Inhibition of Nemo-like Kinase Increases Taxol Sensitivity in Laryngeal Cancer

Ji-Rui Dong, Nan Guo, Jian-Pu Zhao, Pin-Duo Liu, Hui-Hong Feng, Yan Li*

Abstract

Several studies have shown that nemo-like kinase (NLK) plays a vital role in apoptosis of cancer cells. The present research concerned effects and mechanisms of Taxol on NLK knockdown human laryngeal cancer Hep-2 cell lines in vitro. Using RNAi, methyl-thiazoltetrazolium (MTT) assays, real-time RT-PCR, Western blotting and flow cytometry analysis, growth and the cell cycle progression of NLK knockdown Hep-2 cells and expression of downstream molecules were observed. Cell growth was obviously suppressed in the Taxol treated group \( (P<0.001, \ 48 \ \text{hours}) \). Cell numbers of combined Taxol-based chemotherapy with lentivirus mediated RNAi treatment group \( (\text{Lv-shNLK}+\text{Taxol group}) \) were significantly different from NLK-specific siRNA lentivirus infected group \( (\text{Lv-shNLK group}) \) \( (p<0.001) \). Flow cytometry analysis revealed that \( \text{Lv-shNLK}+\text{Taxol} \) caused the G0/G1-phase DNA content to decrease from 44.1 to 3.33% \( (p<0.001) \) and the S-phase DNA content to increase from 38.4 to 82.0% \( (p<0.001) \), in comparison with the \( \text{Lv-shNLK}+\text{Taxol group} \). Immunoblot analysis showed that knockdown of NLK led to significant reduction in the levels of cyclin D1, PCNA and PARP, whereas cyclin B1 was elevated in. Cell growth was also obviously suppressed in the Hep-2 cell line, knockdown of NLK making them more sensitive to Taxol treatment. NLK is expected to become a target of new laryngeal cancer gene therapies.

Keywords: Taxol - sensitivity - nemo-like kinase - Hep-2 cells - laryngeal cancer

Asian Pac J Cancer Prev, 14 (12), 7137-7141

Introduction

Over 500,000 patients worldwide are diagnosed as squamous cell carcinoma of head and neck each year (Nestle et al., 1998). 95% of that is laryngeal squamous cell carcinoma which accounts for 5% of malignant tumors in developed countries and represents a serious threat to human health (Sanderson and Ironside, 2002).

Taxol is one of the most effective antitumor agents developed in the past three decades. However, a major obstacle to achieving successful treatment is the development of cellular taxol drug resistance (Zhang et al., 2012).

Nemo-like kinase (NLK) is a pro-mitogen-acNemo-like kinase (Nemo-like kinase NLK), a member of serine/threonine protein kinase superfamily and also a classic mediator of Wnt/beta-catenin signaling pathway (Brott et al., 1998; Harada et al., 2002). The Wnt/beta-catenin signaling pathway regulates various aspects of development and plays important role in tumor formation in multiple tissues and organs (such as the colon, breast, ovarian, liver, etc.) (Lee et al., 2006; Kim et al., 2008; Yuzugullu et al., 2009).

In the classic Wnt/beta-catenin signaling pathway, NLK is downstream gene of Wnt/beta-catenin signaling pathway (Ishitani et al., 1999). This study aims at the relationship between NLK and laryngeal carcinoma. And we try to clarify the mechanism of Taxol resistance in laryngeal carcinoma. Down-regulate NLK expression could be developed for the management of laryngeal cancer.

Materials and Methods

Cell culture

Laryngeal squamous cell carcinoma cell line Hep-2 was obtained from Cell Bank Type Culture Collection of Chinese Academy of Sciences and cultured in Minimum Essential Medium. And the medium was supplemented with 10% fetal bovine serum (Biowest S1580) and penicillin-streptomycin solution (100 U/mL, Hyclone SV30010) and cells were maintained at 37 °C in humidified 5% CO₂.

RNAi

Small interfering RNA against NLK (GATAGACCTATTGGATATG) and a control siRNA (TTCTCCGAACGTGTCACGT) was generated as a 21-nt inverse repeat separated by a 6-nt loop and was inserted downstream of the U6 promoter in the lentiviral vector pFH1, respectively. The resulting lentiviral vector
that expressed the NLK-specific siRNA or negative control siRNA, together with pVSVG and pCMVΔR 8.92plasmids containing the imperative elements for virus packaging, was co-transfected into 293T cells with calcium phosphate. After a 48hours incubation period post transfection, lentiviruses were harvested in serum-free medium, and filtered and concentrated in primed Centric on Plus-20 filter devices (Millipore, Bedford, MA, USA). Hep-2 cells were grown in 6-wellplates at 70–80% confluence and infected with NLK-specific siRNA lentivirus or control lentivirus (MOI of 120), respectively, and harvested at 5 days after infection for characterizing the levels of NLK expression.

For the treatment group, the dosage of Taxol is 0.1 μM respectively (Beijing Wellso Pharmaceutical Co. Ltd., H10980066). NLK was significantly inhibited by Taxol with Taxol (Beijing Wellso Pharmaceutical Co. Ltd., H10980066). NLK was significantly inhibited by Taxol (0, 0.01, 0.1, 1, 10 μM respectively). NLK was gradually decreased as the Taxol dosage’s increment (Figure 1C, 1D). In accordance with the down regulation of NLK in normal Hep-2, NLK protein was also down regulated in the NLK-specific siRNA expressed Hep-2 as evidenced by western blot. Thus, Taxol could inhibit the expression of NLK (Figure 2).

**indicates \( P<0.05 \), **indicates \( P<0.01 \). B: The effects of Taxol on the expression of NLK in Hep-2 at different dosage levels determined by q-PCR. *indicates \( P<0.05 \), **indicates \( P<0.01 \). D: The effects of different dosage Taxol on the expression of NLK tested by western blotting.

**Results**

Taxol inhibits the expression of NLK in Hep-2 cells. Expression of NLK was examined by Real-time PCR. We tested the expression of NLK at 0, 24, 48 hours treated with Taxol (Beijing Wellso Pharmaceutical Co. Ltd., H10980066). NLK was significantly inhibited by Taxol (\( p<0.01 \), Figure 1A, 1B, Taxol dosage: 0.1 μM). Also, the expression of NLK treated with different dosage Taxol (0, 0.01, 0.1, 1, 10 μM respectively) of NLK in Hep-2 determined by q-PCR (0, 0.01, 0.1, 1, 10 μM respectively). NLK in Hep-2 was determined by q-PCR.

**Propidium iodide (PI) flow cytometry analysis**

Cells in each well were harvested after treated with 50μg/mL PI and 50μg/mL RNase in PBS for 30 min at room temperature. Each experiment was performed in triplicate, and analyses were performed by FAC-Scan Flow Cytometer (Becton Dickinson, San Jose, CA, USA) in accordance with the manufacturer’s guidelines.

**Statistical analysis**

Statistical significance was determined by a Student’s t-test using SPSS17.0 software. Differences were considered significant at values of \( P<0.05 \).

**Effect of Taxol on the proliferation of NLK Knockdown Hep-2 cells**

Six groups were used including parent Hep-2 cells
Knockdown of NLK makes Hep-2 cells more sensitive to Taxol treatment. As shown in Figure 4B, sub-G1 phase cell cycle arrest, which virtually inhibits the growth of Hep-2 cells. Knockdown of NLK makes Hep-2 cells more sensitive to Taxol treatment.

In contrast to the control Taxol lentivirus-treated cultures treated with Taxol caused the G0/G1-phase DNA content decreased from 44.07 to 3.33% (p<0.001) and the S-phase DNA content increased from 38.37 to 82.03% (p<0.001), in contrast to the control Taxol lentivirus-treated cultures (Figure 4A). These data indicated that NLK-specific siRNA lentivirus infection and Taxol induced G0/G1 phase cell cycle arrest, which virtually inhibits the growth of Hep-2 cells. Knockdown of NLK makes Hep-2 cells more sensitive to Taxol treatment.

As shown in Figure 4B, sub-G1-phase DNA content in NLK-specific siRNA lentivirus infected group rises from 0.67 to 0.91% compared with the control lentivirus-treated group. Compared with the control Taxol lentivirus-treated group, sub-G1-phase DNA increased from 8.23 to 10.19% (p<0.05) in Taxol NLK-specific siRNA lentivirus infected group. However, the addition of Taxol significantly increased apoptosis in NLK-specific siRNA lentivirus infected cells (from 0.91 to 10.19%), suggesting that combining NLK knockdown inhibition with Taxol treatment.

Detection of the downstream molecules

Furthermore, expression levels of Cyclin B1, Cyclin D1, P21, P27, PCNA and PARP were examined in Hep-2 cells, control lentivirus-infected cells, NLK-specific siRNA lentivirus infected cells, and Hep-2 cells treated with Taxol. Cells treated with Taxol and Taxol+NLK siRNA lentivirus infected group showed strong proliferation. However, the addition of Taxol significantly increased apoptosis in NLK-specific siRNA lentivirus infected cells (from 0.91 to 10.19%), suggesting that combining NLK knockdown inhibition with Taxol treatment.

**Figure 1. The Effect of Taxol on the Cell Cycle Traverse of Hep-2 Cells as Measured by Flow Cytometry.** Con: parent Hep-2 cells, Lv-shCon: control lentivirus-infected cells, Lv-shNLK: NLK-specific siRNA lentivirus infected cells, Con+Taxol: Hep-2 cells treated with Taxol, Lv-shCon+Taxol: control lentivirus-infected cells treated with Taxol, and Lv-shNLK+Taxol: NLK-specific siRNA lentivirus infected cells treated with Taxol. *indicates P<0.05, **indicates P<0.01

**Figure 2. The Effect of Taxol on the NLK Expression in NLK Knockdown Hep-2 Cells Determined by Immunoblotting.** “-” means lacking of Taxol, “+” indicates treating with Taxol

**Figure 3. The Proliferation of Hep-2 Cells was Tested by MTT.** Con: parent Hep-2 cells, Lv-shCon: control lentivirus-infected cells, Lv-shNLK: NLK-specific siRNA lentivirus infected cells, Con+Taxol: Hep-2 cells treated with Taxol, Lv-shCon+Taxol: control lentivirus-infected cells treated with Taxol, and Lv-shNLK+Taxol: NLK-specific siRNA lentivirus infected cells treated with Taxol. **indicates P<0.01

**Figure 4. The Effect of Taxol on the Cell Cycle Traverse of Hep-2 Cells as Measured by Flow Cytometry.** Con: parent Hep-2 cells, Lv-shCon: control lentivirus-infected cells, Lv-shNLK: NLK-specific siRNA lentivirus infected cells, Con+Taxol: Hep-2 cells treated with Taxol, Lv-shCon+Taxol: control lentivirus-infected cells treated with Taxol, and Lv-shNLK+Taxol: NLK-specific siRNA lentivirus infected cells treated with Taxol

**Figure 5. The Expression Levels of Cyclin B1, Cyclin D1, P21, P27, PCNA, PARP and GAPDH were Examined by Western-blotting.** Con: parent Hep-2 cells, Lv-shCon: control lentivirus-infected cells, Lv-shNLK: NLK-specific siRNA lentivirus infected cells, Con+Taxol: Hep-2 cells treated with Taxol, Lv-shCon+Taxol: control lentivirus-infected cells treated with Taxol, and Lv-shNLK+Taxol: NLK-specific siRNA lentivirus infected cells treated with Taxol

**Effect of Taxol on the cell cycle traverse of NLK knockdown Hep-2 cells**

Cell cycle analysis by flow cytometric assay revealed that NLK-specific siRNA lentivirus infected group treated with Taxol caused the G0/G1-phase DNA content decreased from 44.07 to 3.33% (p<0.001) and the S-phase DNA content increased from 38.37 to 82.03% (p<0.001), in contrast to the control Taxol lentivirus-treated cultures. These data indicated that NLK-specific siRNA lentivirus infection and Taxol induced G0/G1 phase cell cycle arrest, which virtually inhibits the growth of Hep-2 cells. Knockdown of NLK makes Hep-2 cells more sensitive to Taxol treatment.

As shown in Figure 4B, sub-G1-phase DNA content in NLK-specific siRNA lentivirus infected group rises from 0.67 to 0.91% compared with the control lentivirus-treated group. Compared with the control Taxol lentivirus-treated group, sub-G1-phase DNA increased from 8.23 to 10.19% (p<0.05) in Taxol NLK-specific siRNA lentivirus infected group. However, the addition of Taxol significantly increased apoptosis in NLK-specific siRNA lentivirus infected cells (from 0.91 to 10.19%), suggesting that combining NLK knockdown inhibition with Taxol treatment.

Detection of the downstream molecules

Furthermore, expression levels of Cyclin B1, Cyclin D1, P21, P27, PCNA and PARP were examined in Hep-2 cells, control lentivirus-infected cells, NLK-specific siRNA lentivirus infected cells, and Hep-2 cells treated with Taxol and Taxol+NLK siRNA lentivirus infected group showed strong proliferation. However, the addition of Taxol significantly increased apoptosis in NLK-specific siRNA lentivirus infected cells (from 0.91 to 10.19%), suggesting that combining NLK knockdown inhibition with Taxol treatment.
with Taxol, control lentivirus-infected cells treated with Taxol, and NLK-specific siRNA lentivirus infected cells treated with Taxol. Immunoblot analysis showed that the knockdown of NLK led to significant reduction in the levels of Cyclin D1, PCNA and PARP; whereas Cyclin B1 was elevated in Taxol treated NLK knockdown cells (Figure 5C).

Discussion

NLK is a pro-mitogen-activated protein kinase and also a classic mediator of Wnt/beta-catenin signaling pathway (Brott et al., 1998; Harada et al., 2002). Recently, a growing number of studies have shown that NLK plays an important role in tumor occurrence and development process, becoming one of the hotspots of the current study.

Emami’s study shows that the expression of NLK is decreased in PCa metastases in comparison to normal prostate epithelium. Their results also show that overexpression of NLK resulted in induction of apoptosis, which was more pronounced in AR-expressing LNCaP versus AR-negative PC-3 cells (Emami et al., 2009). Overexpression of NLK can also be able to induce apoptosis in colon cancer cells. Yasuda’s research suggests that overexpression of NLK may have targets other than TCF for induction of apoptosis in human colon carcinoma cells (Yasuda et al., 2003).

Cui’s results suggest that NLK induces apoptosis in glioma cells via activation of caspases. In glioma, the expression of NLK in glioma is inversely related to the glioma grading. NLK may be a useful independent prognostic indicator for glioma (Cui et al., 2011).

In ovarian cancer, single nucleotide polymorphism analysis shows that the NLK gene rs2125846 single nucleotide polymorphism associated with ovarian cancer risk. Another study shows that NLK expression is higher than in the normal tissue of the ovary than ovarian cancer (Zhang et al., 2011; Stevens et al., 2012).

Jung found that NLK expression in cancer tissues is significantly higher than the corresponding normal liver tissue in hepatocellular carcinoma. Targeted deletion of NLK can suppress the growth of hepatocellular carcinoma through inhibition of CDK2 and Cyclin D1, suggesting that NLK may play a role in promoting the growth of hepatoma cells by promoting cell cycle progression (Jung et al., 2010).

Our study showed that Taxol could inhibit the expression of NLK and the growth of cells. Besides, knockdown of NLK makes Hep-2 cells more sensitive to Taxol treatment and led to significant reduction in the levels of Cyclin D1, PCNA and PARP, whereas Cyclin B1 was elevated in Taxol NLK knockdown cells.

Poly (ADP-ribose) polymerase (PARP) is a family of proteins involved in a number of DNA repair and programmed cell death cellular processes (Satoh and Lindahl, 1992). This protein can be cleaved by ICE-like caspasas in vitro (Lazebnik et al., 1994). The cleavage occurs between Asp214 and Gly215 in human, in which the PARP amino-terminal DNA binding domain (24 kDa) and the carboxy-terminal catalytic domain (89 kDa) were separated (Lazebnik, 1994; Nicholson, 1995). Cleavage of PARP causes cellular disassembly and is cells undergoing apoptosis marker (Tewari et al., 1995). Proliferating Cell Nuclear Antigen, commonly known as PCNA, is present in normal proliferating cells and tumor cells, which plays an important role as a good indicator of cell proliferation. Besides, PCNA is important for both DNA synthesis and DNA repair (Shivji, 1992; Essers, 2005). Cyclin B and Cyclin D are members of the cyclin family, which is involved in regulating cell cycle progression. The amount and the activity of cyclin B (which binds to Cdk1), a mitotic cyclin, rise through the cell cycle (Ito, 2000), until mitosis, where they fall suddenly due to degradation of cyclinB (Hershko, 1999). The synthesis of cyclin D is initiated during G1 and drives the G1/S phase transition, which is synthesized as long as the growth factor is present. Figure 5 showed that PARP, PCNA and Cyclin B were significantly reduced in the NLK knockdown group. Thus, DNA repair and cell cycle were affected as a result of the decreasing of PARP, PCNA and Cyclin. Therefore, the regulation of these molecules may be the mechanism underlying the enhanced anti-tumor effect of Taxol combined with NLK inhibition in laryngeal therapy.

To sum up, we demonstrate that lentivirus-mediated NLK knockdown enhances Taxol therapy sensitivity in human Hep-2 cells. As a result of the enhanced efficacy of NLK inhibition and Taxol therapy on cell apoptosis and cell proliferation in Hep-2 cells, the anticancer efficacy of Taxol is improved by this combined treatment. NLK is expected to become the target of new laryngeal cancer gene therapy. However, the mechanistic study of the relationship between Taxol resistance and NLK need further investigation.

Acknowledgements

The authors are thankful for the financial support from pharmacology laboratory of Beijing Institute of Technology. The authors also want to express thanks to the help of Shanghai Holly biological technology Co., Ltd.

References


