and subsequently at 37°C overnight in a zymogram reaction buffer. The gel was stained for 30 minutes and then destained. Bands of enzyme activity were detected as a white zone in a dark field.

Western blot analysis

Proteins were extracted from the cytoplasm and nucleus separately in MBT-2V cells treated with 10⁻⁶ mol/L of metastin or medium alone (control) for 24 hours. Each extracted protein (20 µg) was subjected to electrophoresis on 12.5% SDS–polyacrylamide gel and transferred to a polyvinylidene fluoride membrane under 200 mA for 1 hour. The filter was blocked with TBS–TWEEN containing 5% nonfat milk, at 4°C overnight, and then incubated for 1 hour with the primary antibodies for NPIB-65, Lamin
A/C (1:300 dilution; Santa Cruz Biotechnology), or α-tubulin (1:2,000 dilution; Santa Cruz Biotechnology). The filters were then incubated for 1 hour with anti-mouse-IgG–horseradish peroxidase or antirabbit-IgG–horseradish peroxidase antibodies (Sigma Chemical). Finally, the proteins were visualized on X-ray film using an electrochemiluminescence (ECL) Western blotting detection kit (PerkinElmer Life Science).

Electrophoretic mobility shift analysis

MBT-2V cells treated with 10^{-6} mol/L of metastin or medium alone (control) were incubated for 24 hours. The binding reaction mixture consisted of 5 µg of extracted nuclear protein, 2 µg of poly deoxyinosinic–deoxyctydilic acid, and 10,000 cpm ³²P-labeled probe. Binding buffer (75 mmol/L NaCl, 1.5 mmol/L EDTA, 1.5 mmol/L dithiothreitol, 7.5% glycerol, 1.5% NP-40, 15 mmol/L Tris-HCl; pH 7.0) was added, and the mixture was incubated for 20 minutes at room temperature and separated from free DNA on 4% native polyacrylamide gels. The following sequence was used as an NF-κB probe: 5'-ATG TTG AGG GGA CTT TCC CAG GCC-3'.

In vivo experiment

All animal experiments were reviewed and approved by our Institutional Animal Care Committees. Lung metastases were generated by injecting 5 × 10⁵ MBT-2V cells in 200 µL of PBS into the tail vein of female C3H/HeN mice on day 0. Daily intraperitoneal administration of metastin (1.17 mg/kg) or PBS to a total volume of 200 µL was started on day 1 (n = 15 in each group). The mice were then sacrificed on day 26, and the number of metastatic nodules on the lung surface was counted macroscopically. Lung tissues were fixed in 10% formalin, embedded in paraffin, and assessed pathologically stained with hematoxylin and eosin (H&E). To evaluate the side effects of metastin, we examined the change in body weight of the mice, as well as laboratory tests. We also analyzed the histologic appearance of major organs, including heart, liver, kidney, spleen, pancreas, and ovary.

The second set of experiments was done for the survival benefit with metastin treatment in established lung metastasis model. Our previous study showed that lung metastases were established 10 days after MBT-2V cell injection. For evaluating survival benefit of metastin in in vivo
KiSS-1 expression (mean ± SE) was 48.3 ± 4.1%, 48.7 ± 4.1%, 28.0 ± 5.7%, 25.9 ± 3.2%, and 22.2 ± 13.5% in Ta, T1, T2, T3, and T4, respectively, whereas the corresponding values for GPR54 expression were 70.4 ± 4.6%, 74.4 ± 5.8%, 78.5 ± 5.2%, 75.7 ± 2.8%, and 65.0 ± 10.8%. The associations between the KiSS-1 and GPR54 expression, and the clinicopathologic features are summarized in Table 1. KiSS-1 expression was associated with tumor grade (P < 0.001), pT stage (P < 0.001), and positive or negative LVI (P < 0.001), whereas GPR54 expression was associated with age (P = 0.020).

Representative samples stained with KiSS-1 and GPR54 antibody are shown in Fig. 1. Strong cytoplasmic KiSS-1 staining was observed in pTa low-grade LVI(−) tumors (Fig. 1C). In contrast, pT3 high-grade LVI(+) tumors showed no apparent cytoplasmic KiSS-1 staining (Fig. 1E). Cytoplasmic GPR54 staining was observed in both pTa and pT3 tumors (Fig. 1D and F).

**Prognostic significance of KiSS-1 expression on tumor metastasis**

Overall, 44 (29.1%) patients had distant metastases during follow-up; 39.1% (27/69) with low KiSS-1 expression compared with 20.7% (17/82) with high KiSS-1 expression. Univariate analysis revealed that pT2 or greater, positive LVI, and low KiSS-1 expression were significant risk factors for metastasis (Table 2). Multivariate analysis showed that pT2 or greater (P = 0.013), positive LVI (P < 0.001), and low KiSS-1 expression (P = 0.028) were independent predictors for metastasis. However, GPR54 expression was not selected. The 5-year metastasis-free survival rates were 60.9% for patients with low KiSS-1 expression and 81.0% for patients with high KiSS-1 expression (P = 0.012; Fig. 1G).

We further evaluated the association between KiSS-1 or GPR54 expression and the initial metastatic pattern. Eleven patients had lymph node metastases, 25 patients had hematogeneous metastases including lung, liver, and bone, and 8 had both metastatic patterns at the initial presentation of metastasis. KiSS-1 expression in patients with hematogeneous metastases was 23.3 ±

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**Table 1. Association between KiSS-1 or GPR54 expression and clinicopathologic features**

<table>
<thead>
<tr>
<th></th>
<th>KiSS-1 expression</th>
<th>GPR54 expression</th>
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<tbody>
<tr>
<td></td>
<td>&lt;25</td>
<td>&lt;85</td>
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<tr>
<td>Overall</td>
<td>69</td>
<td>73</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>33</td>
<td>0.349</td>
</tr>
<tr>
<td>≥65</td>
<td>36</td>
<td>0.497</td>
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<tr>
<td>Gender</td>
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<td>Male</td>
<td>53</td>
<td>0.856</td>
</tr>
<tr>
<td>Female</td>
<td>16</td>
<td>0.180</td>
</tr>
<tr>
<td>Tumor grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>High</td>
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<td>0.488</td>
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<td>Pathologic stage</td>
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<tr>
<td>&lt;T2</td>
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<td>&lt;0.001</td>
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<tr>
<td>T2 ≤</td>
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<td>0.326</td>
</tr>
<tr>
<td>LVI</td>
<td></td>
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</tr>
<tr>
<td>Negative</td>
<td>26</td>
<td>0.732</td>
</tr>
<tr>
<td>Positive</td>
<td>43</td>
<td>0.385</td>
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<tr>
<td>Tumor location</td>
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<tr>
<td>Pelvis</td>
<td>45</td>
<td>0.083</td>
</tr>
<tr>
<td>Ureter</td>
<td>24</td>
<td>0.408</td>
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**Table 2. Univariate and multivariate analyses for metastasis-free survival and overall survival**

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<th>Metastasis-free survival</th>
<th>Overall survival</th>
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<tr>
<td></td>
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<td>Multivariate</td>
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<tr>
<td>Age (65 vs. ≥65)</td>
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<td>Gender (male vs. female)</td>
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<tr>
<td>Tumor grade (high vs. low)</td>
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<tr>
<td>Pathologic stage (T2 ≤ vs. &gt;T2)</td>
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<td>0.013</td>
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<tr>
<td>LVI (yes vs. no)</td>
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<td>&lt;0.001</td>
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<tr>
<td>Ureter vs. ureter</td>
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<td>0.028</td>
</tr>
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<td>GPR54 (≤85 vs. 85 ≤)</td>
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*KiSS-1 expression and clinicopathologic features in 151 upper tract urothelial carcinoma patients*

Cytoplasmic KiSS-1 and GPR54 staining was observed in the nonneoplastic urothelial epithelia (Fig. 1A and B).
4.9%, which was significantly lower than in those with lymph node metastases (44.3 ± 7.4%, P = 0.011). GPR54 expression was 74.4 ± 4.1% with hematogeneous metastases and 79.0 ± 6.4% with lymph node metastases. The difference was not statistically significant (P = 0.427).

Prognostic significance of KiSS-1 expression on overall survival
Overall, 48 (31.8%) patients died during follow-up; 39.1% (27/69) with low KiSS-1 expression compared with 25.6% (21/82) with high KiSS-1 expression. Univariate analysis revealed that high grade, pT2 or greater, positive LVI, and low KiSS-1 expression were significant risk factors of overall survival (Table 2). Multivariate analysis showed that high grade (P = 0.027), pT2 or greater (P = 0.012), positive LVI (P = 0.004), and low KiSS-1 expression (P = 0.021) were independent predictors of overall survival. However, GPR54 expression was not selected. The 5-year survival rates were 63.8% for patients with low KiSS-1 expression and 75.0% for patients with high KiSS-1 expression (P = 0.043; Fig. 1H).

mRNA and protein levels of KiSS-1 and GPR54 in MBT-2V cells
As shown in Fig. 2A, real-time PCR analyses revealed that high levels of KiSS-1 and GPR54 mRNA expression were detected in murine placental tissue, which was used as a positive control for KiSS-1 and GPR54. In MBT-2V cells, no KiSS-1 mRNA expression was observed, however, GPR54 mRNA was detected. Immunohistochemical analyses showed that no KiSS-1 protein expression was observed but cytoplasmic GPR54 protein expression was detected in MBT-2V cells (Fig. 2B). In murine placental tissue, strong staining KiSS-1 and GPR54 expressions were observed (data not shown). These results showed that MBT-2V cells had GPR54 expression but no KiSS-1 expression at the mRNA and protein levels.

Effects of metastin on cell proliferation in MBT-2V cells
As shown in Fig. 3A, the absorbance values in MBT-2V cells 24 hours after exposure to 10⁻⁷, 10⁻⁶, and 10⁻⁵ mol/L of metastin were 0.234 ± 0.013, 0.297 ± 0.014, and 0.289 ± 0.013, respectively. The differences were not significant when compared with the vehicle control (0.300 ± 0.013). Similar results were observed 48 and 72 hours after treatment with metastin. These results showed that metastin itself does not affect MBT-2V cell proliferation at the concentrations tested in the proliferation assay.

Effects of metastin on invasive ability in MBT-2V cells
As shown in Fig. 3B, the number of cells that had invaded was 190.6 ± 4.1, 99.2 ± 2.7, 29.0 ± 1.1, and 4.6 ± 0.4 in vehicle control, 10⁻⁷, 10⁻⁶, and 10⁻⁵ mol/L of metastin, respectively. MBT-2V cell invasion was inhibited by metastin in a dose-dependent manner. Representative samples are shown in Fig. 3C.

Expression and activity of MMP-9 and NF-κB after metastin treatment
We analyzed MMP-9 mRNA expression using real-time PCR and its activity by zymography. MMP-9 mRNA expression in MBT-2V cells was significantly reduced 24 hours after treatment with metastin (10⁻⁶ mol/L) compared with control (P < 0.05; Fig. 4A). The results of zymography revealed that MMP-9 gelatinolytic activity was reduced by metastin treatment (Fig. 4B). We further evaluated the effect of metastin on NF-κB activity by electrophoretic mobility shift assay (EMSA). As shown in Fig. 4C, DNA-binding activity was inhibited 24 hours after exposure to metastin (10⁻⁶ mol/L). Western blot analysis revealed that nuclear NF-κB (p65) protein expression was decreased after 24 hours of exposure to metastin (10⁻⁶ mol/L) compared with that of the vehicle control (Fig. 4D). In contrast, cytoplasmic NF-κB (p65)
expression increased. Translocation of NF-κB into the nucleus was reduced by metastin treatment. Metastin suppressed lung metastasis in murine lung metastasis model

In the first set of in vivo experiments, the number of metastatic lung nodules (mean ± SE) on the lung surface in mice treated with metastin was 6.3 ± 2.3 (n = 15), which was significantly lower than in mice treated with vehicle control (30.4 ± 5.1, n = 15; P < 0.01; Fig. 5A). Representative samples are shown in Fig. 5B. Histopathologic examination of H&E-stained sections also showed that metastatic lung nodules of the lung in control mice were much more prominent compared with those in treated mice (Fig. 5C).

With regard to the side effects of metastin, no abnormal weight loss or abnormal laboratory data were observed in the mice treated with metastin. We also analyzed histologically the major organs, including heart, liver, kidney, spleen, pancreas, and ovary by preparing H&E-stained sections. There were no significant differences in the histologic appearance or damage to these organs between the control and treated mice.

In the second set of in vivo experiments, metastin treatment resulted in a 40% animal survival over a 40-day follow up period compared with a 0% animal survival for control group (P < 0.01).

Discussion

In this study, we investigated 151 cases of upper tract urothelial carcinoma and analyzed the impacts of the expression of KISS-1 and GPR54 by immunohistochemistry. Our results indicated that low KISS-1 expression, which...
was closely related to tumor grade, pT, and LVI, was a significant risk factor for metastasis and overall survival of upper tract urothelial carcinoma. However, GPR54 expression, which was closely related to age and was expressed in most upper tract urothelial carcinoma, was not a prognostic indicator. To the best of our knowledge, this is the first study to examine the expression patterns of KiSS-1 and GPR54 in upper tract urothelial carcinoma specimens. Although GPR54 was not found to be a prognostic indicator of upper tract urothelial carcinoma, KiSS-1 product, metastin, has been reported to affect binding to GPR54, so urothelial carcinoma that express GPR54 to a high degree may become a therapeutic target of metastin. Sanchez-Carbayo and colleagues analyzed the association between KiSS-1 expression and the clinicopathologic characteristics of bladder tumors (15). They showed that low KiSS-1 expression was significantly associated with worse overall survival in 69 bladder cancer patients. KiSS-1 expression was significantly lower in bladder tumors with vascular invasion compared with normal urothelium. Furthermore, they observed that all bladder tumors developing distant metastases showed a complete loss of KiSS-1. In this study, we also evaluated the association between KiSS-1 expression and the initial metastatic pattern in patients with metastatic upper tract urothelial carcinoma. Interestingly, KiSS-1 expression in patients with hematogenous metastases was significantly lower than in those with lymph node metastases. These results confirmed the results of Sanchez-Carbayo and colleagues showing that KiSS-1 expression in tumor specimens might be a biomarker, especially for predicting the occurrence of hematogenous distant metastases after surgical treatment in highly aggressive urothelial carcinoma. Nicolle and colleagues evaluated KiSS-1 and GPR54 expression in 64 human bladder tumors by real-time PCR. They showed that KiSS-1 expression was not associated with tumor grade, but that GPR54 expression was significantly higher in high-grade tumors than in low-grade tumors (22). Their conclusion was not the same as ours, however, as they only evaluated the association between KiSS-1 expression and tumor grade at the mRNA level.

To evaluate the effectiveness of metastin in in vitro and in vivo studies, we used MBT-2V cells, which are highly metastatic murine bladder cancer cells, after confirming GPR54 expression. In the MBT-2V bladder cancer lung metastasis model, metastin treatment dramatically reduced lung metastatic nodules and significantly prolonged mouse survival. The side effects of metastin were also evaluated; however, we did not observe any abnormal weight loss or abnormal laboratory data. There were also no definite differences in the histologic appearance of major organs between the control and treated mice. In this study, metastin inhibited tumor invasion but did not affect cell proliferation. Several other reports have also found that kisspeptin did not affect tumorigenicity (9, 23).

The mechanism by which the KiSS-1/GPR54 system regulates tumor progression is still unclear. Ohtaki and...
colleagues initially showed that metastin changed the cellular morphology of B16-BL6 melanoma cells by forming focal adhesion and stress fibers (9). After their study, various other groups reported downstream signaling pathways activated by kisspeptin (8, 12, 24–27). Yan and colleagues previously revealed that KiSS-1 expression reduced NF-κB binding to the MMP-9 promoter (28). In this study, we evaluated MMP-9 mRNA expression and gelatinolytic activity, as well as NF-κB protein expression and the activity after metastin treatment. Our results indicated that MMP-9 expression and the activity were inhibited, and translocation of NF-κB into the nucleus was reduced by treatment with metastin. We believe the reduction of MMP-9 expression and its activity may be caused by a reduction in the translocation of NF-κB into the nucleus by the metastin treatment.

MMPs are a family of proteolytic enzymes that degrade the extracellular matrix or components of the basement membrane. Several reports have shown that elevated MMP expression in tumor tissues is correlated with tumor stage, grade, or prognosis. With respect to urothelial carcinoma, it was previously shown that MMP-2 and MMP-9 are expressed at higher levels in invasive bladder tumors than in superficial bladder tumors (29). Other reports have shown that MMP-9, but not MMP-2, is correlated with invasive bladder tumors (30, 31). MBT-2V cells, which are highly metastatic murine bladder cancer cells, were established from parent MBT-2 tumors with multiple lung metastases, and it was shown that MMP-9 might be associated with the metastatic ability of MBT-2V cells (20). These findings suggest that metastin inhibits lung metastasis by reducing MMP-9, especially in the MBT-2V lung metastasis model.

NF-κB is associated with resistance to apoptosis, the expression of angiogenic proteins, and carcinogenesis because of its fundamental effects on cellular dedifferentiation and proliferation (32). It was previously noted that NF-κB nuclear expression had a significant effect on

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**Figure 5.** A, lung metastases were generated by injecting $5 \times 10^5$ MBT-2V cells in 200 μL of PBS into the tail vein of female C3H/HeN mice on day 0. Daily intraperitoneal administration of metastin (1.17 mg/kg) or PBS to a total volume of 200 μL was started on day 1 ($n = 15$ in each group). The mice were then sacrificed on day 26, and the number of metastatic nodules on the lung surface was counted macroscopically (***, $P < 0.01$). B, representative extracted lung in mice treated with or without metastin. Their lungs were stained intratracheally with 15% India Black Ink solution. C, microscopic findings in the H&E-stained slides of the lung tissue in mice treated with or without metastin (×20 magnification). D, Kaplan-Meier curve between the metastin treatment group (dotted line) and control group (straight line). In the established lung metastasis model, intraperitoneal administration of metastin (1.17 mg/kg) or PBS was started on day 10 ($n = 15$ in each group).
tumor invasiveness and aggressive behavior in bladder cancer patients (33). Another report showed that highly metastatic bladder cancer cells constitutively express high levels of NF-xB, whereas less aggressive bladder cancer cells express lower levels of NF-xB activity (34). These findings suggest that bladder cancer cells with NF-xB activity have strong malignant potential.

In summary, loss of KiSS-1 expression was a significant risk factor for tumor metastasis and was associated with poor survival among upper tract urothelial carcinoma patients. Metastin inhibited tumor invasive activity in vitro without affecting cell proliferation and dramatically inhibited pulmonary metastasis and prolonged mouse survival in the present bladder cancer lung metastasis model. Translocation of NF-xB into the nucleus was reduced by treatment with metastin, resulting in a reduction in MMP-9 expression and its activity. KiSS-1 expression in tumor specimens might be a clinical biomarker for predicting the outcome of urothelial carcinoma, and metastin might be a novel therapeutic modality for inhibiting the metastasis of urothelial carcinoma that has high metastatic potential.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Prognostic Role of KiSS-1 and Possibility of Therapeutic Modality of Metastin, the Final Peptide of the KiSS-1 Gene, in Urothelial Carcinoma

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