Asian Journal of Andrology (2010) 12: 381–389

www.nature.com/aja

Original Article

Effects of transferred *NK4* gene on proliferation, migration, invasion and apoptosis of human prostate cancer DU145 cells

Dan Yue[1], Yong Wang[2], Ping Ma[1], Yin-Yan Li[3], Hong Chen[1], Ping Wang[2], Chang-Shan Ren[1]

[1]*Cancer Research Institute, First Affiliated Hospital, China Medical University, Shenyang 110001, China*
[2]*Department of Urology, Fourth Affiliated Hospital, China Medical University, Shenyang 110032, China*
[3]*Department of Ultrasound, First Affiliated Hospital, China Medical University, Shenyang 110001, China*

Abstract

We investigated the ability of NK4, an antagonist of human hepatocyte growth factor (HGF), to inhibit the influence of HGF on proliferation, migration, invasion and apoptosis of human prostate cancer cells. Expression vector pBudCE4.1-EGFP-NK4 containing NK4 cDNA was used to transfect human prostate cancer DU145 cells, and the effects of the autocrine NK4 on tumor cell proliferation, migration, invasion and apoptosis were assessed *in vitro*. *In vivo*, we subcutaneously implanted DU145 cells, mock-transfected clone (DU145/empty vector) cells and NK4-transfected clone (DU145/NK4) cells into nude mice, and then evaluated tumor growth, cell proliferation and cell apoptosis *in vivo*. We found that DU145/NK4 cells expressed NK4 protein. In the *in vitro* study, autocrine NK4 attenuated the HGF-induced tumor cell proliferation, migration and invasion, and stimulated apoptosis. Furthermore, autocrine NK4 effectively inhibited the HGF-induced phosphorylation of c-Met, extracellular signal-regulated kinase-1 (ERK1). and protein kinase B 1/2 (Akt1/2). Histological examination revealed that autocrine NK4 inhibited proliferation and accelerated apoptosis of prostate cancer cells. These results show that genetic modification of DU145 cells with NK4 cDNA yields a significant effect on their proliferation, migration, invasion and apoptosis. Molecular targeting of HGF/c-Met by NK4 could be applied as a novel therapeutic approach to prostate cancer.

Asian Journal of Andrology (2010) 12: 381–389. doi: 10.1038/aja.2010.22; published online 19 April 2010.

Keywords: hepatocyte growth factor, human prostate cancer, NK4, DU145 cells

1 Introduction

In European countries and the United States, the incidence of and mortality from prostate cancer are high. Bone and lymph node metastases and the progression from androgen-dependent (AD) to androgen-independent (AI) growth lead to a poor clinical outcome. Thus, we need a better understanding of the mechanisms of prostate cancer growth, and its invasive and metastatic potentials for developing effective therapeutic interventions.

Hepatocyte growth factor (HGF), also known as scatter factor, is a heterodimeric molecule with a number of biological activities, including regulation of migration, invasion and angiogenesis, in cancer [1]. HGF is composed of an α-chain, containing the N-terminal hairpin domain and four kringle domains, and a serine protease-like β-chain. Over-expression of HGF and its receptor, c-Met, in prostate cancer has been reported [2, 3]. Moreover, a higher plasma level

Correspondence to: Dr Chang-Shan Ren, Cancer Research Institute, First Affiliated Hospital, China Medical University, Shenyang 110001, China.
Fax: +86-24-2326-8468 E-mail: csrencmu@yahoo.com.cn
and Dr Ping Wang, Department of Urology, Fourth Affiliated Hospital, China Medical University, Shenyang 110032, China.
Fax: +86-24-6257-1119 E-mail: cmu4h-wp@126.com
Received: 12 September 2009 Revised: 16 November 2009
Accepted: 16 March 201P Published online: 19 April 2010

of HGF in prostate cancer patients is associated with an advanced stage of malignancy and a poor prognosis [3, 4]. It is also known that the adaptive switch of HGF from paracrine to autocrine is intimately involved in the progression of prostate cancer from the AD to the AI state [5]. In addition, Humphrey *et al*. [6] reported a strong relationship between c-Met expression and the progression of prostate cancer. Using these characteristics of prostate cancer, we have found a new approach for investigating prostate cancer.

Recently, the HGF antagonist, NK4, was developed. It is a variant form of HGF, comprising the N-terminal and the subsequent four kringle domains of HGF. As NK4 retains the capacity to bind to the HGF receptor, c-Met, it competes with HGF and inhibits the biological activity of HGF [1]. Competitive inhibitory effects of NK4 on HGF/c-Met have been shown in some types of human cancer cells [7–9].

In this study, we used the pBudCE4.1-EGFP/NK4 expressing *NK4* gene to investigate the antitumor effect of NK4 on human prostate cancer DU145 cells *in vitro* and *in vivo*. This study may supply an experimental basis for the clinical application of NK4 in prostate cancer treatment.

2 Materials and methods

2.1 Cell culture

DU145 prostate cancer cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). DU145 cells were cultured in RPMI 1640 (GIBCO BRL, Gaithersburg, MD, USA). They were supplemented with 10% (v/v) fetal calf serum (Invitrogen, Carlsbad, CA, USA) and incubated at 37ºC with 5% CO_2.

2.2 Plasmid construction

Plasmid pBudCE4.1-EGFP/NK4 was constructed by inserting human NK4 cDNA into the *Hin*dIII and *Xba*I restriction sites of pBudCE4.1-EGFP. The plasmid DNA was amplified in a transformant of *Escherichia coli* bacteria. pBudCE4.1-EGFP/NK4 and the empty vector pBudCE4.1-EGFP were transfected into DU145 cells by Lipofectamine2000 (Invitrogen). The cells were selected in the presence of Zeocin 50 μg mL^{-1} (Invitrogen). Resistant clones were obtained after 4 weeks.

2.3 Western blotting

Cells were cultured in 25 cm^2 culture flask at 1×10^6 cells per flask, with medium containing 10% fetal calf serum for 48 h. The supernatants were centrifuged to remove the cell debris. The conditioned medium was collected, and NK4 in the medium was assayed. Meanwhile, total cellular proteins were extracted. Equal amounts of each protein sample were separated by electrophoresis on sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Invitrogen) using a wet transfer. Immunoblotting was performed with primary rabbit anti-human c-Met polyclonal antibody (diluted in the ratio 1:50) (Thermo Fisher Scientific, Cheshire, UK), rabbit anti-human phosphorylated c-Met (diluted in the ratio 1:200), goat anti-human protein kinase B 1/2 (Akt1/2) (diluted in the ratio 1:200), rabbit anti-human phosphorylated Akt1/2 (diluted in the ratio 1:200), rabbit anti-human extracellular signal-regulated kinase-1 (ERK1) (diluted in the ratio 1:200), mouse anti-human phosphorylated ERK1 (diluted in the ratio 1:200), rabbit anti-human HGF-α polyclonal antibody (diluted in the ratio 1:200) and mouse anti-human β-actin (diluted in the ratio 1:1 000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunocomplexes were detected with the secondary antibodies, and then developed with the enhanced chemiluminescence system (ECL, Amersham, Uppsala, Sweden). The bands were quantified densitometrically using ImageJ software (NIH, Bethesda, MD, USA) and were shown as ratios. This experiment was repeated five times.

2.4 Proliferation assay

Cells were seeded onto 96-well plates at a density of 4.0×10^3 cells per 0.2 mL complete medium and allowed to attach for 24 h. Following this incubation, the cells were washed once with serum-free RPMI 1640 and replaced with a medium containing 5% fetal calf serum and 0 or 10 ng mL^{-1} of HGF in triplicate. Cells were cultured for 24 h, 48 h, 72 h and 96 h. At the last 4 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to a final concentration of 1 mmol L^{-1}. The formazan product was dissolved in dimethyl sulfoxide and absorbances were read on a Dynatech MR 5000 plate reader at 490 nm. This experiment was repeated five times.

2.5 In vitro migration assay

Transwell chamber (Corning Coster, Cambridge, MA, USA) equipped with a filter membrane with 8-μm pores was used to determine the motility of prostate

cancer cells with or without HGF. Cells were harvested with trypsin, washed once with 5% fetal calf serum medium and adjusted to a concentration of 1×10^5 cells per mL. A volume of 0.2 mL of the cell suspension was added to the upper well of a 24-well Transwell chamber. The chambers were placed in lower wells containing 0.5 mL of RPMI 1640 medium supplemented with 10% fetal calf serum and 0 or 10 ng mL^{-1} of HGF for 12 h in 5% CO_2 at 37ºC. At the end of the incubation period, cells in the upper chamber were removed with a cotton swab from the top of the filter, and the migrating cells were fixed with cold methanol/acetic acid (3:1) and then stained with Giemsa. The number of migrating cells was evaluated by random observation of five fields on every section by two investigators. The average number of cells per field was defined as migration index. Five independent series of experiments were performed.

2.6 In vitro invasion assay

Cells were harvested with trypsin, washed once with 5% fetal calf serum medium and adjusted to a concentration of 2×10^5 cells per mL. A volume of 0.2 mL of the cell suspension was added to each upper well of a 24-well Matrigel-coated invasion chambers (Becton Dickinson, Bedford, MA, USA). The chambers were placed in wells containing 0.5 mL of RPMI 1640 medium supplemented with 10% fetal calf serum and 0 or 10 ng mL^{-1} of HGF, and incubated for 24 h in 5% CO_2 at 37ºC. At the end of the incubation period, noninvading cells were removed with a cotton swab from the top of the filter and the invading cells were fixed with cold methanol/acetic acid (3:1), and then stained with Giemsa. The number of invading cells was evaluated by random observation of five fields on every section by two investigators. The average number of cells per field was defined as invasion index. Five independent series of experiments were performed.

2.7 Cell apoptosis assay

Cells were plated on a 75 cm^2 flask at 40%–50% confluence. After 12 h incubation in serum-free RPMI 1640 medium, the old medium was exchanged and a 10% fetal calf serum culture medium containing 0 or 10 ng mL^{-1} of HGF was added, after which the cells were incubated for an additional 48 h. A total of 5×10^5 cells were harvested and resuspended in binding buffer and stained with fluorescein isothiocyanate-coupled annexin V and propidium iodide (Keygen, Nanjing, China). Flow cytometric analysis for quantification of apoptotic cells was performed using a Becton Dickinson FACSCalibur system (Becton Dickinson, San Jose, CA, USA). CellQuest Pro software (Becton Dickinson) was used for the flow cytometric analyses. Five independent series of experiments were performed.

2.8 In vivo tumor growth assay

Athymic male nude mice (BALBc nu/nu; 8-week-old) were obtained from Vital River Laboratories (Beijing, China). The mice were housed in laminar flow cabinets under specific pathogen-free conditions in facilities approved by the China Medical University. All procedures for animal experiments were approved and supervised by the Animal Protection and Usage Committee, and institutional guidelines for animal welfare and experimental conduct were followed. A total of 15 male nude mice were divided into three groups: DU145 group, DU145/empty vector group and DU145/NK4 group. A volume of 0.2 mL of the cell suspension (1×10^7 cells) mixed with matrigel (Becton Dickinson, Bedford, MA, USA) was subcutaneously injected into the left scapular area of mouse. Tumor sizes were recorded in all groups over a 4-week period using digital calipers, and the tumor volume = width × $(\text{length})^2 \times 0.52$ and a tumor growth curve was drawn.

At 3 days after the final treatment, all mice were killed. The tumors were removed, fixed in 10% formalin for 24 h, embedded in paraffin wax and serially sectioned (5 μm). Sections were used for immunohistochemical staining.

2.9 Histopathological examination

Proliferating tumor cells were detected by streptavidin-peroxidase immunohistochemical method [10], using an antibody against proliferating cell nuclear antigen (PCNA) according to the manufacturer's (Maxin Biotech Inc., San Francisco, CA, USA) instructions. Apoptotic cells within the tumor were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (Roche, Mannheim, Germany) according to the manufacturer's instructions. The proliferating cells and apoptotic cells were evaluated by random observation of five fields on every section by two investigators, one field was magnified up to 400-fold. The number of positively labeled nuclei per total nuclei in those fields was calculated (%).

2.10 Statistical evaluation

Statistical values are presented as mean ± SD of the

mean. All comparisons were made using the unpaired two-tailed *t*-test with the SPSS Software 12.0 version (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as $P < 0.05$.

3 Results

3.1 NK4 expression in the DU145 cell culture medium

To confirm the appropriate expression of NK4 in the DU145/NK4 cells, we performed Western blotting. Cells were cultured in 2% serum supplemented RPMI 1640 medium. After 72 h, the culture supernatants were subjected to electrophoresis. A specific NK4 protein band at 50 kDa was detected in the culture medium, showing an efficient expression of the NK4 in the DU145/NK4 cells (Figure 1).

3.2 Inhibition of cell proliferation by autocrine NK4 in vitro

We analyzed the effect of autocrine NK4 on proliferation of prostate cancer cells. Without HGF, the proliferation of the DU145/NK4 group was similar to that of DU145/empty vector and DU145 groups *in vitro* ($P > 0.05$), indicating that NK4 alone has no significant effect on cell proliferation. With the addition of 10 ng mL^{-1} HGF, we saw a dramatic increase in cell proliferation of DU145 and DU145/empty vector groups (DU145 *vs.* DU145 + HGF, $P < 0.01$; DU145/empty vector *vs.* DU145/empty vector + HGF, $P < 0.01$). However, the HGF-induced cell proliferation was suppressed by autocrine NK4 (DU145/NK4 + HGF *vs.* DU145 + HGF or DU145/empty vector + HGF, $P < 0.01$) (Figure 2).

3.3 Inhibition of cell migration by autocrine NK4 in vitro

We used a transwell chamber to determine whether the autocrine NK4 could weaken HGF-induced cell motility in different groups. The results showed that there were no significant differences in migration index of the three groups without HGF ($P > 0.05$). Cell migration index increased after HGF treatment, but this HGF-induced motility was, again, significantly inhibited in the DU145/NK4 group (DU145 + HGF: 79.55 ± 11.16; DU145/empty vector + HGF: 77.05 ± 6.13; DU145/NK4 + HGF: 58.55 ± 8.48; $P < 0.01$) (Figure 3).

3.4 Inhibition of cell invasion by autocrine NK4 in vitro

An important step in metastasis is the invasion of tumor cells through a basement membrane. This invasion can be tested using an in vitro invasion model, in which, cells migrate through an extracellular matrix. The invasion index of all groups was similar without HGF ($P > 0.05$). In the presence of 10 ng mL^{-1} HGF, the cell invasion index within DU145, DU145/empty vector and DU145/NK4 groups increased by 1.8-, 1.7- and 1.3-fold, respectively, compared with cultures without HGF. Furthermore, autocrine NK4 suppressed the HGF-induced cell invasion (DU145 + HGF: 41.75 ± 5.41; DU145/empty vector + HGF: 39.35 ± 6.45; DU145/NK4 + HGF: 30.05 ± 4.22; $P < 0.01$) (Figure 4). This assay shows that the ability of HGF to stimulate DU145 cell invasion can be antagonized by NK4.

3.5 Induction of cell apoptosis by autocrine NK4 in vitro

Induction of apoptosis by NK4 was quantified by flow cytometry using annexin V and propidium io-

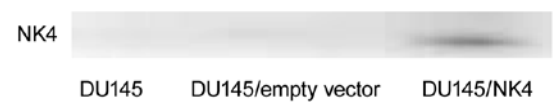

Figure 1. Expression of NK4 in different groups. NK4 expression in culture medium was examined by Western blot analysis.

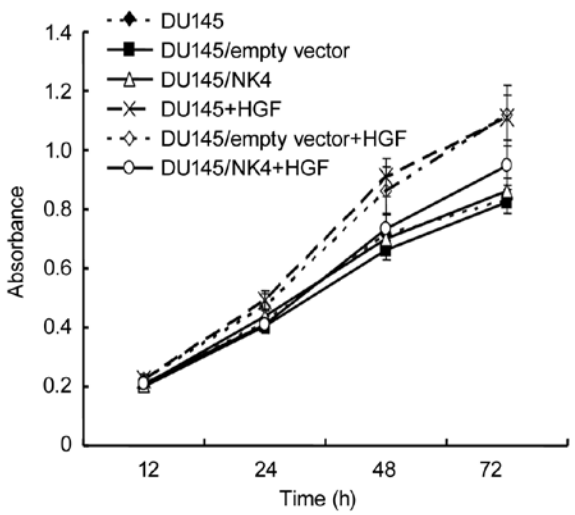

Figure 2. Effect of autocrine NK4 on cell proliferation *in vitro*. In the presence or absence of 10 ng mL^{-1} hepatocyte growth factor (HGF), cell proliferation of different groups was evaluated by MTT assay. The autocrine NK4 suppressed the HGF-induced cell proliferation in DU145/NK4 cells.

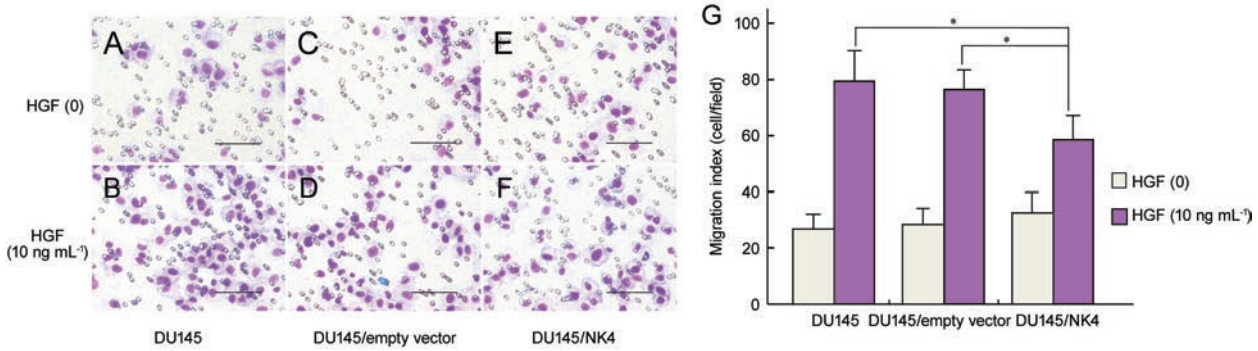

Figure 3. Effect of autocrine NK4 on cell migration *in vitro*. Transwell chamber equipped with a filter membrane with 8-μm pores was used to determine the cell motility in different groups. Migrating cells were fixed with cold methanol/acetic acid and stained with Giemsa. (A)–(F): The migrating cells were microscopically observed and counted. (G): Migration index in different groups. Compared with DU145 (B) and DU145/empty vector (D), the cell migration induced by hepatocyte growth factor (HGF) in DU145/NK4 (F) decreased. Scale bars = 100 μm. Data are presented as mean ± SD ($^*P < 0.01$, compared with DU145 + HGF or DU145/empty vector + HGF group).

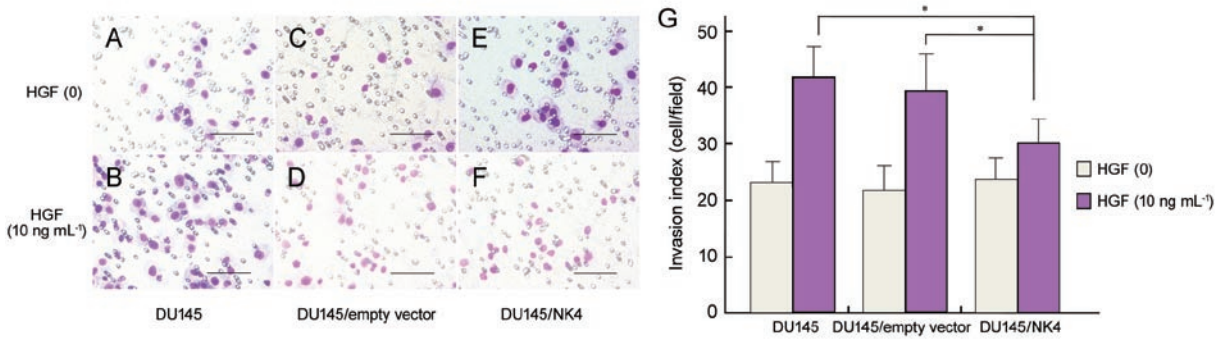

Figure 4. Effect of autocrine NK4 on cell invasion *in vitro*. Using matrigel-coated invasion chambers, cell invasion ability was observed. The invading cells were fixed with cold methanol/acetic acid, and then stained with Giemsa. (A)–(F): Photomicrographs showing representative views of cell invasion assays. (G): Invasion index in different groups. In the presence of hepatocyte growth factor (HGF), the number of invading DU145/NK4 (F) cells was smaller than those of DU145 (B) and DU145/empty vector (D) cells. Scale bars = 100 μm. Data are presented as mean ± SD ($^*P < 0.01$, compared with DU145 + HGF or DU145/empty vector + HGF group).

dide staining. In the absence of HGF, the difference between apoptosis ratios among the three groups was not statistically significant ($P > 0.05$). However, NK4 significantly affected the induction of apoptosis in DU145 cells in the presence of HGF: the apoptosis ratio of DU145/NK4 + HGF group (9.22% ± 1.01%) was higher than the ratios of DU145 + HGF (2.84% ± 0.65%) or DU145/empty vector + HGF groups (2.90% ± 0.59%) ($P < 0.01$) (Figure 5).

3.6 Autocrine NK4 regulation of the activation of c-Met, ERK1 and Akt1/2 in DU145 cells

Because these signal transducers, c-Met, ERK1 and Akt1/2, are key molecules in the biological characteristics of cancer cell, we examined the effect of autocrine NK4 on intracellular signaling by measuring the phosphorylation status of these molecules with western blot analysis. As shown in Figure 6, HGF-induced phosphorylation of c-Met, ERK1 and Akt1/2 was inhibited in DU145/NK4 cells, ($P < 0.05$).

3.7 Inhibition of tumor growth by autocrine NK4 in vivo

To test the impact of NK4 on *in vivo* tumor development, we established the *in vivo* model. No animals died during the treatment and all of them grew normally without dyscrasia. There was no significant difference in body weight among the three groups before or after treatment. At the end of the experiment, we found that

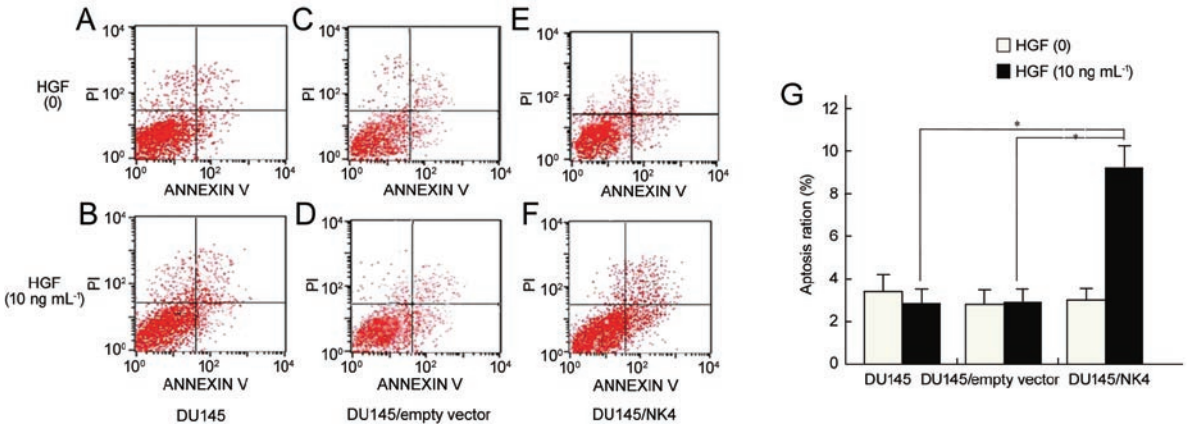

Figure 5. Flow cytometric analysis for quantification of apoptotic cells. (A)–(F): Cells were stained with FITC-coupled annexin V and propidium iodide. Cell apoptosis percentages were evaluated by flow cytometry. (G): Apoptosis ratio between different groups. In the presence of hepatocyte growth factor (HGF) (10 ng mL^{-1}), the apoptosis ratio of DU145/NK4 (F) cells was higher than that of DU145 (B) and DU145/empty (D) vector cells. Data are presented as mean ± SD ($^{*}P < 0.01$, compared with DU145 + HGF or DU145/empty vector + HGF group).

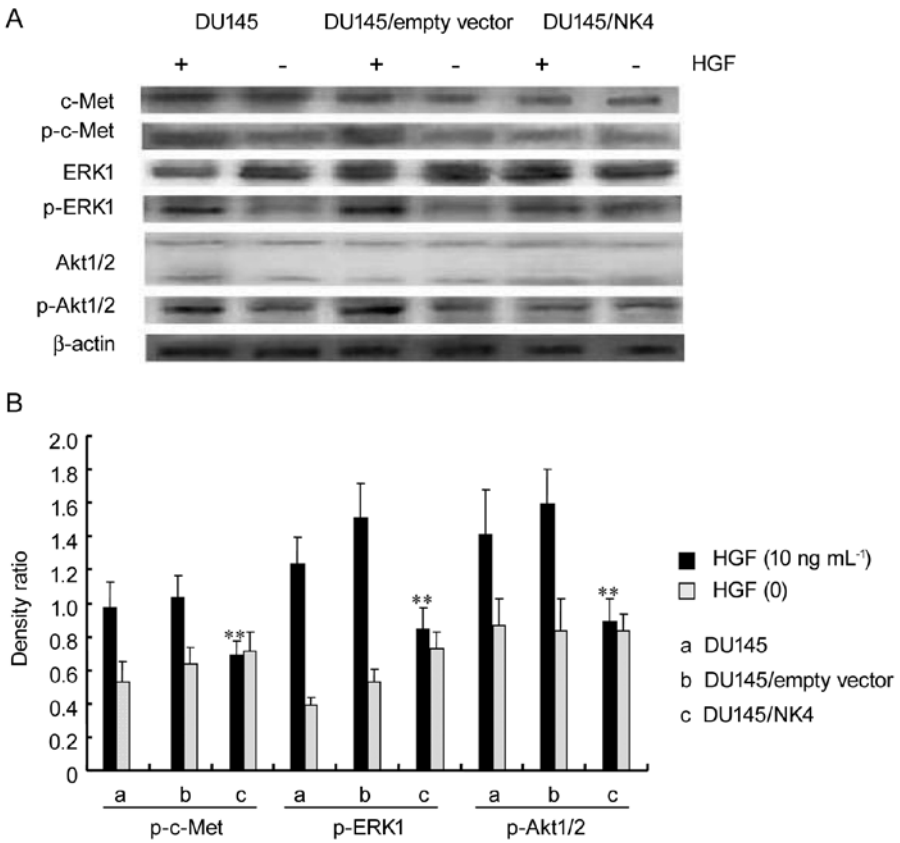

Figure 6. Regulation of c-Met, ERK1 and Akt1/2 activation by autocrine NK4. (A): Western blot analysis on the effects of autocrine NK4 on c-Met, ERK1 and Akt1/2, as described in the Materials and methods section. The cell lysates were subjected to SDS-PAGE, followed by immunoblotting with anti-human c-Met/phosphorylated c-Met, anti-human ERK1/phosphorylated ERK1, anti-human Akt1/2/phosphorylated Akt1/2 and anti-human β-actin antibodies. Hepatocyte growth factor (HGF)-induced phosphorylation of c-Met, ERK1 and Akt1/2 was inhibited by autocrine NK4. The protein expression of β-actin in the same samples was used as a loading control. (B): Densitometric analysis of the activation of c-Met and signal transducers. The band densities are shown as ratios: objective band density/β-actin density. Data are presented as mean ± SD ($^{**}P < 0.05$, compared with DU145 + HGF or DU145/empty vector + HGF group).

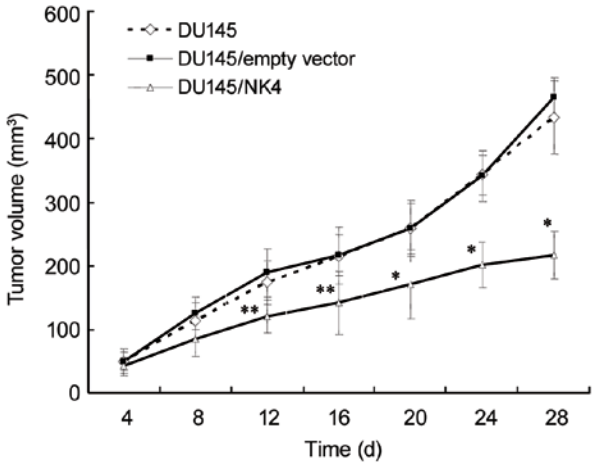

Figure 7. Tumor growth curves. Tumor cells, 1×10^7 cells each, were injected subcutaneously at the left scapular area of each mouse. Tumor sizes were recorded in all groups over a 4-week period: tumor volume = width × length^2 × 0.52. Growth of DU145/NK4 tumors was suppressed compared with DU145 and DU145/empty vector tumors growth. Data are presented as mean ± SD ($^*P < 0.01$, $^{**}P < 0.05$, compared with DU145 or DU145/empty vector group).

the tumors in the DU145/NK4 group grew much slower than those in the other two groups, and the average size of tumors in the DU145/NK4 group was significantly smaller than the other two groups ($P < 0.01$). Meanwhile, no significant difference was observed between the DU145 group and the DU145/empty vector group ($P > 0.05$) (Figure 7).

3.8 Immunohistochemical examination of the tumor

We examined PCNA to assess the inhibitory effect on the cell proliferation in the tumor. There was a lower percentage of PCNA-positive cells in the DU145/NK4 group (76.76% ± 9.30%), compared with the DU145 group (85.72% ± 10.70%) and the DU145/empty vector group (91.72% ± 12.39%) ($P < 0.05$). As for TUNEL staining, the apoptotic index indicated a higher incidence of apoptosis in the DU145/NK4 group (8.08% ± 2.53%) than in the DU145 group (2.88% ± 0.93%) or DU145/empty vector group (3.28% ± 1.02%) ($P < 0.01$) (Figure 8). These results were consistent with the *in vitro* results.

4 Discussion

For this report, we transfected the *NK4* gene into malignant human prostate DU145 cells and established a stable transfectant (DU145/NK4) producing NK4. We

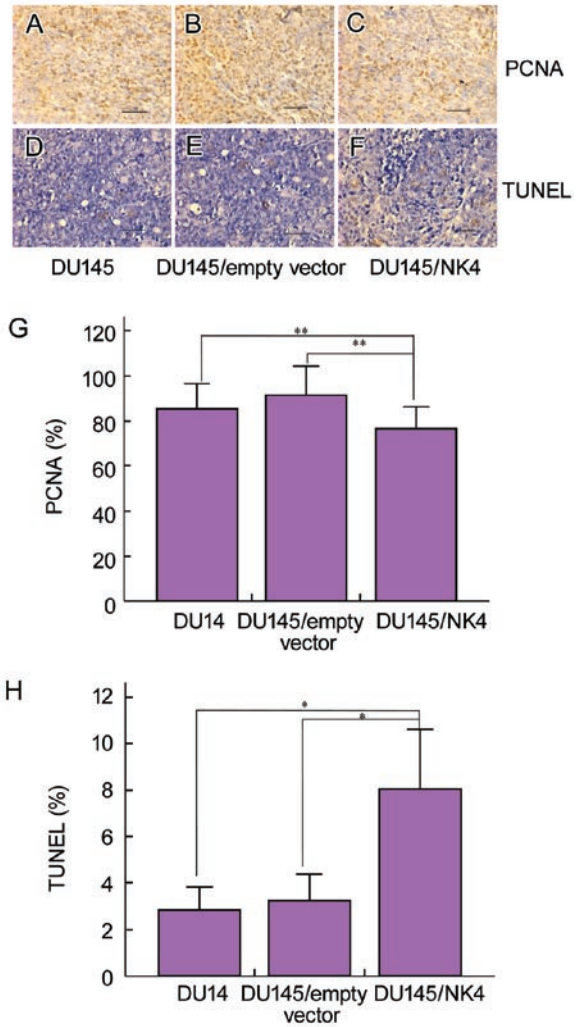

Figure 8. Immunohistochemical analysis of the prostate cancer cells formed in nude mice. Anti-PCNA antibody and TUNEL assay were used to determine the number of proliferating cells (A, B, C and G) and apoptotic cells (D, E, F and H), respectively. Compared with DU145 and DU145/empty vector tumors, the number of proliferating cells decreased and apoptotic cells increased in DU145/NK4 tumors. Scale bars = 50 μm. Data are presented as mean ± SD ($^*P < 0.01$, $^{**}P < 0.05$, compared with DU145 or DU145/empty vector group).

then investigated the *in vitro* behavior of DU145/NK4 and its *in vivo* antitumor effects. This NK4 expression inhibited the proliferation, migration and invasion of DU145 cells and induced their apoptosis *in vitro*. Autocrine NK4 also suppressed tumor growth in mice. Immunoblot analysis revealed that NK4 inhibited the activation of HGF receptor/c-Met and the downstream signaling of ERK1 and Akt1/2. Thus, NK4 is capable of blocking the HGF/c-Met signaling pathway, and

thereby, may have an important role in antagonizing the growth, invasion and metastasis of prostate cancer.

The c-Met protein was detected in the androgen-independent human prostate cancer cell line, DU145. This is consistent with the studies of Davies *et al*. [11]. Furthermore, Western blot analysis indicated that NK4 protein was expressed in the DU145/NK4 cells. These results show the feasibility of using transferred *NK4* gene to investigate its antitumor effect in DU145 cells. *In vitro*, without HGF, the proliferation, migration, invasion and apoptosis of the cells in the DU145/NK4, DU145/empty vector and DU145 groups were not significantly different, suggesting that the plasmid itself does not influence the biological characteristics of DU145 cells.

Malignant tumor cell proliferation is an important characteristic of cancer development, and apoptosis has an important role in the inhibition of oncogenesis, tumor development and growth. In our study, autocrine NK4 inhibited proliferation and induced apoptosis of DU145 cells *in vitro* and *in vivo*. The mechanism by which NK4 promotes apoptosis in DU145 cells may be its inhibitory effect on HGF-induced proliferation, leading to increased apoptosis. Death from cancer is generally associated with metastasis, a biological phenomenon linked to the tumor cell's ability to migrate, seed and colonize distant sites. In our study, we found that autocrine NK4 weakened the HGF-induced cell migration and invasion capacity. Therefore, NK4 may have potential value in the clinical therapy of cancer.

Hepatocyte growth factor has attracted considerable attention as a stromal-derived mediator in tumor–stromal interaction, on the basis of its paracrine action and close involvement in cancer growth, invasion and metastasis. As a competitive antagonist of HGF, NK4 binds to the c-Met receptor, without eliciting any biological influence, thus preventing HGF from activating the receptor [1]. Through our *in vitro* assay, we found that autocrine NK4 could inhibit cell proliferation, migration and invasion, and it induced cell apoptosis in the presence of HGF. However, autocrine NK4 by itself (without HGF) did not have any biological effects, indicating that the antitumor effect of NK4 on DU145 cells was specific for the HGF signal. Activation of Ras/ERK/MAPK (mitogen-activated protein kinase) changes the expression/activation of cell cycle regulators that affect cell proliferation. Meanwhile, Ras/ERK/MAPK activation by HGF changes the gene expression of matrix metalloproteinases and urokinase plasminogen activator that control cell migration and invasion, whereas PI3K/Akt activation by HGF mediates cell survival and resistance to apoptosis [12]. Western blot analysis revealed that NK4 inhibited the activation of c-Met and the downstream signaling of ERK1 and Akt1/2 in the presence of HGF.

Cancer cell would need continuous exposure to NK4 for NK4 to be used as a cancer treatment. This may be achieved by using continuous infusion pumps [13] or by gene transfer [8]. In this study, we evaluated the activity of the *NK4* gene by introducing it into the prostate cancer cells *in vivo*. From a clinical point of view, the efficiency and practicality of liposome transfection is limited. Some problems of replication-deficient adenovirus vectors still need to be overcome, including the specific targeting of malignant cells with less damage to other normal cells and increasing the efficacy of gene transfer [14]. With the advancement of recombinant DNA techniques, a tumor-specific gene can be expressed by using promoter elements of genes usually transcribed by tumors [15, 16]. In this research, using NK4-transfected prostate cancer cells, we showed the dramatic antitumor effect of NK4. This finding should contribute to the development of a new *NK4* gene therapy delivery system that can eliminate the need for continuous administration of recombinant protein and can provide a local source of the molecule.

Studies have shown that NK4 can block HGF, vascular endothelial growth factor and basic fibroblast growth factor-mediated angiogenesis [17, 18]. A putative binding molecule other than c-Met receptor may participate in the antiangiogenic signal transduction of NK4 [1]. Thus, NK4 may be considered as a multipotential, antitumor, therapeutic protein. Because NK4 has the potential to impact many aspects of tumorigenesis, our subsequent work will explore the effect of NK4 on angiogenesis of prostate cancer.

In conclusion, our study showed that NK4 inhibited the proliferation, migration and invasion, and induced apoptosis of DU145 cells by blocking the HGF/c-Met signaling pathway. Therefore, NK4 is a promising therapeutic agent for suppressing the malignant behavior of prostate cancer cells, and its gene delivery may be a new approach to treating prostate cancer.

Acknowledgment

We thank Bill Fong and Bethany Fong for their assistance in manuscript preparation and revision.

References

1 Matsumoto K, Nakamura T. NK4 gene therapy targeting HGF-Met and angiogenesis. Front Biosci 2008; 13: 1943–51.
2 Nakashiro K, Hayashi Y, Oyasu R. Immunohistochemical expression of hepatocyte growth factor and c-Met/HGF receptor in benign and malignant human prostate tissue. Oncol Rep 2003; 10: 1149–53.
3 Hashem M, Essam T. hepatocyte growth factor as a tumor marker in the serum of patients with prostate cancer. J Egypt Natl Canc Inst 2005; 17: 114–20.
4 Yasuda K, Nagakawa O, Akashi T, Fujiuchi Y, Koizumi K, *et al*. Serum active hepatocyte growth factor (AHGF) in benign prostatic disease and prostate cancer. Prostate 2009; 69: 346–51.
5 Nakashiro K, Hara S, Shinohara Y, Oyasu M, Kawamata H, *et al*. Phenotypic switch from paracrine to autocrine role of hepatocyte growth factor in an androgen-independent human prostatic carcinoma cell line, CWR22R. Am J Pathol 2004; 165: 533–40.
6 Humphrey PA, Zhu X, Zarnegar R, Swanson PE, Ratliff TL, *et al*. Hepatocyte growth factor and its receptor (c-MET) in prostatic carcinoma. Am J Pathol 1995; 147: 386–96.
7 Du W, Hattori Y, Yamada T, Matsumoto K, Nakamura T, *et al*. NK4, an antagonist of hepatocyte growth factor (HGF), inhibits growth of multiple myeloma cells: molecular targeting of angiogenic growth factor. Blood 2007; 109: 3042–9.
8 Murakami M, Nagai E, Mizumoto K, Saimura M, Ohuchida K, *et al*. Suppression of metastasis of human pancreatic cancer to the liver by transportal injection of recombinant adenoviral NK4 in nude mice. Int J Cancer 2005; 117: 160–5.
9 Wright TG, Singh VK, Li JJ, Foley JH, Miller F, *et al*. Increased production and secretion of HGF alpha-chain and an antagonistic HGF fragment in a human breast cancer progression model. Int J Cancer 2009; 125: 1004–15.
10 Yue D, Wang Y, Xiao JY, Wang P, Ren CS. Expression of TRPC6 in benign and malignant human prostate tissues. Asian J Androl 2009; 11: 541–7.
11 Davies G, Watkins G, Mason MD, Jiang WG. Targeting the HGF/SF receptor c-met using a hammerhead ribozyme transgene reduces *in vitro* invasion and migration in prostate cancer cells. Prostate 2004; 60: 317–24.
12 Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. Nat Rev Mol Cell Biol 2003; 4: 915–25.
13 Kuba K, Matsumoto K, Date K, Shimura H, Tanaka M, *et al*. HGF/NK4, a four-kringle antagonist of hepatocyte growth factor, is an angiogenesis inhibitor that suppresses tumor growth and metastasis in mice. Cancer Res 2000; 60: 6737–43.
14 Legrand V, Leissner P, Winter A, Mehtali M, Lusky M. Transductional targeting with recombinant adenovirus vectors. Curr Gene Ther 2002; 2: 323–39.
15 Dorer DE, Nettelbeck DM. Targeting cancer by transcriptional control in cancer gene therapy and viral oncolysis. Adv Drug Deliv Rev 2009; 61: 554–71.
16 Stoff-Khalili MA, Rivera AA, Nedeljkovic-Kurepa A, DeBenedetti A, Li XL, *et al*. Cancer-specific targeting of a conditionally replicative adenovirus using mRNA translational control. Breast Cancer Res Treat 2008; 108: 43–55.
17 Ueda K, Iwahashi M, Matsuura I, Nakamori M, Nakamura M, *et al*. Adenoviral-mediated gene transduction of the hepatocyte growth factor (HGF) antagonist, NK4, suppresses peritoneal metastases of gastric cancer in nude mice. Eur J Cancer 2004; 40: 2135–42.
18 Nakabayashi M, Morishita R, Nakagami H, Kuba K, Matsumoto K, *et al*. HGF/NK4 inhibited VEGF-induced angiogenesis in *in vitro* cultured endothelial cells and *in vivo* rabbit model. Diabetologia 2003; 46: 115–23.