Abstract. Breast cancer is the most frequently diagnosed cancer in women. In these studies, a metastasis suppressor gene, KISS1 and its truncated fragment, were overexpressed in the breast cancer cell line MDA-MB-231. In addition, KISS1 expression was downregulated in MDA-MB-157 cell line using a KISS1-specific siRNA. The effects of KISS1 on breast cancer cells both in vivo and in vitro were then identified. Our results indicate that KISS1 can induce apoptosis and inhibit mobility of breast cancer cells. Moreover, the expression of KISS1 in established xenografted tumors was associated with a decrease in tumor size and weight. Accordingly, the survival rate of these mice was significantly higher compared to that of mice bearing tumors that did not express KISS1. We also confirm that KISS1 could decrease the number of circulating tumor cells (CTCs). The plasma levels of metastin and the number of CTCs were significantly positively correlated. Furthermore, we found that KISS1 can inactivate p-MEK and p-ERK. Overall, these studies demonstrate the antitumor activity of KISS1 in the breast cancer cell lines and provide insight into relevant mechanisms that may lead to novel treatments for breast cancer.

Introduction

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries (1). Breast cancer is the most frequently diagnosed cancer in females. Worldwide, more than a million women are diagnosed with breast cancer in 2008, accounting for 14% (458,400) of the total cancer deaths (2). Maintaining a healthy body weight, increasing physical activity and minimizing alcohol intake are the best available strategies to reduce the risk of developing breast cancer (3).

KISS1 was originally identified as a metastasis suppressor gene in human melanoma and breast carcinoma cells (4). The KISS1 gene is located on chromosome 1 near q32.1 with regulatory elements localized in chromosome 6 at 6q16.3-q23 (5). The KISS1 gene encodes a precursor protein that is processed into several related peptides, generically named kisspeptins (6,7), where the major product appears to be a 54-amino acid peptide, named kisspeptin-54 or metastin. In addition, three natural peptides of 14-, 11- and 10-amino acids have been also identified, sharing a common 10-amino acid C-terminal region (8). Cogent data support that loss of KISS1 expression has been associated with the progression and metastasis of various tumors, including esophageal, brain, breast, ovarian and melanoma (9).

KISS1 was recently shown epigenetically silenced by hypermethylation in bladder cancer (10) and colorectal cancer (11).

Circulating tumor cells (CTCs) captured from peripheral blood were recently shown to predict disease outcome and therapy response in cancer patients (12). The enumeration of CTCs at different time-points during treatment has proven to be a reliable surrogate marker of treatment response and a potential alternative for non-invasive therapy monitoring (13). EpCAM as well as cytokeratin expressing cells can be found in peripheral blood of advanced cancer patients but are rare in healthy donors (14). Breast cancer cells of all grades typically express the epithelial cytokeratins CK7, CK8, CK18 and CK19 (15).

In this report, we evaluated whether KISS-1 expression and methylation patterns differed in breast cancer cell lines, MDA-MB-231 and MDA-MB-157. Then we evaluated the inhibition potential of KISS1 protein on tumor growth in a breast cancer model, as evidenced by tumor size, tumor weight and circulating tumor cells. Furthermore, mechanism(s) of an integrated KISS1 protein, as well as one truncated KISS1 protein, were examined in a breast cancer model. It is anticipated that the results obtained will provide novel insight into potential strategies for the treatment of breast cancer.

Materials and methods

Cell culture. MDA-MB-231 and MDA-MB-157 cell lines were purchased from the American Type Culture Collection
(Rockville, MD, USA) and maintained in DMEM supplemented with 10% FCS and antibiotics (100 µM penicillin and 100 µM streptomycin). Cells were maintained in a humidified cell incubator with 5% CO₂ at 37°C.

**DNA extraction, sodium bisulfite conversion and methylation-specific PCR (MSP).** Cells were incubated with medium containing 10 µM 5-aza-2’-deoxycytidine (5-aza-dC) (Sigma-Aldrich, Carlsbad, CA, USA) for 48 h. Then we isolated DNA and carried out MSP as the method described below. Genomic DNA was extracted from cells using a TissueGen DNA kit according to the manufacturer's instructions (CWbiotech, Beijing, China). The concentration and purity of the DNA were determined by absorbance at 260 and 280 nm by NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Wilmington, NC, USA). Sodium bisulfite modification for the extracted DNA was performed using an EZ DNA Methylation™ kit strictly according to the manufacturer's instructions (Zymo Research, Orange, CA, USA). Sequencing results confirmed that >99.0% of cytosine residues were converted. The bisulfite-converted DNA was resuspended in 10 µl elution buffer and stored at -80°C until the samples were ready for analysis. The 5 µl PCR mixture contained 10 ng bisulfite-treated DNA, 25 mM dNTP, 0.2 U of Hot Start Taq DNA polymerase (Takara, Dalian, China) and a 1 µM mixture of forward and reverse primers. The primer sequences for the methylated KISS1 gene were 5’-CGGGTTGGAAGTTTTAGC-3’ (sense) and 5’-GCTTCGACAAACGAAAAAC-3’ (antisense) and for the unmethylated allele were 5’-TTTGGGGTTGGAAGT TTTAGT-3’ (sense) and 5’-ACTTCAACAAACAAAAAAC AAC-3’ (antisense). The PCR products were separated in 2% agarose gel with ethidium bromide and visualized under a UV imaging system (UVP, Upland, CA, USA).

**RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR).** RNA (1 µg) was reverse-transcribed using AMV reverse transcriptase (Promega, Madison, WI, USA) and amplified using specific primers and conditions for KISS1. The KISS1 primers used were: 5’-ATGAACTCAGAGGTTTGGGGAAGT TTTAGT-3’ (sense) and 5’-ACTTCAACAAACAAAAAAC AAC-3’ (antisense). GAPDH was used as an internal normalization control. The GAPDH primers used were: 5’-GAAGGCCT GGGCCTCATATTG-3’ (sense) and 5’-GGGGGCAATCCACA GTCTT-3’ (antisense). PCR amplification of cDNA was performed in reaction volumes of 15 µl. Finally, products were resolved by 1% agarose gel electrophoresis and visualized by ethidium bromide staining and a UV imaging system (UVP).

**siRNA and plasmid transfections.** MDA-MB-231 cells were seeded in 10-cm dishes and grown overnight to 70% confluency, trypsinized and transfected with siRNA targeting KISS1, or a non-targeting construct, using Amaxa nucleofector (Lonza, Portsmouth, NH, USA). For the preparation of truncated KISS1 proteins, the relevant sequences were amplified from full-length KISS1 by PCR using primers that included designed restriction sites (Table 1), then digested with the relevant restriction enzymes and ligated into pcDNA3.1. MDA-MB-231 cells were seeded overnight and transfected with either pcDNA3.1 or pcDNA-3.1-KISS1 fragments, using Lipofectamine™ 2000 according to the procedures of the manufacturer (Invitrogen, Carlsbad, CA, USA).

**Immunofluorescence assays.** Cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized in 2% Triton X-100 for 10 min, then blocked with 5% bovine serum albumin in PBS containing 2% Triton X-100 for 1 h. Anti-KISS1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were incubated with cells for 2 h at room temperature to identify the location of intact KISS1 proteins, or truncated KISS1 fragments. Cells were then washed with PBS and incubated with Alexa Fluor® 488 goat anti-rabbit IgG for 2 h at RT. To determine whether or not a lamellipodium was present, cells were stained for actin and actin-associated proteins using Fluorophore-conjugated phalloidin (Invitrogen). Cells were examined and photographed using a laser confocal microscope (Olympus, Tokyo, Japan).

**Apoptosis assay.** For the apoptosis assay, equal numbers of cells were seeded in 6-cm plates. Following the manufacturer's instructions (Apoptosis Detection kit, KeyGen, Nanjing, China), cells were trypsinized, washed twice with cold PBS, then resuspended in 200 µl binding buffer. Annexin V-FITC was added to a final concentration of 0.5 µg/ml. Additionally samples were incubated at room temperature in the dark. After 20 min, 300 µl binding buffer containing 0.5 µg/ml PI was added and samples were immediately analyzed on a FACS Calibur flow cytometer (Becton-Dickinson Medical Devices, Shanghai, China). Cells in the stages of early apoptosis were defined as FITC+/PI-.

**Gelatin zymography.** Fifty micrograms of protein was applied to 10% polyacrylamide gels with 1% gelatin incorporated as a substrate for gelatinolytic proteases. After running the gel the SDS was removed by washing twice in 2.5% Triton X-100 for 30 min. The gels were incubated overnight in zymography development buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM NaN₃, and 5 mM CaCl₂. After development the gels were stained for 3 h in 45% methanol/10% glacial acetic acid containing 1% (w/v) Coomassie Blue R-250 and subsequently destained with the same solution without dye. The gelatinolytic activity of each MMP was qualitatively evaluated as a clear band against the blue stained gelatin background.

**Xenograft assays.** NOD SCID mice (NOD.CB17-Prkdcscid/NcrCrl) that were 3-5-weeks-old were purchased from Charles River (MA, USA). All animals were housed four to a plastic cage with filter top. The animal room was controlled at 20±2°C, 50±10% humidity and a 12-h light/dark cycle. MDA-MB-231 cells (4x10⁵ in 200 µl) or MDA-MB-157 cells (5x10⁵ in 200 µl) were subcutaneously injected into the axilla of each mouse. After the tumor diameters reached 3-5 mm, the mice injected with MDA-MB-157 cells were divided randomly into three groups and received a 200 µl intratumoral injection of siRNA, or scramble. The mice injected with MDA-MB-231 cells were divided randomly into four groups and received a 200 µl intratumoral injection of pCDNA-3.1, pCDNA-3.1-KISS1-T1 or pCDNA-3.1-KISS1-T2. Two injections were administered at 9 a.m. and 4 p.m. every three days. Tumor growth was then monitored for 30 days. Every five days until the end of
the experiment, one mouse from each group was randomly selected to be sacrificed by CO2 asphyxiation and tumors were harvested and weighed. The tissue was fixed in 4% paraformaldehyde for histopathology analysis. All experiments with animals were performed according to the guidelines of China Medical University Ethics Committee.

**Immunohistochemical staining (IHC).** Immunohistochemical staining was performed on 4-μm sections obtained from formalin-fixed, paraffin-embedded blocks. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 min. Antigen retrieval was carried out in citrate buffer (10 mM, pH 6.0) for 30 min at 95°C in a microwave oven. Sections were incubated with primary antibody at 4°C overnight. Signaling pathway proteins were probed with: anti-ERK, anti-phospho-ERK, anti-MEK and anti-phospho-MEK antibodies (Santa Cruz). Afterward, sections were incubated with a biotinylated secondary antibody and then exposed to a streptavidin complex (HRP). Positive reactions were visualized with 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma), followed by counterstaining with hematoxylin. Sections treated without primary antibodies were used as negative controls.

**Survival curves.** Additional mice (n=140) were used to establish xenografts to obtain survival curves. Mice with xenografted tumors (as described above) that reached 3-5 mm in diameter were divided into seven groups (n=20 for each). Survival was monitored until the experiments were terminated due to heavy tumor burden.

**Isolation and enumeration of circulating tumor cells.** The CellSearch® system (Veridex LLC, Warren, NJ, USA) is the only US Food and Drug Administration-cleared test for CTC enumeration in clinical practice (16). Blood samples (7.5 ml) from breast mouse models were drawn into CellSave® tubes (Veridex LLC), which were maintained at RT and processed within 72 h of collection. CTCs were defined as nucleated EpCAM-positive cells, lacking CD45 but expressing cytoplasmic cytokeratins 8, 18 and 19. All CTC evaluations were performed by qualified and trained personnel.

**Enzyme-linked immuno sorbent assay (ELISA).** Blood samples for metastin determination were collected in ethylenediamine tetraacetate tubes, placed on ice and centrifuged at 3,000 rpm for 20 min. Recovered serum was stored at -80°C in aliquots until assayed. All samples were measured in duplicate. Circulating serum metatin was determined using a sandwich enzyme immunoassay (Human Metastin ELISA Phoenix Pharmaceuticals, Burlingame, CA, USA) with a sensitivity of 0.12 ng/ml.

**Statistical analysis.** Data are presented as the mean ± standard deviation (SD). Differences between groups were analyzed using Student’s t-test for continuous variables. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS, version 17.0; SPSS, Inc.) and significance was established at P<0.05.

**Results**

**The mRNA and protein levels of KISS1 were evaluated in MDA-MB-231 and MDA-MB-157 cells after treatment.** We compared endogenous KISS1 protein level in two breast cancer cell lines, MDA-MB-231 and MDA-MB-157. MDA-MB-231 cells showed relatively low KISS1 expression levels, whereas the MDA-MB-157 lines expressed relatively stronger KISS1 protein levels (Fig. 1A). A further link between methylation and gene silencing was established by the treatment of hypermethylated (MDA-MB-231) and hypomethylated (MDA-MB-157) cell lines with a DNA demethylating drug. Exposure of the two cell lines to 5-aza-2’-deoxycytidine (AZA) increased the expression of KISS1 at the transcript level (Fig. 1B). Based on the level of KISS1 protein in MDA-MB-231 and MDA-MB-157 cells, MDA-MB-231 cells were transfected with KISS1 fragments that included: integrated (aa 1-121) and truncated (aa 20-121). MDA-MB-157 cells were transfected with KISS1 siRNA. After transfection, KISS1 mRNA and protein levels were significantly changed compared to untransfected cells by using RT-PCR and western blotting, respectively (Fig. 1A). These results collectively suggested that the transfection was successful. Interesting, the results of immunofluorescence analysis showed integrated KISS1 protein was localized in the cytoplasm of transfected MDA-MB-231 cells, however, truncated KISS1 protein was mainly localized in the nucleus (Fig. 1C).

**The effects of KISS1 protein on breast cancer cells in vitro.** To detect apoptotic cells, Annexin V-FITC and PI double staining was performed. In MDA-MB-231 cells expressing intact or truncated KISS1, the apoptotic ratio was 4-5 times higher than that of untransfected ones (P<0.05; Fig. 2A). The effect of

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**Table I. Primers used to generate integrated and truncated forms of KISS1.**

<table>
<thead>
<tr>
<th>Region of KISS1 amplified</th>
<th>Primers (5’-3’)</th>
<th>Product (bp)</th>
</tr>
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<tbody>
<tr>
<td>aa 1-121 (integrated)</td>
<td>F, AAGCTTATGAACCTCACTGGTTTCTTGGC R, GGATTCCTTCGCGTCCGGCTTCAAG</td>
<td>381</td>
</tr>
<tr>
<td>aa 20-121 (truncated)</td>
<td>F, AAGCTTGGAGGCCATTA GAAAGGTG R, GGATTCCTTTCGCGTCCGGCTTCAAG</td>
<td>324</td>
</tr>
</tbody>
</table>

Italic indicates *Hin*dIII restriction sites; underlined indicates *Bam*HI restriction sites. F, forward; R, reverse.
KISS1-expression on the motility of MDA-MB-231 cells was determined by wound-healing assay and transwell assay. The percentage wound closure of the MDA-MB-231 cells transfected with intact (89.4%) or truncated KISS1 (88.7%) was decreased when compared to the untreated (66.4%) and the mock transfected (63.6%) cells (P<0.05; Fig. 2B). Transwell assay also showed that less KISS1 positive cells migrated to the lower side of the membrane than negative ones (P<0.05; Fig. 2C). Furthermore, we found the activity of MMP-2 and MMP-9 was inhibited by both intact and truncated KISS1 proteins (Fig. 1A, blue stripe). Conversely, MDA-MB-157 cells treated with KISS1 siRNA showed less apoptotic ratio than untreated ones (P<0.05; Fig. 2A). Motility was significantly increased in KISS1 siRNA treated cells compared to KISS1 scramble treated or untreated cells (P<0.05; Fig. 2B and C). Additionally, forced KISS1 overexpression could cause
Figure 2. The antitumor activities of KISS1 in vitro. (A) The proportion of apoptotic cells (early apoptosis) was determined by double-staining with Annexin V/FITC and PI. (B) Wound closure was quantified in the regions flanked by dotted lines.
weaker lamellipodia formation in MDA-MB-231 cells, labeled with phalloidin immunostaining. Downregulation of KISS1 protein in MDA-MB-157 cells showed stronger lamellipodia formation (Fig. 1C).

The antitumor activity of KISS1 protein in breast cancer mouse model. As shown above, KISS1 mediates an inhibitory effect on breast cancer cells in vitro. The antitumor properties of KISS1 were further evaluated using breast cancer mouse models. As shown in the Fig. 3A, upper panel, from days 10 to 60, compared to untreated MDA-MB-231 cells and mock treated ones, intact or truncated KISS1 treated cells had a significant lower tumor volume (P<0.05). Correspondingly, the weights of intact or truncated KISS1 treated tumors also were lower than that of untreated and mock treated tumors (P<0.05; Fig. 3A lower panel). In addition, the survival rate of mice with tumors treated with intact or truncated KISS1 was significantly improved. By the end of the experiment, 16/20 of mice in the intact or truncated KISS1 group were still alive, while all of the mice in the untreated and mock groups had died (Fig. 3B). Conversely, results using KISS1-knockdown MDA-MB-157 mouse models were comparable to results using the KISS1-upregulation MDA-MB-231 mouse models (P<0.05; Fig. 3).

Correlation of metastin serum levels or mouse model prognosis and CTCs. A statistically significant difference was observed between circulating metastin levels in each group (P<0.05; Fig. 3C). Intact KISS1 treated group showed a higher serum metastin level than untreated group and mock treated group. However, truncated KISS1 treated group showed no changes compared with untreated group (P>0.05; Fig. 3C). CTC detection remains a big technical challenge despite the continuing development of many new exciting technologies (17). In this study, we isolated and enumerated the circulating tumor cells in mouse models by using the CellSearch system. KISS1-positive models showed less CTCs in peripheral blood than KISS1-negative ones (P<0.05; Fig. 3D). The plasma level of metastin and the number of CTCs were significantly positively correlated (r=0.981, P<0.001; Fig. 3E). To further investigate the relationship between CTC number and prognosis we defined 3 risk groups, a low (CTC <10), a medium (CTC 10-30) and a high-risk group (CTC >30). A significantly different survival rate between the low and the medium risk as well as between the medium and the high-risk groups in the Cox model was confirmed (P<0.05; Fig. 3F).

KISS1 fragments suppress the MEK/ERK signaling pathway. To identify the mechanism of apoptosis induced by KISS1, immunohistochemical staining assays were performed to detect changes in possible signaling pathway proteins. While total levels of MEK and ERK showed no changes, the levels of phospho-MEK and phospho-ERK were observed to be significantly lower in KISS1-positive cells versus negative ones (Fig. 4). Interestingly, both intact and truncated KISS1 could inhibit the MEK/ERK signaling pathway in breast cancer cells. Activation of phospho-MEK and phospho-ERK were detected, while total levels of Ras, Raf, MEK and ERK were unchanged after MDA-MB-157 models treated with KISS siRNA (Fig. 4). In combination, these results suggest that KISS1 induced apoptosis in breast cancer cells by suppressing the MEK/ERK signaling pathway.
Discussion

Downregulation of KISS1 expression is able to increase tumor progression and poor prognosis in many cancers, such as pancreatic cancer, breast cancer, bladder cancer, brain cancer, epithelial ovarian cancer and gastric cancer (18-23). A recent study showed that KISS1 expression is decreased in human breast cancer, particularly in patients with aggressive tumors and with mortality (24). As noted in the introduction, kisspeptin is a product of the KISS1 gene which encodes a 145-amino acid precursor. Posttranslational modifications of this peptide result in a C-terminally amidated 54-amino acid peptide and several shorter fragments (e.g. kisspeptin-10, kisspeptin-13, kisspeptin-14) (6). However, the biological activities of these fragments were not very clear. In this study, we aimed to explicate the distinct roles of KISS1 different fragments in breast cancer cell. We studied two breast cancer cell lines, MDA-MB-231 and MDA-MB-157. Consistent with Teng et al (25), we confirmed MDA-MB-231 cells showed relatively low KISS1 expression levels, whereas the MDA-MB-157 expressed relatively stronger KISS1 protein levels. We found that KISS1 was aberrantly silenced by CpG island promoter hypermethylation. In bladder tumors (10) and colorectal tumors (11), the loss of KISS1 expression was attributed to epigenetic silencing by hypermethylation. Then we upregulated KISS1 protein expression in MDA-MB-231 cells by eukaryotic transfection and downregulated KISS1 protein expression in MDA-MB-157 cells by RNA interference. MDA-MB-231 cells after treatment showed a higher apoptotic ratio and a lower

Figure 3. KISS1 suppressed tumor growth in xenograft mouse models. (A) Tumor volume and tumor weights of each group described in Materials and methods that were sacrificed 20 days after the cells were injected. (B) Kaplan-Meier survival curves of the groups described in Materials and methods. (C) Serum metastin levels from xenograft mouse models were measured by using ELISA. (D) Circulating tumor cells (CTCs) in serum of each group were isolated by using the CellSearch system. (E) Negative correlation between serum levels of metastin and CTC in xenograft mouse models. (F) Kaplan-Meier plot estimating overall survival for 3 risk groups (<10 CTCs, 10-30 CTCs, >30 CTCs). All results were statistically significant (P<0.05).
mobility than untreated ones. Previous studies showed that an inverse relationship between of KISS1 and MMP-9 expressions (26,27). We also confirmed that upregulation of KISS1 expression leads to loss of MMP-9 expression in breast cancer cell lines.

The main purpose of this study was to identify the roles of KISS1 protein distinct domains. Especially, nuclear export signal of KISS1 protein has not been formally identified. Interestingly, we found KISS1 protein without residues 1-19 was unable to shift from nucleus to cytoplasm entirely. The results indicated that the nuclear export signal of KISS1 may be included in residues 1-19. However, KISS1 protein without residues 1-19 also showed antitumor activity. Both intact KISS1 protein and KISS1Δ20-121 induced apoptosis, inhibited mobility and caused weaker lamellipodia formation. In vivo, tumor volumes and tumor weights were found to be reduced in xenografts established from treated cells versus untreated cells. Correspondingly, survival times were also longer for treated cell xenograft models. Previous studies have shown that patients with negative expression of KISS1 protein were correlated with poor disease-specific survival by using Kaplan-Meier curve survival analysis (10,11).

Metastasis is a multi-stage process that selects for circulating tumor cells (CTCs) that can infiltrate, survive in and colonize distant organs (28). Several studies have suggested that the presence of CTCs that have survived therapy might reflect a failure of systemic therapy (29,30). Consistent with previous studies, we also found a negative correlation between the plasma level of metastin and the number of CTCs in xenograft models. These results provide valuable insight into potential novel treatments for breast cancer.

Acknowledgements

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