Abstract

Purpose: Neuropilin 1 (NRP1) is a mediator of lung branching and angiogenesis in embryonic development and angiogenesis in cancer. The role of NRP1 in cancer progression is not fully elucidated. We investigated the role of NRP1 in cancer invasion and tumor angiogenesis, its signaling pathways, prognostic significance, and therapeutic implications.

Experimental Design: Sixty patients with non–small cell lung cancer (NSCLC) were studied. NRP1 mRNA expression was measured using real-time quantitative reverse-transcription PCR. NRP1 and cancer cell invasion, angiogenesis, and signaling pathways were studied using NRP1 stimulation by vascular endothelial growth factor 165 (VEGF165) and NRP1 inhibition by small interfering RNAs (siRNA), soluble NRP1 (sNRP1), and NRP1-inhibition peptides. The NRP1-inhibition peptides were identified using a phage display peptide library.

Results: NSCLC patients with high expression of NRP1 had shorter disease-free (P = 0.0162) and overall survival (P = 0.0164; log-rank test). Multivariate analyses showed NRP1 is an independent prognostic factor in overall (HR, 2.37; 95% CI, 1.15 to 4.9; P = 0.0196) and disease-free survival (hazard ratio (HR), 2.38; 95% confidence interval (95% CI), 1.15 to 4.91; P = 0.0195) of NSCLC patients. Knockdown of NRP1 suppressed cancer cell invasion, filopodia formation, tumorigenesis, angiogenesis, and in vivo metastasis. NRP1 signaling pathways involved VEGF receptor 2 and phosphoinositide-3-kinase (PI3K) and Akt activation. Two potent synthetic anti-NRP1 peptides, DG1 and DG2, which block NRP1 signaling pathways and suppress tumorigenesis, cancer invasion, and angiogenesis, were identified.

Conclusions: NRP1 is a cancer invasion and angiogenesis enhancer. NRP1 expression is an independent predictor of cancer relapse and poor survival in NSCLC patients. NRP1 plays a critical role in tumorigenesis, cancer invasion, and angiogenesis through VEGF, PI3K, and Akt pathways. NRP1 may have potential as a new therapeutic target in NSCLC.

Lung cancer is the most common cause of cancer deaths, accounting for 17% of deaths from cancer (1, 2). Non–small cell lung carcinoma (NSCLC) is the predominant type of lung cancer (3). Metastasis is the major cause of treatment failure and cancer deaths (4). The identification of metastasis enhancers and their signaling pathways may improve our understanding of the metastatic process and provide future targeted therapy for NSCLC patients.

Neuropilin 1 (NRP1) was originally identified as a neuronal semaphorin 3A receptor that mediates axonal extension during embryonic development (5, 6). It was later discovered to be present in endothelial cells, mediating angiogenesis during development and in lung cells, controlling lung branching during development (7). NRP1 is a type I transmembrane glycoprotein and a coreceptor for two extracellular ligands, semaphorins/collapsins, and vascular endothelial growth factor (VEGF; refs. 8, 9). VEGF mediates tumor angiogenesis and directly enhances tumor growth via VEGF/VEGFR receptor (VEGFR) autocrine loops in tumors (10–13). NRP1 forms complexes with Flt-1 (VEGFR1) and Flk-1/KDR (VEGFR2) to enhance the binding of VEGF165 to VEGFRs and promotes VEGF165-mediated tumor angiogenesis, cell migration, and tumorigenicity (14–16).

NRP1 has been observed in cancer cells, including PC3 prostate cancer cells and metastatic MDA-MB-231 breast cancer cells (17, 18). Overexpression of NRP1 enhances tumor angiogenesis and tumor growth in vivo (18). NRP1 expression is present in various human cancers (19–22) and is associated with increased tumor aggressiveness and neovascularization; however, its modes of action are not fully understood.
In a previous study, we identified by cDNA microarray that NRP1 expression is positively correlated with the invasion ability of cancer cells in lung cancer cell line models (23, 24). The role of NRP1 in cancer progression in NSCLC patients is not fully elucidated. In this study, we investigated the role of NRP1 as an enhancer for cancer invasion, metastasis, and angiogenesis and its signaling pathways, prognostic significance, and therapeutic implications.

**Materials and Methods**

**Cells and reagents.** Human lung cancer cell lines, CL1-0, CL1-1, CL1-5, and CL1-5-F4, were established by selection of increasingly invasive cell populations from a clonal cell line of human lung adenocarcinoma, CL1 (23). Human umbilical cord endothelial cells (HUVEC) and culture media were purchased from Cell Applications, Inc. Cell culture reagents were from Invitrogen. Human VEGF165 was from PeproTech, Inc. Human anti–phospho-VEGFR2 antibody was from Santa Cruz Biotechnology. Mouse antibody to phosphotyrosine (clone 4G10) was from Calbiochem. Anti-VEGFR2 (sc-504) antibody was from Santa Cruz Biotechnology. Recombinant soluble NRP1 (sNRP1) protein containing His6 and c-Myc domain tags was synthesized in NIH-3T3 murine fibroblast cells. Two cyclic peptides DG1 (CRRPRMLTC) and DG2 (CRSRRIRLC) were synthesized by Digitalgene (Taiwan). Primer sequences for reverse transcription-PCR (RT-PCR) and real-time PCR (RT) analysis are shown in Supplementary Table S1.

**Patients and tissue specimens.** Sixty consecutive patients who underwent surgery for NSCLC at the National Taiwan University Hospital from September 1, 1994, to April 30, 1998, were included in the study. This investigation was approved by the Institutional Review Board of the National Taiwan University Hospital. None of the patients had received neoadjuvant chemotherapy or radiation therapy before surgery. Specimens of lung cancer tissue obtained at surgery were immediately snap-frozen in liquid nitrogen and stored at -80°C until use. The postsurgical pathologic stage of each tumor was classified on the basis of the AJCC staging system. Each of the NSCLC subtypes was classified according to the international tumor-node-metastasis classification (25). The demographic features of the patients are shown in Table 1.

**NRP1 mRNA expression in tumor specimens from NSCLC patients.** NRP1 expression in tumors from NSCLC patients was measured by real-time quantitative RT-PCR, based on TaqMan methodology, using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems; ref. 26). The relative amounts of tissue NRP1 mRNA expression were normalized with TATA-box binding protein mRNA and expressed as -ΔC_T = [C_T(NRP1) - C_T(TBP)]. Patients were included in the high-expression group when -ΔC_T was 0.32 (the median) or greater. The primer probe sets were designed and synthesized by Applied Biosystems. The sequences of primers and small interfering RNAs (siRNA) used in this study are listed in Supplementary Table S1.

**In vitro invasion assay.** A modified Boyden chamber system was used to investigate the invasive capability of CL cells treated with selected peptides, sNRP1, and siRNA of NRP1 (23). The polycarbonate membranes (containing 8-μm pores) of Transwell inserts were coated with Matrigel. The cells were suspended in RPMI 1640 containing 10% NuSerum (Life Science), and 2.5 × 10^4 cells were placed into the upper well of each chamber. After incubation for 48 h at 37°C, the Transwell membrane was fixed with methanol for 10 min at room temperature and stained with a 50-μg/mL solution of propidium iodide (Sigma) for 30 min at room temperature. The number of cells in each membrane was counted under a microscope at a magnification of ×50 using the Analytical Imaging Station software package (Imaging Research Inc.). Each sample was assayed in triplicate.

**Identification of NRP1-binding peptides by phage display.** A phage peptide library displaying cyclic random peptides (Ph.D. C7C from New England Biolabs) was used for biopanning of NRP1. Recombinant human sNRP1 protein was coated onto the wells of polystyrene 96-well plates and incubated with 2 × 10^11 plaque-forming units of the primary library. Bound phages were eluted with glycine-HCl (pH, 2.2) and amplified in Escherichia coli (ER2738). Biopanning was repeated for four rounds, with concentrations of Tween 20 in the wash solution increasing from 0.1% to 0.7%. Randomly selected phage clones from the fourth round of panning were sequenced.

**Surface plasmon resonance.** The binding kinetics of selected peptides with NRP1 were investigated using the surface plasmon resonance-based measuring system (Biacore AB) at 25°C. Recombinant NRP1 was synthesized by Applied Biosystems. The sequences of primers and small interfering RNAs (siRNA) used in this study are listed in Supplementary Table S1.

### Table 1. Clinicopathologic characteristics of 60 NSCLC patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Low NRP1 expression patients (%)</th>
<th>High NRP1 expression patients (%)</th>
<th>P</th>
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<tbody>
<tr>
<td>Age (mean ± SD, y)</td>
<td>64.4 ± 11.5</td>
<td>62.1 ± 11.1</td>
<td>0.435*</td>
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<td>Sex</td>
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<tr>
<td>Male</td>
<td>21 (70)</td>
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<td>Female</td>
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<td>Stage</td>
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</tr>
<tr>
<td>I and II</td>
<td>20 (67)</td>
<td>12 (40)</td>
<td>0.069 †</td>
</tr>
<tr>
<td>III and IV</td>
<td>10 (33)</td>
<td>18 (60)</td>
<td></td>
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<tr>
<td>Histology</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Adenocarcinoma</td>
<td>14 (47)</td>
<td>22 (73)</td>
<td>0.064 †</td>
</tr>
<tr>
<td>Squamous</td>
<td>16 (53)</td>
<td>8 (27)</td>
<td></td>
</tr>
</tbody>
</table>

* Fisher’s exact test.
† Fisher’s exact test.
immobilized on CM5 sensor chips by amine coupling at 400 response units using the amine coupling kit (Biacore) according to the manufacturer's instructions. Binding was detected in resonance units after injecting various concentrations of peptide at a flow rate of 30 µL/min. Sensograms of association and dissociation were recorded and analyzed using BIAssessment software 3.0 (Biacore AB).

VEGFR tyrosine phosphorylation. VEGFR2 phosphorylation was assessed as previously described (27). Briefly, CL1-5 cells were treated with a mixture of hVEGF and sNRP1 for 30 min on ice and then at 37°C for 7 min. Cell lysates were immunoprecipitated with anti-VEGFR2 antibodies. For Western blotting, the membranes were first probed with anti–Flk-1 antibodies and then reprobed with anti–phospho-VEGFR2 antibodies 2/3 (pc460) after being stripped with deblotting buffer. In the angiogenesis assay, HUVECs were pretreated with peptides for 10 min followed by treatment with VEGF for 5 min, and the cells were then immediately extracted with lysis buffer. Activation of Flk-1/KDR was determined by immunoblotting cell extracts with anti–Flk-1 antibodies and then reprobing with anti–phospho-VEGFR2 antibodies (pTyr1214) after the membranes had been stripped with deblotting buffer.

Phosphoinositide-3-kinase activity assay. Phosphoinositide-3-kinase (PI3K) activities were assayed as described previously (28) with some modifications. In brief, CL1-5 cell extracts were incubated with the antiphosphotyrosine antibody and then precipitated with protein A-Sepharose. The immunocomplexes were preincubated with phosphatidylinositol-4,5-P2 (Sigma), and the kinase reaction was initiated by adding 10 µCi of [γ-32P]ATP in reaction buffer for 15 min. Phospholipids were separated by TLC and visualized by phosphorimaging.

Wound healing. Cell migration was measured by the in vitro scratch wound healing assay (29). CL1-5 cells were transfected with 24 nmol/L siRNA-1 in 12-well plates. Twenty-four hours after transfection, cells were scratched with a yellow pipette tip and photographed 18, 21, and 24 h after the scratch. The cell migration at 0, 18, 21, and 24 h was evaluated by counting cells that had migrated from the wound edge.

Filamentous actin staining. For filamentous actin (F-actin) staining, cells were seeded on coverslips in 24-well plates and allowed to attach for 24 h in medium containing 10% FCS. The cells were fixed, washed, and permeabilized in 0.1% Triton-X. The cells were incubated for 30 min with 5 units/mL of rhodamine-conjugated phalloidin (Molecular Probe) and mounted using FluorSave reagent (Calbiochem). The slides were analyzed using a Zeiss Axioplan 2 microscope.

Experimental metastasis in vivo. Cells were washed and resuspended in PBS. Subsequently, a single-cell suspension containing 10⁶ cells in 0.1 mL of PBS was injected into the lateral tail veins of the 6-week-old severe combined immunodeficiency (SCID) mice (supplied by the animal center in the College of Medicine, National Taiwan University, Taipei, Taiwan). Mice were killed after 5 weeks. The lungs were removed, weighed, and fixed in 10% formalin for further examination of metastasis formation. The number of lung tumor colonies was counted under a dissecting microscope. All animal experiments were done in accordance with the animal guidelines at the Department of Animal Care, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan.

In vivo angiogenesis assay. All animal work was done under protocols approved by the Institutional Animal Care and Use Committee of the College of Medicine, National Taiwan University. The effect of peptides on in vivo angiogenesis was evaluated in the murine angiogenesis model using the Matrigel plug assay as described by Passaniti et al. (30).

In vivo tumorigenesis assay. CL1-5 cells (2 × 10⁶) were mixed with or without peptides and then implanted into the flanks of the 6-week-old SCID mice. Injected mice were examined every 5 or 7 days for tumor appearance, and tumor volumes were estimated from the length (a) and width (b) of the tumors, as measured with calipers, using the formula V = ab²/2 (31). Mouse experiments were approved by the Laboratory Animal Center, Institute of Biomedical Sciences, Academia Sinica.

Statistical analyses. All data are presented as the means and 95% confidence intervals (95% CI) of at least three experiments. All statistical analyses were done with the SAS Statistical Program (version 9.1; SAS Institute Inc.). Statistical significance was determined using an one-way ANOVA or as described. Fisher’s exact test was done to test associations between covariates and NRP1 for categorical data, and Student’s t test was used to test continuous variables. Survival curves were obtained by the Kaplan-Meier method. Disease-free and overall survival of patients with low versus high expression of NRP1 was analyzed using the log-rank test. Multivariate Cox proportional-hazards regression was done with overall or disease-free survival as the response variable. P < 0.05 was considered statistically significant.

![Figure 2](https://www.aacrjournals.org/clinicaacancerres/content/13/16/4761/F2.large.jpg)

**Fig. 2.** Kaplan-Meier survival plots for patients with non– small cell lung cancer grouped according to NRP1 mRNA expression. The relative amount of tissue NRP1 mRNA, standardized against the amount of TATA-box binding protein mRNA, was expressed as \( \Delta C_T = C_T(NRP1) - C_T(TBP) \), where \( C_T \) is the threshold cycle. Patients were included in the high-expression group when the \( \Delta C_T \) was 0.32 (the median) or greater. A, the difference in disease-free survival between the high- and low-expression patients was significant (\( P = 0.0162 \)). Tick marks, patients free of recurrence at their last follow-up. B, the difference in overall survival between the high- and low-expression patients was significant (\( P = 0.0164 \)). Tick marks, patients alive at their last follow-up. All statistical tests were two sided.
Results

NRP1 expression correlates with the invasive ability of lung cancer cells. Five distinct lung tumor cell lines with progressive invasiveness were established in our previous study. Microarray analysis showed that NRPI was up-regulated in the highly invasive NSCLC cell lines, CL1-5 and CL1-5-F4 (Fig. 1A). NRPI and its coreceptor VEGFR2 were only expressed in the highly invasive CL1-5 and CL1-5-F4 cells. Expression of the NRPI ligand semaphorin 3A was down-regulated in an opposite direction (Fig. 1A).

Fig. 3. Suppression of NRPI expression inhibited the invasion and migration abilities of CL1-5 cells. A, expression of NRPI shown by Western blotting of tumor cells transfected by NRPI-siRNA-1, NRPI-siRNA-2, or nonsilencing siRNA. B, inhibition of CL1-5 cell-invasive activity by NRPI siRNAs. The invasive activity of cells was detected by invasion assay. CL1-5 cells (2.5 × 10⁴) were seeded on Transwells coated with 30 μg Matrigel, transfected with NRPI-siRNA-1, NRPI-siRNA-2, or nonsilencing siRNA and incubated for 48 h. The cells that had invaded the membrane were then counted. Values were normalized to the relative invasion activity of the reagent control. Experiments were done in triplicate, three independent times. ANOVA revealed that the invasion ability was statistically significantly different among the cells treated with different concentrations of siRNAs, either siRNA-1 or siRNA-2 (siRNA-1 group, P < 0.001; siRNA-2 group, P = 0.002). C, inhibition of tumor cell migration by NRPI-siRNA determined by scratch wound healing assay. CL1-5 cells were transfected with siRNA-1 for 24 h, and cells were scratched with a yellow pipette tip. The wound was photographed after the scratch, and cell numbers were counted within the gap area. Pictures of a representative assay at 0, 18, 21, and 24 h are shown here. D, NRPI-siRNA inhibition of tumor cell migration at various times. The number of migrated cells within the gap area was counted after the scratch. Columns, mean of four independent experiments; bars, SE. *, P < 0.05, which is significantly different from nonsilenced cells. E, siNRPI inhibition of tumor cell invasion ability as shown by invasion assay. The relative invasion ability was statistically significantly different among the cells treated with different concentrations of sNRPI by ANOVA (P = 0.002). F, sNRPI inhibition of F-actin polymerization and filopodia formation in CL1-5 cells. Red, F-actin.

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pattern to NRP1. There were no differences in VEGF or plexin A1 expression in this cell panel (Fig. 1B).

**NRP1 mRNA expression correlates with cancer relapse and survival in NSCLC patients.** Real-time quantitative RT-PCR was used to determine the number of NRP1 transcripts in lung cancer tissues from 60 patients with NSCLC. We arbitrarily used the median value to classify patients into high- or low-expression groups. The clinicopathologic characteristics of the 60 NSCLC patients studied are shown in Table 1. Patients with high NRP1 expression had shorter disease-free ($P = 0.0162$) and overall survival ($P = 0.0164$) compared with low NRP1-expression patients (Fig. 2).

Multivariate Cox’s proportional hazards regression analyses showed that low NRP1 expression was associated with overall survival of NSCLC patients independent of clinicopathologic stage, age, sex, and cell type [0 for low and 1 for high NRP1 expression, respectively; hazard ratio (HR), 2.37; 95% CI, 1.15-4.9; $P = 0.0196$]. Similarly, the hazard ratio for disease-free survival remained significant only for the expression of NRP1 (HR, 2.38; 95% CI, 1.15-4.91; $P = 0.0195$).

**Endogenous NRP1 expression knockdown suppresses cancer cell invasion.** To knock down NRP1 expression, two individual siRNAs directed against the NRP1 gene were transfected into NRP1-positive lung cancer cells CL1-5. Significant suppression of NRP1 expression was achieved by siRNA-1 and siRNA-2 (Fig. 3A). Both NRP1 siRNAs decreased the invasion ability of CL1-5 cells in a dose-dependent manner compared with the nonsilencing siRNA control (Fig. 3B).

To examine whether the anti-invasion activity of the NRP1-specific siRNAs is associated with suppression of cell mobility, the effects of NRP1-specific siRNA1 on the migration capability of cells were analyzed. CL1-5 cells were transfected with siRNA-1 or nonsilencing control siRNA; the migration ability was determined by the scratch wound healing assay. That NRP1 siRNAs can suppress CL1-5 cell mobility, and migration capability was shown by the scratch wound healing assay (Fig. 3C). NRP1-siRNAs significantly inhibited the migration of CL1-5 cells at 24 h (Fig. 3D).

**Soluble NRP1 inhibits cancer cell invasion and filopodia formation.** Recombinant sNRP1 was expressed in human fibroblast (NIH-3T3) cells and was secreted into cultured medium. sNRP1 proteins were purified from the conditioned medium by ammonium sulfate precipitation and then by Ni-NTA column purification on a fast protein liquid chromatography (FPLC) system. The binding affinity of the recombinant sNRP1 to VEGF165 was determined by surface plasmon resonance analysis. The average dissociation constant ($K_D$) of the human VEGF165 binding to sNRP1 was 125 nmol/L, consistent with previous results obtained using the same technology (10). sNRP1 is expressed differently from intact NRP1 and seems to be a VEGF165 antagonist (32). We found a dose-dependent decrease in the invasion ability of CL1-5 cells after treatment with sNRP1 (Fig. 3E). The F-actin of CL1-5 cells was stained with rhodamine-conjugated phalloidin and examined by fluorescence microscopy. sNRP1 inhibited F-actin polymerization and filopodia formation in CL1-5 cells in a dose-dependent manner (Fig. 3F).

**Knockdown of endogenous NRP1 expression suppresses cancer metastasis in vivo.** Knockdown of endogenous NRP1 expression in CL1-5 cells by shRNA lentivirus significantly reduced the invasive activity by 50% (Fig. 3G). Mice injected with CL1-5/shNRP1 cells developed significantly fewer pulmonary metastatic nodules than those with CL1-5/shLuc cells (Fig. 3H).
NR1 signaling pathways involve VEGFR2, PI3K, and Akt activation. To identify the signaling pathways affected by NR1, we treated CL1-5 cells with VEGF165 for various periods and analyzed signaling intermediates. CL1-5 cells were treated with VEGF165 and sNRP1, and the phosphorylation of VEGFR2 was determined by immunoprecipitation with an anti-VEGFR2 antibody followed by Western blotting with an anti-phospho-VEGFR2 antibody. VEGF165-induced VEGFR2 activation was decreased by sNRP1 in a dose-dependent manner and was totally blocked by high concentrations of sNRP1 (Fig. 4A). VEGF165 induced PI3K activation with peak phosphorylation at 30 min, which returned to the baseline levels by 60 min. The addition of siRNA-1 decreased VEGF165-induced PI3K activation in CL1-5 cells compared with the nonsilencing siRNA-1 control (Fig. 4B). Phosphorylation of Akt, a downstream mediator of PI3K, is involved in NR1 modulation of VEGF actions. VEGF165-induced phosphorylation of Akt at Ser473 in CL1-5 cells was decreased to less than one third in the presence of sNRP1 (Fig. 4C). Both PI3K inhibitors, wortmannin and LY294002, decreased the invasion ability of CL1-5 cells (ANOVA: wortmannin, \( P < 0.001 \); LY294002, \( P < 0.001 \); Fig. 4D and E).

RRXR-containing peptides can inhibit NR1-mediated VEGFR2 phosphorylation. To identify whether any new signature motif can bind and inhibit NR1-mediated invasion, we used mammalian cell–expressed NR1 proteins as bait to screen a random cyclic 7-mer peptide library for NR1-binding peptides. A Ph.D. C7C phage display library containing 10\(^{11}\) random cyclic 7-amino acid peptides was applied for biopanning. After four rounds of biopanning, 63 clones were isolated. DNA sequencing showed that almost all selected peptides contained arginine (R) residues. A consensus motif, -RRXR-, was found in nine clones by MULTALIN program alignment (Table 2). The two most potent peptides (cyclic 9-mer peptides, DG1, and DG2) were selected and chemically synthesized for further analysis of their binding kinetics and NR1 inhibition. Surface plasmon resonance was used to measure the real-time association and dissociation of the binding of RRXR-containing peptides to NR1. The average dissociation constants (\( K_D \)) for the binding of DG1 and DG2 to NR1 were 1.40 ± 0.23 and

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**Fig. 4.** VEGF165-induced NR1 signaling involves VEGFR2 phosphorylation, activation of PI3K and Akt phosphorylation. A, sNRP1 inhibits phosphorylation of VEGFR2. Phosphorylation of VEGFR2 was determined by immunoprecipitation with an anti-VEGFR2 antibody followed by Western blotting with an anti-phospho-VEGFR2 antibody. Total VEGFR2 was determined by Western blot with an anti-VEGFR2 antibody. B, the effect of siRNA-1 on PI3K activity. After siRNA-1 (NR1) transfection for 48 h, CL1-5 cells were treated in RPMI SF medium with 1.3 nmol/L VEGF165 for the indicated times. PI3K activity was detected as described in Materials and Methods. C, inhibition of Akt phosphorylation by sNRP1 in CL1-5 cells. CL1-5 cells were treated with 1.3 nmol/L VEGF165 in the presence of (a) or absence of (b) 10 nmol/L of sNRP1 for the indicated times. Akt and phospho-Akt proteins were detected by Western blotting. The ratio of phosphorylated to total Akt is represented by Akt-p/Akt. D, the invasion ability was statistically significantly different among the cells treated with different concentrations of wortmannin by ANOVA (\( P \leq 0.001 \)). E, the invasion ability was statistically significantly different among the cells treated with different levels of LY294002 by ANOVA (\( P \leq 0.001 \)).
5.37 ± 0.49 μmol/L, respectively (Table 3). The slightly higher binding affinity of DG1 to NRP1 was due to a more favorable $k_a$. No binding was observed to either the immobilized VEGFR1 or VEGFR2 sensor chips (data not shown). DG1 and DG2 specifically inhibited VEGF$_{65}$-induced phosphorylation of VEGFR2 at Tyr$^{1214}$ in a concentration-dependent manner with a significant effect at 40 μmol/L and almost complete inhibition at 120 μmol/L (Fig. 5A).

### Table 2. The RRXR motif of the peptides selected by binding to NRP1

<table>
<thead>
<tr>
<th>Peptide number</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>4-1</td>
<td>RRPRLMT</td>
</tr>
<tr>
<td>4-2</td>
<td>QLRQRQRR</td>
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<td>4-3</td>
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<tr>
<td>4-4</td>
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<td>4-28</td>
<td>PIRRQRRL</td>
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<tr>
<td>4-53</td>
<td>HKRRIRQ</td>
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<tr>
<td>Consensus</td>
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RRXR-containing peptides inhibit cancer cell invasion, tumorigenesis, and tumor angiogenesis. The in vitro invasion assay was done using the highly invasive CL1-5 cells to investigate the effects of DG1 and DG2 on the invasiveness of the lung carcinoma cells. Treatment with DG1 or DG2 peptides inhibited CL1-5 cell invasion in a dose-dependent manner (Fig. 5B). DG1 reduced the number of cancer cells invading through the Matrigel by 70%, and DG2 reduced the number of cancer cells by 50%. This suggests that the interaction of the peptides with NRP1 may be associated with NRP1-mediated cancer cell invasion. The treatment was not cytotoxic, suggesting that the decreased number of invading cells was due to the inhibitory effect of the RRXR-containing peptides on the invasive phenotype.

To understand whether DG1 can reduce angiogenesis or tumorigenesis, in vivo angiogenesis and xenograft tumor assays were done. DG1 inhibited tumor angiogenesis in vivo (Fig. 5C). The tumor microvascular count from DG1-treated CL1-5 cells (75 ± 4; in ×200 fields) was significantly less than that of the untreated tumor cells (227 ± 33; in ×200 fields). The tumor angiogenesis activity of DG1-treated CL1-5 cells decreased significantly by 3-fold compared with the untreated tumor cells.

Next, we investigated the effect of DG1 on tumorigenicity in vivo using the xenograft tumor assay. DG1 treatment reduced tumor volume to 60.1 mm$^3$ (95% CI, 27.6-92.6 mm$^3$) in mice 21 days after the inoculation of the CL1-5 cells, compared with the tumor volume of 464.1 mm$^3$ (95% CI, 200.1-728.2 mm$^3$; $P = 0.003$) without DG-1 treatment (Fig. 5D).

### Discussion

Our results indicate that NRP1 is an enhancer of cancer invasion and angiogenesis and is an independent predictor of cancer relapse and poor survival in NSCLC patients. Suppression of NRP1 signaling inhibits cancer invasion, tumorigenesis, angiogenesis, and in vivo metastasis. The protumorigenic effect of NRP1 involves VEGF, PI3K, and Akt pathways. Two potent synthetic anti-NRP1 peptides (DG1 and DG2), which can block NRP1 signaling pathways, inhibit tumorigenesis, cancer invasion, and angiogenesis, were identified (Fig. 5E).

Our findings on NRP1 as a cancer invasion or angiogenesis enhancer in NSCLC are consistent with the previously reported association of NRP1 with tumorigenesis or angiogenesis in various cancers (19–22) in vitro and in vivo. Similarly, a significant correlation between VEGF and NRP1 has been observed in high-grade preneoplastic lesions, microinvasive and corresponding invasive squamous cell carcinoma, and basaloid carcinoma (33). The association of NRP1 with clinical outcome is less clear. Coexpression of NRP1 and NRP2 associated with neovascularization and tumor progression in NSCLC patients were reported in one study (34). To the best of our knowledge, this is the first report that NRP1 is an independent predictor of cancer relapse and poor survival in NSCLC patients. This may have important clinical implications. NRP1 may be a potential biomarker for the selection of high-risk NSCLC patients for adjuvant chemotherapy, antiangiogenesis therapy, or other new targeted therapies. This may allow the maximization of potential therapeutic benefits for high-risk patients and spare low-risk patients from unnecessary treatment or toxicity.

In this study, we showed that NRP1 interacts with VEGF2 to mediate VEGF-induced tumor invasion. VEGF mediates tumor angiogenesis and promotes migration and invasion of tumor cells by directly acting on its receptors via an endothelial cell–independent pathway (8). Previous studies have shown that NRP1 alone can mediate breast cancer cell migration in a VEGF2-independent manner (12), and in vitro studies have shown that VEGF1 activation by VEGF-A or VEGF-B in colorectal cancer cells leads to an increase in cell migration and invasion (13). VEGF competes with semaphorin 3A for NRP1/plexin A1 complex binding and enhances breast carcinoma migration via an autocrine pathway (35). NRP1 also inhibits migration, independent of semaphorin 3A, in pancreatic adenocarcinoma cells (36). One explanation for the different results may be the presence or lack of VEGF2. In pancreatic adenocarcinoma cells lacking plexin A1 and VEGF2, the repression mediated by NRP1 may result through other ligands yet to be identified. In this study, the endogenous expression level of VEGF2 was correlated with that of NRP1 in the CL1 cell panel, being expressed in CL1-5 and CL1-5-F4 cells, but not in CL1-0 and CL1-1 cells, whereas there was no difference in plexin A1 expression. Our studies on neuropilin function highlight its role in lung cancer cell invasion as a critical VEGF2 coreceptor that facilitates VEGF-mediated signaling through this tyrosine kinase–linked receptor.

We found that NRP1 functions as a cancer invasion and angiogenesis enhancer through the VEGF, PI3K, and Akt pathways. VEGF activates protein kinase C, mitogen-activated protein kinase, and phosphatidylinositol 3-kinase pathways. VEGF activates protein kinase C, mitogen-activated protein kinase, and phosphatidylinositol 3-kinase pathways.

### Table 3. Kinetic constants for the interaction of the selected peptides with NRP1

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K_D$ (μmol/L)</th>
<th>$K_a$ (mol/L)$^{-1}$ s$^{-1}$</th>
<th>$k_d$ × $10^{-4}$ s$^{-1}$</th>
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</thead>
<tbody>
<tr>
<td>DG1</td>
<td>1.40 ± 0.23</td>
<td>1,229 ± 246</td>
<td>17.2 ± 2.25</td>
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<tr>
<td>DG2</td>
<td>5.37 ± 0.49</td>
<td>123.4 ± 24</td>
<td>6.63 ± 0.81</td>
</tr>
</tbody>
</table>

NOTE: The kinetic constants were determined using the Biacore system as described in Materials and Methods.
protein kinase/extracellular signal-regulated kinase, p38 mitogen-activated protein kinase, phospholipase C \(\gamma\), PI3K, and Akt/protein kinase B (37–39). PI3K plays a key role in the signal transduction of angiogenesis. PI3K catalyzes the formation of phosphoinositides, which are potent mediators of angiogenesis, cell adhesion, and proliferation (40). Akt is well known for its role as a mediator of cancer cell mobility and invasion (41). VEGF can promote cell movement and tubulogenesis via the Akt pathway (42). These pathways may explain how NRP1 functions as a cancer invasion, metastasis, and angiogenesis enhancer. In endothelial cells, phosphorylation of Tyr\(^{1214}\) of VEGFR2 is required for VEGF-induced angiogenesis.}

![Cyclic 7-mer peptides bind NRP1 and inhibit CL1-5 invasion and angiogenesis in vivo](image)

**A**. The selected peptides reduce phosphorylation of VEGFR2. HUVECs were pretreated with peptides for 10 min followed by treatment with VEGF for 5 min. Phosphorylation of VEGFR2 was determined by Western blotting with an anti-phospho-VEGFR2 antibody. Total VEGFR2 was determined by Western blotting with an anti-VEGFR2 antibody. The effect of peptides on VEGFR2 phosphorylation was assessed using image analysis software.

**B**. The effect of peptides on the invasion activity of CL1-5 cells. Values were normalized to the relative invasion activity of the nontreated control cells. Experiments were done in triplicate, three independent times.

**C**. Immunohistochemical staining of the Matrigel plug sections with an anti-CD31 antibody showed a significant decrease in CD31-positive vessels in plugs containing DG1 peptide compared with mock-treated plugs. Original magnification, \(\times 200\).

**D**. Effect of peptides on tumor angiogenesis in vivo. Volumes of tumors from control CL-15 cells (\(\bullet\)) and DG1-treated cells (\(\blacklozenge\)) were measured at the indicated times as described in Materials and Methods. Means and 95% CI are shown (\(n = 5\) mice per group).

**E**. Summary diagram showing that VEGF\(_{165}\) can bind to NRP1 and trigger the NRP1/VEGFR2/PI3K/Akt signaling pathways and result in tumor angiogenesis, cancer cell invasion, and tumorigenesis. The synthetic peptides DG1/DG2 can specifically block this signaling pathway and may have therapeutic potential.
activation of Cdc42 and cell migration (43). Rho GTPase Cdc42 was the first signaling protein to be shown to induce filopodia (44). DG1 and DG2 specifically inhibited phosphorylation of VEGFR2 at Tyr1214 induced by VEGF165 in a concentration-dependent manner. Our finding that NRP1 expression can induce filopodia formation in cancer cells is new and may explain how NRP1 increases cancer cell migration and invasion.

The role of NRP1 in enhancing tumor angiogenesis and tumor growth suggests that antagonizing NRP1 in tumor cells may be a feasible anti-tumor strategy (45). Schuch et al. (46) reported that sNRP1, as an antiangiogenesis factor, offers a potential treatment for acute myeloid leukemia. Similarly, we found that several small peptides with the consensus RRXX sequence motif could specifically block NRP1 signaling and suppress cancer cell invasion, tumorigenesis, and tumor angiogenesis, implying that NRP1 may be developed as target for lung cancer therapy. The minimal NRP1-binding synthetic peptide DG1 inhibited the invasive activity and in vivo angiogenesis of lung cancer cells without affecting cell viability. DG1 can inhibit VEGF165-mediated downstream signal and VEGFR2 phosphorylation. Although no sequence homology was found between the selected peptides and VEGF165, we found that peptides with positive net charges and a cysteine-linked cyclic conformation are essential for NRP1 binding (data not shown). Our results are consistent with the report that the cysteine residue at position 22 in exon 7, which is Cys137 in VEGF, is crucial for maintaining the specific structure required for NRP1-binding inhibition (47). It was recently reported that a 24-mer exon 7–encoded peptide can induce apoptosis of human breast carcinoma cells and endothelial cells by blocking VEGF autocrine or paracrine signaling (37). Some of the major advances in cancer treatment are in signal transduction inhibitors using small molecules such as imatinib mesylate in chronic myeloid leukemia (48). The development of NRP1-targeted therapy for lung cancer using small-molecule signal transduction inhibitors such as DG1 and DG2 may be worthy of further investigations.

In conclusion, NRP1 is a cancer invasion and angiogenesis enhancer. NRP1 is an independent predictor of cancer relapse and poor survival in NSCLC patients. NRP1 plays a critical role in tumorigenesis, cancer invasion, metastasis, and angiogenesis through VEGF, PI3K, and Akt pathways. NRP1 may be a potential new therapeutic target in NSCLC. Synthetic anti-NRP1 peptides with conserved RRXX sequence motifs can block NRP1 signaling pathways and suppress tumorigenesis, cancer invasion, and angiogenesis.

References


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