· Original Article in English ·

Effects of RNA interference on NPM-ALK fusion gene expression in anaplastic large-cell lymphoma cells

Yan-Xia ZHAO, Long-Jun GU, Qi-Dong YE, Jin-Cai ZHAO

Department of Hematology/Oncology, Shanghai Children's Medical Center, Xinhua Hospital, Shanghai Second Medical University, Shanghai 200127, *China*

Abstract: Objective To evaluate two small interfering RNAs (siRNAs) on the NPM-ALK fusion gene expression in anaplastic large-cell lymphoma cell line Karpas299, and to study the effect of RNA interference on Karpas299 cells proliferation. **Methods** Two siRNAs sequences (siRNA- I and siRNA-II) were designed to target the NPM-ALK fusion site in anaplastic large-cell lymphoma cell line Karpas299. An siRNA U6 expression system including U6 RNA-based polymerase III promoter was set up. The two siRNAs designed for down-regulation of the NPM-ALK fusion mRNA were transfected into Karpas299 cells by liposomal transfection reagents. The effect of RNAi on NPM-ALK mRNA expression was detected by real-time RT-PCR and Western blot. The anti-proliferative effects of the siRNA U6 system were assessed using MTT. Apoptosis was observed by fluorescence microscopy. **Results** The mRNA level of NPM-ALK in Karpas299 cells transfected with siRNA-I and siRNA-II decreased by approximately 75% and 35% respectively. The NPM-ALK protein expression was inhibited in Karpas299 cells at 72 hrs of siRNA-I transfection. The siRNA-II treatment had no effect on NPM-ALK protein expression. siRNA-I had inhibitory effects on Karpas299 cells proliferation and induced the cells apoptosis, while siRNA-II did not. **Conclusions** Sequence specific siRNAs targeting NPM-ALK was capable of suppressing NPM-ALK expression and inhibiting cellular proliferation. RNA interference may be a suitable technique for studying the function of NPM-ALK gene and may be used to develop siRNA-based targeted gene therapeutic approaches against NPM-ALK-positive lymphomas. [**Chin J Contemp Pediatr**, **2005**, **7**(**3**):**202 - 206**]

Key words: RNA interference; RNA, small interfering; Gene fusion; NPM-ALK; Anaplastic large-cell lymphoma

RNAi 阻断 NPM-ALK 基因表达及对大细胞间变性淋巴瘤细胞的影响

赵艳霞，顾龙君，叶启东，赵金彩 上海第二医科大学附属新华医院上海儿童医学中心血液/肿瘤科，上海 200127

[**摘 要**] **目的** 应用 RNA 干扰技术抑制大细胞间变性淋巴瘤细胞系(Karpas299)中 NPM-ALK 融合基因表达，观察其对肿瘤细胞生长的影响。**方法** 针对 NPM-ALK 融合位点设计两个 siRNA 序列 siRNA-I 与 siRNA-II，经 PCR 反应构建含 U6 启动子 siRNA 正义和反义线性表达载体，通过脂质体转染 Karpas299 细胞，应用实时荧光定量 RT-PCR、Western blot 检测 siRNA 片段对 NPM-ALK mRNA 和蛋白表达的抑制作用，MTT、Hoechst 荧光染色检测 siRNA对肿瘤细胞生长的影响。**结果** siRNA-I 可导致 NPM-ALK mRNA 下降约 75% ($P < 0.05$)，转染 72 h 后可导致蛋白表达下降；转染siRNA-II细胞 NPM-ALK mRNA 下降为 35% ($P < 0.05$)，但蛋白水平无明显改变。转染 siRNA-I的细胞可抑制 Karpas299 细胞的增殖和诱导凋亡发生，siRNA-II 则无明显的抑制增殖和诱导凋亡作用。**结论** 含有针对 NPM-ALK 融合位点特异 siRNA 序列 的 U6 表达载体，可特异地抑制 NPM-ALK 基因 mRNA 和蛋白的表达，并能抑制大细胞间变性淋巴瘤肿瘤细胞株 Karpas299 细胞的增殖，导致肿瘤细胞凋亡增加，提示 NPM-ALK 融合基因的异常表达与大细胞间变性淋巴瘤形成密切相关，为研究 NPM-ALK 基因功能和大细胞间变性淋巴瘤基因靶向治疗提供了新策略。 [**中国当代儿科杂志，2005，7(2):202-206**]

[**关 键 词**] RNA 干扰；siRNA；融合基因；NPM-ALK；大细胞间变性淋巴瘤

[**中图分类号**] R73 [**文献标识码**] A [**文章编号**] 1008 - 8830(2005)03 - 0202 - 05

[Received] December 18, 2004; [Revised] February 28, 2005

[Foundation Item] Supported by the Technology Commission of Shanghai Municipal Government of China under Grant No. 02DJ14011.

[Biography] Yan-Xia ZHAO (1976-), Female, MD, Specializing in pediatric hematology/oncology.

[Correspondence Author] Long-Jun GU, Department of Hematology/Oncology, Shanghai Children's Medical Center, Xinhua Hospital, Shanghai 200127, China (Email: liguscmo@hotmail.com).

Human anaplastic large-cell lymphoma (ALCL) represents a distinct sub-group of non-Hodgkin's lymphomas (NHL). The majority of ALCL cases arising in childhood have a characteristic chromosomal translocation t (2;5) (p23;q35)[1], associating the anaplastic lymphoma kinase (ALK) gene at 2p23 and the nucleophosmin gene at 5q35. This translocation leads to the expression of a chimeric NPM-ALK protein, linking the N-terminal region of NPM, an ubiquitous nucleolar phosphoprotein, to the C-terminal region of ALK containing its kinase domain. The NPM-ALK fusion protein codes for a constitutively active tyrosine kinase. The fusion gene and its products serve as useful kinds of molecular targets for ALCL therapy.

The discovery of post-transcriptional genetic interference phenomena—RNA interference (RNAi)—is one of the most exciting advances in molecular genetics in recent years. Small interfering RNAs (siRNAs) can affect the expression of homologous genes in a sequence specific manner in plants and Caenorhabditis elegans. It was also shown to exist in mammalian cells, including human cells[2], and paves the way for the development of novel RNA therapeutics. In this study, two siRNAs sequences (siRNA- I and siRNA-II) were designed targeting to the NPM-ALK fusion site in anaplastic large-cell lymphoma cell line karpas299. Anti-NPM-ALK siRNAs specifically inhibit NPM-ALK mRNA expression in Karpas299 cells. They reduce NPM-ALK protein expression and inhibit NPM-ALK-dependent, but not cytokine-dependent, cell proliferation.

Materials and methods

Cell cultures

Karpas299(NPM-ALK positives) was kindly provided by Dr. S. Morris, St. Jude's Children's Research Hospital, Memphis, TN. The K562 cells were obtained from the Shanghai Institute of Cell Biology. RPMI 1640 medium and fetal bovine serum were purchased from Gibco-BRL. Cells were cultured in RPMI 1640 containing 10% fetal calf serum without antibiotics in a humidified atmosphere of 5% CO_2 at 37℃.

siRNA design

Two siRNA sequences that directed against the fusion sequence of NPM-ALK were chosen according to the guideline described in the protocol of LineSilenceTM RNAi Transcription Kit (Allele Biotechnology & Pharmaceuticals, San Diego, USA). The sense and antisense sequences were: siRNA-I: 5′-ACAGCACTTAGTAGTGTAC-3′ 5′-GTACACTACTAAGTGCTGT-3′; siRNA-II: 5′-AGTGTACCGCCGGAAGCAC-3′ 5′-GTGCTTCCGGCGGTACACT-3′. The oligoribonucleotide with two point mutations were designed and the sequences were: siRmut: 5′-ACAGCACggAGTAGTGTAC-3′ 5′-GTACACTACTccGTGCTGT-3′. The U6 RNAi cassettes for NPM-ALK were generated through PCR according to the protocol of the LineSilenceTM RNAi Transcription Kit. PCR products were column-purified from the primers using QIAGEN PCR purification kit (Qiagen GmbH, Hilden, Germany) and their concentrations were estimated by ultraviolet spectroscopy. 1 μg each sense or antisense LineSilenceTM cassettes for NPM-ALK was used for per well of a 24-well plate (Costar, Corning Inc., Acton, NY).

Transfection

Cells were transfected in 24-well plates using DMRIE-C (Invitrogen AB, Lidingo, Sweden) according to the manufacturer's instructions. Except the U6 RNAi cassettes for NPM-ALK, siRNA-I, siRNA-II and siRNA mutation cells were transfected with single sense or antisense RNAi transcription template and treated with DMRIE-C only. Untreated cells were used as controls.

RNA isolation, cDNA synthesis and real-time PCR

Total RNA was extracted by Trizol reagent (Gibco BRL, MD, USA) according to the manufacturer's instructions. RNA was reversely transcribed to cDNA by the kit (Promega, Madison, WI, USA). Real-time PCR reactions were carried out using real time PCR kit (Tarkara, Japan). Levels of GAPDH mRNA were quantified as an internal standard. The RT mixture was diluted to 100 μL with glass distilled water. The sequence of primer and Taqman probe of NPM-ALK and GAPDH are shown in Table 1. The PCR products were detected using a real-time system ABI7 000 (Applied Biosystems, Foster City, CA, USA). Possible contamination with genomic DNA was eliminated by performing real-time PCR on mock reverse transcription reactions in which the reverse transcriptase was omitted. PCR reactions were performed in triplicate for each sample and the mean value at which the PCR product crossed the threshold (Ct) was calculated. The ratio of NPM-ALK mRNA expression to that of the internal standard mRNA (GAPDH) was calculated using the method of REST© software[3] according to Equation 1.

$$\text{Ratio} = \frac{E_{\text{NPM-ALK}}^{\triangle \text{CtNPM-ALK(control-treated)}}}{E_{\text{GAPDH}}^{\triangle \text{CtGAPDH(control-treated)}}}$$

Table 1 Sequences of GAPDH and NPM-ALK primers and probes

Sequence	5′primer	3′primer	probe(5′FAM-3′Tamra)
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	CAAGCTTCCCGTTCTCAGCC
NPM-ALK	GGGCCAGTGCATATTAGTGGA	TGTACTCAGGGCTCTGCAGCT	AGCACTTAGT AGTGTACCGCCGGAAGCACC

Cell extraction and Western blot

Samples were centrifuged at 4℃ in a microfuge to precipitate the cells. For protein extraction, the cells were re-suspended in hypotonic cell lysis buffer lysed by pipetting and incubated for 30 minutes on ice. The samples were diluted into 1∶4 with non-reducing sample buffer (1 mL: 60 μL 1 M Tris-HCl pH 6.8; 312 μL 80% Glycerol; 200 μL 10% SDS; 428 μL H_2O; grains of Bromphenol blue) and electrophoresed on an 8% SDS-polyacrylamide gel. In a standard Western blot protocol, for detection of the fusion proteins, the rabbit polyclonal antibody ALK11 against NPM-ALK (1∶4 000), the rabbit antibody against human β-actin (1∶5 000, BD), and goat antibody against rabbit IgG (1∶2 000) were used. The protein was detected with ECL system (Amer-sham, Uppsala, Sweden) by chemoluminescence.

Detection of cell proliferation and apoptosis

Cell viability was determined by the MTT assay (Sigma, MO, USA). Cells were replated onto 96 well plates (per well) at a density of 0.1×10^5 cells/well after transfection, and the same number cells cultured without drugs were taken as the controls. Cell viability was determined by an absorbance spectrophotometer (at 492 nm). Each experiment was performed at least three times. The percentage of cell growth inhibition was calculated as follows: Inhibition (%) = [A492 (control) - A492 (drug)] / A492 (control) ×100.

The cells were collected and stained with Hoechst 33258 (YT, China). At the end of incubation, the cells were washed and resuspended in PBS for observation of nuclear morphology using fluorescence microscope (Olibath, German).

Statistical analysis

Statistical analysis was performed using statistical package SAS 6.12. One-way ANOVA and q-test were used to analyze differences between groups. Differences were considered significant at $P<0.05$.

Results

Reduction of NPM-ALK mRNA expression by U6 RNAi cassettes

Real-time RT-PCR analysis showed an approximately 75% ($P = 0.001$) and 35% ($P = 0.023$) decrease in the mRNA level of NPM-ALK, respectively, in Karpas299 cells transfected with siRNA-I or siRNA-II, but no decrease was detected in the cells following transfection of control siRNA or only sense or antisense sequences (Figure 1). These results demonstrated that siRNA-I and siRNA-II were highly specific for suppression of the fusion NPM-ALK protein.

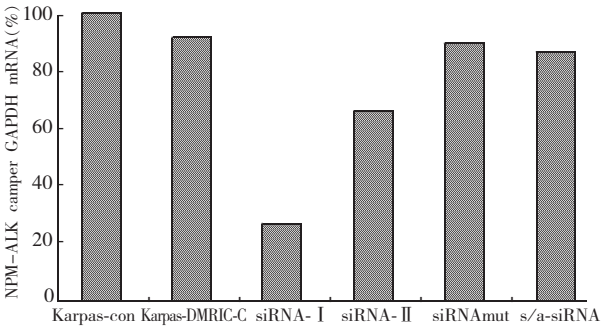

Figure 1 Effects of siRNA vector treatment on NPM-ALK mRNA expression

Knockdown of NPM-ALK protein by U6 RNAi cassettes

Western blot analysis demonstrated a suppression of NPM-ALK protein expression in Karpas299 cells at 72 hours of siR-I transfection. Neither siR-II nor the sense or antisense siRNA treatment had effects on the fusion protein expression (Figure 2). To ensure the sequence specificity of the siRNA, the mutated siRNA (the siRmut) was used and was unable to reduce the NPM-ALK protein level, indicating the extraordinary sequence of RNAi.

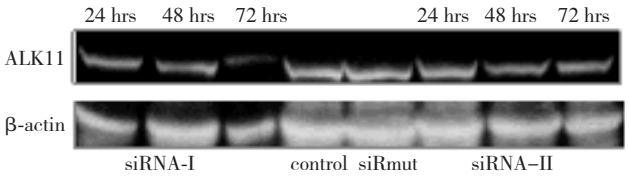

Figure 2 Western blot analysis of NPM-ALK protein expression after siRNA vector transfection

Cell growth inhibition on Karpas299 cells

siRNA-I had inhibitory effects on Karpas299 cells growth compared with the controls ($F = 98.55$, $P = 0.0001$). The cell growth inhibition rate reached as high as (45.7 ±4.4)% after siRNA-I treatment for

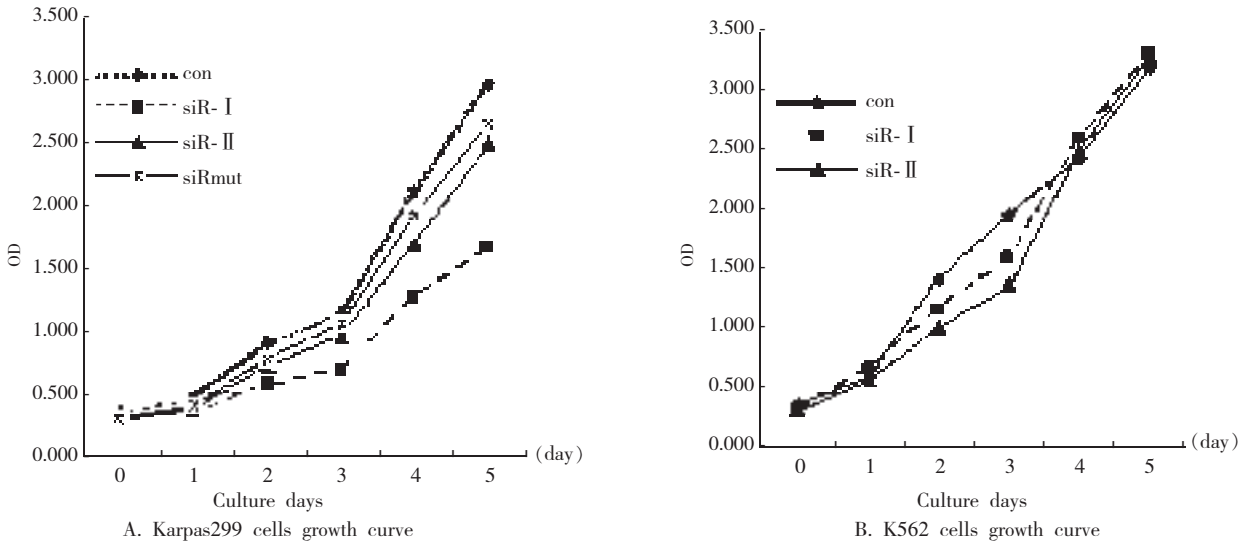

Figure 3 The growth of the tumor cells after siRNA transfection

72 hours. siRNA-II treatment did not affect the growth of the cells ($F = 1.15, P = 0.3245$). The growth of K562 cells which lacked the expression of NPM-ALK was not inhibited after siR-I transfection.

RNAi-induced morphological changes of Karpas299 cells

The nuclear morphological changes were observed by Hoechst 33258 staining. In untreated controls, Karpas299 cells were round in shape and stained homogeneously. After 72 hours of siRNA-I treatment, blebbing nuclei and granular apoptotic bodies appeared. However, the cells treated with siRNA-II, siRNA mutation, sense or antisense siRNA and DMRIE-C had not been obviously changed.

Discussion

In ALCL, the chromosomal translocation t (2;5) (p23;q35) resulted in the NPM/ALK fusion gene that encodes a constitutively activated protein-tyrosine kinase (PTK). Evidence has demonstrated that NPM-ALK is a causative agent of ALCL and its transforming potential leads to oncogenicity as now well established in vitro and in vivo. NPM-ALK can cause many cell lines transformation (including NIH3T3, Rat-1, Ba/F3)[4]. Transfer of NPM-ALK-transduced bone marrow cells into irradiated recipient mice led to the formation of large B cell lymphomas, and transgenic mice with NPM-ALK transcription targeted to T cells developed malignant T cell lymphomas[4,5]. NPM-ALK enhances factor-independent proliferation and inhibits apoptosis predominantly via activation of the phospholipase C-γ (PLC-γ), Jak/Stat and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways[6,7]. But the complete mechanism of transduction and its precise signaling pathways remain unknown. The specific inhibitor of NPM-ALK just like STI571 for BCR-ABL has also not been found. So the molecule targeting NPM-ALK by gene-silencing mechanisms can be used to analyze its oncogenic potential and may form a basis for the development of a specific gene therapeutic approach toward the treatment of ALCL.

Gene silencing by small interfering RNA (siRNA) has become a powerful and rapidly evolving experimental method for studying gene function in mammalian cells. RNAi is thought to be an evolutionarily conserved surveillance mechanism that responds to double-stranded RNA by sequence specific silencing of homologous genes. Because of the high stability of siRNAs and the effectiveness of the silencing process induced, RNA interference has been shown to be superior to conventional antisense strategies. Recently, many publications described the effective inhibition of fusion genes generated by chromosomal translocation such as BCR-ABL involving siRNAs against the fusion site[8]. The siRNA of pp60c-src and Bcl-XL gene successfully inhibited ALK^+ ALCL cells growth and caused cell apoptosis[9,10]. Therefore, LineSilenceTM RNAi cassettes (Allele) which use U6 RNA-based polymerase III promoter and modified terminator for high level, precise siRNA expression inside target cells were selected. It is equivalent or better than the multiple transfection of siRNA. This study showed that NPM-ALK mRNA and protein levels were significantly decreased 72 hours after siRNA-I transfection and that

the Karpas299 cell growth was inhibited and the amount of apoptotic cells significantly increased after transfection. The siRNA mutation and sense/antisense cassettes were used and unable to reduce the NPM-ALK protein level, indicating the sequence specificity of the siRNA. They also had no effect on the cell growth or apoptosis. The siRNA-I was also transfected into K562 cells which has no expression of NPM-ALK protein and had no effect on cell growth. All of these data showed that siRNA-I had specifically targeted the NPM-ALK gene and had biological effects on Karpas299 cells. The results also indicated that RNAi may be a suitable technique for studying the function of NPM-ALK gene and may be used to develop RNAi-based targeted gene therapeutic approaches against NPM-ALK-positive lymphomas. However, the mechanism of RNAi is still unclear and further study is needed.

[**References**]

[1] Morris SW, Xue L, Ma Z, Kinney MC. Alk$^+$ CD30$^+$ lymphomas: a distinct molecular genetic subtype of non-Hodgkin's lymphoma [J]. Br J Haematol, 2001, 113(2): 275-295.

[2] Rye PD, Stigbrand T. Interfering with cancer: a brief outline of advances in RNA interference in oncology [J]. Tumour Biol, 2004, 25(5-6): 329-336.

[3] Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR [J]. Nucleic Acids Res, 2002, 30(9): e36.

[4] Chiarle R, Gong JZ, Guasparri I, Pesci A, Cai J, Liu J, Simmons WJ, et al. NPM-ALK transgenic mice spontaneously develop T-cell lymphomas and plasma cell tumors [J]. Blood, 2003, 101(5): 1919-1927.

[5] Miething C, Grundler R, Fend F, Hoepfl J, Mugler C, von Schilling C, et al. The oncogenic fusion protein nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) induces two distinct malignant phenotypes in a murine retroviral transplantation model [J]. Oncogene, 2003, 22(30): 4642-4647.

[6] Lai R, Rassidakis GZ, Medeiros LJ, Ramdas L, Goy AH, Cutler C, et al. Signal transducer and activator of transcription-3 activation contributes to high tissue inhibitor of metalloproteinase-1 expression in anaplastic lymphoma kinase-positive anaplastic large cell lymphoma [J]. Am J Pathol, 2004, 164(6): 2251-2258.

[7] Gu TL, Tothova Z, Scheijen B, Griffin JD, Gilliland DG, Sternberg DW. NPM-ALK fusion kinase of anaplastic large-cell lymphoma regulates survival and proliferative signaling through modulation of FOXO3a [J]. Blood, 2004, 103(12): 4622-4629.

[8] Wohlbold L, van der Kuip H, Miething C, Vornlocher HP, Knabbe C, Duyster J, et al. Inhibition of bcr-abl gene expression by small interfering RNA sensitizes for imatinib mesylate (STI571) [J]. Blood. 2003,102(6):2236-2239.

[9] Cussac D, Greenland C, Roche S, Bai RY, Duyster J, Morris SW, et al. Nucleophosmin-anaplastic lymphoma kinase of anaplastic large-cell lymphoma recruits, activates, and uses pp60c-src to mediate its mitogenicity [J]. Blood, 2004, 103(4): 1464-1471.

[10] Coluccia AM, Perego S, Cleris L, Gunby RH, Passoni L, Marchesi E, et al. Bcl-XL down-regulation suppresses the tumorigenic potential of NPM/ALK in vitro and in vivo [J]. Blood, 2004, 103(7): 2787-2794.

(Edited by Xia WANG)