Nek2 as an Effective Target for Inhibition of Tumorigenic Growth and Peritoneal Dissemination of Cholangiocarcinoma

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Abstract
We investigated the role of Nek2, a member of the serine/threonine kinase family, Nek, in the tumorigenic growth of cholangiocarcinoma cells. Expression of Nek2 is elevated in cholangiocarcinoma in a tumor-specific manner as compared with that of normal fibroblast cells. Expression of exogenous Nek2 did not perturb the growth of cholangiocarcinoma cells, whereas suppression of the Nek2 expression with siRNA resulted in the inhibition of cell proliferation and induced cell death. In xenograft-nude mouse model, s.c. injection of Nek2 siRNA around the tumor nodules resulted in reduction of tumor size as compared with those of control siRNA injection. In peritoneal dissemination model, Nek2 siRNA–treated mice showed statistically longer survival periods in comparison with those of the control siRNA–treated mice. Taken together, our data indicate a pivotal role of Nek2 in tumorigenic growth of cholangiocarcinoma. [Cancer Res 2007;67(20):9637–42]

Introduction
Cholangiocarcinoma, one of the most common liver tumors in Southeast Asian countries (1, 2), is a primary malignancy derived from the bile duct epithelium. Despite the progress of the combined therapy (3, 4), its prognosis is still extremely miserable among all malignancies (5). To search for the molecular targets for the treatment of cholangiocarcinoma, we compared the gene expression pattern of cholangiocarcinoma with that of a normal genome. Using cDNA expression array containing 1,176 unique genes (6). Among these explored genes, we identified Nek2 as a candidate that showed high expression in a tumor-specific manner.

Nek2 is a member of the serine/threonine kinase family, Nek, structurally related to the essential mitotic regulator NIMA (3). Functional studies have implicated Nek2 in regulation of centrosome separation and spindle formation (4, 7). Moreover, increased expression of Nek2 has been reported in cell lines derived from breast, cervical, and prostate carcinomas, as well as lymphomas (8), suggesting the involvement of Nek2 in tumorigenesis. Indeed, forced expression of Drosophila Nek leads to abnormalities in cleavage furrow formation and cytokinesis failure (9), which can cause chromosome instability and aneuploidy (10). Despite its importance, however, the role of Nek2 in tumorigenesis remains largely unclear.

To obtain clues, we investigated the role of Nek2 in cholangiocarcinoma by use of siRNA against Nek2. We show for the first time that Nek2 depletion in cholangiocarcinoma cell lines causes growth suppression and cell death. Moreover, we show that administration of siRNA against Nek2 into tumor-bearing mice induces substantial elongation of survival length.

Materials and Methods

Cell culture and plasmid construction. The cell lines HuCCT1, TFK1, HuE28, ETK1, BBE, and IHGGK were obtained from Tohoku University and Riken Cell Bank. CCKS1 is kindly supplied by Y. Nakamura (Department of Human Pathology, Kanazawa University Graduate School of Medicine, Takamachi, Kanazawa, Japan). HA-Dsred Nek2 plasmid was made by ligotage of COOH-terminally hemagglutinin (HA)-tagged Nek2 gene into a pDsrred express vector (Clontech).

Preparation of siRNAs. The sense and antisense strands of Nek2 siRNAs obtained from Qiagen were as follows: Nek2 siRNA 19, 5′-GGAGGG-GACUCUGCACUGA-GdTDdT-3′ (sense), 5′-ACAUCUGCCAUCUGCU-3′ (antisense); Nek2 siRNA 27, 5′-GGAAUGCCACAGAAGA-GdTDdT-3′ (sense), 5′-ACUCUGCU-3′ (antisense); and Nek2 siRNA 65, 5′-GGAGGGCAAAUUGAGAGdTDdT-3′ (sense), 5′-UCUCUAAUUGUCGCC-CUCdTdT-3′ (antisense).

Luciferase GL2 siRNA (Dharmacon) and negative control siRNA (Qiagen) were used as controls. Transfection of siRNA in vitro was carried out with GenePORTER (Genlantis).

Semiquantitative reverse transcription-PCR. Semiquantitative PCR in a GeneAmp PCR system 9700 (Applied Biosystems) was previously described (11). Primer sequences were as follows: Nek2, 5′-AACGAGGTCACAGACAGGGA-3′ (sense), 5′-TGCATTATTTGCTACAGGA-3′ (antisense); β-actin, 5′-CTCTCGTGTTGGCAGTTACAGG-3′ (sense), 5′-TCATCACCATTGGCAATGAG-3′ (antisense).

Immunoblotting and immunocytochemistry. SDS-PAGE and immunoblotting analysis were done as previously described (12). Mouse monoclonal anti-Nek2 (Transduction laboratories), and rabbit anti-mouse immunoglobulin Gs (Biosource) were used for the analysis. Immunocytochemistry was done as previously described (13). Nuclear DNA was counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Biosource).

Cell proliferation assay and dye exclusion test. Cell proliferation was determined by a colormetric assay of cell viability that is based on the cleavage of tetrazolium salt by mitochondrial dehydrogenases (Tetra Color One, Seikagaku Corp.). The absorbance of the formazan dye was measured at 450 and 630 nm 1.5 h after adding the reagent. To assess the viability of cells after siRNA treatment, dye exclusion test was done.

Live cell imaging. Cells, grown on a glass-base dish (Iwaki), were mounted in the CO2 microincubator regulated at 37°C and placed on the microscope stage. Images were captured at intervals of 2 or 5 min with inverted microscope IX81 (Olympus) and the MetaMorph software (Universal Imaging Corp.).

Animal efficacy studies. Male nude mice, 8 weeks old and 20 to 25 g in weight (CREA Japan, Takaorr, Kanazawa, Japan), were used as controls. Transfection of siRNA in vivo was carried out with GenePORTER (Genlantis).

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dUTP nick end labeling (TUNEL) analysis (DeadEnd TUNEL system, Promega). For the analysis of survival rate, mice were inoculated with HuCCT1 cells ($1 \times 10^5$) into the abdominal cavity. Nek2 siRNA 27 (20 $\mu$mol/L) dissolved in 100-$\mu$L cell matrix was directly injected into abdominal cavity once a week, thrice. Animal experiments were done in compliance with the guideline of the Institute for Laboratory Animal Research, Nagoya University Graduate School of Medicine.

**Statistical analysis.** All data were presented as means $\pm$ SE. The statistical differences were analyzed by the Student’s $t$ test, the repeated measures ANOVA, or the Kaplan-Meier method with Statview for Windows. The results were considered significant when $P < 0.05$.

**Results and Discussion**

We compared the gene expression pattern of cholangiocarcinoma with that of a normal liver using cDNA expression array containing 1,176 unique genes. Among these genes, the expression of Nek2 was substantially increased in cholangiocarcinoma in a tumor-specific manner (data not shown). We therefore compared the expression levels of Nek2 in cholangiocarcinoma cell lines, HuCCT1, TFK1, HuH28, CCKS1, ET1K1, RBE, and IHGGK, with that in HFF, a cell line derived from normal human fibroblast. Cholangiocarcinoma cells expressed higher levels of Nek2 mRNA and protein than did HFF cells (Fig. 1A and B). Nek2 has three spliced variants, Nek2A, Nek2B, and Nek2A-T (14). The predominant isoform shown in Fig. 1B corresponds to Nek2A/Nek2A-T and the smaller band is Nek2B (14). Cholangiocarcinoma cells expressed relatively larger amounts of Nek2A/Nek2A-T and Nek2B, as compared with those of HFF. In HuCCT1, we confirmed the localization of Nek2 to the centrosomes (Fig. 1C).

**Figure 1.** Expression of Nek2 in cholangiocarcinoma. A, detection of Nek2 expression in cholangiocarcinoma cell lines (HuCCT1, TFK1, HuH28, and CCKS1) and a normal fibroblast cell line (HFF) by semiquantitative PCR. The quantity of cDNA was assessed by amplification of $\beta$-actin. B, detection of Nek2 expression in cholangiocarcinoma cell lines (HuCCT1, TFK1, HuH28, CCKS1, ET1K1, RBE, and IHGGK) and HFF by immunoblotting analysis. $\beta$-Actin was used as a control. Top, Nek2A and Nek2B; bottom, $\beta$-actin. C, detection of Nek2 expression in HuCCT1 by immunocytochemistry. $\gamma$-Tubulin was used as a marker of centrosome. DNAs were counterstained with DAPI. Left, Nek2 (arrowhead); middle, $\gamma$-tubulin (asterisk); right, DAPI. D, proliferation of HuCCT1 stably transfected with Nek2 (HuCCT1/Nek2). Left, expression of HA-Dsred Nek2 in HuCCT1/Nek2 stable clone by immunoblotting with anti-Nek2. Right, cell proliferations with 0.5% or 10% serum that were quantified by cell proliferation assays (see Materials and Methods). Similar results were obtained in three independent experiments. $P < 0.05$, Student’s $t$ test.
To assess the role of Nek2 in the growth of cholangiocarcinoma cells, we examined the effect of exogenous Nek2 expression in HuCCT1. HA-tagged Nek2 ligated with Dsred vector (HA-Dsred Nek2) was transfected into HuCCT1 (HuCCT1/Nek2). Immuno-blotting with anti-Nek2 showed that substantial amount of HA-Dsred Nek2 was expressed in HuCCT1/Nek2 to the level similar to that of endogenous Nek2 (Fig. 1D, left). Expression of HA-Dsred Nek2 did not perturb the growth of HuCCT1 but rather stimulated it (Fig. 1D, right). With the medium containing 0.5% serum, the growth-promoting effect of HA-Dsred Nek2 became more evident.

To study the function of Nek2, we next treated the cholangiocarcinoma cells with double-stranded siRNA oligonucleotides against Nek2. Three types of siRNA, Nek2 siRNA 65, Nek2 siRNA 27, and Nek2 siRNA 19, were designed and transfected into HuCCT1 cells. As shown in Fig. 2A, expression of Nek2 in HuCCT1 was substantially suppressed by treatment with Nek2 siRNA 65, Nek2 siRNA 27, and Nek2 siRNA 19. Control siRNA was used as a nonsilencing siRNA. Cell proliferations were quantified by cell proliferation assays (see Materials and Methods). Similar results were obtained in three independent experiments. 

P < 0.05, Student’s t test.
siRNA, but not with control siRNA. Surprisingly, we found that treatment of HuCCT1 with Nek2 siRNA caused concomitant suppression of cell growth to the level of ~50% of control siRNA-treated cells (Fig. 2B). In contrast to HuCCT1, the effect of Nek2 siRNA on the growth of HFF cells was limited.

To study further the effect of Nek2 siRNA on cell growth, we studied the living siRNA-transfected cells by time-lapse microscopy. The cells were transfected with either rhodamine-labeled Nek2 siRNA or FITC-labeled control siRNA, and both cells were subsequently incubated for 12 h. As shown in Fig. 2C, Nek2 siRNA, but not control siRNA, caused suppression of cell division and induced cell death. We confirmed the Nek2 siRNA–dependent cell death by trypan blue dye exclusion test. As shown in Fig. 2D, all three types of Nek2 siRNA could induce cell death in HuCCT1 and TFK1 cells. In contrast, the effect of Nek2 siRNA on the survival of HFF cells was at limited levels.

We next examined the effect of Nek2 siRNA treatment on the growth of cholangiocarcinoma tumor in xenograft-nude mouse model. Mice were s.c. inoculated with HUCCT1. Four weeks after inoculation, 20 μmol/L of either Nek2 siRNA in cell matrix or control siRNA in cell matrix, as well as cell matrix alone, was s.c. injected around the tumor nodules of mice once a week for 3 weeks. After the treatment, tumor size was measured once a week until 12 weeks after tumor inoculation. Surprisingly, mean tumor volumes in Nek2 siRNA–treated mice were substantially reduced in comparison with those of the control mice (Fig. 3A and B). The Nek2 expression in the tumor tissues was dramatically suppressed in Nek2 siRNA–treated tumors as compared with control siRNA–treated tumors (Fig. 3C).

Tumor sections of the xenografts were analyzed by H&E staining followed by TUNEL staining. Multiple empty spaces occupied with debris were observed in the Nek2 siRNA–treated mice were substantially reduced in comparison with those of the control mice (Fig. 3A and B). The Nek2 expression in the tumor tissues was dramatically suppressed in Nek2 siRNA–treated tumors as compared with control siRNA–treated tumors (Fig. 3C).

Figure 3. In vivo consequences of Nek2 siRNA. A, photos of HuCCT1 xenograft. Four weeks after tumor inoculation, nude mice bearing HuCCT1 tumors were treated with Nek2 siRNA 27 (20 μmol/L) once a week for 3 wk (arrows). On the 28th day after the final dose of Nek2 siRNA, HuCCT1 tumors were photographed. Control was used as placebo and control siRNA was used as nonsilencing siRNA. B, the volume of HuCCT1 tumors shown in A. C, detection of Nek2 expression in established HuCCT1 tumors after Nek2 siRNA treatment. Immunoblot analysis confirmed the expression of Nek2 in tumors. Four weeks after tumor inoculation, nude mice bearing tumors were treated with Nek2 siRNA (20 μmol/L) once a week for 3 wk and, 48 h after final administration of siRNA, tumors were resected. Control was used as placebo and luciferase pGL2 siRNA was used as nonsilencing siRNA. Top, Nek2; bottom, β-actin. D, right, H&E staining (top) and TUNEL staining (bottom) of resected tumor sections from HuCCT1 xenografts in A. Left, analysis of siRNA induction in HuCCT1 xenografts by direct injection of siRNA. Fluorescent images were captured in 48 h after injection of rhodamine-conjugated siRNA. DNAs were counterstained with DAPI. Control siRNA was used as nonsilencing siRNA.
To further evaluate the effects of Nek2 siRNA treatment, we challenged siRNA in the peritoneal dissemination model in which HuCCT1 was injected i.p. It should be noted that peritoneal dissemination is one of the major undesirable complications frequently associated with inoperable cases (15). To confirm the incorporation of siRNA into cancer cells in the peritoneal cavity, rhodamine-labeled Nek2 and control siRNA in atelocollagen complexes were administered i.p. into mice after the injection of HuCCT1. As shown in Fig. 4A, similar levels of both siRNAs were incorporated into cancer cells. Nek2 siRNA or control siRNA (20 μmol/L) was administrated once a week i.p. for 3 weeks. All controlled mice (n = 5) died within 40 days (mostly within 14 days) following the final treatment. Mice treated with control siRNA showed similar lengths of survival with control mice. In contrast, the Nek2 siRNA–treated mice showed statistically longer survival periods as with those of the control mice (Fig. 4B). Survival rates of the Nek2 siRNA–treated mice were ~1.5 times higher than those of the control siRNA–treated mice. It should be noted that these mice tolerated the siRNA treatment well and showed no decrease in their body weight. We observed no sign of toxicity in this treatment during the experimental period of up to 12 weeks.

Among all malignancies, the prognosis of cholangiocarcinoma is extremely miserable. For the improvement of its therapy, identification of specific molecular targets critical for its tumorigenicity is an important issue. In this report, we showed that Nek2 is abundantly expressed in cholangiocarcinoma and required for the tumorigenic growth and survival of the cells. In normal adult mouse, expression of Nek2 is specifically strong in the testis but weak in other tissues, suggesting that expression of Nek2 in cholangiocarcinoma is abnormally controlled. Surprisingly, suppression of Nek2 expression in cholangiocarcinoma subsequently caused apoptotic cell death. Although the precise mechanism by which suppression of Nek2 expression causes cell death remains to be clarified, our results strongly suggest that Nek2 is required for the survival of cholangiocarcinoma cells. Nek2 siRNA did not induce massive cell death in normal fibroblasts, suggesting that cholangiocarcinoma cells have specific dependence on Nek2 for
their growth and survival, which is called by Weinstein (16) as “oncogene addiction.”

Many reports suggest that delivery, especially in vivo delivery, is the most important obstacle for siRNA-based gene therapy. In this report, we showed that cell matrix was an effective delivery system for siRNA treatment. The synthetic siRNA resisted biodegradation for at least 48 h in tumor. A significant number of cancer cells in the peritoneal cavity have accepted the siRNAs. Direct injection with cell matrix is less insensitive to nuclease than an i.v. injection (17) and is insufficiently immunogenic to stimulate IFN expression in vivo (18).

In summary, this is the first demonstration that Nek2 plays a critical role in tumorigenic growth of cholangiocarcinoma, and inhibition of Nek2 expression with its siRNA causes suppression of cholangiocarcinoma growth and survival. Our data in vivo provide a strong rationale for the further investigation of Nek2 inhibitors as a new cancer therapy, holding the potential to provide regression of multiple human malignancies.

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