RESEARCH ARTICLE

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 cancerHep-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 hours). Cell numbers of combined Taxol-based chemotherapy with lentivirus mediated RNAi treatment group (Lv-shNLK+Taxol goup) were significantly different from NLK-specific siRNA lentivirus infected group (Lv-shNLK group) ($p<0.001$). Flow cytometry analysis revealed that Lv-shNLK+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 Lv-shNLK+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 transforming growth factor beta kinase (TAK1). In the Wnt signaling pathway of mammalian cells, NLK is considered as inhibitory factor of β-catenin/TCF/LEF, playing as negative regulator 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% CO_2.

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

*School of Life Science, Beijing Institute of Technology, Beijing, China *For correspondence: jr.dong.china@gmail.com*

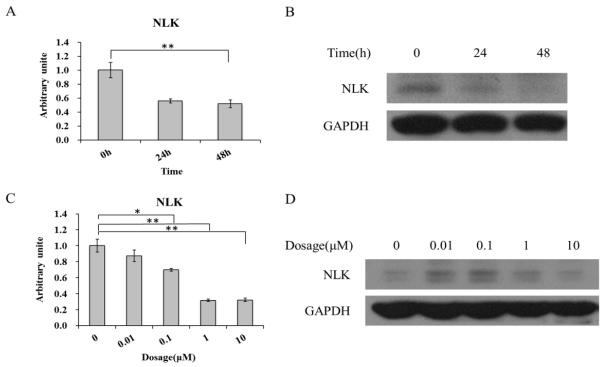

Figure 1. The Effects of Taxol on the Expression of NLK in Hep-2. A: The effects of Taxol on the expression of NLK in Hep-2 at different hours determined by q-PCR. **indicates $P<0.01$. B: The effects of Taxol on the expression of NLK in Hep-2 at different hours tested by immunoblotting. C: The effects of different dosage Taxol on the expression of NLK determined by q-PCR (0, 0.01, 0.1, 1, 10 μM respectively). *indicates $P<0.05$, **indicates $P<0.01$. D: The effects of different dosage Taxol on the expression of NLK tested by western-blotting

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).

Real-time RT-PCR

Total RNA was prepared using Trizol (Gibco RL, Grand, and Island, NY, USA) and reverse-transcribed according to the manufacturer's instructions. 5 μg of the cDNA was used to synthesize the first strand of cDNA using Super Script II RT 200 U/μl (Invitrogen, Carlsbad, CA, USA). The gene was amplified with SYBR Master Mixture (Takara, Japan) on IQ5 (BioRad). An initial denaturation was compromised at 95°C for 15seconds, then 45 cycles at 95°C for 5 seconds, and 60°C for 30seconds. The following primers were used: NLK -Foward: ATCATCAGCACTCGCATCATC; NLK -Reverse: GACCAGACAACACCAAAGGC. The expression of NLK mRNA was quantified and normalized to the level of the control reference, actin.

Western blot

After transfection for 8 days, cells were harvested by RIPA buffer supplemented with protease and phosphatase inhibitor cocktails. The protein content was assessed by BCA Protein Assay Kit (HyClone-Pierce, South Logan, UT, USA). Proteins were separated by SDS-PAGE, transferred to poly-binylidenedifluoride (PVDF) membranes (Millipore, Bedford, MA). And then the proteins were stained by anti-NLK antibody (Sigma, HPA018192). Besides, Cyclin B1 (Medical&biological laboratories Co., Ltd, MD-17-3), CyclinD1 (Medical&biological laboratories Co., Ltd, K0128-3), P21 (Cell signaling, #2947), P27 (Cell signaling, #3686), PCNA (Medical&biological laboratories Co., Ltd, MH-12-3S) and PARP (cell signaling, #9542) were tested. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies. Labeled proteins were detected by ECL Western blotting system. Anti-GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-32233) was used to demonstrate equal protein loading.

Methyl-thiazoltetrazolium (MTT) assay.

Exponentially growing cells were inoculated into 96-well plates with 2×10^3 cells per well. Following incubation for 0, 24, 48hours, respectively, 20 μL of sterile MTT (5mg/mL; Sigma-Aldrich Corp) was added to each well. Following a further incubation at 37°C for 4 hours, 100 μL of acidic isopropanol was added to stop the reaction. After being well mixed overnight, the formazan production was determined by measurement of the spectrometric absorbance at595nm.

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 $P<0.05$

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). 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-2as evidenced by western blot. Thus, Taxol could inhibit the expression of NLK (Figure 2).

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

Six groups were used including parent Hep-2 cells

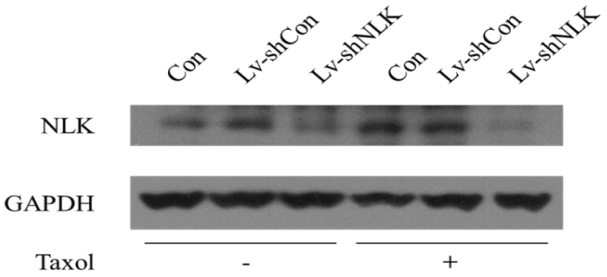

Figure 2. The Effect of Taxol on The Nlk Expression in Nlk Knockdown Hep-2 Cells Determined By Immunoblotting. "-" means lacking of Taxol. "+" indicates treating with Taxol

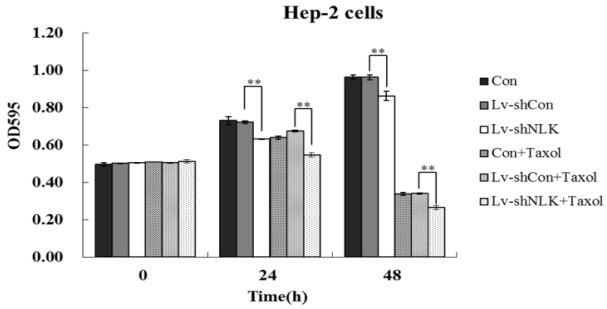

Figure 3. The Proliferation of Hep-2 Cells was Tested by MTT. Con: parent Hep-2 cells, Lv-shCon: control lentivirus-infected cells, Lv-shNLK: NLK-specific siRNA lentivirus infected cells, Con+Taxol: Hep-2 cells treated with Taxol, Lv-shCon+Taxol: control lentivirus-infected cells treated with Taxol,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

(Con), control lentivirus-infected cells (Lv-shCon), NLK-specific siRNA lentivirus infected cells (Lv-shNLK), Hep-2 cells treated with Taxol (Con+Taxol), control lentivirus-infected cells treated with Taxol (Lv-shCon+Taxol), and NLK-specific siRNA lentivirus infected cells treated with Taxol (Lv-shNLK+Taxol). In order to investigate the possible function of Taxol in the proliferation of Hep-2 cells, the dynamics of cell growth in six groups, all groups were determined by MTT assay (Figure 3). Following a 2 dayperiod, the growth of NC cells was indistinguishable from Con cells and showed strong proliferation. However, significantly lower number of cells (Con+Taxol) were observed on day 2 ($p<0.001$), and the viable cells were reduced about 65%compared to Con group on day 2. Cell numbers of Lv-shCon+Taxol group had a significantly difference from Lv-sh Con group ($p<0.001$), which was reduced about 65%compared toLv-shCongroup on day 2.Cell numbers of Lv-shNLK+Taxol group were significantly different from Lv-shNLK group ($p<0.001$, 70% reduced compared to Lv-shNLK group on day 2). Numbers of cells inLv-shNLK+Taxol group was less than Lv-shCon+Taxol group (23% less) on day 2, indicating that NLK play a role in the proliferation of Hep-2 cell. In sum, all these results indicated that the growth of cells was greatly inhibited by Taxol.

Effect of Taxol on the cell cycle traverse of NLK knockdownHep-2 cells

Cell cycle analysis by flow cytometric assay revealed that NLK-specific siRNA lentivirus infected group

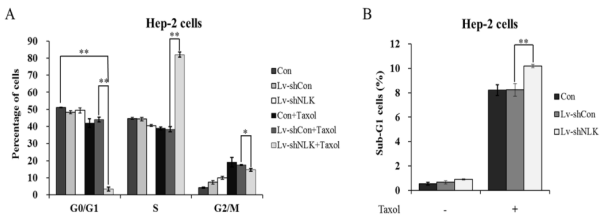

Figure 4. The Effect of Taxol on the Cell Cycle Traverse of Hep-2 Cells as Measured by Flow Cytometry. Con: parent Hep-2 cells, Lv-shCon: control lentivirus-infected cells, Lv-shNLK: NLK-specific siRNA lentivirus infected cells, Con+Taxol: Hep-2 cells treated with Taxol, Lv-shCon+Taxol: control lentivirus-infected cells treated with Taxol, and Lv-shNLK+Taxol: NLK-specific siRNA lentivirus infected cells treated with Taxol. *indicates $P<0.05$, **indicates $P<0.01$

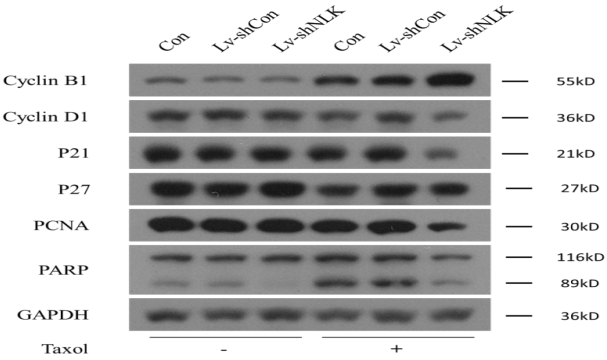

Figure 5. The Expression Levels of Cyclin B1, Cyclin D1, P21, P27, PCNA, PARP and GAPDH were Examined by Western-blotting. Con: parent Hep-2 cells, Lv-shCon: control lentivirus-infected cells, Lv-shNLK: NLK-specific siRNA lentivirus infected cells, Con+Taxol: Hep-2 cells treated with Taxol, Lv-shCon+Taxol: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

treated with Taxol caused theG0/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 combiningNLK knockdown inhibition with Taxol.

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, Hep-2 cells treated

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 shows that over-expression 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). Over-expression 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 caspases 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

Brott BK, Pinsky BA, Erikson RL (1998). Nlk is a murine protein kinase related to Erk/MAP kinases and localized in the nucleus. *Proc Natl Acad Sci USA*, **95**, 963-8.

Cui G, Li Z, Shao B, et al (2011). Clinical and biological significance of nemo-like kinase expression in glioma. *J Clin Neurosci*, **18**, 271-5.

Essers J, Theil AF, Baldeyron C, et al (2005). Nuclear dynamics of PCNA in DNA replication and repair. *Mol Cell Biol*, **25**, 9350-59.

Emami KH, Brown LG, Pitts TE, et al (2009). Nemo-like kinase induces apoptosis and inhibits androgen receptor signaling in prostate cancer cells. *Prostate*, **69**, 1481-92.

Hershko A (1999). Mechanisms and regulation of the degradation of cyclin B. *Philos Trans R Soc Lond B Biol Sci*, **354**, 1571-5.

Harada H, Yoshida S, Nobe Y, et al (2002). Genomic structure of the human NLK (nemo-like kinase) gene and analysis of its promoter region. *Gene*, **285**, 175-82.

Ishitani T, Ninomiya-Tsuji J, Nagai S, et al (1999). The TAK1-NLK-MAPK-related pathway antagonizes signalling between beta-catenin and transcription factor TCF. *Nature*, **399**, 798-802.

Ito M (2000). Factors controlling cyclin B expression. *Plant Mol Biol*, **43**, 677-90.

Jung KH, Kim JK, Noh JH, et al (2010). Targeted disruption of Nemo-like kinase inhibits tumor cell growth by simultaneous suppression of cyclin D1 and CDK2 in human hepatocellular carcinoma. *J Cell Biochem*, **110**, 687-96.

Kim M, Lee HC, Tsedensodnom O, et al (2008). Functional interaction between Wnt3 and Frizzled-7 leads to activation of the Wnt/beta-catenin signaling pathway in hepatocellular carcinoma cells. *J Hepatol*, **48**, 780-91.

Lazebnik YA, Kaufmann SH, Desnoyers S, et al (1994). Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. Nature, **371**, 346-7, 1994.

Lee JY, Marston DJ, Walston T (2006). Wnt/Frizzled signaling controls C. elegans gastrulation by activating actomyosin contractility. *Curr Biol*, **16**, 1986-97.

Nicholson DW, Ali A, Thornberry NA, et al (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature*, **376**, 37-43.

Nestle FO, Alijagic S, Gilliet M, et al (1998). Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med*, **4**, 328-32.

Satoh MS and Lindahl T (1992). Role of poly (ADP-ribose) formation in DNA repair. *Nature*, 356, 356-8.

Shivji KK, Kenny MK and Wood RD (1992). Proliferating cell nuclear antigen is required for DNA excision repair. *Cell*, **69**, 367-74.

Sanderson RJ and Ironside JA (2002). Squamous cell carcinomas of the head and neck. *BMJ*, **325**, 822-7.

Stevens KN, Kelemen LE, Wang X, et al (2012). Common variation in Nemo-like kinase is associated with risk of ovarian cancer. *Cancer Epidemiol Biomarkers Prev*, **21**, 523-8.

Tewari M, Quan LT, O'Rourke K, et al (1995). Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly (ADP-ribose) polymerase. *Cell*, **81**, 801-9.

Yasuda J, Tsuchiya A, Yamada T (2003). Nemo-like kinase induces apoptosis in DLD-1 human colon cancer cells. *Biochem Biophys Res Commun*, **308**, 227-33.

Yuzugullu H, Benhaj K, Ozturk N, et al (2009). Canonical Wnt signaling is antagonized by noncanonical Wnt5a in hepatocellular carcinoma cells. *Mol Cancer*, **8**, 90.

Zhang Y, Peng C, Wu G, et al (2011). Expression of NLK and its potential effect in ovarian cancer chemotherapy. *Int J Gynecol Cancer*, **21**, 1380-7.

Zhang X, Li W, Li H, et al (2012). Genomic methylation profiling combined with gene expression microarray reveals the aberrant methylation mechanism involved in nasopharyngeal carcinoma taxol resistance. *Anticancer Drugs*, **23**, 856-64.