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Honokiol suppresses metastasis of renal cell carcinoma
by targeting KISS1/KISS1R signaling

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Abstract. Renal cell carcinoma (RCC) is a common urological cancer worldwide and is known to have a high risk of metastasis, which is considered responsible for more than 90% of cancer-associated deaths. Honokiol is a small-molecule biphenol isolated from Magnolia spp. bark and has been shown to be a potential anticancer agent involved in multiple facets of signal transduction. In this study, we demonstrated that honokiol inhibited the invasion and colony formation of highly metastatic RCC cell line 786-0 in a dose-dependent manner. DNA-microarray data showed the significant upregulation of metastasis-suppressor gene KISS1 and its receptor, KISS1R. The upregulation was confirmed by qRT-PCR analysis. Overexpression of KISS1 and KISS1R was detected by western blotting at the translation level as well. Of note, the decreased invasive and colonized capacities were reversed by KISS1 knockdown. Taken together, the results first indicate that activation of KISS1/KISS1R signaling by honokiol suppresses multistep process of metastasis, including invasion and colony formation, in RCC cells 786-0. Honokiol may be considered as a natural agent against RCC metastasis.

Introduction

Metastasis is the tendency of cancer cells to spread to distant organs in the body, which is considered responsible for more than 90% of cancer-associated deaths (1-4). It involves a multistep process including migration from the primary tumors, invasion to surrounding tissues, and proliferation leading to the colonization at distant sites (1,4). Accordingly, 25-30% of patients with renal cell carcinoma (RCC) have metastatic spread by the time they are diagnosed (5-7) and in these cases, the 5-year survival rate of patients is <10% (8,9). Moreover, 20-25% of suffers remain unresponsive to all treatments and the disease progresses rapidly (10,11). Honokiol, a small-molecule biphenolic compound isolated from Magnolia spp. Bark, has been shown to exhibit anticancer effects in different cancer types (12-17). The most widely investigated mechanism of its anticancer activities is apoptosis, which is induced in vitro and in vivo through multiple facets of signal transduction (12,14,16-25). Recently, several studies demonstrated that honokiol could also inhibit metastasis of breast, brain, gastric, lung and prostate cancer cells (13,21,26-32). However, only one study shows the metastasis suppression of RCC cells A-498 by honokiol through reversing epithelial-mesenchymal transition and blocking cancer stem cell properties (33). Definitely, there are other important targets involved in the process of RCC metastasis suppression by honokiol.

In this study, we found that honokiol inhibits the invasion and colony formation of highly metastatic RCC cells 786-0 (34) in a dose-dependent manner. DNA-microarray data showed significant upregulation of metastasis-suppressor gene KISS1 and its receptor, KISS1R. Both of the upregulation were confirmed by qRT-PCR analysis. Overexpression levels of KISS1 and KISS1R were detected by western blotting at the translation level as well. Of note, inhibition of invasion and colony formation were reversed by KISS1 knockdown. Taken together, our results indicate that honokiol suppresses the multistep process of metastasis, including invasion and colony formation, in RCC cells 786-0 via stimulation of KISS1/KISS1R signaling pathway.

Materials and methods

Cell culture and reagents. Human RCC cells 786-0 were obtained from ATCC (Manassas, VA, USA). Cancer cells were maintained according to the ATCC procedures. Honokiol (98%) (HonoPure®) was provided by Econugenics Inc. (Santa Rosa, CA, USA) and dissolved in DMSO at a concentration of 80 mM then stored at -20°C. DMSO was purchased from Sigma (St. Louis, MO, USA). Anti-KISS1, anti-KISS1R and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell invasion assay. Cell invasion of 786-0 cells treated with honokiol (0-20 µM) was performed as previously described (35).
Data points represent the mean ± SD of three individual filters within one representative experiment repeated at least twice.

Colony formation assay. Colony formation of the 786-0 cells incubated in the presence of honokiol (0–40 µM) was evaluated as previously described (36). Data points represent the mean ± SD in one representative experiment repeated at least twice.

**DNA-microarray and quantitative RT-PCR analysis.** The 786-0 cells were treated with honokiol (0–40 µM) for 24 h and TaqMan® Array Human Tumor metastasis was performed as previously described (37). In qRT-PCR analysis, the 786-0 cells were treated with honokiol (0–40 µM) for 24 h. Isolation, quantification, reverse transcription of RNA and PCR were performed as previously described (37). Relative quantity (RQ) of gene expression was normalized to β-actin and performed using the 2−ΔΔCT method (38).

**Western blot analysis.** The 786-0 cells were treated with honokiol (0–40 µM) for 24 h. Whole protein extracts isolated from cells were prepared and western blot analysis with KISS1 and KISS1R antibodies were performed as previously described (39). Western blots were quantified with HP-Scanjet 550c and analyzed by UN-SCAN-IT software (Silk Scientific, Orem, UT, USA).

**siRNA transfection.** The 786-0 cells were transfected with human KISS1 siRNA or control siRNA-A as previously described (37). After 48 h of transfection, the cells were harvested and KISS1 knockdown was verified by western blot analysis.

**Statistical analysis.** All the statistical analysis was performed using SigmaPlot 11.2.0 (Systat Software Inc., San Jose, CA, USA). Data are presented as mean ± SD. Statistical comparisons were carried out using ANOVA with the significance level adjusted using the repeated t-tests with Bonferroni correction. P-value <0.05 was considered to be significant.

## Results

**Honokiol inhibits invasion and colony formation of highly metastatic RCC cells.** To evaluate whether honokiol (Fig. 1) suppresses invasive behavior of highly metastatic RCC cells, the 786-0 cells were treated with honokiol (0-20 µM) for 24 h and cell invasion was determined as described in Materials and methods. As shown in Fig. 2A, honokiol inhibits cell invasion through Matrigel in a dose-dependent manner. Moreover, honokiol significantly decreases the number of anchorage-independent colonies formed, which is a key step in cancer metastasis (Fig. 2B and C). In summary, honokiol significantly inhibits invasion as well as colony formation of highly metastatic RCC 786-0 cells in a dose-dependent manner.

**Effect of honokiol on the expression of genes related to human tumor metastasis.** In order to gain further mechanistic insight into the molecular events underlying metastasis inhibition of the 786-0 cells treated with honokiol, DNA-microarray analysis of 92 tumor metastasis-associated genes and 4 candidate endogenous control genes was performed. Table I summarizes the genes with large recurring expression differences compared with control. For example, significant upregulation was observed including the expression of metastasis suppressor gene (KISS-1, 28.56±11.17), genes encoding TIMP metalloproteinase inhibitor 4 (TIMP4, 14.25±4.04) and KISS-1 receptor (KISS1R, 13.33±5.11). In addition, honokiol markedly suppresses expression of genes encoding chemokine (C-X-C motif) ligand 12 (CXCL12, 0.13±0.05), chemokine (C-C motif) ligand 7 (CCL7, 0.14±0.04), interleukin-18 (IL18, 0.23±0.04) and Matrix metalloproteinase 7 (MMP7, 0.26±0.09).

**Honokiol activates KISS1/KISS1R signaling in highly metastatic RCC cells.** Since recent studies showed that activation of KISS1/KISS1R signaling by kisspeptin treatment decreases the motility and invasive capacity of conventional RCC, and overexpression of KISS1 inhibits invasion of RCC cells Caki-1 (40,41), we confirmed the significant upregulation of KISS1 and KISS1R in the 786-0 cells treated with honokiol by qRT-PCR (Fig. 3). In accordance with the change in mRNA, western blot analysis showed that honokiol stimulates expression of KISS1 and KISS1R in the 786-0 cells dose-dependently at the protein level (Fig. 4).
Silencing KISS1 reverses suppression of invasion and colony formation. To determine whether the suppression of invasion and colony formation by honokiol are associated with the activation of KISS1/KISS1R signaling in the 786-0 cells, we silenced KISS1 with siRNA as described in Materials and methods. As shown in Fig. 5, knockdown of KISS1 partially rescues the effect of honokiol on cell invasion by more than 40%. Moreover, the effect of honokiol on colony formation of the 786-0 cells is markedly reversed by KISS1 silencing (Fig. 6). These results further indicate that KISS1/KISS1R signaling is a major target of honokiol in suppressing metastasis of RCC cells.

Discussion

In the present study, we investigated the role of honokiol in the metastasis of RCC cells. Our results showed that honokiol significantly inhibited the invasion and colony formation of highly metastatic RCC cells 786-0 in a dose-dependent manner. Moreover, honokiol markedly upregulated metastasis-suppressor gene KISS1 and its receptor, KISS1R, at both transcription and translation levels. Interestingly, knockdown of KISS1 partially rescued the effect of honokiol on cell invasion and its effect on colony formation of the 786-0 cells is reversed as well, indicating that KISS1/KISS1R signaling
is a major target of honokiol in suppressing metastasis of RCC cells.

Metastasis suppressors are defined as molecules whose expression results in the suppression of metastasis processes and since 1986, more than 13 metastasis suppressors have been identified (42). The *KISS1* gene, initially discovered as a novel human malignant melanoma metastasis-suppressor gene (43), has been validated as an anti-metastatic gene by preclinical and clinical evidence in various types of cancer (44). The encoded KISS1 protein can be processed to a C-terminally amidated peptide termed metastin binding and activating the G-protein coupled receptor GPR54 (*KISS1R*) (45). Shoji et al found that metastin inhibited migration and invasion of RCC with overexpression of *KISS1R* (46). In addition, a recent study demonstrated that an absence of *KISS1R* expression was associated with rapid progression of conventional RCC in patients (40), suggesting KISS1/KISS1R signaling as a promising target in RCC.
Honokiol targets multiple signaling pathways such as nuclear factor kB (NF-κB), signal transducers and activator of transcription 3 (STAT3), mammalian target of rapamycin (mTOR) and epidermal growth factor receptor (EGFR), which have great relevance during cancer initiation and progression (47). Moreover, pharmacokinetic studies revealed that honokiol crossed the blood-brain barrier (BBB), the blood-cerebrospinal fluid barrier (BCSFB) and had a desirable bioavailability after intravenous administration in animal models (48) thus making it a suitable agent for clinical trials.

In summary, our results indicate that activation of KISS1/KISSIR signaling by honokiol decreases the invasiveness and colonized capacity of highly metastatic RCC cells. Furthermore, we confirmed that honokiol stimulated the expression of TIMP4 dose-dependently (data not shown). It is in accordance with the finding that metasin suppresses the motility and invasive ability of RCC cells which possess KISSIR through the downregulation of MMP-2 (49). As emerging studies show that KISSIR activates a series of signaling molecules such as protein kinase C (PKC), extra-cellular signal-regulated kinases 1 and 2 (ERK1/2), p38, and phosphatidylinositol-3-kinase (PI3K) (50), further studies are in progress to investigate the specific mechanism of honokiol, which may have the potential for use as a natural agent against RCC metastasis.

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References

1. Mehlen P and Puisieux A: Metastasis: A question of life or death. Nat Rev Cancer 6: 449-458, 2006.
2. Nguyen DX and Massagué J: Genetic determinants of cancer metastasis. Nat Rev Genet 10: 703-713, 2009.
3. Monteiro J and Fodde R: Cancer stemness and metastasis: Therapeutic consequences and perspectives. Eur J Cancer 46: 1198-1203, 2010.
4. Deep G and Agarwal R: Antimetastatic efficacy of silybin: Molecular mechanisms and therapeutic potential against cancer. Cancer Metastasis Rev 29: 447-463, 2010.
5. Gupta K, Miller JD, Li J, et al: Honokiol inhibits U87MG human glioblastoma cell invasion through endothelial cells by regulating membrane permeability and the epithelial-mesenchymal transition. Int J Oncol 44: 187-194, 2014.
6. Lai KH, Chiu HC, Lin PC and Lai GM: Honokiol inhibits SGC-7901 human gastric cancer cell invasion by enhancing apoptosis and the epithelial-mesenchymal transition. Anticancer Drugs 24: 535-554, 2013.

We refer to the original reference for further details.
31. Wen J, Fu AF, Chen LJ, Xie XJ, Yang GL, Chen XC, Wang YS, Li J, Chen P, Tang MH, et al: Liposomal honokiol inhibits VEGF-D-induced lymphangiogenesis and metastasis in xenograft tumor model. Int J Cancer 124: 2709-2718, 2009.

32. Shigemura K, Arbiser JL, Sun SY, Zayzafoon M, Johnstone PA, Fujisawa M, Gotoh A, Weksler B, Zhou HE and Chung LW: Honokiol, a natural plant product, inhibits the bone metastatic growth of human prostate cancer cells. Cancer 109: 1279-1289, 2007.

33. Li W, Wang Q, Su Q, Ma D, An C, Ma L and Liang H: Honokiol suppresses renal cancer cells' metastasis via dual-blocking epithelial-mesenchymal transition and cancer stem cell properties through modulating miR-141/EB2 signaling. Mol Cells 37: 383-388, 2014.

34. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A and Rath M: Modulation of human renal cell carcinoma 786-0 MMP-2 and MMP-9 activity by inhibitors and inducers in vitro. Med Oncol 23: 245-250, 2006.

35. Lloyd FP Jr, Slivova V, Valachovicova T and Sliva D: Aspirin inhibits highly invasive prostate cancer cells. Int J Oncol 23: 1277-1283, 2003.

36. Slivova V, Valachovicova T, Jiang J, et al: Ganoderma lucidum inhibits invasiveness of breast cancer cells. J Cancer Integr Med 2: 25-30, 2004.

37. Cheng S, Eliaz I, Lin J, Thyagarajan-Sahu A and Sliva D: Triterpenes from Poria cocos suppress growth and invasiveness of pancreatic cancer cells through the downregulation of MMP-7. Int J Oncol 42: 1869-1874, 2013.

38. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.

39. Jiang J, Slivova V, Harvey K, Valachovicova T and Sliva D: Ganoderma lucidum suppresses growth of breast cancer cells through the inhibition of Akt/NF-kappB signaling. Nutr Cancer 49: 209-216, 2004.

40. Chen Y, Yusenko MV and Kovacs G: Lack of KISS1R expression is associated with rapid progression of conventional renal cell carcinomas. J Pathol 223: 46-53, 2011.

41. Zhang H, Guo Y, Shang C, Song Y and Wu B: miR-21 down-regulated TCF21 to inhibit KISS1 in renal cancer. Urology 80: 325-329.e1, 2012.

42. Hurst DR and Welch DR: Metastasis suppressor genes at the interface between the environment and tumor cell growth. Int Rev Cell Mol Biol 286: 107-180, 2011.

43. Lee JH, Miele ME, Hicks DJ, Phillips KK, Trent JM, Weissman BE and Welch DR: KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. J Natl Cancer Inst 88: 1731-1737, 1996.

44. Beck BH and Welch DR: The KiSS-1 metastasis suppressor: A good night kiss for disseminated cancer cells. Eur J Cancer 46: 1283-1289, 2010.

45. Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, Terao Y, Kumanos K, Takatsu Y, Masuda Y, et al: Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. Nature 411: 613-617, 2001.

46. Shoji S, Tang XY, Umemura S, Itoh J, Takekoshi S, Shima M, Usui Y, Nagata Y, Uchida T, Osamura RY, et al: Metastin inhibits migration and invasion of renal cell carcinoma with overexpression of metastin receptor. Eur Urol 55: 441-449, 2009.

47. Arora S, Singh S, Piazza GA, Contreras CM, Panyam J and Singh AP: Honokiol: A novel natural agent for cancer prevention and therapy. Curr Mol Med 12: 1244-1252, 2012.

48. Wang X, Duan X, Yang G, Zhang X, Deng L, Zheng H, Deng C, Wen J, Wang N, Peng C, et al: Honokiol crosses BBB and BCSFB, and inhibits brain tumor growth in rat 9L intracerebral gliosarcoma model and human U251 xenograft glioma model. PLoS One 6: e18490, 2011.

49. Yoshioka K, Okuno Y, Horiguchi Y, Ozu C, Namiki K and Tachihi H: Effects of a KiSS-1 peptide, a metastasis suppressor gene, on the invasive ability of renal cell carcinoma cells through a modulation of a matrix metalloproteinase 2 expression. Life Sci 83: 332-338, 2008.

50. Cvetković D, Babwah AV and Battacharya M: Kisspeptin/KISS1R System in breast cancer. J Cancer 4: 653-661, 2013.