Selective inhibition of nuclear factor-κB by nuclear factor-κB essential modulator-binding domain peptide suppresses the metastasis of highly metastatic oral squamous cell carcinoma

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Nuclear factor-κB (NF-κB) activation contributes to the development of metastasis, thus leading to a poor prognosis in many cancers, including OSCC. However, little in vivo experimental data are available about the effects of NF-κB inhibition on OSCC metastasis. OSCC sublines were established from a GFP-expressing parental cell line, GSAS, and designated GSAS/N3 and N5 according to the in vivo passage number after cervical lymph node metastasis by a serial orthotopic transplantation model. In vitro migration and invasion were assessed in these cells, and the NF-κB activities and expression of NF-κB-regulated metastasis-related molecules were also examined. In in vivo experiments, the metastasis and survival of tumor-engrafted mice were monitored. Furthermore, the effects of a selective NF-κB inhibitor, NEMO-binding domain (NBD) peptide, on metastasis in GSAS/N5-engrafted mice were assessed, and engrafted tongue tumors were immunohistochemically examined. Highly metastatic GSAS/N3 and N5 cells showed an enhanced NF-κB activity, thus contributing to increased migration, invasion, and a poor prognosis compared with the parent cells. Furthermore, the expression levels of NF-κB-regulated metastasis-related molecules, such as fibronectin, β1 integrin, MMP-1, -2, -9, and -14, and VEGF-C, were upregulated in the highly metastatic cells. The NBD peptide suppressed metastasis and tongue tumor growth in GSAS/N5-inoculated mice, and was accompanied by the downregulation of the NF-κB-regulated metastasis-related molecules in engrafted tongue tumors. Our results suggest that the selective inhibition of NF-κB activation by NBD peptide may provide an effective approach for the treatment of highly metastatic OSCC. 

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Oral cancer, predominantly OSCC, is a significant public health problem, and approximately 30% of patients are found to have lymph node metastasis.1 Despite advances in treatment, the 5-year survival rate is <50% for patients with a single unilateral lymph node metastasis, and <25% for patients with bilateral metastases.2 Therefore, to elucidate the crucial molecular pathways associated with the metastasis of OSCC is significant for the development of more effective treatments to improve patient survival.

Cancer metastasis consists of a complex, multistep succession of events, and these events are regulated by a wide variety of molecules that contribute to cell adhesion, migration, invasion, and angiogenesis/lymphangiogenesis.3 We previously reported that an increased expression of some extracellular matrix molecules, including fibronectin,4,5 their receptors, some integrins (including β1 integrin),6 and MMPs such as MMP-1, -2, -9, and -147,8 are involved in the invasive and metastatic properties of OSCC. VEGF-C expression is also reported to correlate with lymphangiogenesis and lymphatic metastasis in OSCC.9

The NF-κB signaling pathway is activated in many cancers, including head and neck cancer, contributing to the acquisition of malignant characteristics, such as increased invasion, survival, chemoresistance, and angiogenesis.10,11 We have previously shown that a high expression level of NF-κB correlates with enhanced invasion and metastasis of OSCC.12 The phosphorylation of IkB by the IKK complex is a critical step in pathways leading to NF-κB activation. The IKK complex contains two catalytic subunits (IKKα and IKKβ) and a regulatory subunit named NEMO or IKKγ.13 NEMO is reported to associate with a segment in the C-terminus of both IKKα and IKKβ that is named the NBD.14 Previous studies have shown that a cell-permeable NBD peptide disrupted the association of NEMO with both IKKs in vitro, blocked TNF-α-induced NF-κB activation in various cell types, and effectively ameliorated the responses in animal models of inflammation.14,15

We and others have shown that NF-κB inhibition in OSCC reduces the metastatic potential by downregulating metastasis-related molecules in vitro.16–18 However, to the best of our knowledge, there has been no previous report to show that targeting NF-κB suppresses the regional lymph node metastasis of OSCC by using an orthotopic tumor transplantation model which mimics the human metastatic processes. Furthermore, the utility of a selective NF-κB inhibitor, the NBD peptide, as a potential anticancer agent has not yet been fully elucidated.

In this study, we developed highly lymph node-metastatic OSCC cell lines by an orthotopic nude mouse model, and found that enhanced NF-κB activity plays a crucial role in the metastasis of OSCC. Then we investigated the effect of targeting NF-κB by the NBD peptide on the metastatic potential of OSCC in this model.

Materials and Methods

Establishment of a GFP-expressing OSCC cell line. A human OSCC cell line, SAS cells were initially transfected with the pAcGFP1-C1 vector (Clontech Laboratories, Mountain View, CA, USA) using Fugene 6 (Roche Diagnostics, Indianapolis, IN, USA), then the transfectants that showed strong GFP fluorescence were isolated by a flow cytometer. The isolated transfectants were further selected by culture in 1 mg/mL G418 (Life Technologies, Grand Island, NY, USA) supplemented

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medium. The resultant cells with bright GFP, designated GSAS, were used as the parental cells in this study. All other materials and methods are described in detail in Data S1.

Results

Establishment of metastatic OSCC cell lines, and their effects on survival in a mouse model. To analyze the mechanism underlying the metastasis of OSCC, we established five metastatic OSCC sublines, which were designated GSAS/N1, N2, N3, N4, and N5 according to the in vivo passage number after cervical lymph node metastasis. The in vivo GFP imaging findings of the tumors at 21 days after orthotopic transplantation are shown in Figure 1(a-1–a-3). All tumor-inoculated nude mice had local tumor growth in the tongue (Fig. 1b). The metastatic potential of the GSAS/N sublines during the five rounds of transplantation is shown in Figure 1(b). After the first and second transplantation, cervical lymph node metastases were observed in four of 10 (40%) mice. During the third, fourth, and fifth rounds of transplantation, the cervical lymph node metastatic rate increased to 60%, 70%, and 100%, respectively. The average number of metastatic lymph nodes increased from 0.5 after the first transplantation to 2.3 after the fifth. Liver and lung metastases were observed after more than three rounds of transplantation. No significant differences

Fig. 1. Properties of OSCC cell lines with high metastatic potential to the cervical lymph nodes and the effects of the cells on survival in an orthotopic nude mouse model. (a-1–a-3) In vivo GFP imaging of tumors at 21 days after orthotopic transplantation. The tongue tumor growth (a-1,a-2, arrow) and the development of metastases to the cervical lymph nodes (a-1, arrow heads), liver (a-2, arrow head), and lung (a-3, arrow head) are clearly visible. (b) Tumorigenesis and the metastatic potential of GSAS sublines during five rounds of transplantation. *P < 0.05. (c) In vitro growth of GSAS sublines. Cell proliferation in DMEM containing 10% FBS was monitored for 7 days. The results represent the means ± SD of three independent experiments. (d) Effects of GSAS sublines on the survival of nude mice after transplantation.
in the in vitro cellular growth activity were found between the parental (GSAS) and the metastatic (GSAS/N1, N3, N5) sublines (Fig. 1c).

We next examined the effects of the metastatic sublines on survival in this model. The mice inoculated with GSAS/N3 and N5 cells died significantly earlier than those inoculated with GSAS cells (Fig. 1d), suggesting that the in vivo malignancy of the GSAS/N3 and N5 cells was significantly higher than that of the parental GSAS cells.

**Highly metastatic OSCC cells show increased expression levels of cell migration-, tumor invasion-, and lymphangiogenesis-related molecules in vivo.** We immunohistochemically investigated the expression levels of metastasis-related molecules in the tongue tumor tissue after transplantation of GSAS and GSAS/N5 cells. As shown in Figure S1, highly metastatic GSAS/N5 cells expressed higher levels of metastasis-related molecules involved in cell migration (β1 integrin, fibronectin), tumor invasion (MMP-1, -2, -9, and -14), and lymphangiogenesis (VEGF-C) than the parental GSAS cells. In addition, positive staining for GSAS and GSAS/N5 for AE1/AE3 provided evidence that these cells show the characteristics of epithelial cells.

As these molecules overexpressed in the highly metastatic cells were all NF-κB-regulated molecules, we hypothesized that the highly metastatic OSCC cells possess enhanced NF-κB activity.

**Highly metastatic OSCC cells show enhanced NF-κB activity in vivo and in vitro.** To obtain further evidence of the involvement of NF-κB in the metastasis of OSCC, we next examined the expression status of NF-κB (p65) in the tongue tumors after transplantation of GSAS, GSAS/N3, and N5 cells. As shown in Figure 2(a), nearly all of the expression of NF-κB was found in the cytoplasm in GSAS tumors. In contrast, most of it was found in the nucleus of GSAS/N3 and N5 tumors. These findings suggest that NF-κB is constitutively activated in highly metastatic OSCC cells in vivo.

We next evaluated the nuclear translocation of NF-κB after TNF-α stimulation by immunofluorescence assays (Fig. 2b). The subcellular localization of p65 in GSAS/N3 and N5 cells cultured without TNF-α stimulation was mainly identified in the cytoplasm, unlike that in tumor tissues. This finding seemed to reflect the differences in the cellular states between serum-free cultured cells and in vivo tumor cells exposed to various environmental stimuli. At 20 min after TNF-α stimulation, the subcellular localization of p65 was found to be approximately 50%, 75%, and 90% in the nuclei of GSAS, GSAS/N3, and GSAS/N5 cells, respectively. Furthermore, pretreatment of the GSAS/N5 cells with the NBD peptide inhibited the translocation of p65 into the nucleus.

We next examined the DNA binding activity of NF-κB by EMSA (Fig. 2c). In untreated cells, NF-κB activity was the strongest in the GSAS/N5 cells, but the activity was inhibited by a selective NF-κB inhibitor, the NBD peptide. In addition, TNF-α enhanced the NF-κB DNA binding activity in both the parental (GSAS) and the highly metastatic (GSAS/N3, N5) cells.

We further examined the transcriptional activity of NF-κB (Fig. 2d), which increased according to the in vivo passage number after metastasis in the untreated cells. TNF-α markedly enhanced the NF-κB transcriptional activity, and the NBD peptide almost completely inhibited it in both the parental GSAS and highly metastatic (GSAS/N3, N5) cells. Additionally, the NBD peptide inhibited the TNF-α-induced transcriptional activity of NF-κB by almost half.

We examined the status of JNK/c-Jun/AP-1 activation after treatment of GSAS/N5 with NBD peptide to confirm that the NBD peptide specially blocked NF-κB activation. The NBD peptide did not affect AP-1, one of other key transcription factors involved in cancer progression (Fig. S2).

Enhanced NF-κB activity in highly metastatic OSCC cells contributes to increased cell migration and tumor invasion. Increased cell migration and tumor invasion are the critical features of highly metastatic cancer cells. To evaluate the migration of the cell lines, we carried out the wound healing assay (Fig. 3a, b). The wounded areas in the GSAS/N5, N3, and GSAS cells recovered in this order. Furthermore, the migration of GSAS/N5 cells was augmented by TNF-α, and was inhibited by the NBD peptide. These results suggest that increased cell migration depends on enhanced NF-κB activity.

Next, to evaluate the invasion of the cells, we carried out an in vitro invasion assay. The invasive potential of each cell line was assessed using two types of inner wells coated with collagen I or Matrigel, which is rich in laminin and collagen IV. As shown in both the collagen I (Fig. 3c, d) and Matrigel (Fig. S3a, b) invasion assays, the GSAS/N5, N3, and GSAS cells showed more invasive properties in this order. Furthermore, the invasion of each cell line was augmented by treatment with TNF-α, and was inhibited by the NBD peptide. These results suggest that increased tumor invasion also depends on an enhanced NF-κB activity.

Enhanced NF-κB activity in highly metastatic OSCC cells contributes to increased expression of NF-κB-dependent metastasis-related molecules. Next, we examined whether the highly metastatic OSCC cells with enhanced NF-κB activity overexpress NF-κB-regulated metastasis-related molecules at both the gene and protein levels. As shown in Figure 4, the GSAS/N5 cells expressed higher levels of metastasis-related molecules involved in cell migration (β1 integrin, fibronectin), tumor invasion (MMP-1, -2, -9, and -14), and lymphangiogenesis (VEGF-C) at both the gene and protein levels compared with GSAS and GSAS/N3 cells. Furthermore, the expression level of each molecule in the GSAS/N5 cells was enhanced by TNF-α, and was inhibited by the NBD peptide. These results suggest that an enhanced NF-κB activity contributes to an increased expression of metastasis-related molecules in metastatic OSCC.

Selective inhibition of NF-κB activation using NBD peptide suppresses metastatic potential of highly metastatic OSCC cells in vivo. We next investigated whether the inhibition of NF-κB activation using the NBD peptide suppresses the metastatic potential of highly metastatic OSCC in vivo. We first compared the antimitastatic effects using different concentrations (2, 8, or 40 μg/mouse/day, on day 3, 6, 9, 12, and 15) of the NBD peptide after orthotopic inoculation of the GSAS/N5 cells. The NBD peptide given at 16 μg/mouse/day showed the most potent antimitastatic effect (data not shown). Therefore, we chose this concentration for the in vivo NF-κB blockade experiments. A group of 20 mice was orthotopically inoculated with GSAS/N5 cells, and given either the NBD peptide or a control peptide. Figure 5(a) shows that the NBD peptide clearly suppressed the metastasis of the cells to the cervical lymph nodes compared with the control. Treatment with the NBD peptide also showed growth-inhibitory effects on engrafted tongue tumors (Fig. S4).

We next immunohistochemically examined the expression of AE1/AE3 in the metastatic lymph node sections from the control peptide-treated mice. As a result, positive staining of the metastatic tumors for AE1/AE3 provided evidence that the metastatic tumors show the characteristics of epithelial cells (Fig. S5).

As shown in Figure 5(b), although the mice in both groups had 100% of local tumor growth in the tongue, treatment with the NBD peptide reduced the metastasis to the cervical lymph nodes (control, 100% vs NBD, 40%; P < 0.05), liver (control, 40% vs NBD, 20%), and lungs (control, 10% vs NBD, 0%). We did not observe any apparent toxic side effects or lethality in mice treated with the NBD peptide, and did not find any drug-induced severe damage to either the liver or kidneys of the mice (Fig. S6).
As shown in Figure 5(c), an immunohistochemical analysis of GSAS/N5 tumors engrafted in the tongues revealed that the NBD peptide exerts its antimetastatic effect through decreased expression of β1 integrin, fibronectin, MMP-1, -2, -9, and -14, and VEGF-C, accompanied by decreased nuclear localization of p65. Additionally, the number of LYVE-1-positive lymphatic vessels in NBD-treated tumors were reduced in parallel with the reduced expression of VEGF-C, suggesting that tumor-associated lymphangiogenesis was suppressed by the NBD peptide, and that this was mediated through the inhibition of VEGF-C expression.

**Discussion**

Our data demonstrated that NF-κB plays a crucial role in the metastasis of OSCC, contributing to increased migration and
Enhanced NF-κB activity in highly metastatic OSCC cells contributes to increased cell migration and tumor invasion into the stromal matrices. (a,b) Wound healing assay. The monolayer GSAS subline cells were wounded by scratching, and incubated in serum-free DMEM with vehicle or 1 ng/mL TNF-α or 100 μmol/L NBD peptide. Cell migration into the wound area was photographed under a phase-contrast microscope. Original magnification, ×200. One representative photograph is shown (a), and the quantitative results represent the means ± SD of three independent experiments (b). **P < 0.01. (c,d) In vitro tumor invasion assay. Cells suspended in serum-free DMEM were seeded into the upper chamber with an 8 μm-pore size membrane coated with collagen I, were treated with vehicle, 1 ng/mL TNF-α, 100 μmol/L NBD peptide, or 1 ng/mL TNF-α plus 100 μmol/L NBD peptide. After 24 h, the invaded cells on the bottom of the membrane in the outer chamber containing serum-supplemented DMEM were stained and quantified. A representative photograph of each group is shown (c, original magnification, ×100), and the quantitative results represent the means ± SD of three independent experiments (d). **P < 0.01.
invades. With regard to increased cell migration in the highly metastatic cells, fibronectin and β1 integrin seemed to be important factors. The migration of cancer cells on a fibronectin-coated dish has been reported to be promoted. On the other hand, the integrin-ECM interaction initiates signal transduction, resulting in cell migration and the release of MMPs.

With regard to the increased tumor invasion of the highly metastatic cells, MMP-1, -2, -9, and -14 are proteolytic enzymes that have a significant role in facilitating tumor invasion and metastasis through degradation of the ECM. As MMP-1 can degrade stromal fibrillar collagen I, and MMP-2 and -9 can degrade collagen IV, the augmented invasiveness found in the collagen I and Matrigel invasion assays were likely due to upregulation of MMP-1, and MMP-2 and -9, respectively. MMP-14 (MT1-MMP) is a membrane-bound MMP that has been reported to drive invasion by functioning as an activator of proMMP-2, and is also linked to cell migration, angiogenesis, and metastasis.

Our data showing both the upregulation of β1 integrin and MMP-14 in highly metastatic OSCC cells is consistent with a previous study by Zhang et al. They showed data from a comparative gene expression analysis between poorly metastatic and highly metastatic OSCC cells and their highly metastatic derivatives. Therefore, β1 integrin and MMP-14 may play a major role in the metastasis of OSCC to the lymph nodes.

Recently, there have been an increasing number of in vivo studies that have shown that the effects of the NBD peptide against inflammatory disorders, such as inflammatory arthritis, inflammatory bowel disease, and inflammatory osteolysis. Although NBD peptide is reported to inhibit the constitutive NF-κB activity in tumor cell lines, including head and neck SCC, pancreatic cancer, melanoma, and various lymphomas, there has only been one report which showed the blockade of NF-κB by the NBD peptide to reduce cancer metastasis, using a liver metastatic model of lung carcinoma cells. In that study, Maeda et al. described that the liver metastasis induced by NF-κB activation was associated with interleukin-6-mediated angiogenesis.

There is only one in vivo study on OSCC cells clearly showing that targeting NF-κB reduces metastasis in an animal model. In that report, Yan et al. transplanted OSCC cells into the foot pads of mice, thus confirming the occurrence of popliteal fossa lymph node metastases. However, the effects of NF-κB inhibition on OSCC metastasis in their study did not closely mimic the human processes. On the other hand, we demonstrated, for the first time, that the selective inhibition of NF-κB by the NBD peptide reduces the metastasis of highly metastatic OSCC cells in an orthotopic nude mouse model. In addition, we confirmed the metastasis-inhibitory effect of BAY11-7082, another specific NF-κB inhibitor, which selectively inhibits the phosphorylation of IκBα (data not shown). Taken together, these results suggest that selective inhibition of NF-κB by the NBD peptide may have novel therapeutic potential for not only inflammatory diseases, but also cancer metastasis.

Cell proliferation was suppressed, and the expression of NF-κB-dependent genes involved in cell growth (cyclin D1) and cell survival (cIAP1, 2) was downregulated in NBD-treated cultured cells (data not shown). Therefore, our in vitro and in vivo data support the speculation that the reduction in metastatic potential in highly metastatic OSCC cells was due to the combined effects of attenuated migration, invasion, angiogenesis/lymphangiogenesis, growth, and survival due to the inhibition of NF-κB-dependent targets.

In the in vivo NF-κB blockade experiments, we did not observe any severe side-effects or liver or kidney toxicity. These results are in agreement with those of previous reports. However, prior to the clinical use of the NBD peptide in cancer patients, the optimization of both drug design and drug delivery based on pharmacological assessments will be required to prevent detrimental side-effects and ensure optimal activity.

The major limitation of our study is that the present data are based on the findings restricted to a parental and the derivative OSCC cell lines. Therefore, further studies will be required to confirm the role of NF-κB in the metastatic potential of OSCC using other highly metastatic OSCC cell lines. However, in our assessment, it is noteworthy that the NF-κB activity increased

![Fig. 4.](https://example.com/fig4.png) Enhanced NF-κB activity in highly metastatic OSCC cells contributes to increased expression of NF-κB-dependent metastasis-related molecules at both the gene and protein levels. Cells were incubated in serum-free DMEM with vehicle, 1 ng/mL TNF-α, 100 µmol/L NBD peptide, or 1 ng/mL TNF-α plus 100 µmol/L NBD peptide. After 24 h, the samples were extracted for RT-PCR or Western blotting. (a) RT-PCR. The total RNA of each of the cell lines was isolated, and complementary DNA was synthesized. The PCR was carried out using gene-specific primers, and the amplification conditions are shown in Table S1. Expression of GAPDH was used as an internal control. (b) Western blot analysis. The whole cell proteins were separated by SDS-PAGE, transferred onto membranes, and probed with antibodies against fibronectin, β1 integrin, MMP-1, -2, -9, and -14, VEGF-C, and β-actin. After incubation with HRP-conjugated secondary antibodies, the membranes were visualized using the ECL Plus detection kit.
according to the enhancement of the metastatic potential of the OSCC cells, and selective inhibition of NF-κB activation using the NBD peptide reduced metastasis in a model that mimics human metastatic processes.

In conclusion, our results suggest that the selective inhibition of NF-κB activation by the NBD peptide may provide an effective approach for the treatment of highly metastatic OSCC.

Fig. 5. Selective inhibition of NF-κB activation using NBD peptide suppresses the metastatic potential of highly metastatic OSCC cells in vivo by downregulation of NF-κB-regulated metastasis-related molecules. GSAS/N5 inoculated mice were treated with 16 μg/mouse/day NBD peptide or control peptide by i.p. injection on days 3, 6, 9, 12, and 15. After 21 days, the mice were killed, in vivo GFP imaging of the tumor was carried out using an illumination device, and a necropsy was carried out. (a) GFP imaging of the tumors that metastasized to the cervical lymph nodes in control or NBD peptide-treated mice. Arrowheads indicate the GFP imaging of the cervical lymph node metastases in a control mouse. (b) The percentage of mice that developed tongue tumor, cervical lymph node metastasis, liver metastasis, and lung metastasis. **P < 0.01. (c) Representative microscopic images of H&E and immunohistochemical staining of the tongue tumor sections from control or NBD peptide-treated mice. The expression levels of human NF-κB (p65), fibronectin, β1 integrin, MMP-1, -2, -9, and -14, VEGF-C, and murine LYVE-1 were immunohistochemically examined. Original magnification, ×200.
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Disclosure Statement

The authors have no conflicts of interest.

Abbreviations

AP-1 activator protein-1
ECM extracellular matrix

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GFP green fluorescent protein
IKK inhibitor of NF-kappaB
INKL c-Jun NH2-terminal kinase
LYVE-1 lymphatic vessel endothelial hyaluronan receptor-1
MMP matrix metalloproteinase
NBD peptide NEMO-binding domain peptide
NEMO NF-kappaB essential modulator
MMP-2 matrix metalloproteinase-2
NF-kappaB nuclear factor-kappaB
VEGF vascular endothelial growth factor

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Immunohistochemical staining of metastasis-related molecules in tongue tumor tissue after transplantation.

Fig. S2. Examination of AP-1 activation after NBD peptide treatment.

Fig. S3. Matrigel invasion assay.

Fig. S4. Growth-inhibitory effect of NBD peptide on engrafted tongue tumor.

Fig. S5. Immunohistochemical staining for AE1/AE3 of metastatic lymph node sections.

Fig. S6. Hematoxylin and eosin (H&E) staining of liver and kidney sections from mice treated with the NBD peptide.

Table S1. Primer sequences and amplification conditions for RT-PCR.

Data S1. Supplementary materials and methods.

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