Original Article

NK4 suppresses cholangiocarcinoma angiogenesis and invasion through targeting HIF-1α pathway

Rui Xie1,2, Quan-Peng Li3, Xian-Xiu Ge3, Jun-Jie Nie1, Fei Wang3, Bo-Ming Xu4, Zhen-Guo Pan2, Lin Miao1

1Institute of Digestive Endoscopy and Medical Center for Digestive Diseases, Second Affiliated Hospital of Nanjing Medical University, Nanjing 210011, Jiangsu, P. R. China; 2Department of Gastroenterology, Huai’an First People’s Hospital, Nanjing Medical University, Huai’an 223300, Jiangsu, P. R. China

Abstract: Cholangiocarcinoma (CCA) is a deadly biliary tract and hepatic malignancy with complicated genetics. This study aims to investigate the effect of hepatocyte growth factor (HGF) antagonist NK4 in inhibiting CCA cell hypoxic growth and invasion in vitro and in vivo. Human CCA cells stably transfected with NK4 (Hu-NK4) or control plasmid (Hu-Em) were treated with HGF under normoxia or hypoxia. The growth, apoptosis, and invasion of CCA cells were analyzed by MTT, flow cytometry, and transwell invasion assays, respectively. Tumor growth of human CCA in xenograft mouse model was measured by tumor size and weight. Tumor angiogenesis was assayed by cluster of differentiation 31 (CD31) positive microvessels. The expression of hypoxia-inducible factor 1-alpha (HIF-1α), vascular endothelial growth factor (vegf), and CD31 was analyzed by quantitative real-time PCR and western blot. The expression of NK4 significantly inhibited HGF-induced CCA cell growth and invasion while induced apoptosis, and hypoxia enhanced these changes. NK4 strongly inhibited tumor growth and angiogenesis of human CCA in vivo through inhibiting HIF-1α and vegf expression. These results implicated that NK4 might be an effective therapeutic reagent for CCA.

Keywords: NK4, hepatocyte growth factor, cholangiocarcinoma, hypoxia, tumor angiogenesis

Introduction

Cholangiocarcinoma (CCA) is the most common biliary tract and hepatic malignancy [1, 2]. While the incidence of CCA has been increasing [1, 3], the 5 year survival rate has not reminded at about 10% [4, 5]. The genetics of CCA is very complicated. TP53, kras, and Smad4 are the most frequently mutated genes but MLL3, ROBO2, RNF43, PEG3, GNAS, BAP1 ARID1A, IDH1, IDH2 and many other genes are also found mutated in CCA with different frequencies [6-8]. Moreover, a number of receptor tyrosine kinases pathways are heavily implicated in the carcinogenesis and progression of CCAs, including ERBB family of receptor tyrosine kinases (ERBB1 or EGFR, ERBB2 or HER-2/neu, and ERBB3 and 4) [9], fibroblast growth factor receptor (FGFR) [10], and the hepatocyte growth factor (HGF) receptor, c-met [11].

HGF/c-Met pathway is dysregulated in many cancers to promote the growth, invasion, and dissemination of tumors, leading to poor prognostics and drug resistance in cancer patients [12]. MET amplification was identified in many cancers, which is associated with a poor clinical outcome and linked to resistance to EGFR and ERBB2 inhibitors [13-17]. The expression of HGF and MET is elevated in CCAs, which is associated with activation of ERBB family members, especially ERBB2 [1]. NK4, a peptide fragment containing the four kringle domains of the HGF α-chain and specific antagonist for HGF/c-MET signaling, has been shown to inhibit tumor growth, invasion and metastasis [18, 19]. The proliferation and invasion of human CCA cells was inhibited by NK4 in vitro [20].

Hypoxia inducible factor 1α (HIF1α) is a master regulator of transcriptional response to low oxygen and associated with resistance to radiotherapies and chemotherapies, leading to a poor clinical outcome [21, 22]. Under cellular hypoxia, HIF1α protein is accumulated due the inhibition of prolyl hydroxylation proline residues 402 and 564 and ensuing ubiquitination
and degradation of HIF1α [22]. HIF1 drives the transcription of a large number of target genes that involved in tumor angiogenesis, glucose metabolism, survival, invasion and metastasis [21-23]. This study aims to investigate whether HIF1α plays a role in the inhibition of CCA cells growth by NK4 in vitro and in vivo.

Materials and methods

Cell cultures

NK4 overexpressing HuCC-T1 cells (Hu-NK4) and control Hu-Em cells were previously described [20] and were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Shanghai, China) supplemented with 10% fetal bovine serum (Life Technologies) in a humidified 5% CO₂, 95% ambient air incubator. For hypoxia treatment, cells were incubated in humidified Innova CO-170 incubator (New Brunswick Scientific, Edison, NJ) with 95% N₂/5% CO₂ for 16 hr (unless otherwise specified). All in vitro experiments were performed in the presence of 10 ng/ml of HGF (ProSpec, East Brunswick, NJ).

Cell growth assay

Approximately 2000 cells per well were seeded in 96-well plates under normoxic condition or hypoxia condition for 48 h. During the last 3 hr, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added to a final concentration of 1 mmol/l. Then 150 μl dimethyl sulfoxide was added into each well and incubated for 30 min with shaking, and read on a microplate reader (Bio-Rad Laboratories, Hercules, CA) at 490 nm.

Cell invasion assays

For cell invasion assays, 1×10⁴ cells were seeded on a fibronectin-coated polycarbonate membrane pre-coated with matrigel (R&D systems, Minneapolis, MN) insert in transwell apparatus (Corning Costar, Acton, MA) that lower chambers containing 600 μl of DMEM containing 10% FBS and cultured under normoxic condition or hypoxia condition for 12 hr. The cells on the top surface of the insert were gently detached with a cotton swab. Cells adhering to the lower surface were stained with 0.1% crystal violetin methanol and counted under a microscope in five random fields (200×).

Flow cytometry for cell apoptosis

Hu-Em and Hu-NK4 cells were seeded in a 6-well plate at a density of 1×10⁵ cells/well and cultured overnight under normoxic or hypoxic condition. Apoptotic cells were measured by flow cytometry using an Annexin V-PE/7-AAD Apoptosis Detection Kit (KGA1015-1018, Key-GEN BioTech, Nanjing, China) according to the manufacturer’s instructions.

Nude mouse xenograft cancer model

BALB/C nude mice (6-8 weeks old) were purchased from the Animal Core Facility of Nanjing Medical University (Nanjing, China) and kept under standard pathogen-free conditions with a temperature of 24°C and 12:12 hr light/dark cycle. Mice were give free access to food and water. All animal protocols were approved by the institutional animal care and usage committee of Nanjing Medical University. Xenografts were initiated by subcutaneous injection of 1×10⁷ Hu-Em or Hu-NK4 cells in 200 μl PBS into the right flank near the axillary fossa (n = 10). Tumors were measured every 3 days with calipers. When tumors were 1.5 cm in the longest diameter, mice were euthanized, and the tumor tissues were collected.
Immunohistochemistry and microvessel density

Serial sections of 4 µm thick were dewaxed and endogenous peroxidase was quenched with 3% H$_2$O$_2$ in methanol for 30 min. Before staining, nonspecific binding was blocked by incubation with 10% bovine serum albumin (BSA) in PBS at 37°C for 1 hr. Then, all incubations with 1:50 anti-HIF-1α (ab51608, Abcam, Cambridge, MA), 1:50 anti-VEGF (ab46154, Abcam) and 1:100 anti-CD31 (ab28364) antibodies in PBS containing 1% BSA were carried out at 4°C overnight. Slides were briefly washed in PBS, incubated with horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody (Jackson Immuno Research, West Grove, PA) at room temperature for 60 min, and developed with 3,3′-diaminobenzidine (DAB) (ThermoFisher Scientific, Hudson, NH). Nuclei were counterstained with Meyer’s hematoxylin (Sigma).

Real-time reverse transcription (RT)-PCR

Total RNA was isolated from cells or tumor tissues using the TRI zol total RNA isolation kit (Invitrogen, Shanghai, China) according to the manufacturer’s protocol. RT-PCR was performed using the Transcriptor First Strand cDNA Synthesis kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s protocol. Real-time quantitative PCR was carried out using the SYBR-Green Master Mix (Roche Molecular Biochemicals) on a Prism 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA). The cycling conditions were as follows: 94°C for 2 min followed by 40 cycles of 94°C for 15 sec, 58°C for 15 sec, and 72°C for 30 sec. The primers used were CTCGTCTGAGGGGACAGGA and CTCAGGTGGCTTGTCAGGG for HIF-1α; ACGAAAGCGCAAGAAATCCC and CTCCAGGGGATTAGACAGA for VEGF; CACAGATGAGAACCACGCCT and GCCCTCAGAAGACAACAT for CD31; and TCACCCACTGTGCCCATCTACGA and CAGCAGAA-CGGCTATTGCCAAATGG for β-actin, which were synthesized by Sangon (Shanghai, China). The relative mRNA levels were calculated using 2$^{-\Delta\Delta CT}$ method with β-actin as the internal control.

Western blot analysis

Total proteins (40 µg) extracted from cells or tumor tissues were run on a 10% polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore,
Billerica, MA). The membranes were blocked with 5% non-fat milk for 2 h followed by incubation with the primary antibodies at 4°C overnight. After being washed, the membrane was incubated with HRP-conjugated secondary antibodies for 2 h at room temperature and visualized with an enhanced chemiluminescence kit (Amersham, Piscataway, NJ).

Statistical analysis

Data are presented as the means ± SE. Statistical analysis was carried out using GraphPad 5 or Microsoft Excel. The differences between groups were analyzed by one-way ANOVA or Student T-test. A p value less than 0.05 was considered statistically significant.

Results

The effects of NK4 inhibiting human CCA cell growth and promoting apoptosis were enhanced by hypoxia

NK4 overexpression significantly inhibited the HGF-induced growth of human cholangiocarcinoma cells (Figure 1) and promoted apoptosis (Figure 2) under both normoxia and hypoxia conditions. Moreover, hypoxia strongly enhanced NK4-induced HuCC-T1 cell growth inhibition (Figure 1) and apoptosis (Figure 2).

NK4 inhibited hypoxia-strengthened invasion of human CCA cells

The number of invading control CCA cells (Hu-Em) was increased about 2.3-fold by hypoxia whereas hypoxia resulted in about 60% increase of invading Hu-NK4 cells (Figure 3). Under normoxia, NK4 overexpression reduced the number of invading cells by about 66% while it caused 76% reduction of invading cells under hypoxia (Figure 3).

NK4 inhibited the expression of HIF-1α and angiogenic factors

NK4 significantly inhibited the expression of hypoxia induced factor 1α (Figure 4A, 4D), CD31 (Figure 4B, 4D), and VEGF (Figure 4C, 4D) under both hypoxia and normoxia conditions. Moreover, hypoxia markedly increased the mRNA (Figure 4A-C) and protein (Figure 4D) levels of HIF-1α, CD31, and VEGF in human CCA cells.

Tumor growth in vivo was substantially inhibited by NK4

In human CCA cells xenograft mouse model, Hu-NK4 cells produced significantly smaller tumors (Figure 5A). NK4 reduced the tumor volume (Figure 5B) and tumor weight (Figure 5C) by more than 50% compared to control Hu-Em cells.
NK4 inhibits CCA

Figure 4. NK4 inhibited HGF and hypoxia induced expression of genes involved in angiogenesis in vitro. The mRNA (A-C) and protein levels of HIF-1α (A, D), vegf (B, D), and CD31 (C, D) were analyzed by quantitative PCR and western blot. Experiment was independently performed 3 times with triplicates. The data were expressed as mean ± standard error. *P < 0.01 compared to Hu-Em normoxia; #P < 0.05 compared to Hu-NK4 normoxia; ^P < 0.01 compared to Hu-Em hypoxia.

Figure 5. NK4 reduced tumor burden. (A) Representative photograph showing the size of tumor from mice grafted with Hu-Em or Hu-NK4 cells. Average tumor size (B) and tumor weight (C) of Hu-Em or Hu-NK4 cells inoculated mice. The data were expressed as mean ± standard error. *P < 0.01 compared to Hu-Em tumors.

Discussion

NK4 markedly inhibited the expression of HIF-1α, CD31, and VEGF in xenograft human CCA tumor tissues (Figure 6A-C). The mRNA levels of HIF-1α, CD31, and VEGF in Hu-NK4 tumors were 40-70% less than those in Hu-Em tumors (Figure 6A) while the protein levels were 50-90% less in Hu-NK4 tumors compared to Hu-Em tumors (Figure 6B, 6C). The number of positive cells and intensity of HIF-1α and CD31 (Figure 6D) were drastically reduced by NK4.

NK4 inhibited tumor angiogenesis

NK4 inhibited the growth and hypoxia responses of human cholangiocarcinoma cells in vitro and in vivo. Overexpressing NK4 inhibited the growth and invasion of HuCC-T1 cells as well as the expression of HIF-1α, CD31, and VEGF in Hu-NK4 cells. Mean-
NK4 inhibits CCA

while, NK4 strongly promoted CCA cell apoptosis, especially under hypoxia. NK4 suppressed tumor growth and angiogenesis in vivo, resulted in much smaller tumor burden and less vasculatures in tumor tissues.

NK4 has long been shown to inhibit the growth and invasion of cancers [18-20], especially NK4 was able to inhibit HGF-stimulated growth, migration, and invasion of human cholangiocarcinoma cells in vitro [20]. Moreover, NK4 sensitized mouse colon cancer and human CCA cells to the killing of 5-fluorouracil (5-FU) by enhancing 5-FU activation of caspases [24, 25] and modulating the balance of Bcl-2 family members [24] or suppressing 5-FU induced phosphorylation of AKT and Erk1/2 [25]. The current study clearly demonstrated that NK4 not only inhibited CCA cells growth and invasion in vitro but also significantly reduced cholangiocarcinoma tumor growth in vivo, which paved the way for further studies using NK4 to treat CCA as NK4 gene therapy for mesothelioma has been proposed [26].

The rapid growth of tumors produced a hypoxic environment which impacted aspects of the biology of tumors and their responses to therapy including: favoring survival; suppressing apoptosis; switching metabolism; enhancing receptor tyrosine kinase-mediated signaling; suppressing immune reactivity; increasing genomic instability; promoting tumor angiogenesis and vasculogenesis, the epithelial-to-mesenchymal transition, invasiveness, and metastasis [27]. Hypoxia promoted MET expression

Figure 6. NK4 inhibited tumor angiogenesis. The mRNA (A) and protein (B, C) levels of HIF-1α, vegf, and CD31 were analyzed by quantitative real-time PCR and western blot respectively. (D) Immunohistochemical staining showed that NK4 significantly inhibited tumor HIF-1α and CD31 protein levels. The data were expressed as mean ± standard error. *P < 0.01 compared to Hu-Em tumors.
and amplified HGF signaling to induce invasive growth of cancers [28]. Our data showed that NK4 inhibited HIF-1α expression and HIF-1-induced CCA cells invasion in vitro and tumor angiogenesis in vivo, which might mainly be contributed to antagonizing HGF/MET signaling by NK4 but other MET-independent anti-angiogenic mechanisms could also be involved [29].

In hypoxic condition, HIF-1α could activate vegf and other angiogenic factors to facilitate the formation of new microvessels in tumors [30]. Meanwhile, HGF/c-Met regulates the expression of vegf in colon cancer through its downstream signaling [31]. The interplay between HIF-1α and HGF/c-Met forges onto vegf to promote angiogenesis. The ability of NK4 to block HGF/c-Met signaling and to inhibit HIF-1α expression and activity makes it a very promising candidate for receptor tyrosine kinase positive cancers including CCA and other diseases with similar etiology such as rheumatoid arthritis [32, 33].

In conclusion, HGF antagonist NK4 strongly inhibited hypoxia induced growth and invasion of human cholangiocarcinoma cells in vitro and induced apoptosis of CCA cells. NK4 also inhibited hypoxic HIF-1α and vegf expression. In xenograft human CCA mouse model, NK4 significantly reduced tumor growth and tumor angiogenesis by inhibiting HIF-1α and vegf levels in tumors.

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Disclosure of conflict of interest

None.

Address correspondence to: Lin Miao, Institute of Digestive Endoscopy and Medical Center for Digestive Diseases, Second Affiliated Hospital of Nanjing Medical University, 121 Jiangjia Yuan, Nanjing 210011, Jiangsu, P. R. China. Tel: +862558509900; E-mail: miaolinxh@163.com

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