ELSEVIER

European Journal of Cancer 40 (2004) 2135–2142

European Journal of Cancer

www.ejconline.com

# Adenoviral-mediated Gene transduction of the hepatocyte growth factor (HGF) antagonist, NK4, suppresses peritoneal metastases of gastric cancer in nude mice

Kentaro Ueda [a], Makoto Iwahashi [a,*], Ichiro Matsuura [a], Mikihito Nakamori [a], Masaki Nakamura [a], Toshiyasu Ojima [a], Teiji Naka [a], Koichiro Ishida [a], Kunio Matsumoto [b], Toshikazu Nakamura [b], Hiroki Yamaue [a]

[a] *Second Department of Surgery, School of Medicine, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-8510, Japan*
[b] *Division of Molecular Regenerative Medicine, Course of Advanced Medicine, Osaka University, Graduate School of Medicine, Suita, Osaka 567-0871, Japan*

Received 15 December 2003; received in revised form 27 April 2004; accepted 7 May 2004
Available online 2 July 2004

**Abstract**

The competitive inhibitory effects of NK4 (a specific hepatocyte growth factor (HGF)-antagonist) on the interaction between HGF and the c-Met/HGF receptor has been shown in HGF-mediated invasion of some distinct types of human cancer cells. Furthermore, NK4 has inhibitory effects on the angiogenic pathways driven by basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), as well as by HGF. In this study, to evaluate the therapeutic efficacy of adenoviral-mediated NK4 gene treatment, we employed animal models of peritoneal metastasis using two gastric cancer cell lines, the strongly c-Met expressing MKN45 cell line and the weakly c-Met-expressing cell line, TMK1. In both models, the total number and weight of peritoneal tumours per mouse and ascites treated early with AxCANK4 (administered 3 times 2, 7 and 12 days after the tumour inoculation) were significantly reduced compared with those treated with phosphate-buffered solution (PBS) and AxCALacZ ($P < 0.05$). In Factor-VIII-related-antigen-stained sections from peritoneal metastatic tumours, the inhibition of intratumour vessels was observed in tissues from tumours of MKN45 and TMK1 treated with AxCANK4. We also compared the therapeutic effect of early AxCANK4 treatment with that of late treatment (at 7, 12 and 17 days). Peritoneal metastases and ascites treated late with AxCANK4 showed less of an improvement than those treated early with AxCANK4 in both models. In addition, the inhibitory effect of cisplatin (CDDP) on peritoneal metastasis was significantly enhanced by AxCANK4, suggesting that the combination of intraperitoneal (i.p.) chemotherapy with NK4 gene therapy might be effective, even in cases of advanced peritoneal metastasis from gastric cancer. To conclude, these results show clearly that NK4 gene therapy inhibits peritoneal metastases from gastric cancer, regardless of the level of c-Met/HGF receptor expression in the tumour cells, and especially in the early stages of peritoneal metastasis.
© 2004 Elsevier Ltd. All rights reserved.

*Keywords:* Gene therapy; NK4; HGF antagonist; Adenoviral vector; Peritoneal metastasis; Gastric cancer; CDDP

## 1. Introduction

Peritoneal metastasis is the most common type of metastasis associated with gastric cancer [1], and is most likely caused by the presence of free cancer cells exfoliated from the serosal surface of the primary cancer in the abdominal cavity [2]. Because the rate of recurrence in the form of peritoneal metastasis after surgery for advanced gastric cancer is 44.2%, this mode of metastasis severely affects the prognosis of cancer patients [3]. Although trials to prevent the peritoneal metastasis of gastric cancer have been performed, including

* Corresponding author. Tel.: +81-73-441-0613; fax: +81-73-446-6566.
*E-mail address:* makoto@wakayama-med.ac.jp (M. Iwahashi).

0959-8049/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2004.05.006

chemotherapy and radiotherapy, no effective prolongation of survival has been obtained [4]. Therefore, the prevention and treatment of peritoneal metastasis are important therapeutic targets for gastric cancer patients.

Hepatocyte growth factor (HGF), originally identified and cloned as a potent mitogen for hepatocytes [5,6], greatly enhances cell motility and the breakdown of extracellular matrix in a variety of cancer cells leading to their dissemination [7–10]. c-Met, a specific HGF receptor which is a membrane-spanning tyrosine kinase, is overexpressed in many kinds of cancer cells [11–13]. In fact, HGF is mainly produced by stromal fibroblasts, and acts on cancer cells to promote their proliferation and on stromal cells to promote neovascularisation [14]. Therefore, the HGF/c-Met system plays a definitive role in the peritoneal invasion and metastasis of gastric cancer.

Recently, specific HGF-antagonist termed NK4 has been prepared. It is composed of the N-terminal hairpin domain and four kringle domains of the α-subunit of HGF. NK4 binds to the c-Met/HGF receptor, without inducing tyrosine phosphorylation of c-Met [15]. This competitive effect of NK4 on the interaction between HGF and the c-Met receptor has been shown in the HGF-mediated invasion of certain types of human cancer cells [13,16–18]. Furthermore, it has been reported that NK4 has inhibitory effects on angiogenic pathways driven by the basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) [19]. Of note, the anti-angiogenic effect of NK4 is independent of the HGF/c-Met system. Considering these two-way inhibitory effects, HGF/NK4 gene therapy could potentially be an ideal treatment for patients with peritoneal metastases from gastric cancer.

In this study, we examined the therapeutic efficacy of adenoviral-mediated HGF/NK4 gene therapy for heating peritoneal metastasis of gastric carcinoma in cells with both high- and low-levels of c-Met expression. We also compared the effect of early and late treatments. Considering the potential clinical application, we additionally investigated the efficacy of combining NK4 gene therapy with chemotherapy using cisplatin (CDDP), which is one of the most effective treatments for peritoneal metastases from gastric cancer [20].

## 2. Materials and methods

### 2.1. Cell lines and cell culture

The human gastric carcinoma cell lines, MKN45, obtained from Riken Gene Bank (Ibaraki, Japan), and TMK1, kindly provided by Dr. Yasui (First Department of Pathology, Hiroshima University School of Medicine, Hiroshima, Japan), were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Nikken Bio Medical Laboratory, Kyoto, Japan) containing 10% fetal bevine serum (FBS) (GIBCO, NY, USA) and 5% penicillin/streptomycin (GIBCO). It has been reported that the **c-met** gene was amplified 14-fold in MKN45 cells [21,22]. The expression of **c-met** mRNA, examined by Northern blotting in gastric cancer cell lines, showed a remarkably high expression level, at least ten-fold higher than that observed in TMK1 cells [23]. Other investigators reported that MKN45 exhibited the highest c-Met expression compared with several other gastric cancer cell lines and grew in response to HGF in a dose-dependent manner. By contrast TMK1 showed a low c-Met expression and did not proliferate in response to HGF [12].

The human embryonic kidney cell line, 293, purchased from the American Type Culture Collection (Rockville, MD, USA) was cultured as previously described in [24,25].

### 2.2. Construction of the recombinant adenoviral vector

The NK4 fragment was excised from the plasmid pcDNA/hNK4 containing the complete human NK4 cDNA by *Hind III* and *Xba I*, blunt-ended, and ligated into the *Swa I* site of the cassette cosmid pAxCAwt (Takara Adenovirus Expression Vector Kit, Takara, Shiga, Japan) to yield pAxCANK4. An adenoviral vector, AxCANK4, expressing the NK4 gene under the control of the CAG promoter was generated using the cosmid cassettes and adenovirus DNA-terminal protein complex (COS-TPC) method by transfecting 293 cells with pAxCANK4, and the *EcoT221*-digested Ad5-dlX DNA-terminal protein complex [24–26]. AxCALacZ expressing the *LacZ* gene under the control of the CAG promoter was also generated using pAxCALacZ (Takara, Shiga, Japan) by the COS-TPC method.

### 2.3. Drug

CDDP was provided from Bristol Pharmaceuticals K.K. (Tokyo, Japan) as BRIPLATIN, a 0.5 mg/ml CDDP solution in 0.9% sodium chloride.

### 2.4. Experimental design of NK4 gene therapy for peritoneal metastases models of gastric cancer

To investigate the efficacy of systemic adenoviral-mediated NK4 gene therapy, animal models of peritoneal metastasis from gastric cancer were developed. Briefly, six to seven-week-old athymic female BALB/c *nu/nu* mice (CLEA, Tokyo, Japan) received an intraperitoneal injection (i.p.) of $3 \times 10^6$ MKN45 or TMK1 cells per mouse [27]. These mice were randomly divided into the following five groups (each group: $n = 5$): Group A, treated with PBS; Group B, treated with AxCALacZ (early treatment); Group C, treated with

AxCANK4 (early treatment); Group D, treated with AxCANK4 (late treatment). The adenoviral vectors ($1.0 \times 10^9$ plague-forming units (pfu)) were administered i.p. three times 2, 7 and 12 days after tumour inoculation for early treatment or 7, 12 and 17 days for late treatment. The mice were all sacrificed on day 28 after tumour inoculation, and the total number and the total weight of peritoneal tumours in each mouse were examined. We also examined the volume of ascites being produced from the peritoneal metastases.

To assess the inhibitory effect on angiogenesis, disseminated tumours (larger than 3 mm) of Groups A, B and C were randomly selected, and then subjected to immunohistochemical staining with an antibody against Factor-VIII-related antigen (von-Willebrand factor) (Dako, Carpinteria, CA, USA). The average number of immunopositively-stained spots from five fields at a 200-fold magnification ($n = 4$) was used to represent the density of the intratumour vessels.

### 2.5. Evaluation of combination therapy of Adenoviral-mediated NK4 with CDDP

Six to seven-week-old athymic female BALB/c *nu/nu* mice received an i.p. injection of $3 \times 10^6$ MKN45 cells. These mice were randomly divided into the following four groups: Group A, no treatment ($n = 5$); Group B, treated with CDDP alone ($n = 5$); Group C, treated with CDDP and AxCANK4 ($n = 5$). The mice were injected with 8 mg/kg of CDDP into the abdominal cavity on day 3 after tumour inoculation (Groups B and C), and AxCANK4 ($1.0 \times 10^9$ pfu) was administered i.p. three times on day 5, 10 and 15 (Group C). The mice were all sacrificed on day 28 after tumour inoculation, and the total number and total weight of peritoneal tumours of each mouse were examined.

### 2.6. Statistics

Quantitative results were expressed as the mean ± the standard deviation (SD) of the mean. Statistical analysis was performed by using the oneway analysis of the variance (ANOVA) and Fisher's test or Student's *t*-test, as appropriate. $P < 0.05$ was considered statistically significant. StatView 5.0 software (Abacus Concepts, Inc., Berkeley, CA) was used for all of the statistical analyses.

## 3. Results

### 3.1. Therapeutic efficacy of adenoviral-mediated NK4 gene transduction in animal models of peritoneal metastasis

In the *in vivo* models employing MKN45 (Fig. 1A-a and A-b) and TMK1 (Fig. 1A-c and A-d), on day 28 after tumour inoculation, the mesenterium of the mice treated early with AxCANK4 on days 2, 7 and 12 had only a few metastatic tumours and were almost normal (Fig. 1A-b and A-d), while those of mice treated early with AxCALacZ had many tumours and were remarkably shortened (Fig. 1A-a and A-c).

In the model using MKN45, in which c-Met is highly expressed, the total number and weight of peritoneal tumours of each mouse treated early with AxCANK4 were significantly reduced compared with the mice treated with PBS and AxCALacZ ($P < 0.005$ and $P < 0.05$), respectively. In addition, the total number and weight of peritoneal tumours of each mouse treated late with AxCANK4 were decreased compared with the mice treated with PBS ($P < 0.05$) and AxCALacZ ($P < 0.05$). However, this decrease was less compared with the mice treated early with AxCANK4 (Table 1). Values for numbers and weights were approximately 2–3 times higher. Moreover, in the mice treated early with AxCANK4, there was no ascites at all, whereas in mice from the other groups, some ascites was observed (Table 1).

In the model using TMK1, which weakly expresses c-Met, the total number and weight of each mouse treated early with AxCANK4 were significantly decreased compared with mice treated with PBS or AxCALacZ ($P < 0.005$). The total number of tumours in mice treated late with AxCANK4 was also decreased compared with mice treated with PBS or AxCALacZ ($P < 0.05$). However, the inhibitory effect on peritoneal metastases resulting from early treatment with AxCANK4 was much stronger than that of late treatment ($P < 0.01$). There was a large volume of ascites with remarkable abdominal dissemination in the mice treated with PBS (Fig. 1B-a), treated with AxCALacZ and even treated late with AxCANK4. On the other hand, little ascites was observed in mice treated early with AxCANK4 (Fig. 1B-b) (Table 2).

### 3.2. Microvessel density in tumours disseminated from the abdominal cavity

In the model using MKN45, the microvessel density in the disseminated tumour treated with AxCANK4 was $2.0 \pm 0.9$/high powered fields (hpf), and significantly decreased compared with that of the PBS- or AxCALacZ-treated mice ($8.9 \pm 3.4$, $7.8 \pm 4.3$/hpf) ($P < 0.05$) (Fig. 2A). In the model using TMK1, the microvessel density of disseminated tumours was also suppressed in the tumours by AxCANK4 ($P < 0.05$) (Fig. 2B).

### 3.3. Therapeutic efficacy of the combination therapy CDDP with Adenoviral-mediated NK4

In the MKN45 model, the total number and weight of peritoneal tumours in each mouse treated with CDDP

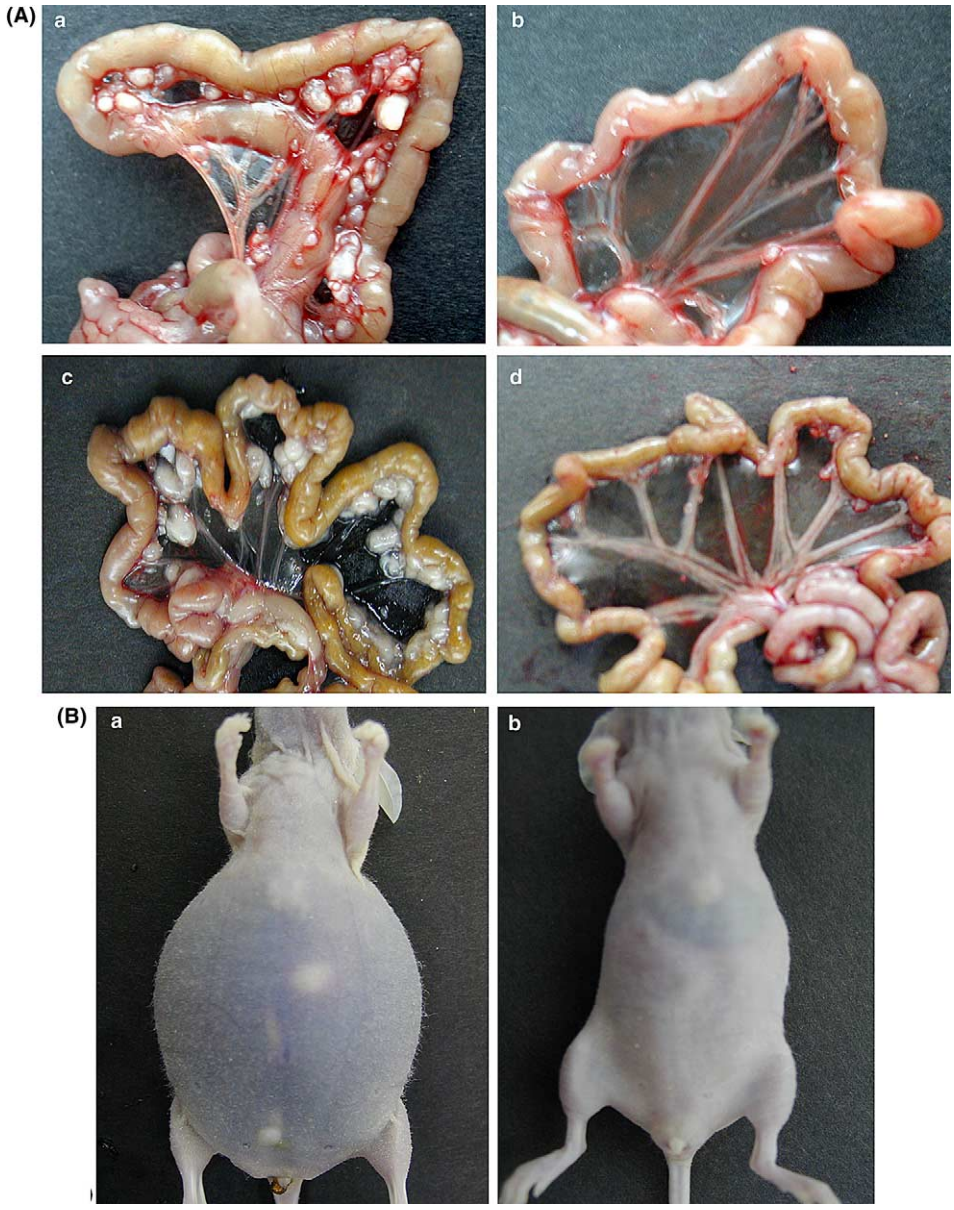


Fig. 1. The nude mice received an intraperitoneal (i.p.) injection of $3 \times 10^6$ MKN45 or TMK1 cells. AxCALacZ (a and c) or AxCANK4 (b and d) ($1.0 \times 10^9$ plaque-forming units (pfu)) was administered i.p. three times at 2, 7 and 12 days after tumour inoculation. To evaluate the therapeutic efficacy, the mice were sacrificed on day 28 after the tumour inoculations. A, Macroscopic findings of disseminated tumours in the mesenterium: MKN45 (a, b) and TMK1 (c, d). B, Photograph of abdomen in the peritoneal metastases model of TMK1: the mouse treated with AxCALacZ (a) and the mouse treated with AxCANK4 (b).

Table 1
Therapeutic efficacy of NK4 gene treatment for peritoneal metastasis from MKN45 cells

| Treatment group | Number[a] | Weight[b] (mg) | Ascites[c] |
|---|---|---|---|
| (A) PBS | $86.0 \pm 37.8$ | $1067.0 \pm 729.7$ | + |
| (B) AxCALacZ | $57.2 \pm 12.5$ | $818.8 \pm 274.3$ | + |
| (C) AxCANK4 early treatment | $8.6 \pm 2.7$[d] | $172.6 \pm 80.9$[e] | − |
| (D) AxCANK4 late treatment | $26.4 \pm 6.8$[e] | $278.0 \pm 38.6$[e] | ± |

[a] Data on the total number of disseminated tumours per mouse are expressed as the mean ± standard deviation (SD) for five mice.
[b] Data on the total weight of disseminated tumours per mouse are expressed as the mean ± SD for five mice.
[c] Data are presented as: −, no ascites; ±, little ascites; +, ascites not having distended abdomen; ++, substantial ascites having a distended abdomen.
[d] $P < 0.005$ compared with Groups A and B.
[e] $P < 0.05$ compared with Groups A and B.

Table 2
Therapeutic efficacy of NK4 gene treatment for peritoneal metastasis from TMK1 cells

| Treatment group | Number[a] | Weight[b] | Ascites[c] |
|---|---|---|---|
| (A) PBS | $108.8 \pm 18.3$ | $3664 \pm 1011$ | ++ |
| (B) AxCALacZ | $88.8 \pm 19.9$ | $3038 \pm 738$ | ++ |
| (C) AxCANK4 early treatment | $26.6 \pm 11.1$[d] | $1196 \pm 677$[e] | ± |
| (D) AxCANK4 late treatment | $60.8 \pm 20.9$[f] | $1753 \pm 1273$[g] | +∼++ |

[a] Data on the total number of disseminated tumours per mouse are expressed as the mean ± SD for five mice.
[b] Data on the total weight of disseminated tumours per mouse are expressed as the mean ± SD for five mice.
[c] Data are presented as: −, no ascites; ±, little ascites; +, ascites not having distended abdomen; ++, substantial ascites having a distended abdomen.
[d] $P < 0.0001$ compared with Groups A and B, and $P < 0.01$ compared with Group D.
[e] $P < 0.005$ compared with Groups A and B.
[f] $P < 0.05$ compared with Groups A and B, and $P < 0.01$ compared with Group C.
[g] $P < 0.05$ compared with Groups A and B.

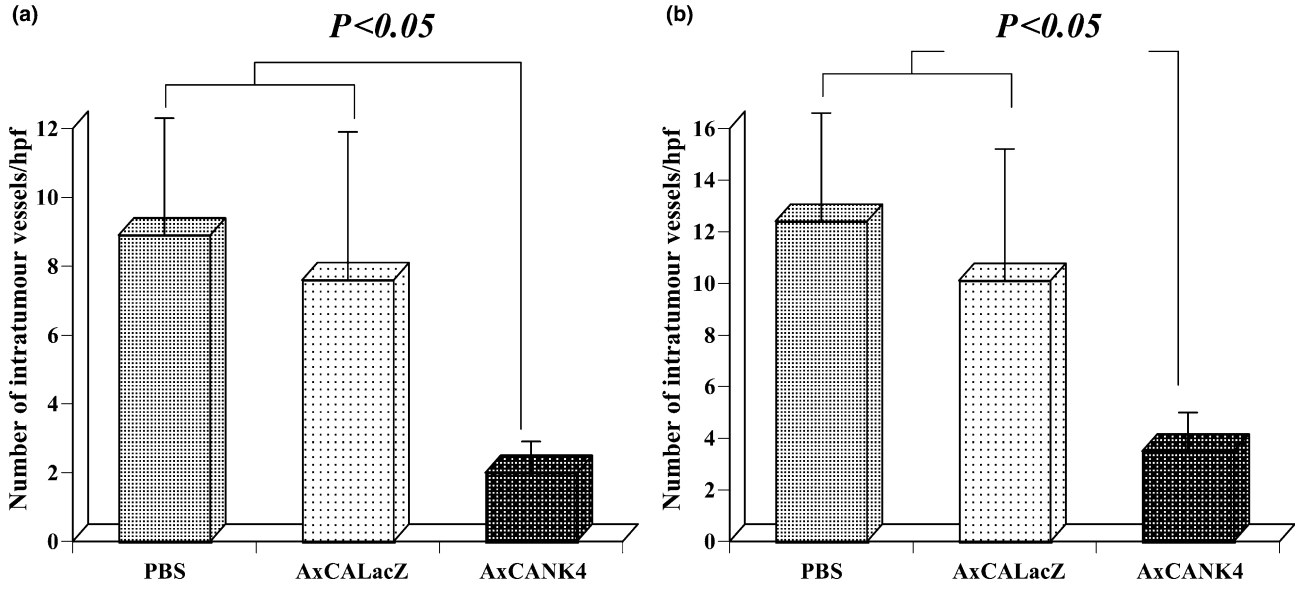


Fig. 2. The anti-angiogenic effect on metastatic tumours of MKN45 and TMK1 larger than 3 mm in diameter was assessed by immunohistochemical staining with an antibody against Factor-VIII-related antigen. The average number of immunopositively-stained spots from five fields at 200-fold magnification was used to represent the density of the intratumour vessels. (a): MKN45 tumours, (b): TMK1 tumours. The statistical analysis was performed using the Fisher's test.

were significantly reduced ($34.0 \pm 11.0$, $440.0 \pm 128.5$ mg) compared with the controls ($96.0 \pm 32.1$, $1187.0 \pm 492.4$ mg) ($P < 0.05$). Of note, the inhibitory effect of CDDP on peritoneal metastasis was significantly enhanced by AxCANK4 ($7.0 \pm 2.9$, $86.0 \pm 65.8$ mg) ($P < 0.01$) (Fig. 3).

## 4. Discussion

NK4 is a useful tool for multistep molecular targeting in cancer tissue formation. It has been showed that continuous infusion of the NK4 protein in nude mice inhibits tumour invasion, motility and growth, and increases apoptotic tumour cell death [16,19]. Furthermore, NK4 suppresses tumour growth and metastases acting as an angiogenesis inhibitor as well as an HGF antagonist [19,28]. However, it is necessary to administer NK4 frequently to achieve an antitumour effect *in vivo*. This would be a problem for any clinical applications. Recently, it has been reported that the adenoviral-mediated gene transfer of NK4 makes it possible to obtain a high concentration of NK4 *in vivo* resulting in the inhibition of tumour growth [14,28].

In this study, AxCANK4 ($1.0 \times 10^9$ pfu) were administered i.p. three times at an interval of five days, and both peritoneal dissemination and ascites were significantly suppressed. We previously demonstrated that serum levels of aspartate aminotransferase (AST) and alanine aminotranferase (ALT) in mice treated with a total of $3.0 \times 10^9$ pfu of adenoviral vectors were similar to those in normal mice, except for the administration of AxCACD expressing the cytosine deaminase gene driven by the CAG promoter [25]. In addition, it was re-

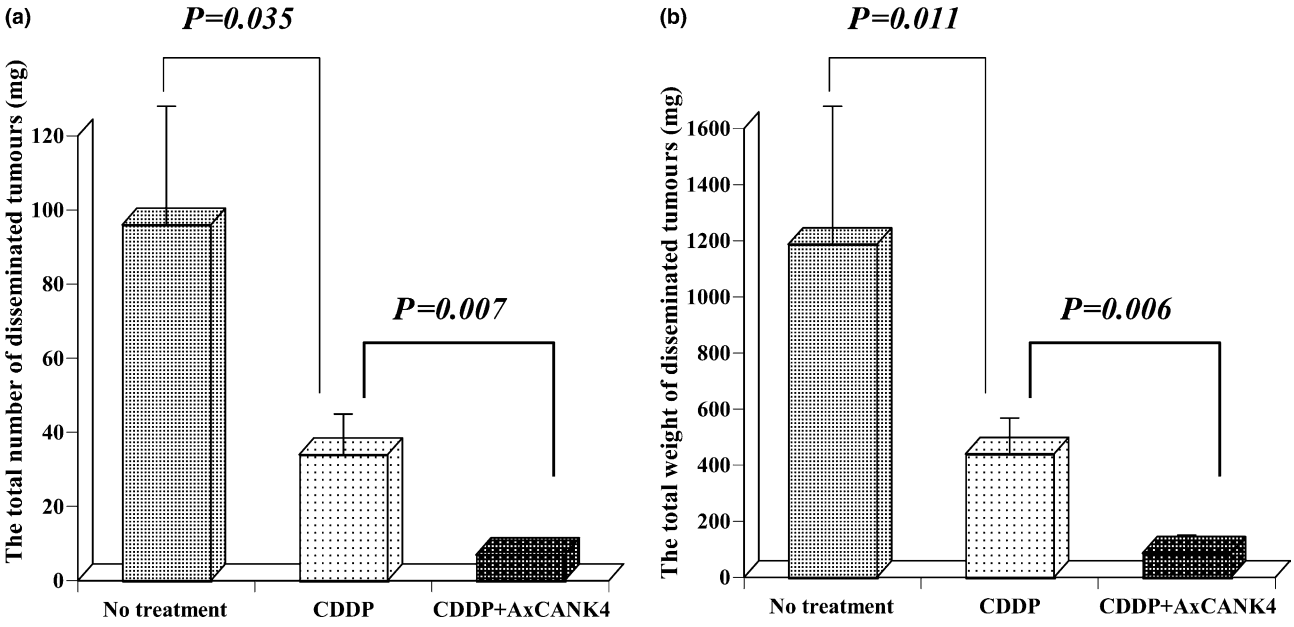


Fig. 3. The total tumour number and weight of the disseminated tumours. The mice received intraperitoneally (i.p.) an injection of MKN45 ($3 \times 10^6$ cells). The mice were injected with cisplatin (CDDP) (8 mg/kg) into the abdominal cavity on day 3, and AxCANK4 ($1.0 \times 10^9$ pfu) was administered i.p. three times at 5, 10 and 15 days. The mice were sacrificed on day 28. No treatment: mice not treated with CDDP or AxCANK4; CDDP: mice treated with CDDP alone; CDDP + AxCANK4: mice treated with CDDP and AxCANK4. Bars represent the SD for five mice. Statistical analysis was performed using the Student's *t*-test.

cently reported that only a minimal elevation of liver enzyme levels following the i.p. injection of Adenovial-mediated NK4 was observed in contrast to Mock injections [14]. Therefore, we assumed that no remarkable side-effects are associated with treatment with this amount of adenoviral vector.

Peritoneal metastasis is established by the attachment of cancer cells to mesothelial cells and invasion of the cells into the tissues beneath the peritoneum. It was reported that HGF, produced by peritoneal fibroblasts and mesothelial cells in monolayers, facilitates invasion of the disseminated tumour cells into the peritoneum [29]. Moreover, the HGF-c-Met signalling pathway is involved in the malignant behaviour of various tumour cells [9]. Indeed, in the peritoneal metastasis model using MKN45 which overexpressed the c-Met receptor, AxCANK4 treatment significantly inhibited the progression of peritoneal metastasis. However, it has been reported that c-Met overexpression was detected in only 46.1% of gastric cancer cases and gene amplification in 10.2% [30].

Recently, it has been shown that NK4 suppresses tumour growth and metastases, both as an angiogenesis inhibitor as well as an HGF antagonist, and this anti-angiogenic activity is likely to be exhibited through mechanisms that are independent of its HGF-antagonist activity [19,31]. Furthermore, deletion of the N-terminal hairpin domain (an essential domain for c-Met receptor binding in NK4 and HGF) in NK4 led to the selective loss of HGF-antagonist activity, while the remaining four-kringle variant of HGF (K1-4) retained the angio-inhibitory effects [32]. Tumours are always dependent on the development of an adequate blood supply through angiogenesis for growth at both primary and secondary sites such as peritoneal metastases [33]. In fact, several studies have reported the therapeutic efficacy of gene therapy using inhibitory molecules of endogenous angiogenesis, such as Flt-1 [27], angiostatin [34] and endostatin [35]. The present study demonstrated that AxCANK4 gene therapy was remarkably effective in the peritoneal metastasis models, and this therapeutic effect was independent of the amount of c-Met expression in the tumour cells. The inhibitory effect of AxCANK4 administered i.p. was thought to be mainly due to the anti-angiogenic effect of NK4, which not only antagonises HGF-induced angiogenesis, but also inhibits the angiogenesis induced by other angiogenic inducers, because the inhibition of intratumour vessels was observed in both MKN45 and TMK1 dissemination tumours treated with AxCANK4.

Many gene therapy studies for peritoneal metastasis have been reported. In most of these studies, treatments started within three days after inoculation of tumour cells in the abdominal cavity [27,28,31]. In this study, we compared the therapeutic effect of early treatment (AxCANK4 was administrated three times at 2, 7 and 12 days) with that of late treatment (at 7, 12 and 17 days) to examine whether the timing of treatment could affect efficacy. Although the late treatment of AxCANK4 obviously inhibited abdominal metastases compared with controls in both models, the inhibitory effect of late

treatment was not as strong as that of early treatment. In our preliminary experiments, there were no disseminated tumours and no abdominal effusion on day 2 after i.p. inoculations of MKN45 or TMK1. On the other hand, ≈10 metastatic tumours and some ascites were recognised on day 7. This means that this therapy is more effective for microscopic metastases established in the early phase than for macroscopic metastases. Therefore, considering the clinical application, patients with advanced gastric cancer, who have positive peritoneal washes without macroscopic disseminated tumours, would be good candidates for NK4 gene therapy. This therapy would not be sufficient for patients with macroscopic peritoneal metastases.

One of the strategies to overcome this disadvantage and enhance the inhibitory effect of NK4 gene therapy on peritoneal metastasis is to use i.p. chemotherapy. The i.p. administration of CDDP has been widely used for the treatment of gastric carcinoma with peritoneal metastases [36,37]. Indeed, in the present study, i.p. chemotherapy with CDDP (8 mg/kg) alone showed remarkable therapeutic efficacy against peritoneal metastases from gastric cancer. A dose of 8 mg/kg CDDP is thought to be almost the maximum tolerable dose (MTD) in mice, because it has been reported that the 50% lethal dose of CDDP in mice is 13.5 mg/kg (11.9–15.3, 95% Confidence Interval) and mice injected with 10 mg/kg of CDDP do not die from toxicity [38]. Of note, the combination treatment of CDDP (8 mg/kg) with AxCANK4 strongly enhanced the therapeutic efficacy of CDDP alone (8 mg/kg), suggesting that combination therapy of i.p. chemotherapy with NK4 gene therapy might be effective, even for macroscopic peritoneal metastases from gastric carcinoma.

In conclusion, we demonstrated that adenoviral-mediated NK4 gene therapy inhibited peritoneal metastases from gastric cancer. This suggests it may have future clinical applications for the treatment of highly advanced gastric carcinoma with peritoneal metastasis in combination with i.p. chemotherapy.

## Acknowledgements

We thank Dr. W. Yasui (Hiroshima University, Hiroshima, Japan) for the human gastric carcinoma cell line, TMK1.

## References

1. Benevolo M, Mottolese M, Cosimelli M, *et al*. Diagnostic and prognostic value of peritoneal immunocytology in gastric cancer. *J Clin Oncol* 1998, **16**, 3406–3411.
2. Koga S, Kaibara N, Iitsuka Y, Kudo H, Kimura A, Hiraoka H. Prognostic significance of intraperitoneal free cancer cells in gastric cancer patients. *J Cancer Res Clin Oncol* 1984, **108**, 236–238.
3. Katai H, Maruyama K, Sasako T, Okajima K, Kinoshita T, Naparkov A. Mode of recurrence after gastric cancer surgery. *Dig Surg* 1994, **11**, 99–103.
4. Hallissery MT, Dunn JA, Ward LC, Allum WH. The second British stomach cancer group trial of adjuvant radiotherapy or chemotherapy in respectable gastric cancer: five-year follow-up. *Lancet* 1994, **343**, 1309–1312.
5. Nakamura T, Nawa K, Ichihara A. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem Biophys Res Commun* 1984, **122**, 1450–1459.
6. Nakamura T, Nishizawa T, Hagiya M, *et al*. Molecular cloning and expression of human hepatocyte growth factor. *Nature* 1998, **342**, 440–443.
7. Zarnegar R, Michalopoulos GK. The many faces of hepatocyte growth factor: from hepatopoiesis to hematopoiesis. *J Cell Biol* 1995, **129**, 1177–1180.
8. Vande Woude GF, Jeffers M, Cortner J, Alvord G, Tsarfaty I, Resau J. Met-HGF/SF: tumourigenesis, invasion and metastasis. *Ciba Found Symp* 1997, **212**, 119–130.
9. Stella MC, Comoglio PM. HGF: a multifunctional growth factor controlling cell scattering. *Int J Biochem Cell Biol* 1999, **31**, 1357–1362.
10. Matsumoto K, Namamura T. NK4 (HGF-antagonist/angiogenesis inhibitor) in cancer biology and therapeutics. *Cancer Sci* 2003, **94**, 321–327.
11. Di Renzo MF, Narsimhan RP, Olivero M, *et al*. Expression of the Met/HGF receptor in normal and neoplastic human tissues. *Oncogene* 1991, **6**, 1997–2003.
12. Kaji M, Yonemura Y, Harada S, Liu X, Terada I, Yamamoto H. Participation of c-met in the progression of human gastric cancers: anti-c-met oligonucleotides inhibit proliferation or invasiveness of gastric cancer cells. *Cancer Gene Ther* 1996, **3**, 393–404.
13. Maehara N, Matsumoto K, Kuba K, Mizumoto K, Tanaka M, Nakamura T. NK4, a four-kringle antagonist of HGF, inhibits spreading and invasion of human pancreatic cancer cells. *Br J Cancer* 2001, **84**, 864–873.
14. Maemondo M, Narumi K, Saijo Y, *et al*. Targeting angiogenesis and HGF function using an adenoviral vector expressing the HGF antagonist NK4 for cancer therapy. *Mol Ther* 2002, **5**, 177–185.
15. Date K, Matsumoto K, Shimura H, Tanaka M, Nakamura T. HGF/NK4 is a specific antagonist for pleiotrophic actions of hepatocyte growth factor. *FEBS Lett* 1997, **420**, 1–6.
16. Date K, Matsumoto K, Kuba K, Shimura H, Tanaka M, Nakamura T. Inhibition of tumour growth and invasion by a four-kringle antagonist (HGF/NK4) for hepatocyte growth factor. *Oncogene* 1998, **17**, 3045–3054.
17. Hiscox S, Parr C, Nakamura T, Matsumoto K, Mansel RE, Jiang WG. Inhibition of HGF/SF-induced breast cancer cell motility and invasion by the HGF/SF variant, NK4. *Breast Cancer Res Treat* 2000, **59**, 245–254.
18. Parr C, Hiscox S, Nakamura T, Matsumoto K, Jiang WG. NK4, a new HGF/SF variant, is an antagonist to the influence of HGF/SF on the motility and invasion of colon cancer cells. *Int J Cancer* 2000, **85**, 563–570.
19. Kuba K, Matsumoto K, Date K, Shimura H, Tanaka M, Nakamura T. HGF/NK4, a four-kringle antagonist of hepatocyte growth factor, is an angiogenesis inhibitor that suppresses tumour growth and metastasis in mice. *Cancer Res* 2000, **60**, 6737–6743.
20. Jeung HC, Rha SY, Jang WI, Noh SH, Chung HC. Treatment of advanced gastric cancer by palliative gastrectomy, cytoreductive therapy and postoperative intraperitoneal chemotherapy. *Br J Surg* 2003, **89**, 460–466.
21. Yokozaki H. Molecular characteristics of eight gastric cancer cell lines established in Japan. *Pathol Int* 2000, **50**, 767–777.
22. Kuniyasu H, Yasui W, Kitadai Y, Yokozaki H, Ito H, Tahara E. Frequent amplification of the c-met gene in scirrhous type

stomach cancer. *Biochem Biophys Res Commun* 1992, **189**, 227–232.
23. Kuniyasu H, Yasui W, Yokozaki H, Kitadai Y, Tahara E. Aberrant expression of c-met mRNA in human gastric carcinoma. *Int J Cancer* 1993, **55**, 72–75.
24. Ueda K, Iwahashi M, Nakamori M, Nakamura M, Yamaue H, Tanimura H. Enhanced selective gene expression by adenovirus vector using Cre/loxP regulation system for human carcinoembryonic antigen-producing carcinoma. *Oncology* 2000, **59**, 255–265.
25. Ueda K, Iwahashi M, Nakamori M, *et al*. Carcinoembryonic antigen-specific suicide gene therapy of cytosine deaminase/5-fluorocytosine enhanced by Cre/loxP system in the orthotopic gastric carcinoma model. *Cancer Res* 2001, **61**, 6158–6162.
26. Miyake S, Makimura M, Kanegae Y, *et al*. Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. *Proc Natl Acad Sci* 1996, **93**, 1320–1324.
27. Mori A, Arii S, Furutani M, *et al*. Soluble Flt-1 gene therapy for peritoneal metastases using HVJ-cationic liposomes. *Gene Ther* 2000, **7**, 1027–1033.
28. Saimura M, Nagai E, Mizumoto K, *et al*. Intraperitoneal injection of adenovirus-mediated NK4 gene suppresses peritoneal dissemination of pancreatic cancer cell line AsPC-1 in nude mice. *Cancer Gene Ther* 2002, **9**, 799–806.
29. Yashiro M, Chung YS, Inoue T, *et al*. Hepatocyte growth factor (HGF) produced by peritoneal fibroblasts may affect mesothelial cell morphology and promote peritoneal dissemination. *Int J Cancer* 1996, **67**, 289–293.
30. Nakajima M, Sawada H, Yamada Y, *et al*. The prognostic significance of amplification and overexpression of c-met and c-erb B-2 in human gastric carcinoma. *Cancer* 1999, **85**, 1894–1902.
31. Tomioka D, Maehara N, Kuba K, *et al*. Inhibition of growth, invasion, and metastasis of human pancreatic carcinoma cells by NK4 in an orthotopic mouse model. *Cancer Res* 2001, **61**, 7518–7524.
32. Kuba K, Matsumoto K, Ohnishi K, Shiratsuchi T, Tanaka M, Nakamura T. Kringle 1-4 of hepatocyte growth factor inhibits proliferation and migration of human microvascular endothelial cells. *Biochem Biophys Res Commun* 2000, **279**, 846–852.
33. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1995, **1**, 27–31.
34. Tanaka T, Cao Y, Folkman J, Fine HA. Viral vector-targeted antiangiogenic gene therapy utilizing an angiostatin complementary DNA. *Cancer Res* 1998, **58**, 3362–3369.
35. Chen Q-R, Kumar D, Stass SA, Mixson AJ. Liposomes complexed to plasmids encoding angiostatin and endostatin inhibit breast cancer in nude mice. *Cancer Res* 1999, **59**, 3308–3312.
36. Jeung HC, Rha SY, Jang WI, Noh SH, Chung HC. Treatment of advanced gastric cancer by palliative gastrectomy, cytoreductive therapy and postoperative intraperitoneal chemotherapy. *Br J Surg* 2002, **89**, 460–466.
37. Tsujitani S, Fukuda K, Saito H, *et al*. The administration of hypotonic intraperitoneal cisplatin during operation as a treatment for the peritoneal dissemination of gastric cancer. *Surgery* 2002, **131**, S98–104.
38. Hagiwara A, Takahashi T, Kojima O, *et al*. Pharmacologic effects of cisplatin microspheres on peritoneal carcinomatosis in rodents. *Cancer* 1993, **71**, 844–850.