

## NK4 gene therapy targeting HGF-MET and angiogenesis

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### TABLE OF CONTENTS

1. Abstract
2. Significance of HGF-Met in molecular target therapy of cancer
3. NK4 as HGF-antagonist
4. Biological activity of NK4 as angiogenesis inhibitor
5. Experimental cancer treatment by gene expression of NK4
  - 5.1. Inhibition of invasion-metastasis associated with Met inactivation by NK4 gene therapy
  - 5.2. NK4 gene therapy using recombinant adenovirus for metastatic cancers
6. Conclusion and perspective
7. References

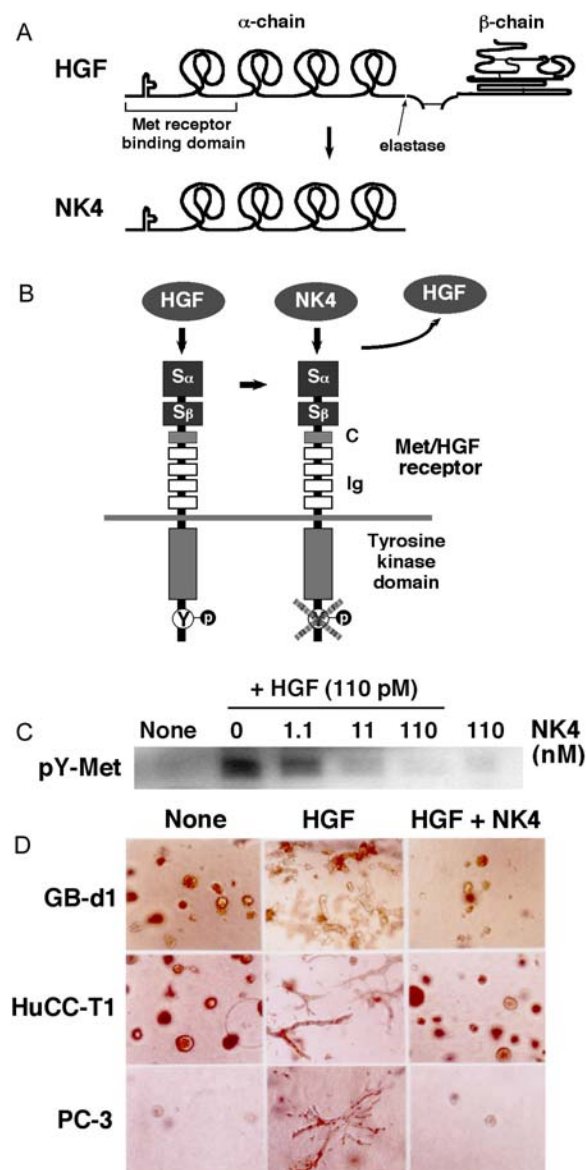
## 1. ABSTRACT

Based on the background that hepatocyte growth factor (HGF) and Met/HGF receptor tyrosine kinase play a definite role in tumor invasion and metastasis, NK4 was isolated as a competitive antagonist against functional association between HGF and Met. NK4 is an internal fragment of HGF and composed of the N-terminal and four kringle domains. Independently on its HGF-antagonist action, NK4 inhibited angiogenesis induced by vascular endothelial cell growth factor and basic fibroblast growth factor, as well as HGF, indicating that NK4 is a bifunctional molecule that acts as an HGF-antagonist and angiogenesis inhibitor. In experimental models of distinct types of cancers, NK4 gene therapy inhibited Met receptor activation and this was associated with inhibition of tumor invasion and metastasis. Likewise, NK4 gene therapy inhibited tumor angiogenesis, thereby suppressing angiogenesis-dependent tumor growth. Cancer treatment with NK4 suppresses malignant tumors to be 'static' in both tumor growth and spreading. NK4 warrants further investigation and attention as potential cancer therapy for humans.

## 2. SIGNIFICANCE OF HGF-MET IN MOLECULAR TARGET THERAPY OF CANCER

It has been established that development of a carcinoma is due to the accumulation of somatic mutations that occur in oncogenes and tumor suppressor genes in epithelial cells. A better understanding of genetic changes that occur in cancer cells would also facilitate progress in new therapeutic approaches such as 'molecular target therapy'. On the other hand, the neoplastic transformation of epithelial cells and the malignant behavior of carcinoma cells such as invasion and metastasis are influenced by their interactions with neighboring stromal components, including fibroblasts, blood vessels, inflammatory cells, and the extracellular matrix (1, 2). An understanding of substances that mediate mutual interactions between epithelial cells of normal or neoplastic and surrounding stromal cells would provide new insights into tumor biology and therapeutics.

Hepatocyte growth factor (HGF) was first identified and cloned as a mitogenic protein for hepatocytes (3-5). HGF is a heterodimeric molecule



**Figure 1.** Structure and biological activities of NK4 as an antagonist against HGF. (A) Schematic structures of HGF and NK4. NK4 was initially obtained after elastase-treatment of HGF. (B) Outline for antagonistic action of NK4 against HGF and Met/HGF receptor. (C) Inhibition of Met receptor tyrosine phosphorylation by NK4. MC-38 murine colon carcinoma cells were stimulated with HGF and varying concentrations of NK4. Tyrosine phosphorylation of the Met receptor was detected with Western blot. (D) *In vitro* inhibition of cancer cell invasion by NK4. Human cancer cell lines (GB-d1, gallbladder carcinoma; HuCC-T1, cholangiocarcinoma; PC-3, lung carcinoma) were cultured in collagen gel in the absence or presence of HGF (110 pM) + NK4 (110 nM).

composed of a 69 kDa alpha-chain and a 34 kDa beta-chain. The alpha-chain contains the N-terminal hairpin domain and subsequent four kringle domains, while the beta-chain contains a serine protease-like domain (Figure

1A). Subsequent studies revealed that HGF has multiple biological effects on a wide variety of cells, including mitogenic, motogenic, morphogenic and anti-apoptotic activities (6, 7). The receptor for HGF is a c-met protooncogene product of transmembrane tyrosine kinase (8). HGF plays roles in dynamic construction and reconstruction of tissues during development and tissue regeneration. In mature tissues, HGF has an organotrophic role in regeneration and protection of various tissues, including the liver, lung, kidney, gastrointestinal tissues, nervous, and cardiovascular tissues (6).

In malignant tissues, however, tumor cells utilize the biological actions of HGF for their invasive and metastatic behavior. HGF potently stimulates invasion of cancer cells and aberrant expression/activation of the Met receptor is closely associated with the progression of tumors toward metastatic tumors (9-11). Particularly, HGF has attracted considerable attention as a stromal-derived mediator in tumor-stromal interactions, based on its paracrine action and close involvement in cancer invasion and metastasis. Since the fatal nature of carcinoma is the result of invasion and subsequent metastasis, new therapeutic approaches targeting HGF and Met tyrosine kinase are ongoing. NK4 was first identified as a competitive antagonist targeting the HGF-Met system (12, 13).

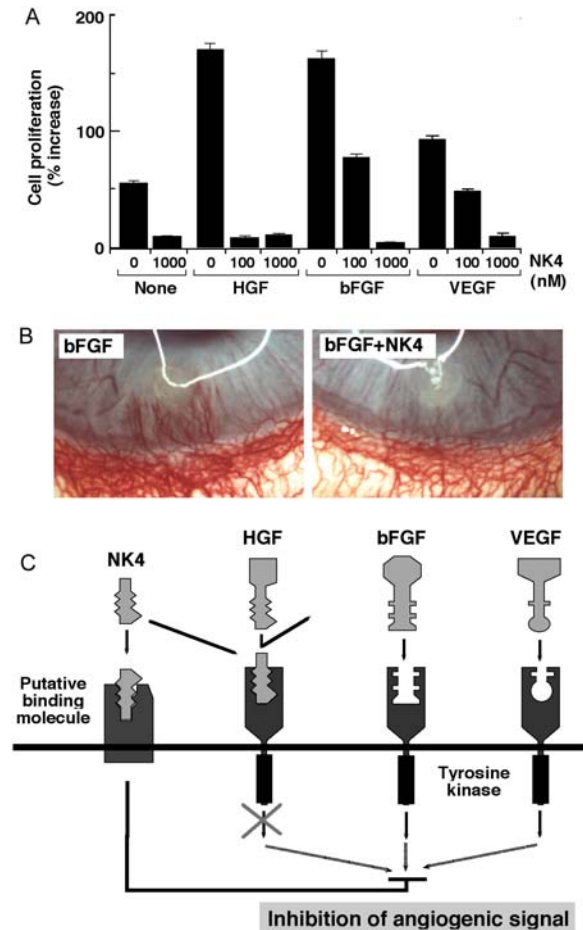
### 3. NK4 AS HGF-ANTAGONIST

NK4 was initially purified from fragments obtained after digestion of HGF with elastase (12). NK4 is composed of the N-terminal 447 amino acids of the alpha-chain of HGF and contains the N-terminal hairpin domain and four kringle domains (thus designated NK4) (Figure 1A). Binding domains responsible for high affinity binding to Met receptor are the N-terminal hairpin and the first kringle domains in NK4 and HGF. NK4 thus binds to Met receptor, but does not activate the Met receptor, thereby competitively inhibiting the Met receptor activation induced by HGF (Figure 1B). It is noteworthy that NK1, NK2, and NK3, all smaller variants of the alpha-chain containing binding domains to the Met receptor, retain agonist activity. NK4 is a complete competitive antagonist for the HGF-Met receptor system, being devoid of agonistic activity in activating the Met receptor.

Figures 1C and D respectively demonstrate the effect of NK4 on HGF-induced Met receptor tyrosine phosphorylation and invasion of cancer cells. HGF induces tyrosine phosphorylation of the Met receptor, whereas simultaneous addition of NK4 and HGF dose-dependently inhibited Met receptor tyrosine phosphorylation. Likewise, HGF induced invasion of human cancer cells cultured in collagen gel, whereas NK4 almost completely inhibited invasion of the cancer cells. Similar effects of NK4 were also demonstrated in distinct types of cancer cells (12, 14-18).

### 4. BIOLOGICAL ACTIVITY OF NK4 AS ANGIOGENESIS INHIBITOR

Because vascular endothelial cells express the Met receptor and HGF acts as an angiogenic



**Figure 2.** Antiangiogenic activity of NK4 and putative mechanism responsible for angioinhibitory action of NK4. (A) Inhibitory effect of NK4 on proliferation of human endothelial cells. Human microvascular endothelial cells were cultured in the absence or presence of HGF (3 ng/ml), bFGF (3 ng/ml), VEGF (10 ng/ml), and/or NK4 for 72 h. (B) Inhibition of bFGF-induced angiogenesis by NK4 in rabbit cornea. A pellet containing 100 ng bFGF or 100 ng bFGF plus 1000 ng NK4 was implanted in the rabbit cornea. (C) Putative mechanism responsible for angioinhibitory action of NK4.

growth factor, it was predicted that NK4 might inhibit the angiogenic responses induced by HGF. However, when effects of NK4 on human vascular endothelial cells in culture were examined, NK4 unexpectedly inhibited proliferation and migration of endothelial cells enhanced by basic fibroblast growth factor (bFGF) and vascular endothelial cell growth factor (VEGF), as well as by HGF (Figure 2A) (19, 20). Likewise, when a pellet containing bFGF or VEGF was implanted under the rabbit cornea, bFGF induced extensive angiogenesis, whereas the co-existence of NK4 with bFGF or VEGF in the pellet inhibited angiogenesis induced by bFGF or VEGF (Figure 2B). These results suggested that NK4 has an angioinhibitory as well as antagonizing action against HGF and the Met receptor. Because NK4 competitively inhibits biological actions of HGF through high-affinity binding to the Met receptor, we asked if the binding of NK4 to the

Met receptor is involved in the antiangiogenic activity of NK4. To address this issue, we prepared a smaller variant of NK4 incapable of binding to the Met receptor. Based on knowledge that both the N-terminal domain and the first kringle domain in HGF are responsible for high-affinity binding to the Met receptor, the N-terminal domain was deleted from NK4. Deletion of the N-terminal domain in NK4 led to a loss of HGF-antagonist activity, whereas the remaining variant composed of four kringle domains retained antiangiogenic activity (21). The result strongly suggests that NK4 is bifunctional, as it is HGF-antagonist and an angiogenesis inhibitor.

Given that the binding of NK4 to the Met receptor is not involved in angioinhibitory action of NK4, how NK4 inhibits signal transduction driven by angiogenic growth factors remained to be addressed. In human endothelial cells in culture, NK4 inhibited tyrosine phosphorylation of the Met receptor induced by HGF, whereas it did not inhibit tyrosine phosphorylation of the VEGF receptor-2 induced by VEGF (19). Likewise, NK4 did not inhibit activation of ERK-1/2 induced by VEGF and bFGF. These results indicated that NK4 allowed for activation of receptors and subsequent signal transduction, at least, leading to activation of ERK-1/2. Therefore, NK4 seems to inhibit angiogenic signal transduction downstream of ERK-1/2 or through a different pathway from ERK-1/2. Taken together with the finding that the binding of NK4 to the Met receptor is not required to exert angioinhibitory actions, association of NK4 to a putative binding molecule other than Met receptor may participate in the antiangiogenic signal transduction of NK4 (Figure 2C).

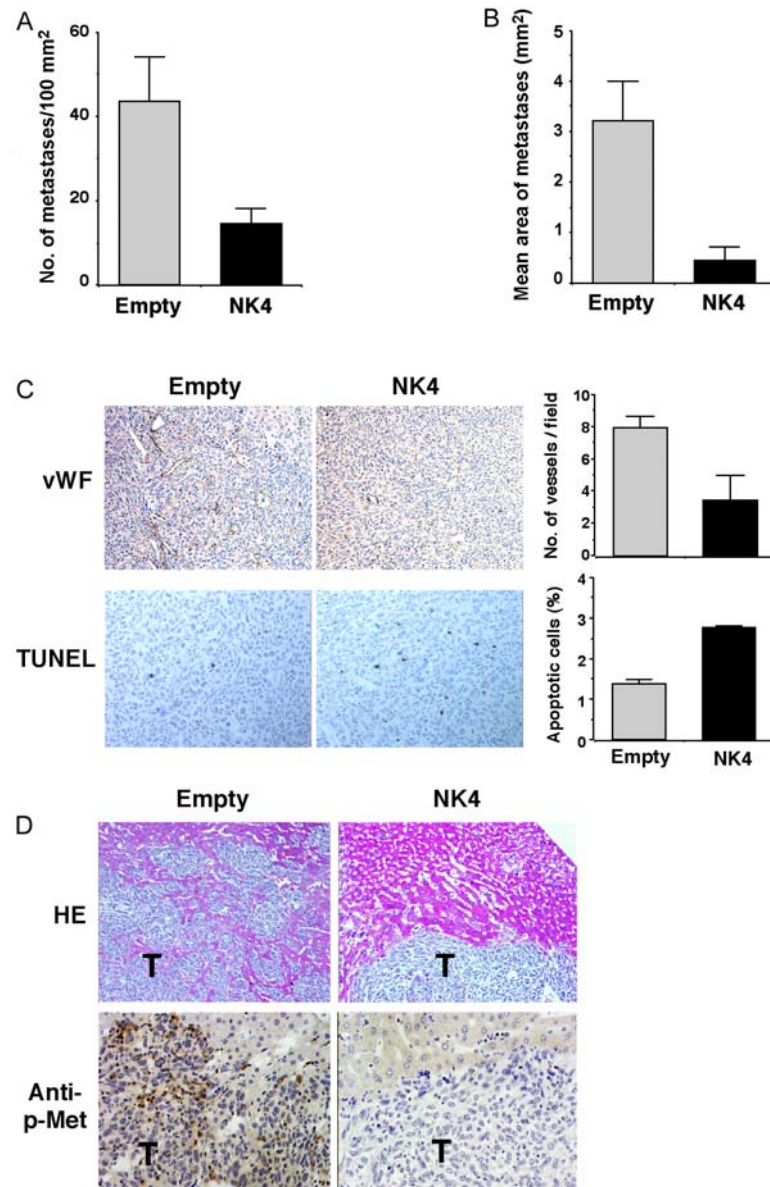
## 5. EXPERIMENTAL CANCER TREATMENT BY GENE EXPRESSION OF NK4

Angiogenesis inhibitors are in clinical development for treatment of diseases associated with unusual angiogenesis, including malignant tumors, because growth of a solid tumor depends on angiogenesis. NK4 as an angiogenesis inhibitor and HGF-antagonist suggests that treatment of patients with NK4 might effectively suppress the malignant behavior of a tumor.

### 5.1. Inhibition of invasion-metastasis associated with Met inactivation by NK4 gene therapy

Colon carcinoma represents one of the most frequently occurring cancers and is associated with high mortality. The majority of deaths from colon cancer are secondary to metastatic disease, with the liver being overwhelmingly the most frequent site of metastasis. Inhibition of liver metastasis and subsequent intrahepatic spreading and invasive growth may prove to be a new therapeutic approach for the treatment of colon carcinoma.

When murine colon carcinoma cells (MC-38 cell line) were inoculated into the spleens of mice, the cancer cells metastasized to the liver and formed a number of metastatic nodules 21 days after inoculation of cells (22). In this model, the human NK4 gene was expressed predominantly in the liver, using hydrodynamics-based delivery of plasmid. The number of intrahepatic metastatic



**Figure 3.** Inhibition of tumor metastasis, angiogenesis, and invasive growth in metastatic colon cancer by NK4 gene expression in mice (22). (A) Inhibition of liver metastasis. (B) Suppressed growth of hepatic metastases of colon cancer. (C) Inhibition of angiogenesis and promoted apoptosis in hepatic metastases of colon cancer. (D) Inhibition of tumor invasion and *in situ* activation of the Met receptor in hepatic metastases of colon cancer. The tyrosine-phosphorylated Met receptor was immunohistochemically detected using an anti-tyrosine phosphorylated Met antibody. T, tumor; HE, hematoxylin and eosin; Anti-p-Met, anti-tyrosine-phosphorylated Met. Empty or expression plasmid for human NK4 was introduced into mice by hydrodynamics-based gene delivery. Murine colon cancer cells were inoculated into the spleen on day 1 after plasmid delivery and mice were killed on day 21.

nodules was inhibited by NK4 gene expression to 33.6% of a value in control mice (Figure 3A). Likewise, the mean area of each intrahepatic metastasis in mice given the NK4 plasmid was much smaller than that in control mice (Figure 3B), indicating that hepatic expression of NK4 suppressed growth of metastases. Blood vessel density in metastatic nodules in mice given the NK4 plasmid was decreased to 44% of the control value and size of blood vessels in tumor

tissue in mice given the NK4 plasmid was much smaller than that seen in control mice (Figure 3C). Instead, the number of cancer cells undergoing apoptotic cell death was enhanced by hepatic expression of NK4. These results indicated that expression of NK4 inhibited metastasis of colon cancer to the liver and angiogenesis in intrahepatic metastatic tissues, thereby inhibiting growth of hepatic metastases.

Histological appearances of intrahepatic metastases indicated that expression of NK4 strongly inhibited spreading and invasion of cancer cells to surrounding hepatic tissue. In control mice, tyrosine-phosphorylated Met was detectable in colon cancer cells in peripheral regions of metastases, hence the Met receptor was activated *in situ* in these cancer cells (Figure 3D). In contrast, in mice given the NK4 plasmid, tyrosine phosphorylated Met was mostly undetectable in cancer cells. NK4 expressed in the liver inhibited activation of the Met receptor in colon cancer cells, thereby inhibiting the spreading and invasion of cancer cells as an HGF-antagonist. The life span of the mice was also prolonged by NK4 gene therapy. Taken together, experimental results obtained in metastatic colon cancer model indicate that simultaneous blocking of tumor angiogenesis and Met receptor activation by the bifunctionality of NK4 deserves attention for therapeutic strategy to inhibit growth, invasion, and metastasis in malignant tumors.

### 5.2. NK4 gene therapy using recombinant adenovirus for metastatic cancers

Experimental cancer treatment with recombinant adenovirus for gene expression of NK4 was first demonstrated by Maemondo *et al.* (23). The expression cassette composed of the promoter and human NK4 gene was inserted into the replication incompetent type V adenovirus. Human lung cancer cells were subcutaneously implanted into mice and recombinant adenovirus for expression of NK4 (Ad-NK4) was administered intratumorally. Gene expression of NK4 decreased blood vessel density and this was associated with the increase in the number of apoptotic cancer cells and inhibition of tumor growth.

When the Matrigel plug containing HGF or bFGF was subcutaneously implanted into the back of mice, intraperitoneal administration of Ad-NK4 inhibited angiogenesis in the Matrigel plug, suggesting that NK4 expressed in intraperitoneal organs and tissues systemically inhibits angiogenesis in distant Matrigel plug. Consistently, intraperitoneal administration of Ad-NK4 suppressed growth of distantly implanted lung cancer. The result indicates that NK4 inhibits angiogenesis-dependent tumor growth even by systemic delivery of NK4.

In a model of intraperitoneal inoculation of gastric cancer cells, cancer cells showed disseminated metastasis in intraperitoneal regions (24). Intraperitoneal administration of Ad-NK4 decreased the number of disseminated metastases, tumor angiogenesis, and tumor growth. Administration of cisplatin was also effective in suppressing the number of disseminated metastases and tumor growth, whereas combined therapy of cisplatin and Ad-NK4 remarkably inhibited disseminated metastasis and tumor growth. Likewise, the peritoneal dissemination and subsequent growth of pancreatic cancer was suppressed by intraperitoneal administration of Ad-NK4, and this was associated with prolongation of the life span (25).

Antitumor effects of NK4 have been demonstrated in various types of tumors (Table 1). For

distinct types of tumors, NK4 gene therapy or administration of NK4 protein showed similar biological activities, including inhibition of tumor angiogenesis, growth, invasion, and metastasis. Through these biological activities, prolonged survival of mice was seen in some models. Inhibition of tumor metastasis might be attributable to both antiangiogenic and HGF-antagonist activities of NK4, while inhibition of tumor invasion and growth might be respectively attributable to HGF-antagonist and antiangiogenic activities of NK4. Among these studies, mice were well tolerated in NK4 gene therapy or NK4 protein administration, suggesting that NK4 is likely to be highly safe material.

## 6. CONCLUSION AND PERSPECTIVE

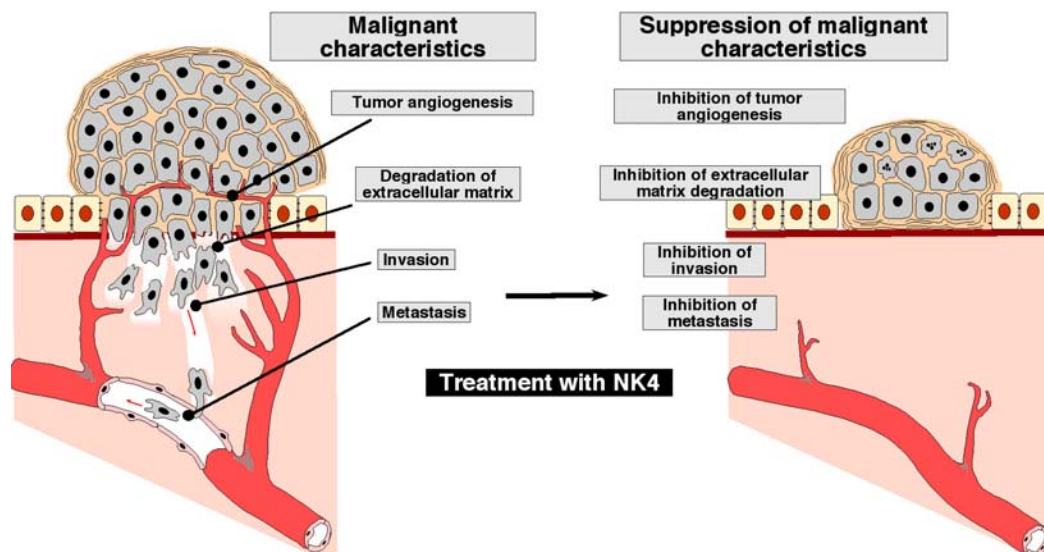
Dependency of tumor growth on neovascularization in tumor tissues is predominantly attributed to primary functions of blood vessels to supply oxygen and nutrients. Angiogenesis inhibition seems to be reasonable way to inhibit growth of a cancer mass, without directly killing tumor cells. However, several clinical studies have shown that the presence of hypoxic regions within tumors correlates with a poor prognosis and an increased risk of metastasis (47), hence a drawback for antiangiogenic strategies. Pennacchietti *et al.* (48) showed that hypoxia led to up-regulation of Met receptor gene expression in cancer cells, thereby leading to amplification of HGF-Met signaling and acquisition of invasive growth potential of cancer cells. Simultaneous inhibition of tumor angiogenesis and HGF-Met signaling by NK4 seems to have a considerable significance in overcoming potential drawbacks in antiangiogenic strategy in cancer treatment. Likewise, many of angiogenic growth factors can be expressed by a single tumor and such redundancy has long been suspected as a potential cause of acquired resistance when tumors are treated with specifically targeted anti-angiogenic drugs. A preclinical evidence for anti-angiogenic drug evasion by alternate pathways of angiogenesis in tumor cells was recently reported (49). In this relevance, inhibitory action of NK4 on angiogenesis driven by different types of angiogenic growth factors such as HGF, bFGF, and VEGF is superior action of NK4 than mono-specific antiangiogenic drugs.

Past experimental approaches and recent clinical trials have established that inhibition of angiogenesis in tumor tissues has become a practical strategy in current/coming cancer treatment. On the other hand, invasive and metastatic characteristics have definitive significance in the prognosis of cancer patients. The HGF-Met receptor system plays definitive role in cancer invasion and metastasis. NK4 was originally identified as a competitive inhibitor of functional association of HGF and the Met receptor, while subsequent studies elucidated angioinhibitory activities of NK4. For a variety of malignant tumors in experimental animals, simultaneous blocking of tumor angiogenesis and HGF-Met system by NK4 gene therapy or NK4 protein administration inhibited malignant characteristics of tumors, including invasion, metastasis, and angiogenesis-dependent growth (Figure 4). Cancer treatment with NK4 is likely to be a way to



**Table 1.** Therapeutic effects of NK4 on distinct types of tumors in experimental models

Type of tumor (species of tumor)	Inoculation site	Therapeutic material	Delivery method	Observed effects	Ref
Breast cancer (human)	Subcutaneous	NK4 protein	Subcutaneous near tumor	Inhibition of angiogenesis and growth	26
Colon carcinoma (murine)	Cervical vein	NK4 gene expression with adenovirus	Intraperitoneal	Inhibition of metastasis (to lung) Prolonged survival	23
Colon carcinoma (murine)	Subcutaneous	NK4 gene expression by adenovirus	Intratumoral, combination with dendritic cells	Inhibition of angiogenesis and growth Induction of tumor-specific cytotoxic T-lymphocytes	27
Colon carcinoma (murine)	Spleen	NK4 gene expression with plasmid	Intravenous	Inhibition of metastasis , Inhibition of angiogenesis, invasion, and growth in metastases, Prolonged survival	22
Colon carcinoma (murine)	Subcutaneous	NK4 gene expression by stable transfection	Stable expression by cancer cells	Inhibition of angiogenesis and growth Prolonged survival	28
Colon carcinoma (murine)	Subcutaneous	NK4 gene expression with plasmid	Intravenous	Inhibition of angiogenesis and growth Inhibition of liver metastasis	45
Gallbladder carcinoma (human)	Subcutaneous	NK4 protein	Subcutaneous, near tumor	Inhibition of invasion and growth	14
Gallbladder carcinoma (human)	Intraperitoneal	NK4 gene expression with adenovirus	Intraperitoneal	Inhibition of growth and metastasis (to peritoneal cavity)	29
Gastric carcinoma (human)	Subcutaneous	NK4 gene expression by stable transfection NK4 gene expression with adenovirus	Stable expression by cancer Intratumoral	Inhibition of growth  Inhibition of growth	30
Gastric carcinoma (human)	Subcutaneous	NK4 gene expression with adenovirus	Intratumoral	Inhibition of angiogenesis and growth	31
Gastric carcinoma (human)	Intraperitoneal	NK4 gene expression with adenovirus	Intraperitoneal, combination with cisplatin	Inhibition of angiogenesis, growth, metastasis (to peritoneal cavity), and ascites accumulation	24
Gastric carcinoma (human)	Intraperitoneal	NK4 gene expression with adenovirus	Intraperitoneal	Inhibition of peritoneal dissemination Prolonged survival in prophylactic administration	32
Gastric carcinoma (human)	Intraperitoneal	NK4 gene expression with cationic liposome HMG-1, 2 complex	Intraperitoneal	Prolonged survival. Inhibition of ascites accumulation, angiogenesis, and growth Suppression of gefitinib-resistance	33
Glioblastoma (human)	Orthotopic (brain)	NK4 protein	Intratumoral	Inhibition of angiogenesis and growth	34
Hepatoma (human)	Subcutaneous	NK4 gene expression with adenovirus	Intratumoral	Inhibition of angiogenesis and growth	35
Hepatoma (human)	Subcutaneous or liver via portal vein	NK4 gene expression with adenovirus	Intratumoral or intravenous	Inhibition of angiogenesis and growth Prolonged survival	36
Lung carcinoma (murine)	Subcutaneous	NK4 protein	Subcutaneous, near tumor	Inhibition of angiogenesis, growth, and metastasis (to the lung)	19
Lung carcinoma (human)	Subcutaneous	NK4 gene expression with adenovirus	Intratumoral or intraperitoneal	Inhibition of angiogenesis and growth	23
Lung carcinoma (murine)	Subcutaneous	NK4 gene expression with adenovirus	Intratumoral, combination with dendritic cells	Inhibition of growth	27
Lung carcinoma (murine)	Subcutaneous	NK4 gene expression by plasmid in cationized gelatin	Subcutaneous, around tumor	Inhibition of angiogenesis, growth, and metastasis (to lung) Prolonged survival	37
Lung carcinoma (human)	Subcutaneous	Cancer cell-specific NK4 gene expression with adenovirus	Intratumoral	Inhibition of angiogenesis and growth Reduction in tumor volume	38
Lymphoma (murine)	Subcutaneous	NK4 gene expression with adenovirus	Intratumoral, combination with dendritic cells	Inhibition of angiogenesis and growth Induction of tumor-specific cytotoxic T-lymphocytes	27
Malignant melanoma (murine)	Subcutaneous	NK4 gene expression with adenovirus	Intratumoral, combination with dendritic cells	Inhibition of growth Induction of tumor-specific cytotoxic T-lymphocytes	27
Mammary carcinoma (murine)	Subcutaneous	NK4 protein	Subcutaneous, near tumor	Inhibition of growth and metastasis (to lung)	19
Multiple myeloma (human)	Subcutaneous	NK4 gene expression with adenovirus	Intramuscular	Inhibition of angiogenesis and growth	46
Ovarian carcinoma (human)	Intraperitoneal	NK4 gene expression by stable transfection	Stable expression by cancer cells	Inhibition of metastasis (to peritoneal cavity)	39
Pancreatic carcinoma (human)	Orthotopic (pancreas)	NK4 protein	Intraperitoneal	Inhibition of invasion, angiogenesis, growth, metastasis (to peritoneal cavity), and ascites accumulation	40
Pancreatic carcinoma (human)	Intraperitoneal	NK4 gene expression with adenovirus	Intraperitoneal	Inhibition of angiogenesis, growth, and metastasis (peritoneal cavity) Prolonged survival	25 41
Pancreatic carcinoma (human)	Orthotopic (pancreas)	NK4 gene expression by stable transfection	Stable expression by cancer cells	Inhibition of angiogenesis, growth, and metastasis (to peritoneal cavity and liver)	42
Pancreatic carcinoma (human)	Subcutaneous	NK4 protein released by cell sheets	Implantation of cell sheet producing NK4	Inhibition of angiogenesis and growth	43
Prostate cancer (human)	Subcutaneous	NK4 protein	Subcutaneous, near tumor	Inhibition of angiogenesis and growth	44



**Figure 4.** Outline for antitumor effects of NK4-treatment. Bifunctionality of NK4 as an HGF-antagonist and as an angiogenesis inhibitor suppresses typical malignant characteristics of a tumor.

suppress malignant tumors and make them 'static' in both growth and spreading. NK4 warrants further investigation and attention as potential cancer therapy for humans.

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**Abbreviations:** HGF: hepatocyte growth factor; bFGF: basic fibroblast growth factor; VEGF: vascular endothelial cell growth factor

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