Nattokinase Crude Extract Inhibits Hepatocellular Carcinoma Growth in Mice

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Nattokinase (NK, E.C. 3.4.21.62) is a serine protease produced by *Bacillus subtilis* natto that shows promise for the treatment of thrombotic disease. In this study, we assessed the effects of NK on the development of hepatocellular carcinoma (HCC), a principal malignancy of the liver that causes morbidity and mortality worldwide. Crude extracts of NK (NCE) were isolated from fermentation medium by centrifugation and separated into three fractions (<10 K, 100~30 K and >30K). Orthotopic HCC mouse models were established and NCE was administered by oral gavage. H&E staining was performed to examine the pathology of HCC livers. Immunohistochemistry and immunofluorescence were used to evaluate FOXM1, CD31, CD44 and vimentin expression in the liver. Compared to PBS groups, NCE increased the survival rates of HCC-bearing mice to 31% and decreased ascites. Low-intensity ultrasound imaging showed that the hypoechoic mass area was lower in NCE-treated mice and that tumor growth significantly decreased. IHC staining showed that the expression of FOXM1 was inhibited by NCE treatment. Immunofluorescence results revealed lower levels of CD31, CD44 and vimentin in the NCE groups. Taken together, these data demonstrate that NCE from *Bacillus subtilis* natto improves survival and inhibits tumor growth in HCC mice.

Keywords: Hepatocellular carcinoma, nattokinase, tumor growth, cancer

Introduction

Hepatocellular carcinoma (HCC) is one of the most common neoplasms and a leading cause of cancer-related death [1]. The incidence of HCC continues to rise due to the prevalence of hepatitis B and hepatitis C infection [2]. Resection (LR), liver transplantation (LT) and radiofrequency ablation are curative treatment options [3]. However, metastasis or recurrence are frequent leading to poor prognosis [4]. New complementary anti-HCC therapies are thus urgently required.

Nattokinase (NK, E.C. 3.4.21.62) is a serine protease composed of 275 amino acids that is produced by *Bacillus subtilis* natto, a traditional soybean food of *B. subtilis* natto fermentation [5]. NK has reported fibrinolytic activity [6] and is used in the treatment of thrombotic disease due to its safety, low-cost, efficiency and stability [7]. NK-degradation products reduce hypertension by preventing elevated plasma angiotensin II levels [8]. The consumption of NK in

the North American hypertensive population is associated with beneficial changes in blood pressure. Randomized and controlled clinical trials also show that the increased intake of NK can prevent and treat hypertension [9]. Other applications of NK include the treatment of cerebral ischemia, hypertension, Alzheimer's disease and vitreoretinal disorders. For example, intravitreal injection of NK alleviates pharmacological vitreolysis due to the induction of posterior vitreous detachment (PVD) [10]. The oral administration of NK has therapeutic applications for the treatment of AD through modulating brain acetylcholinesterase (AChE) activity, TGF- β , Fas and IL-6 levels [11]. The effects of NK in cancer prevention have not been investigated to date.

In this study, NK was produced from *B. subtilis* natto and its ability to prevent HCC in mouse models was investigated. Nattokinase crude extracts (NCE) were isolated from the fermentation medium of cultured *B. subtilis* natto by centrifugation. We found that oral administration of

NCE significantly improved the survival of HCC mice and decreased tumor growth. Furthermore, NCE treatment led to a reduction of FOXM1, CD31 and vimentin expression. FOXM1 and CD31 are neovascularization markers whilst CD44 and vimentin are proliferation-associated factors essential for HCC tumorigenesis [12–15]. This highlights the benefits of NCE for HCC treatment.

Materials and Methods

Medium and Culture Conditions

NCE was produced as previously described [16]. Briefly, *B. subtilis* natto was maintained in Luria-Bertani (LB) medium (1 g of sodium chloride, 1 g of peptone, 0.5 g of yeast extract and 1.5 g of agar in 100 ml of deionized water) (pH 7.0) at 37°C. Seed cultures were inoculated (0.5%, v/v) into fermentation medium for NK production. The fermentation medium was comprised of 50 g of red bean powder, 20 g of glucose, 5 g of sodium chloride, 1 g of dipotassium hydrogen phosphate, 1 g of potassium dihydrogen

phosphate, 0.1 g of calcium chloride and 0.5 g of magnesium sulfate. Cells were grown in 500 ml flasks containing 100 ml of medium and were incubated in a shaker at 225 rpm at 37°C. The human HCC cell line Hepa1-6 (H-2b) was purchased from Boster Biological Technology Ltd. (Wuhan, China) and cultured in DMEM containing 2 mM of glutamine and 10% FBS as per the manufacturer's instructions.

Isolation of NCE

NCE supernatants were extracted from the fermentation medium by centrifugation for 8 min at 12,000 ×g. Supernatants were separated with 10 K and 30 K ultrafiltration membranes (Millipore, Ireland) according to molecular weight. NCE fractions were divided into <10 K, 10~30 K, and >30 K. The molecular weight of NK is ~28 K and 10~30 K fractions were defined as NK extracts.

Fibrinolytic Activity of NCE

The fibrinolytic activity of NCE was measured using the fibrin degradation method. Briefly, fibrin-substrate solution was mixed

Fig. 1. Nattokinase enhanced survival of HCC-bearing mice.

(A) Fibrinolytic activity of NCE with different molecular weight components from the culture supernatants of *B. subtilis* ($n = 5$, *** $p < 0.001$). (B) Survival of HCC-bearing mice on each day (** $p < 0.01$). (C) Ascites levels of HCC-bearing mice on each day ($n = 8$, ** $p < 0.01$). (D). Average appetite of HCC-bearing mice on each day ($n = 8$, ** $p < 0.01$). <10 K, 10~30 K, and >30 K: fractions of NCE with different molecular weights. (E) SDS-PAGE analysis of 10~30 K fractions of NCE from *Bacillus subtilis* natto inoculated fermentation medium at 6, 8, 12, and 24 h.

with 0.1 ml NK fractions and incubated at 37°C for 60 min. PBS and unseparated NCE were used as controls. Trichloroacetic acid (TCA, 2 ml) solution was added to stop the reaction. Samples were centrifuged at 13,000 ×g for 10 min and the absorbance at 275 nm was measured. The fibrinolytic activity (FU) of NCE was defined as the amount of enzyme leading to a 0.01 increase in absorbance in 1 min.

Establishment of Orthotopic HCC Mouse Models and NCE Treatment

All the animal experiments were authorized and approved by the Ethics Committee of Jiangsu University (2012258). C57BL/6 wild-type mice (6–8 weeks old and weighing 20 ± 2 g) were purchased from the Animal Centre of Jiangsu University (China). HCC models *in situ* were established through the intrahepatic inoculation of HCC cells. HCC cells (1×10^5) were suspended in 5 µl of PBS and injected into the liver with a syringe at an angle of 30°. To avoid bleeding and tumor cell leakage from the injection site, the liver surface was covered with Gelfoam for 5 min and the peritoneum and skin were closed. Subsequently, HCC mouse models were established. Different fractions of NCE (<10 K, 10~30 K, and >30 K) and unseparated NCE were administered by oral gavage at a dose of 7.5 µl/g according to body weight. Mice were randomized into five groups: PBS ($n = 16$), <10 K ($n = 16$), 10~30 K ($n = 16$), >30 K ($n = 16$), and unseparated NCE ($n = 14$) by oral gavage. Survival of the mice was assessed after 20 days.

Hematoxylin and Eosin (H&E) Staining

H&E staining was used to examine the pathology of the livers from HCC mouse models. Briefly, liver tissues were fixed with 4% formaldehyde and sections were embedded in paraffin (4 µm). Sections were stained with hematoxylin and eosin according to standard protocols and analyzed by microscopy.

Immunohistochemistry and Immunofluorescence

Following deparaffination and rehydration, liver sections were steamed in citrate buffer (10 mM, pH 6.0) for 30 min for antigen retrieval and exposed to 3% hydrogen peroxide for 30 min to inhibit endogenous peroxidase activity. Slides were blocked in 5% BSA for 1 h and incubated with primary antibodies against FOXM1 (USA), CD31 (Bioworld, USA), CD44 (Bioworld) and vimentin (Bioworld) overnight. For immunohistochemistry assays of FOXM1, slides were incubated in secondary antibodies for 30 min at 37°C and visualized with 3, 3'-diaminobenzidine followed by counterstaining with hematoxylin (Nikon Eclipse Ti-S, Japan). Sections were imaged at 200 × magnification. For immunofluorescence assays, slides were labeled with CD31 and stained with Cy3-labeled anti-rabbit IgG secondary antibodies (1:800) at 37°C for 45 min. Nuclei were counterstained with Hoechst 33342 (1:200; Sigma-Aldrich). Images were acquired sequentially on a fluorescent microscope at 200 × (Nikon Eclipse Ti-S).

SDS-PAGE

Fractions (10~30 K) of NCE were collected from *B. subtilis* natto inoculated fermentation medium at 6, 8, 12, and 24 h respectively. The concentration of collected 10~30 K fractions was detected by Bradford assay. Proteins (10–30 µg) were separated on 10% SDS-PAGE gels. For the analysis of NK expression, coomassie brilliant blue-stained PAGE gels were scanned using the SynGene Bioimaging System.

Statistical Analysis

Data are expressed as the mean ± SEM. Statistical differences were analyzed by a Student's t-test (two-tailed) with Prism software (GraphPad, USA). Fibrinolytic activity and tumor volume assessments were repeated five times. The ascites levels and average appetite were examined in 8 mice. P-values < 0.05 were considered significant.

Results

NCE Enhances the Survival of Hepatocellular Carcinoma (HCC) in Mice

NK activity was detected in the supernatants of *B. subtilis*. The results showed that NK activity was undetectable in

Fig. 2. Nattokinase suppresses tumor growth in HCC-bearing mice.

(A) Low-intensity ultrasound imaging of the tumor area in HCC-bearing mice treated with PBS, NCE and 10K~30K fractions at 20 days. (B) Tumor nodules on the surface of the liver from HCC-bearing mice treated with PBS, NCE and 10K~30K fractions at 20 days ($n = 5$, ** $p < 0.01$).

PBS, <10 K and >30 K fractions. NCE from the *B. subtilis* culture media had enzyme activity of ~14,500 u/ml, which increased to 24,300 u/ml in 10~30 K fractions (Fig. 1A). To assess the therapeutic potential of NK in HCC, NCE was administered to carcinoma *in situ* models for HCC in C57BL/6J mice by oral gavage. In response to the administration of 10~30 K fractions and NCE, 62.5% and 57.14% of HCC mice survived respectively. However, the survival rates decreased to 25%, 25% and 31% in PBS, <10 K fraction and >30 K fractions, respectively (Fig. 1B). Compared to PBS groups and <10 K and >30 K groups (Fig. 1C), the ascites levels of HCC-bearing mice decreased in 10~30 K fractions and the NCE groups. The average appetite of HCC-bearing mice consistently increased in 10~30 K fractions and the NCE group compared to PBS and <10 K and >30 K fractions (Fig. 1D). SDS-PAGE also showed that NK of 28 KDa was expressed in 10~30 K fractions, the expression of which increased with fermentation time from 6 to 24 h (Fig. 1E). These results revealed that NCE enhances the survival of HCC-bearing mice with hepatic carcinoma *in situ*.

NK Suppresses Tumor Growth in HCC-Bearing Mice

To investigate the role of NK in liver tumor growth, low-intensity ultrasound imaging was used to examine the tumor area of HCC *in situ*. Ultrasounds showed that the hypoechoic mass area of liver cancer was inhibited for 15 days after 10~30 K fractions and NCE treatment (Fig. 2A). Tumor nodules visible on the surface of the liver were measured after 20 days of treatment with 10~30 K fractions and NCE treatment (Fig. 2B). The results showed that tumor diameter significantly decreased in 10~30 K fractions and the NCE group. These findings indicated that NCE can suppress *in situ* tumor growth.

NCE Inhibits HCC Cell Proliferation In Vivo

To further investigate the components of NCE that inhibit liver tumor growth, H&E staining was used to examine the pathological changes of NCE-treated liver tumor tissue. In comparison to PBS control groups, the tumor cell area was inhibited and hepatocyte growth was enhanced in the liver tissues from 10~30 K-fractions and NCE-treated mice (Fig. 3A). Forkhead box M1 (FOXM1),

Fig. 3. Immunohistochemistry of FOXM1 in liver tumors.

(A) H&E staining of tumor tissues from HCC-bearing mice treated with PBS, NCE and 10K~30K fractions at 20 days (200 ×, scale bar =). (B) Expression of FOXM1 in tumor tissue from HCC-bearing mice treated with PBS, NCE and 10K~30K fractions at 20 days were investigated by immunohistochemistry (200 ×).

Fig. 4. Immunofluorescence staining of CD31, CD44 and vimentin in liver tumors.

Expression of CD31, CD44 and vimentin in tumor tissue from HCC-bearing mice treated with PBS, NCE and 10K~30K fractions at 20 days were investigated by immunofluorescence (200 ×).

CD44 and vimentin are considered principal regulators of cell differentiation and proliferation and are overexpressed in liver cancer [12–14]. CD31 is an auxiliary neo-vascularization marker. IHC staining revealed decreased expression of FOXM1 in liver tumor samples from 10~30 K-fractions and NCE-treated mice (Fig. 3B). Immunofluorescence staining showed that CD31, CD44 and vimentin expression were reduced in liver tumor samples from 10~30 K-fractions and NCE-treated mice (Fig. 4). These results demonstrate that NCE suppresses tumor growth through inhibiting cell proliferation in the tumors.

Discussion

NK was extracted from traditional fermented food using *B. subtilis* natto (Sumi *et al.* 1987) [17]. The effects of NK on thrombosis have been confirmed in previous studies. This study investigated the efficacy of NK in hepatic cancer prevention in a mouse models. Amongst the components of *B. subtilis* culture medium, only 10~30 K and NCE exhibited NK activity. The molecular weight of NK is 28 Kda. Thus, it can be inferred that NK is present in the

10~30 K fractions and represents the NCE component of *B. subtilis* supernatants. In comparison to a placebo, beneficial changes were found for survival and tumor growth inhibition in NK-treated mice. In this study, improved survival rates in mice with HCC were observed in 10~30 K and NCE groups. The inhibition of ascites levels and improved appetites were found in 10~30 K and NCE groups. The survival status of mice with HCC was associated with tumor growth.

Tumor growth is a complex physiological process involving interactions between cells, growth factors, cancer stem cells and cell cycle regulators [18–20]. Cancer therapy studies have highlighted the importance of vascular and vessel proliferation in tumor tissue [21–23]. The supplementation of dietary natto extracts can suppress intimal thickening in response to endothelial injury in rat femoral arteries [24]. Natto extracts suppress intimal thickening after vascular injury as a result of the inhibition of mural thrombi formation. This suggests that NK plays a role in angiogenesis. The results of this study indicated that NK suppresses the expression of the crucial neovascularization markers CD31, FOXM1, CD44 and vimentin [12–15]. These

transcription factors regulate the proliferation, survival and drug resistance of various cancers [25]. Current cancer therapy focuses on new targets for cancer genesis. The depletion of FOXM1 promotes the anti-tumor activity of PF in colorectal cancer cells [26]. Inhibition of FOXM1 in human PIMAs inhibited mucinous characteristics and reduced tumor growth and invasion [15]. FOXM1 is an anti-tumor target due to its role in tumorigenesis. Similarly, inhibition of CD44 function blocks tumor invasion and metastatic colonization [27]. Vimentin downregulation inhibits the invasion of breast cancer cells [28, 29]. Our results demonstrate that the anticancer effects of NCE may partially be attributable to the inhibition of CD31, FOXM1, CD44 and vimentin.

In conclusion, this is the first report to reveal the anti-HCC effects of NCE from the supernatants of *B. subtilis*. NK treatment could increase survival and inhibit tumor growth in HCC mice, highlighting its potential as a novel anti-HCC therapeutic.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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