Angiotensin-(1-7) Decreases Cell Growth and Angiogenesis of Human Nasopharyngeal Carcinoma Xenografts

Nana Pei1,2, Renqiang Wan3, Xinglu Chen1, Andrew Li4, Yanling Zhang1, Jinlong Li1, Hongyan Du1, Baihong Chen1, Wenjin Wei1, Yanfei Qi6, Yi Zhang7, Michael J. Katovich8, Colin Sumners6, Haifa Zheng5,*, Hongwei Li1,*

1 School of Biotechnology, Southern Medical University, Guangzhou, Guangdong, China; 2Department of Clinical Pathology, The First Affiliated Hospital of Jinan University, Guangzhou, Guangdong, China; 3Department of Otolaryngology-Head and Neck Surgery, Guangdong NO.2 Provincial People’s Hospital, Guangzhou, Guangdong, China; 4Department of Biomedical Engineering, The Johns University School of Medicine, Baltimore, USA; 5Beijing Minhai Biotechnology CO. LTD, Beijing, China; Departments of 6Physiology and Functional Genomics, 7Pharmacology and 8Pharmacodynamics, University of Florida, Gainesville, Florida, USA.

Running title: Ang-(1-7) Inhibits Proliferation of NPC Cells

Key words: Ang-(1-7); nasopharyngeal cancer; angiogenesis; proliferation; Mas

Financial support: This work was supported by National 863 High Technique Development Project of China Grant 2012AA02A403 (H. Li, W. Wei and H. Zheng), National Natural Science Foundation of China Grant 81072113 (H. Li), as well as by awards 20134433120020 and 81401920 to H. Du and B2011171 to Y. Zhang from the Chinese government.

*Correspondence:

Haifa Zheng, Ph.D., Beijing Minhai Biotechnology CO.LTD, No.1 Simiao Road, Biotechnology and Pharmaceuticals Industrial Base, Daxing District, Beijing 102600, China
Phone: 86-10-59613588
E-mail: zhenghaifa@sina.com

Hongwei Li, Ph.D., School of Biotechnology, Southern Medical University, 1023 South Shatai Road, Guangzhou, Guangdong 510515, China.
Phone: 86-20-61648555
Fax: 86-20-61648555
E-mail: hongwei1@yahoo.com

**Competing Interests:** The authors have declared that no competing interests exist.

**Figures and Tables:** 9

**Word in text:** 4,820
Abstract

Angiotensin-(1-7) [Ang-(1-7)] is an endogenous, heptapeptide hormone acting through the Mas receptor (MasR), with anti-proliferative and anti-angiogenic properties. Recent studies have shown that Ang-(1-7) has an anti-proliferative action on lung adenocarcinoma cells and prostate cancer cells. In this study, we report that MasR levels were significantly upregulated in nasopharyngeal carcinoma (NPC) specimens and NPC cell lines. Viral vector-mediated expression of Ang-(1-7) dramatically suppressed NPC cell proliferation and migration in vitro. These effects were completely blocked by the specific Ang-(1-7) receptor antagonist A-779, suggesting that they are mediated by the Ang-(1-7) receptor Mas. In the current study, Ang-(1-7) not only caused a significant reduction in the growth of human nasopharyngeal xenografts, but also markedly decreased vessel density, suggesting that the heptapeptide inhibits angiogenesis to reduce tumor size. Mechanistic investigations revealed that Ang-(1-7) inhibited the expression of the pro-angiogenic factors VEGF and PlGF. Taken together, the data suggest that upregulation of MasR could be used as a diagnostic marker of nasopharyngeal carcinoma and Ang-(1-7) may be a novel therapeutic agent for nasopharyngeal cancer therapy since it exerts significant anti-angiogenic activity.

Introduction

Nasopharyngeal carcinoma (NPC) is a common head and neck cancer, which is highly malignant with local invasion and early distant metastasis. NPC is a multifactorial disease. Both genetic predisposition (1) and epigenetic alterations (2) are important for the initiation and progression of NPC. In addition, the pathogenesis of NPC is closely linked to Epstein–Barr virus infection. Tobacco and alcohol consumption are critical risk factors as well (3). In southern China, the incidence of
NPC has remained very high, with a 5-year overall survival rate of approximately 70% (4). Although its prognosis has improved due to advances in diagnostic and surgical techniques, 30% to 40% of patients will develop distant metastases within 4 years (5). Once metastasis occurs, the prognosis is very poor and so novel treatment strategies for nasopharyngeal cancer are urgently needed.

Angiotensin-(1-7) [Ang-(1-7)], either produced in the circulation or in tissues, is a biologically active peptide hormone of the renin-angiotensin system with vasodilator, antiproliferative, and antithrombotic properties (6, 7) that are mediated by MasR, a unique G-protein-coupled receptor (8, 9). It has been shown that Ang-(1-7) reduced the growth of human lung tumor xenografts, with a concomitant decrease in vascular endothelial growth factor (VEGF) and reduced vessel density, as well as decreasing the growth of orthotopic human estrogen receptor positive or HER2 over-expressing breast tumor xenografts (10-13). A Phase I clinical trial demonstrated that Ang-(1-7) is a first-in-class antiangiogenic drug with activity for treating cancer that is linked to reduction of plasma placental growth factor (PlGF) levels (14). Recent studies showed that Ang-(1-7) attenuates metastatic prostate cancer and reduces osteoclastogenesis (15), and also reduces proliferation and angiogenesis of human prostate cancer xenografts with a decrease in angiogenic factors and an increase in sflt-1(16). These findings suggest that Ang-(1-7) reduces tumor size by attenuating proliferation and angiogenesis. Until now, no functional evidence of Ang-(1-7) in NPC has been documented.

Transgenic expression of Ang-(1-7) can be mediated by viral vectors through the use of synthetic fusion protein expression cassettes which are expressed intracellularly and result in cleavage and secretion of active peptides (17-19). In this study, we investigated the potential involvement of Ang-(1-7) in NPC using lentiviral or AAV vectors expressing fusion proteins which secrete the heptapeptide. First, we tested its effects on cell growth and migration. Second, we investigated the potential role of Ang-(1-7) on NPC tumorigenesis in a murine model. Finally, we explored the underlying mechanism of Ang-(1-7) actions in NPC. We believe our study will provide a better understanding of NPC pathogenesis.

Material and Methods
Cell cultures

5-8F, HNE-1, CNE1, CNE2 and C666-1, derived from human nasopharyngeal squamous cell carcinoma, and an immortalized nasopharyngeal epithelial cell NP69, were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) in 2011. Human Embryonic Kidney 293T cells were purchased from the American Type Culture Collection in 2009. All cultured cells retained the characteristic phenotype as shown on the commercial providers’ repository. Cells were immediately expanded, and multiple aliquots were cryopreserved and used within 3 months after resuscitation. No further independent authentication was carried out by the authors. Human nasopharyngeal carcinoma cell lines were cultured in RPMI-1640 (Invitrogen) medium supplemented with 10% FBS under 5.0% CO2. NP69 cell line was cultured in Keratinocyte-SFM (Invitrogen) supplemented with bovine pituitary extract (BD Biosciences). Sera and media were purchased from Invitrogen and American Type Culture Collection. HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen).

Viral vector construction and preparation

The lentiviral vector Lenti-Ang-(1–7) construct used to produce the Ang-(1–7) was designed, prepared, and titrated as previously described (17, 20, 21). Both IgG2b and Ang-(1–7) from the fusion protein can be detected intracellularly. The enhanced green fluorescent protein (eGFP) lentiviral vector Lenti-eGFP was used as a control.

Two adeno-associated viral vectors (AAVs), AAV2-chicken β-actin promoter (CBA)-Ang-(1-7) and AAV2-CBA-eGFP, were constructed as detailed previously (22). The vector plasmid was packaged in AAV serotype 8 containing a Y733F mutation by transfection of HEK 293T cells according to previously published methods (23, 24). Vector doses were expressed as genome copies.

Clinical specimens

Primary NPC biopsy specimens and normal biopsies of the nasopharynx were obtained from Guangdong NO.2 Provincial People’s Hospital (Guangzhou, Guangdong, China). Both tumor and normal tissues were histologically confirmed by H&E (hematoxylin and eosin) staining. Informed consent was obtained from each
patient, and the research protocols were approved by the Ethics Committee of Guangdong NO.2 Provincial People’s Hospital.

### Quantification of secreted Ang-(1–7) by ELISA

The NPC cells were plated at 50% confluence in 24-well culture plates; they were then transduced with Lenti-Ang-(1–7) at a concentration of 200 multiplicities of infection (MOI) in the presence of 8 μg/ml polybrene (Sigma-Aldrich, St Louis, MO, USA). After 6 h of viral transduction, the media was replaced with fresh growth medium. After 3 days, the secreted levels of Ang-(1–7) were measured using a commercially available ELISA kit (Bachem, San Carlos, CA, USA). The same ELISA kits were also used to determine the secreted levels of Ang-(1–7) in sera from mice in vivo.

### Quantification of cell proliferation

For the cell counting assay, cells were infected with Lenti-Ang-(1-7) to stably overexpress Ang-(1-7). After 72 hours of transduction, the cells were plated in 24 well plates at 1.0 ×10⁴ per well. Cells were harvested on days 1, 2, 3, and 4 from triplicate wells and counted using a hemocytometer, to quantify cell proliferation.

For the MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay, cells transduced with Lenti-Ang-(1-7) were plated in 96-well plates at 2×10³ per well in a final volume of 100 μl and transfected with Ang-(1-7). The cells were cultured for 24, 48, 72, and 96 hours. The effect of Ang-(1-7) on cell growth and viability was determined by MTS assay.

BrdU incorporation assay. Lenti-Ang-(1-7) transduced NPC cells and control cells (Lenti-eGFP or mock transduced cells) were seeded into 6-well plates (3×10⁵ cells/well) with or without the Mas receptor antagonist A-779 (1.0 μmol/L) (Bachem) for 24 h. One hour prior to fixing the cells, 10 μmol/L BrdU (Sigma chemicals) were added to the cultures. Next, a BrdU incorporation assay was performed as described previously (25). Labeling indices were calculated as the number of positively stained cells divided by the number of total cells.

### Colony formation assay

...
Cells were infected with Lenti-Ang-(1-7) to stably overexpress Ang-(1-7). After 72 hours of transduction, the cells were plated in 6-well plates at $2 \times 10^2$ per well and grown for 2 weeks. After 2 weeks, the cells were washed twice with PBS, fixed with methanol/acetic acid (3:1, v/v), and stained with 0.5% crystal violet. The number of colonies was counted under the microscope (26).

**In vitro migration assay**

Confluent monolayers of Lenti-Ang-(1-7) transduced CNE-1 and CNE-2 cells were scraped down the center of a 35 mm tissue culture dish with a sterile micropipette tip to create a denuded zone of constant width. Cellular debris was removed with PBS and the adherent cells were incubated in regular media for 48 h. Next, a migration assay was performed as described previously (27).

**Transwell migration assay**

A total of $1 \times 10^5$ normal or transduced NPC cells were resuspended in serum free RPMI with or without 1.0 μmol/L A-779 and placed in the top portion of a Transwell chamber with 8-μm pores. The lower portion of the chamber contained 10% FBS as a chemoattractant. Next, a transwell migration assay was performed as detailed previously (28).

**RNA isolation, reverse transcription, and quantitative real-time RT-PCR**

Total RNA was extracted using an RNeasy Mini-Kit (Qiagen) according to the manufacturer’s instructions. Quantitative real-time RT-PCR was performed on an ABI 7500 real-time PCR system (Applied Biosystems) as described previously (29). The primers are listed in Supplemental Table S1. The samples were quantified by the comparative $\Delta \Delta C_T$ method by using human GAPDH as the internal standard.

**Western blot analysis**

Western immunoblots were run as described previously (30). Primary antibodies and their sources were as follows. Anti-total p38 MAPK, anti-total JNK MAPK, anti-total p44/42 MAPK, anti-phosphorylated p44/42 (pp44/42) MAPK, anti-phosphorylated JNK (pJNK), and anti-phosphorylated p38 (pp38) MAPK were from
Cell Signaling Technology. Anti-β-actin and the secondary antibodies horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were from Sigma-Aldrich.

**Tumor growth assay**

Female BALB/c nude mice aged 4 to 5 weeks were purchased from the Institute of Comparative Medicine and Center of Laboratory Animals of the Southern Medical University (SMU). Animal handling and experimental procedures were approved by the Animal Experimental Ethics Committee of SMU. Athymic mice were subjected to s.c. injections of human CNE-1 nasopharyngeal cancer cells (1.0×10^6) in Matrigel (50:50) into the lower flank to induce tumor growth. After the tumors reached ∼50 mm^3, the mice were placed into three groups at random and the animals received tail vein injections of AAV8 (Y733F)-CBA-Ang-(1-7), AAV8 (Y733F)-CBA-eGFP (5×10^{11} vg/mouse) or PBS. Each group contained 6 mice and the experiment was repeated 3 times. Tumor size was measured every 3 days. The mice were anesthetized on day 35 and euthanized by decapitation and tumors were dissected. Tumor volumes were calculated as follows: volume = (D × d^2)/2, where D is the longest diameter and d is the shortest diameter. Both livers and tumors were isolated for Western blot and quantitative real-time RT-PCR analysis, or fixed in 10% buffered formalin and used for histologic and immunohistochemical analysis. Sera were also collected for quantification of Ang-(1-7) by ELISA (Bachem).

**Immunohistochemistry**

Tumors were fixed in 4% paraformaldehyde for 24 h and incubated in 70% ethanol for 48 h before embedding in paraffin. The embedded tumors were cut into 5-μm-thick sections and stained with H&E to determine morphology. Cell proliferation in the transplanted tumors was analyzed for Ki67 (1:200; Abcam) and PCNA (Proliferating Cell Nuclear Antigen) (1:500; Abcam) expression. Angiogenesis was determined by immunostaining with antibodies to VEGF (1:100; Abcam) and CD31 (1:100, Dako, Germany). Visualization was achieved using the EnVision+ peroxidase system (Dako) according to the manufacturer’s protocols. Ki67 or PCNA immunoreactive cells were expressed as a percentage of the total cell number of examined fields. Blood vessels were visualized by the presence of CD31-
immunostained endothelial cells and identified by their morphology, as vessels cut in cross-section with visible lumens or vessels cut longitudinally with tube-like morphology (31). The vessel density was assessed by counting vessels from 5 random fields per tumor. Counts were done by an individual who was blinded as to the treatment.

**Statistical analysis**

SPSS 19.0 software was used for statistical analysis. Data are presented as mean ± standard deviation (SD) from 3 to 6 independent experiments. Statistical differences were evaluated by one-way ANOVA followed by Dunnett's post hoc test. The criterion for statistical significance was set at P < 0.05.

**Results**

**MasR was upregulated in human NPC cell lines and clinical specimens**

The G-protein coupled, seven transmembrane protein encoded by the MasR gene has been identified as an Ang-(1-7) receptor (8, 9). A panel of human NPC cell lines was first analyzed to quantitate the expression level of MasR. The results showed that the expression of MasR was increased in 5 NPC cell lines compared with immortalized nasopharyngeal epithelial cell line NP69 (Fig. 1A and 1B). We further examined the expression level of MasR in 26 NPC specimens and 23 normal nasopharyngeal epithelial tissues. Consistent with the data obtained from NPC cell lines, the average expression level of MasR was significantly higher in NPC specimens than in normal nasopharyngeal epithelial tissues (Fig. 1C; P < 0.01). This suggests that MasR may serve as the receptor that mediates the anti-proliferative responses to Ang-(1-7) in human nasopharyngeal cancer cells.

**Ang-(1-7) inhibited NPC cell growth and migration**

To explore the effect of Ang-(1-7) on cell growth, CNE-1 and CNE-2 cells were transduced with Lenti-Ang-(1-7) or Lenti-eGFP. The expression of Ang-(1-7) was increased in CNE-1 and CNE-2 cells respectively in a dose-dependent manner and reached a very high level at 200 MOI (data not shown). Therefore, 200 MOI was utilized in subsequent experiments. The intracellular expression of fusion protein containing Ang-(1-7) was determined by Western blot in Lenti-Ang-(1-7) transduced...
CNE-1 and CNE-2 cells compared with Lenti-eGFP or mock transduced cells (Fig. 2A). Three days after transduction of CNE-1 and CNE-2 cells with 200 MOI of Lenti-Ang-(1–7), robust expression of Ang-(1–7) (~ 60 ng/ml; Fig. 2B) was detected in the culture media. However, there was no significant Ang-(1–7) expression in the cultured media from Lenti-eGFP or mock transduced cells.

The cell growth curve and the MTS assay demonstrated that Ang-(1-7) inhibited cell growth in Lenti-Ang-(1-7) transduced cells compared to the Lenti-eGFP or mock transduced cells (Fig. 2C, 2D). As demonstrated in the colony formation assay, Lenti-Ang-(1-7) transduced CNE-1 and CNE-2 cells showed many fewer colonies compared with Lenti-eGFP infected cells (Fig. 2E; P < 0.01). Confluent monolayers of Lenti-Ang-(1-7) transduced CNE-1 and CNE-2 cells were scraped with a micropipette tip, to evaluate the effect of Ang-(1-7) on cell migration. Cells in media alone migrated into the “wounded” area during the subsequent 48 h incubation (Supplemental Fig. S1). The cell monolayers overexpressing Ang-(1-7) exhibited a significant reduction in cell migration—a 16% reduction at 24 h and a 20% reduction at 48 h in Lenti-Ang-(1-7) transduced CNE-1 cell migration vs. controls (Lenti-eGFP or mock transduced cells). The Lenti-Ang-(1-7) transduced CNE-2 cells showed a 20% reduction in migration vs. controls at 48 h, but there was no significant difference from the control cells at 24 h.

**Effects of Mas receptor antagonists on the inhibition of proliferation and cell migration by Ang-(1-7)**

A Mas receptor antagonist, A-779, was used to study whether it could block the effects of Ang-(1-7) including inhibition of proliferation and cell migration in NPC cells. The results showed that Ang-(1-7) inhibited DNA synthesis and cell migration in Lenti-Ang-(1-7) transduced cells compared to the control cells while the addition of A-779 (1 μmol/L) completely blocked the effects of Ang-(1-7) treatment (Fig. 3A&B). Incubation of NPC cells with A-779 alone exerted no effects on DNA synthesis and cell migration. This suggests that MasR may serve as the receptor that mediates the anti-proliferative and migration inhibition responses to Ang-(1-7) in NPC cells.

**Effects of Ang-(1-7) on VEGF, PI GF, HIF-1α and VEGF receptors in NPC cells**
VEGF is one of the major pro-angiogenic factors secreted by cancer cells and it acts to stimulate the growth of new blood vessels from pre-existing vessels. VEGF mRNA was measured by real-time RT-PCR in transduced NPC cells, to determine whether the over-expression of Ang-(1-7) reduces expression of this angiogenic factor. VEGF mRNA was reduced by 57% and 53%, respectively, in Lenti-Ang-(1-7) treated CNE-1 and CNE-2 cells compared to control cells (Fig. 4A, 4B). Similarly, CNE-1 and CNE-2 cells transduced with Lenti-Ang-(1-7) had respective ~70% and 80% reductions in PlGF mRNA compared to control cells (Fig. 4A, 4B). Under hypoxic conditions, angiogenesis is primarily regulated by hypoxia inducible factor (HIF-1α)-dependent transcription of VEGF (32). HIF-1α is one of the primary transcription factors that control this mechanism. In addition, PlGF activates downstream target genes in endothelial cells via HIF-1α in a hypoxia independent manner (33). Here, HIF-1α mRNA was also shown to be significantly decreased in Lenti-Ang-(1-7) transduced NPC cells compared to control cells (Fig. 4A, 4B).

VEGF and PlGF bind to and activate two distinct VEGF receptors-VEGF receptor 1 or Flt-1, and VEGF receptor 2 or Flk-1-to initiate intracellular signaling cascades (34). Flt-1 and Flk-1 were quantified by real-time RT-PCR in Lenti-Ang-(1-7) treated CNE-1 and CNE-2 cells to determine the effect of the heptapeptide on VEGF receptor expression. Flt-1 and Flk-1 were significantly reduced in Lenti-Ang-(1-7) treated NPC cells compared to controls (Fig. 4C, 4D), suggesting that this heptapeptide may also attenuate VEGF and PlGF signaling by reducing the number of receptors available to these ligands. VEGF receptor 1 or Flt-1 can be processed to soluble Flt-1 or sFlt-1 by alternative splicing (35). In contrast to the Ang (1-7)-mediated reduction in membrane associated VEGF receptors, sFlt-1 was significantly increased in Lenti-Ang-(1-7) treated CNE-1 and CNE-2 cells compared to control cells (Fig. 4E). sFlt-1 is secreted into the circulation and binds to VEGF and PlGF, thereby limiting interaction of these ligands with membrane-bound VEGF receptors. These results suggest that Ang-(1-7) may also attenuate angiogenesis by increasing sFlt-1 to reduce circulating VEGF and PlGF.

Ang-(1-7) inhibited p44/42 MAPK and p38MAPK signaling pathway

MAPK signaling pathways are implicated in cell survival, growth and proliferation. Phosphorylated p44/42, p38 and JNK were measured in transduced NPC cells using
phospho-specific antibodies to determine whether Ang-(1-7) inhibited NPC cell growth by attenuating MAP kinases. Western blot results showed that the p44/42 phosphorylation level in Lenti-Ang-(1-7) treated cells was decreased. The phosphorylation of p38, another MAPK pathway, showed a similar response pattern. However, the level of JNK phosphorylation was not changed in NPC cells treated with Lenti-Ang-(1-7) (Fig. 4F). These results suggest that Ang-(1-7) reduces tumor growth, in part, by attenuating p44/42 and p38 MAP kinase activities.

**Ang-(1-7) suppressed nasopharyngeal tumor growth in nude mice**

CNE-1 cells were injected subcutaneously into the dorsal flank of nude mice to assess the effect of Ang-(1-7) on nasopharyngeal tumor growth. When xenograft tumors were about 50 mm³, mice were randomized for tail intravenous injection with AAV8 (Y733F)-CBA-Ang-(1-7), AAV8 (Y733F)-CBA-eGFP (5×10¹¹ vg/mouse) or PBS to attain a high level of Ang-(1-7) stable expression. The dose selection of the AAV vector was based on preliminary experiments showing a high efficiency and a powerful specificity for targeting the liver (Supplemental Fig. S2). Secreted Ang-(1-7) in mouse sera was quantified by ELISA. The results indicate that the amount of Ang-(1-7) secreted into the sera in the mice treated with AAV-Ang-(1-7) was significantly increased when compared to AAV-eGFP or PBS treated mice, as shown in Figure 5A. At this dose, Ang-(1-7) was shown to effectively inhibit tumor growth with no side effects. Tumors in the control group of mice continued to grow faster over the 30 day period (Fig. 5B, 5C), whereas administration of the AAV8 (Y733F)-CBA-Ang-(1-7) resulted in a significant reduction in tumor volume. By the end of the 30 day treatment period, tumors from mice in the control group were about 2.5-fold larger than tumors from AAV8 (Y733F)-CBA-Ang-(1-7)-treated mice. There were no differences in body weight or pathological abnormalities between the groups (data not shown). The mice were euthanized at the end of the study and the tumors were dissected and weighed. As shown in Figure 5D, the tumors from mice treated with AAV8 (Y733F)-CBA-Ang-(1-7) weighed 60% less than the tumors from mice infused with AAV8 (Y733F)-CBA-eGFP or PBS, demonstrating that Ang-(1-7) reduces tumor growth.

**Ang-(1-7) reduces cell proliferation in CNE-1 xenograft tumors**
Tumor sections from mice infused with AAV8 (Y733F)-CBA-Ang-(1-7), AAV8 (Y733F)-CBA-eGFP (5×10^{11} vg/mouse) or PBS underwent immunostaining using antibodies against the Ki67 and PCNA which are markers of cell proliferation. The results showed that both the staining intensity and the number of hyper-proliferative Ki-67 and PCNA positive tumor cells were significantly decreased compared with both control groups (Fig. 5E; P < 0.05), suggesting that Ang-(1-7) reduces cell proliferation in vivo.

Effect of Ang-(1-7) on VEGF, PlGF, HIF-1α and VEGF receptors in nasopharyngeal tumor xenografts

Consistent with the in vitro results, VEGF mRNA and protein levels were significantly reduced in tumors from nude mice administered AAV8 (Y733F)-CBA-Ang-(1-7) when compared to tumors from AAV8 (Y733F)-CBA-eGFP or PBS treated animals (Fig. 6A, 6B). Similarly, PlGF and HIF-1α were also significantly reduced in tumors from nude mice administered AAV8 (Y733F)-CBA-Ang-(1-7) compared to tumors from AAV8 (Y733F)-CBA-eGFP or PBS treated animals (Fig. 6B). Flt-1 and Flk-1 were quantified by real-time RT-PCR in tumors from mice treated with AAV8 (Y733F)-CBA-Ang-(1-7), AAV8 (Y733F)-CBA-eGFP or PBS to determine the effect of the heptapeptide on VEGF receptor expression. Flt-1 and Flk-1 were significantly reduced in tumors from mice introduced with Ang (1-7) compared to tumors from control mice (Fig. 6C), suggesting that the heptapeptide may also attenuate VEGF and PlGF signaling by reducing the number of available receptors. In contrast to the Ang-(1-7)-mediated reduction in membrane associated VEGF receptors, sFlt-1 was significantly increased in tumor tissue following introduction of the heptapeptide (Fig. 6D). These results suggest that decreases in VEGF, PlGF, HIF-1α and VEGF in response to Ang-(1-7) contribute to decreases in cell and tumor growth both in vivo and in vitro.

Ang-(1-7) Reduces Vessel Density in NPC Xenograft Tumors

Blood vessel density was quantified in NPC xenograft tumors from female BALB/c nude mice to determine whether the heptapeptide reduces angiogenesis. Vessels were identified by positive immunoreactivity to CD31 in combination with vessel morphology. A marked reduction in immunoreactive CD31-stained vessels was
observed in tumor tissue sections from mice treated with AAV8 (Y733F)-CBA-Ang-(1-7) (11.2 ± 1.5 vessels/field) when compared to mice treated with AAV8 (Y733F)-CBA-eGFP (30.0 ± 2.8 vessels/field) or PBS (29.3 ± 2.5 vessels/field) (Fig. 6E), suggesting that Ang-(1-7) significantly attenuates tumor vascularization in xenograft nasopharyngeal tumors.

Discussion

The objective of this study was to investigate the effects of Ang-(1-7) on the growth of nasopharyngeal carcinoma in vitro and in vivo, by using Lentiviral or AAV vectors expressing fusion proteins which release the heptapeptide. The first set of experiments indicated that MasR was significantly upregulated in NPC cell lines and in nasopharyngeal carcinoma tissues (Figure 1). Consistent with our results, a recent study has demonstrated that MasR was significantly increased in colonic adenocarcinomas (36). Higher expression of MasR had also been reported in hepatic colorectal (CRC) metastases compared with the surrounding liver tissue (37). MasR activation by Ang-(1-7) has been demonstrated to mediate anti-angiogenic and antiproliferative effects in many cells and tumors (6, 7, 10, 12, 13). It is possible that the increase in MasR in the cancer cells or tumors is a response to the increased cell number or tumor size, and then serves as a growth inhibitory mechanism to reduce tumor size.

The second set of experiments clarified that Ang-(1-7) caused a marked decrease in cell proliferation and cell migration (Fig. 2, Supplemental Fig. S1) of cultured nasopharyngeal cancer cells. These results are in agreement with findings observed in lung, breast or prostate cancers, in which Ang-(1-7) suppressed cell proliferation and tumor xenografts (11, 13, 15, 16). However, Ang-(1-7) had no effect on cell growth in human colon adenocarcinoma (36). These inconsistent results suggested that the role of Ang-(1-7) was possibly tumor specific and highly dependent on its targets in different cancer cells. In our experiments, the growth and migration inhibitory effect of Ang-(1-7) in cultured human NPC cells was completely blocked by A-779 (Fig. 3). Ang-(1-7) also reduced the phosphorylation and activation of p44/42 MAPK and p38 MAPK (Fig. 4), suggesting that the heptapeptide attenuated the growth and migration of NPC cells by inhibiting this signaling pathway. Our experiments provide the first evidence that Ang-(1-7) inhibits the proliferation of human nasopharyngeal cancer.
cells and support the hypothesis that Ang-(1-7) serves as an endogenous regulator of cell growth. Our studies are also in agreement with the previous studies demonstrating that Ang-(1-7) decreases the ERK signal transduction pathway in lung and prostate cancer cell growth arrest (10, 16). A growing number of studies have shown that p38 MAPK mediated cell migration and invasion (38-41) pathways are involved in Ang II-stimulated migration of vascular adventitial fibroblasts in spontaneously hypertensive rats (42). A recent study demonstrated that the anti-migration and anti-invasion effect of Ang-(1-7) was mediated through inactivation of the PI3K/Akt, p38 MAPK and JNK signaling pathways (43). However, in our study JNK signaling pathways were not involved in Ang-(1-7) induced inhibition of cell growth and migration. Thus, based on our data we may conclude that anti-proliferation effect of Ang-(1-7) is associated with p44/42 MAPK and anti-migration effect is associated with p38 MAPK. The precise regulation of this MAPK signal transduction pathway following treatment of NPC cells with Ang-(1-7) will be further elucidated in future studies.

More importantly, we found that Ang-(1-7) markedly reduced VEGF, PlGF and VEGF receptors in both human nasopharyngeal tumor xenografts and the NPC cells, suggesting that multiple growth factors are involved in the antiangiogenic response to the heptapeptide. VEGF, a robust stimulator of angiogenesis, has a variety of roles in this process, including stimulation of vascular permeability, induction of endothelial cell migration and division, promotion of endothelial cell survival, and stimulation of cell proliferation (44). A significant decrease of VEGF mRNA and VEGF immunoreactivity was observed in the tumors from mice treated with AAV8 (Y733F)-CBA-Ang-(1-7) compared with tumors from control animals (Fig. 6). We also observed significant VEGF reduction in NPC cell lines treated with Lenti-Ang-(1-7). These results are in agreement with previous studies showing decreased VEGF mRNA levels in lung cancer and prostate cancer cell lines treated with the heptapeptide (12, 15, 16). However, the molecular mechanisms for the Ang-(1-7)-mediated decrease in VEGF are not completely established. One of the primary regulators of VEGF transcription is HIF-1α which is up-regulated in tumors in response to oxygen deprivation (32). HIF-1α is increased in response to hypoxia or conditions of low oxygen tension. Low oxygen tension in tumor cells located a distance away from blood vessels results in the production and release of VEGFA to
initiate angiogenesis. Here, we observed decreased HIF-1α mRNA in the tumors from mice treated with AAV8 (Y733F)-CBA-Ang-(1-7) and NPC cells treated with Lenti-Ang-(1-7). Perhaps, as a consequence of the antiproliferative effects of Ang-(1-7), metabolic activity and oxygen consumption may be reduced leading to decreased HIF-1α, VEGF, and finally angiogenesis. HIF-1α expression can also be regulated by other factors such as mitogen-activated protein kinases which are essential for the direct phosphorylation and activation of HIF-1α and VEGF expression (45).

Hypoxia and the associated induction of HIF-1α also regulate the production of other pro-angiogenic genes including PlGF (32) and play a vital role in tumor vascularization (46). A significant decrease of PlGF mRNA was observed in xenografts from mice treated with AAV8 (Y733F)-CBA-Ang-(1-7) when compared with tumors from control animals (Fig. 6). We also observed a parallel and significant reduction in NPC cell lines treated with Lenti-Ang-(1-7). These data demonstrated that the heptapeptide hormone reduced PlGF production as part of this anti-angiogenic effect. These observations are supported by the findings in a Phase I clinical trial for prostate cancer in which responsive patients administered Ang-(1-7) had reduced circulating PlGF (14).

Furthermore, we observed significant reductions in both VEGF receptor 1 (Flt-1) and VEGF receptor 2 (Flk-1) and an increase in sFlt-1 in the tumors of mice treated with AAV8 (Y733F)-CBA-Ang-(1-7) and in Lenti-Ang-(1-7) transduced NPC cells when compared to their respective controls. sFlt-1, produced by alternative splicing of Flt-1, retains the extracellular ligand-binding domain but lacks the transmembrane and intracellular signaling domains (35). sFlt-1 is secreted into the circulation where it binds VEGF and PlGF sequestering these ligands from membrane-bound VEGF receptors. This result is in agreement with a recent study which demonstrated that Ang-(1-7) increased sFlt-1 in prostate cancer cells and orthotopic tumors in association with a reduction in angiogenesis (16), suggesting that sFlt-1 may be an antiangiogenic factor by which Ang-(1-7) regulates tumor angiogenesis. Taken together, our results demonstrate that Ang-(1-7) reduces human nasopharyngeal xenograft growth and tumor angiogenesis, by decreasing the expression of pro-angiogenic factors and VEGF receptors as well as increasing the anti-angiogenic factor sFlt-1.
Ang-(1-7) has previously been investigated for gene therapy approach via viral vector mediated delivery (17-19). These studies highlighted the potential for gene transfer of angiotensin peptides for molecular investigations and therapeutic approaches. The present study provides clear evidence that intravenous administration of AAV-based therapeutic gene delivery causes long-term strong and stable gene expression in vivo. Further, our study showed that administration of AAV8 (Y733F)-CBA-Ang-(1-7) significantly attenuated the growth of nasopharyngeal carcinoma tumors, suggesting that AAV-based gene therapy is potentially effective and useful for nasopharyngeal cancer treatment. In conclusion, our results demonstrate that Ang-(1-7) inhibits the proliferation of human nasopharyngeal cancer cells, and reduces human nasopharyngeal xenograft growth and tumor angiogenesis, which may be mediated by decreasing the expression of pro-angiogenic factors and VEGF receptors as well as increasing the anti-angiogenic factor sFlt-1. These data suggest that the heptapeptide may serve as a novel, anti-angiogenic treatment and a potential target gene for nasopharyngeal carcinoma gene therapy.

Acknowledgments

We acknowledge Shengyao Wang, Renhe Yan, Ye Li and Wanyan Zhou for their technical assistance and help with Western blot and real-time RT-PCR.

References


Figure Legends

**Figure 1. Mas receptor expression was increased in NPC cell lines and NCP clinical specimens.** Protein (A and B) and mRNA expression levels (C) of the Mas receptor in 5 NPC cell lines (HNE-1, 5-8F, C666-1, CNE-1 and CNE-2) in comparison to immortalized nasopharyngeal epithelial NP69 cells. Expression of Mas receptor (C) in nasopharyngeal epithelial tissues obtained from human subjects with NPC (n=26) and from patients with normal nasopharyngeal epithelial tissues (n=23). Mas receptor abundance was normalized to GAPDH. *, \( P < 0.01 \) vs. immortalized nasopharyngeal epithelial NP69 cells or normal tissues.

**Figure 2. Ang-(1-7) inhibited growth of NPC cells.** (A), intracellular Ang-(1-7) was determined by measuring the levels of the Ang-(1-7)-IgG fusion protein in lentiviral vector transduced CNE-1 and CNE-2 cells by western blot; (B), secreted Ang-(1–7) in the culture media collected from Lenti-Ang-(1-7)-IgG transduced NPC cells three days after viral transduction was quantified with an ELISA kit. (C) and (D), effect of Ang-(1-7) on cell proliferation was measured over 96 hours by cell counting and MTS assay in NPC cells; (E), colonies were evaluated and values were reported as the ratio of Lenti-Ang-(1-7) transduced cells to Lenti-eGFP and mock transduced cells. **, \( P < 0.01 \) vs. the control groups (Lenti-eGFP and mock transduced cells).

**Figure 3. A-779 blocked Ang-(1-7)-induced inhibition of DNA synthesis and cell migration.** (A), effect of the MasR antagonist, A-779 on Ang-(1-7)-mediated DNA synthesis. NPC cells (CNE-1 and CNE-2) transduced with Lenti-Ang-(1-7) were treated with 1.0 \( \mu \)mol/L A-779. Cells were incubated with BrdU for 1 h after 24 h of incubation with A-779. BrdU- positive cells were then identified by immunofluorescence staining and observed under a fluorescence microscope. (B), transwell migration assay. Non-transduced or Lenti-Ang-(1-7) transduced NPC cells
were resuspended in serum free RPMI with or without 1.0 \( \mu \)mol/L A-779, and placed in the top portion of a transwell chamber. The cells that migrated to the lower portion of the chamber were fixed and stained with trypan blue and 5 fields per well were counted. Columns, mean of three experiments; bars, SD. **, \( P < 0.01 \).

**Figure 4. Effects of Ang-(1-7) on angiogenic factors, VEGF receptors and MAPK family enzymes in NPC cells.** NPC cells were transduced with Lenti-Ang-(1-7) (200 MOI). After 3 days of incubation, cells were collected and subjected to real-time RT-PCR and Western blot analyses (representative of three different experiments). Panels (A) and (B) show the expression levels of VEGF, PIGF and HIF-1\( \alpha \) in CNE-1 and CNE-2 cells after Lenti-Ang-(1-7) transduction were compared to the control groups (eGFP and mock). Flt-1(Panel C), Flk-1 (Panel D), and sFlt-1 (Panel E) were also measured in these cells; (F), expression of total p44/42, p38, and JNK, and pp44/42, pp38, and pJNK along with \( \beta \)-actin protein bands in NPC cells. Western blots are representative of three different experiments.

**Figure 5. Ang-(1-7) decreases nasopharyngeal tumor growth in nude mice.** (A), Levels of Ang-(1-7) in the sera of control (AAV-eGFP and PBS-treated mice) and AAV8 (Y733F)-CBA-Ang-(1-7)-treated mice were determined by ELISA. *, \( P < 0.01 \) compared with the control groups. Data are representative of 6 mice/group. (B), size of human nasopharyngeal tumor xenografts from mice injected with AAV8 (Y733F)-CBA-Ang-(1-7), AAV8 (Y733F)-CBA-eGFP or PBS was measured using a caliper and volume was calculated as follows: volume = \( (D \times d^2)/2 \), where D is the longest diameter and d is the shortest diameter. *, \( P < 0.01 \); \( n = 6 \). ① tumor cell injection, ② AAV virus or PBS 8 injection. (C), growth curve of tumor volumes. (D), tumors from mice treated with AAV8 (Y733F)-CBA-Ang-(1-7), AAV8 (Y733F)-CBA-eGFP or PBS were weighed at the time of sacrifice. *, \( P < 0.01 \); \( n = 6 \). Each data point represents the mean ± SD of 6 mice. (E), sections of transplanted tumors infused with AAV8 (Y733F)-CBA-Ang-(1-7), AAV8 (Y733F)-CBA-eGFP or PBS were stained with Ki-67 and PCNA. Representative photomicrographs are shown to the left (magnification ×200). *, \( P < 0.01 \) vs. the control groups.

**Figure 6. Effect of Ang-(1-7) on angiogenic factors and vessel density in nasopharyngeal tumor xenografts.** (A), Ang-(1-7) decreases VEGF expression in human nasopharyngeal tumor xenografts. VEGF expression levels in tumor sections
from AAV8 (Y733F)-CBA-Ang-(1-7), AAV8 (Y733F)-CBA-eGFP or PBS-treated human nasopharyngeal cancer xenografts were determined by VEGF immunostaining. (B), levels of VEGF, PIGF and HIF-1α in tumor tissue from nasopharyngeal tumor xenografts were measured by real-time RT-PCR; (C) and (D), levels of Flt-1, Flk-1 and sFlt-1 were also determined in the tumors. E, blood vessels in tumor sections from nasopharyngeal tumor xenografts were identified by CD31 immunoreactivity and vessel morphology, and quantified as the average of 5 fields selected per tumor (magnification ×200). Columns, means from 6 separate experiments; bars, SD. **, P <0.01 vs. the control groups (AAV-eGFP and PBS-treated mice).
Figure 2

A

CNE-1

Lenti-Ang-(1-7) Lenti-eGFP Mock

CNE-2

Lenti-Ang-(1-7) Lenti-eGFP Mock

Ang-(1-7) fusion protein

\( \beta \)-actin

B

<table>
<thead>
<tr>
<th>CNE-1</th>
<th>CNE-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lenti-Ang-(1-7)</td>
<td>80 ± 5</td>
</tr>
<tr>
<td>Lenti-eGFP</td>
<td>60 ± 3</td>
</tr>
<tr>
<td>Mock</td>
<td>40 ± 2</td>
</tr>
</tbody>
</table>

C

CNE-1

Cell Number

0 24 48 72 96

Time (Hours)

CNE-2

Cell Number

0 24 48 72 96

Time (Hours)

D

CNE-1

Absorbance value (OD)

0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6

Time (Hours)

CNE-2

Absorbance value (OD)

0 0.2 0.4 0.6 0.8 1.0 1.2 1.4

Time (Hours)

E

CNE-1

Relative colony number (%)

CNE-1

CNE-2

Downloaded from mct.aacrjournals.org on November 6, 2017. © 2015 American Association for Cancer Research.
Figure 3

A  CNE-1  Ang-(1-7)  Ang-(1-7)+A-779  eGFP  A-779

BrdU  

DAPI

CNE-1

BrdU-positive cells(%)

**

CNE-2

BrdU-positive cells(%)

**

B  CNE-1  Ang-(1-7)  Ang-(1-7)+A-779  eGFP  A-779

CNE-2

Number of cells

**

Number of cells

**
Molecular Cancer Therapeutics

Angiotensin-(1-7) Decreases Cell Growth and Angiogenesis of Human Nasopharyngeal Carcinoma Xenografts

Nana Pei, Renqiang Wan, Xinglu Chen, et al.

Mol Cancer Ther Published OnlineFirst December 15, 2015.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-14-0981

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2015/12/15/1535-7163.MCT-14-0981.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.