Knockdown of survivin gene by vector-based short hairpin RNA technique induces apoptosis and growth inhibition in Burkitt's lymphoma Raji cell line

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Survivin is the smallest member of mammalian IAP (inhibitor of apoptosis) family. It is ubiquitous during embryonic development but is not expressed in normal post-natal tissues, except the thymus, colonic epithelial cells and CD34+ hematopoietic stem cells. However, its expression is upregulated during neoplastic transformation in both solid organ and hematological malignancies, including leukemia and lymphoma. In this study, we used RNA interference with short hairpin RNA (shRNA) technique to inhibit survivin expression in a Burkitt's lymphoma cell line Raji and validated its effects on apoptosis and cell proliferation.

A survivin-shRNA expression vector were constructed and introduced into Raji cells. Expression of survivin mRNA and protein was assessed by RT-PCR and western blot analysis. Apoptosis index of transfected cells was quantified by flow cytometry and cell proliferation was enumerated by trypan blue exclusion.

In Raji cells treated with survivin-shRNA expression vector, survivin mRNA levels were significantly reduced by 67.14% (transient transfection) and 64.28% (stable transfection) respectively, compared with control-shRNA treated group and PBS treated group ($p<0.05$). The levels of survivin protein were significantly reduced by 62.50% (transient transfection) and 60.93% (stable transfection), compared with the two control groups ($p<0.05$). Apoptosis index was significantly increased during transient transfection and stable transfection, respectively 31.20±2.45% and 29.40±1.72% ($p<0.05$). Survivin-shRNA inhibited the proliferation of Raji cells of stable transfection.

In conclusion, the vector-based survivin-shRNA can effectively reduce the expression of survivin gene and induce apoptosis and growth inhibition of transfected Raji cells. We suggest that survivin can be regarded as an ideal target for new anticancer intervention of NHL.

Key words: survivin, RNAi, shRNA, apoptosis, non-Hodgkin's lymphoma, Raji

Survivin is the smallest member of the mammalian inhibitors of the apoptosis protein (IAP) family. It is undetectable in most normal adult tissues, but abundantly expressed in most solid tumors and hematological malignancies, including leukemia and lymphoma [1]. Furthermore, expression of survivin has been detected in a number of preneoplastic lesions including breast adenoma, polyps of colon, hypertrophic actinic keratosis and Bowen's disease, and its expression increases from pre-cancerous to cancerous lesion [2, 3] suggesting that it may be involved during early malignant transformation. Survivin suppresses apoptosis induced by Fax, Bax, caspases and anticancer drugs [4] and it blocks the common downstream component of two major apoptosis pathways: the mitochondrial and the death receptor pathways, through inhibition of terminal effecter caspase-3 and caspase-7 [5]. Its expression is directly related to the grow index and inversely related to apoptotic index of tumor. Survivin regulates the G(2)/M phase of the cell cycle by associating with mitotic spindle microtubules. Over-expression of survivin is associated with carcinogenesis, histological classification of carcinoma, metastasis, poor prognosis, resistance to x-irradiation or chemotherapeutic drugs, as well as short patient survival [6, 7]. As a result of its selective expression in malignant but not normal tissues, survivin has been regarded as "an ideal target gene" for anticancer therapy.

Previous studies showed that de-regulation of apoptosis

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plays an important role in malignant lymphopoiesis. Defects in pathways that regulate apoptosis can promote development of lymphomas [8, 9]. Survivin was upregulated in non-Hodgkin's lymphoma (NHL) and its over-expression was correlated with the aggressiveness of B-NHL [10, 11]. Over-expression of survivin has also been determined in most human leukemia/lymphoma cell lines. Therefore, we regarded survivin as an ideal target gene for anticancer therapy of NHL.

RNA interference (RNAi) is a genetic interference phenomenon directed by the double-stranded RNA (dsRNA). It could specifically and efficiently degrade mRNA, resulting in post-transcriptional gene silencing (PTGS). It is a conserved mechanism that mediates resistance to endogenous parasitic and exogenous pathogenic nucleic acids, and it regulates the expression of protein-coding genes. Inhibition of gene expression by RNAi has been successfully observed in both rat and human cells *in vitro* [12]. Currently, two kinds of RNAi technology – chemically synthesized small interfering RNA (siRNA) and vector-mediated expression of short hairpin RNA(shRNA) are mainly used to block gene expression in mammalian cells [13, 14]**.** Both of them have their respective particularities. According to previous study [15], chemically synthesized siRNA is more easily transfected into cancer cell lines and is apparently more effective in silencing targeted genes, compared with shRNA vectors. But siRNA synthesized *in vitro* suppresses gene expression for only a short period (less than a week) and is often limited to cells that are easily transfected. However, shRNA expression vectors can be used to transcribe and generate shRNA *in vivo* and its advantage is that the expression of target genes can be reduced for weeks or even months, which allows analysis of the consequences of stably silencing gene [16]. Therefore, the approach of shRNA expression vectors would bring a longer period for further investigations and provide extensive uses for functional analysis of genes and anticancer therapy.

In this study, we utilized vector-based shRNA technique and constructed a recombinant plasmid p*Silencer*^{TM} 4.1neo-CMV-survivin-shRNA with a RNA Pol II-mediated promoter, then transfected the vector-based survivin-shRNA to Burkitt's lymphoma Raji cells in which survivin is over-expressed and explored the following effects on cell apoptosis and proliferation.

Material and methods

Cell culture. The following human cell line was used: Raji, derived from a Burkitt lymphoma. Cell line was purchased from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium (GibcoBRL, USA) containing 10% heat-inactivated fetal calf serum (FBS; HyClone, USA), 2 mmol/l glutamine and 50 µg/ml penicillin-streptomycin, at 37 °C in a humidified atmosphere containing 5% carbon dioxide.

ShRNA design and construction of recombinant plasmid expressing survivin-shRNA. A shRNA target sequence was designed to be homologous to wild-type survivin cDNA sequence (GenBank accession NM_001168) by the shRNA Target Finder and Design Tool system at http://www.ambion.com. The selected sequence was submitted to BLAST search to assure the only selected gene was targeted. The forward and reverse chains of survivin-shRNA gene were chemically synthesized (Shanghai Sangon, China). Each chain was respectively flanked with *BamHI* at 5' and *HindIII* (Takara, Biochem. Japan) at 3' end for a cloning purpose.

Forward chain sequence of survivin-shRNA:
5'-GATCCGGCTGGCTTCATCCACTGCTTCAAGAGAG CAGTGGATGAAGCCAGCCTCA-3'.

Reverse chain sequence of a survivin-shRNA:
5'-AGCTTGAGGCTGGCTTCATCCACTGCTCCTTGAA GCAGTGGATGAAGCCAGCCG-3.

A pair of complementary oligos were synthesized and annealed to form a double-stranded fragment. The complementary oligos encoded a hairpin structure with a 19-mer stem derived from the mRNA target site. A 9-bp loop sequence "TTCAAGAGA" separated the two complementary domains. The 5' end of the two oligonucleotides was *BamHI* and *HindIII* restriction site overhangs (Fig. 1)**.** This complementary oligos was then cloned into vector p*Silencer*^{TM} 4.1-CMVneo (Ambion, Inc. USA) completely digested with *BamHI* and *HindIII* (50 ng linearized vector, 1 µl shRNA, 1 µl T4 DNA ligase and 2.5 µl 10x ligase buffer were incubated at 16 °C overnight) to obtain the final construct survivin-shRNA, which could produce the designed shRNA in mammalian cells. The clone of survivin-shRNA was confirmed by DNA sequence. An insignificant control-shRNA was purchased from Ambion and was used as a control as well as PBS reagent.

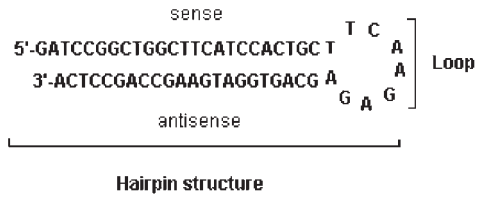

Figure 1. The hairpin structure of survivin-shRNA of the forward strand.

Transfection with the shRNA expressing vector. 8 µl OPTI-MEM (Invitrogen Life Tech. USA) containing 1.0 µl lipofectamine 2000 reagent (Invitrogen) was mixed with 42 µl OPTI-MEM containing 5 µl survivin-shRNA expressing plasmid DNA in 6 of a 24 wells plate at room temperature for 30 minutes, with another 4 wells respectively containing Control-shRNA and PBS reagent as negative controls. Raji cells were maintained in RPMI 1640 supplemented with 10% FBS, and incubated in a humidified incubator containing 5%

CO_2 at 37 °C. Cells were washed by OPTI-MEM for 3 times to replace the culture medium and $1x10^5$ cells/200 µl were seeded respectively in each well. The plate was incubated at 37 °C in a moist atmosphere containing 5% CO_2 for 4 hours, then 750 µl RPMI containing with 20% FBS, 1 µl PHA and PMA were added into each well. Transiently transfected cells were harvested at 48 h after transfection and analyzed by RT-PCR, western blot analysis and flow cytometric assays. Cells stably expressing shRNA were established by selection with medium first containing 500 µg/ml geneticin (G418; Invitrogen). The medium was renewed every 3 days. After 15–20 days selection, the resistant colonies were combined in pools in selective medium. Then, the resistant colonies were further selected by huge dose G418 (2000 µg/ml) for one week in order to exclude the possibility of non-transfected but G418-resistant colonies. After huge dose selection of G418 and 10–20 days amplifying cultured, the colonies of stably transfected with G418-resistantance were obtained and also analyzed by RT-PCR, western blot and flow cytometric assays.

Flow cytometric analysis. Cells of Control-shRNA treated group, PBS-treated group, survivin-shRNA treated (48 h) and survivin-shRNA treated (stable-over 30 days) were harvested by centrifugating at 1,000xg for 5 min and washed with PBS 3 times to remove the culture medium. Cell suspension was fixed in ice-cold 70% ethanol in PBS, and stored at 4 °C. Prior to analysis, the cells were washed and resuspended in PBS, and incubated with 1 g/l of RnaseI and 20 g/l of propidium iodide (PI) at 37 °C for 30 min. Apoptosis was analyzed with flow cytometer (Coulter. Co, USA). For each sample, at least $1x10^4$ cells were analyzed by flow cytometry. Cell cycle distributions were determined by measuring the cellular DNA content using flow cytometry. The percentage of apoptotic cells in sub-G1 phase was calculated using Multicycle Software.

Assay of cell proliferation. For evaluation of cell proliferation, cells were stained with trypan blue and counted using a hemocytometer. Cell numbers of survivin-shRNA stably treated group, control-shRNA treated group and PBS-treated group were detected on day 1 to 7. Each experimental condition was performed six times and all data presented as the mean ±S.E. for each group was determined to compose the growth curve.

RT-PCR analysis. Total RNA was extracted from Raji cells using the Trizol reagent (GibcoBRL, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was generated from total RNA using Reverse Transcription kit (Ferment AG, Switzerland). Taq DNA polymerase, dNTPs, DNA Marker DL-2000, λDNA/Ecol301, T4 DNA ligase were bought from Takara. PCR of the cDNA was performed in a final volume of 50 µl containing 25 µl Premix Taq (Taq DNA polymerase 1.25u, dNTP Mixture 0.04 mM, Ex Taq Buffer 4 mM), 2 µl cDNA, 1 µl of each primer, and 21 µl H_2O. The samples were amplified 35 cycles at 94 °C for 1 min, at 58 °C for 30 s, at 72 °C for 45 s, and finally at 72 °C for 10 min. The PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. Sense primer of wild type survivin gene was 5'-CCA GAT GAC GAC CCC ATA GA-3', and its antisense primer was 5'-CCT CTG GTG CCA CTT TCA AG-3'. The length of amplified fragments was 401 bps. Amplification of human GAPDH gene served as a control to assure the accuracy for a sample loading and integrity. The upstream and downstream primers were 5'-TCC ATG ACA ACT TTG GTA TCG TG-3' and 5'-TGC AGC GTA CTC CCC ACA T-3', respectively. The length of amplified fragments was 208 bps. Normal Human Peripheral blood lymphocytes were used as negative control for survivin mRNA expression. Signal intensities were quantified in a densitometer system by Gene tools from Syngene.

Western blotting analysis. $2x10^6$ cells were washed 3 times with phosphate-buffered saline (PBS) and lysed at 150 µl cold buffer (50 mmol/l Tris-HCL PH 7.4, 150 mmol/l NaCl, 1% Triton X-100, 1% deoxycholie acid, sodium salt, 0.1% SDS, 100 µg/ml phenylmethylsulfonyl fluride, 1 µg/ml aprotinin and 1 mmol/l DTT) for 30 min, and scraped. Cells extract were centrifuged at 20,000 x g at 4 °C for 30 min and supernatant was used for western blot analyses. Up to 50 µg of total protein from each sample was heated at 95 °C for 5 min after mixing with an equal volume of 2x SDS loading buffer. Samples were separated on 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to PVDF membranes. The membrane was blocked in 5% Skimmed milk in TBST buffer (20mM Tris-HCL, PH 7.5, 0.05% TWEEN 20) at room temperature for 1 to 2 h with a gentle shaking, and then incubated overnight at 4 °C in TBST buffer containing 5% BSA and survivin polyclonal antibody (1:500, Santa Cruz, Biotech., USA). After washing with TBST, the membrane was incubated in 5% skimmed milk in TBST buffer containing goat anti-rabbit IgG (1:5000, Santa Cruz) for 60 min at room temperature. Protein level was detected by an ECL Test Kit and enhanced chemiluminescent agent (Sigma, Co. USA) and visualized by autoradiography. For normalization of protein loading, the membranes were stripped by stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, PH 6.7) and used for western blot analysis with a monoclonal antibody against β-actin (1:2000, Santa Cruz) as reference. The density of the bands on the membrane was scanned and analyzed with an image analyzer system of Gene tools from Syngene.

Statistical analysis. The software package SPSS 11.0 was used for statistical analysis. Values were expressed as mean ±S.E. and statistical analysis of the data was carried out by Student's t-test. $p<0.05$ were considered significant.

Results

Effect of survivin-shRNA treatment on survivin mRNA expression. Survivin mRNA expression was examined by the methods of RT-PCR. As shown in Figure 2A, Raji cells of

both transient (48 h) and stable transfection with survivin-shRNA exhibited significantly decreased survivin mRNA content, compared with Control-shRNA and PBS treated control groups which presented almost the same brightness strip at 408 bps. Analyzed by Gene tools from Syngene (Fig. 2B), mRNA expression was significantly reduced by 67.14% (transient transfection) and 64.28% (stable transfection) respectively, compared with two control groups ($p<0.05$). There was no significant difference in reduction of survivin mRNA expression between transient transfection and stable transfection.

Effect of survivin-shRNA treatment on survivin protein expression. The effect of survivin-shRNA on survivin protein expression was evaluated by Western blot analysis. As shown in Figure 3A, protein levels of survivin in Control-shRNA treated group and PBS-treated group were almost similar, but significant reductions in survivin protein were observed in cells both transiently and stably transfected with survivin-shRNA. Analyzed by Gene tools from Syngene (Fig. 3B), survivin protein expression was significantly reduced by 62.50% (transient transfection) and 60.93% (stable transfection), compared with two control groups ($p<0.05$). There was no significant difference in reduction of survivin protein expression between transient transfection and stable transfection.

Effect of survivin-shRNA treatment on apoptosis by flow cytometry. Raji cells transitorily (48 h) and stably (over 30 days) transfected with survivin-shRNA showed a signifi-

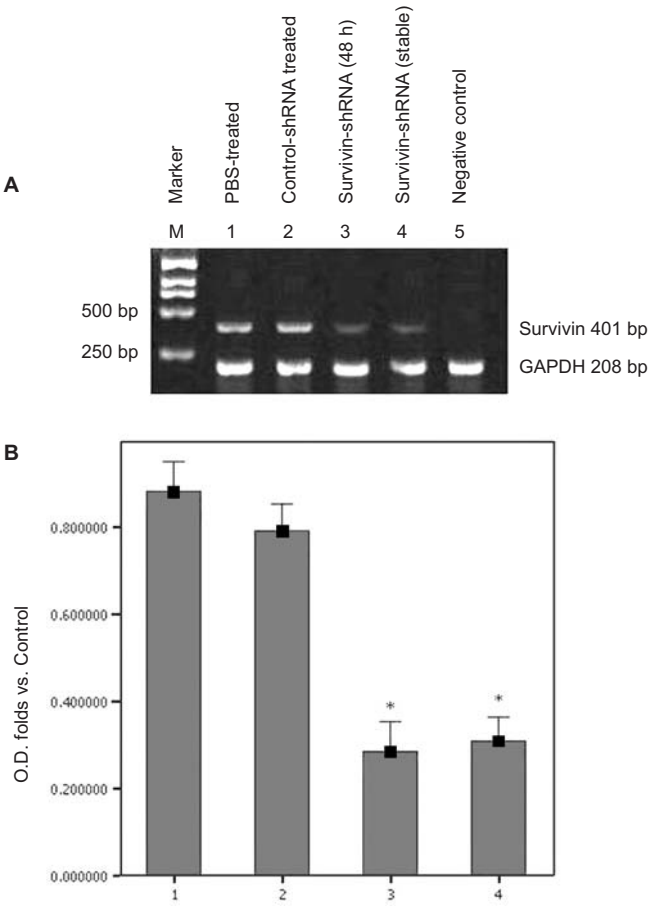

Figure 2. Effect of shRNA on survivin mRNA expression. Survivin mRNA levels were quantified by RT-PCR. (A) RT-PCR analysis of survivin mRNA levels in Raji cells treated with PBS reagent, control-shRNA (stable), survivin-shRNA (48 h) and survivin-shRNA (stable). (Negative control-peripheral blood lymphocytes in normal person). (B) Quantitative representation of survivin mRNA levels. Bands corresponding to survivin and GAPDH were scanned and the intensity was determined by optical density (O.D.) measurements. Data expressed as folds vs. control were the mean ±S.E. and analyzed by bars with standard error. *p<0.05.

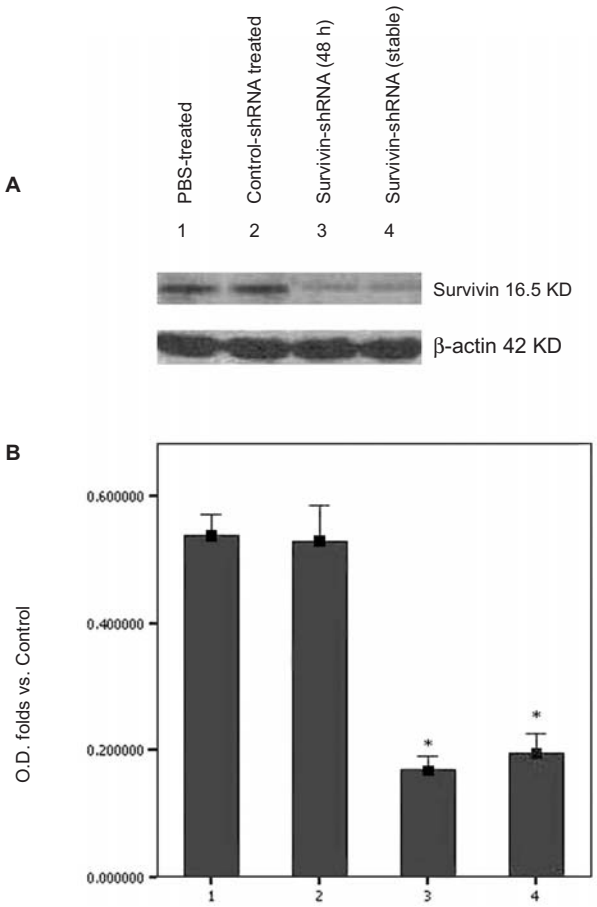

Figure 3. Effect of shRNA on survivin protein expression. Survivin protein levels were quantified by Western blot. (A) Western blot analysis of survivin protein levels in Raji cells treated with PBS reagent, control-shRNA (stable), survivin-shRNA (48 h) and survivin-shRNA (stable). (B) Quantitative representation of survivin protein levels. Bands corresponding to survivin and ß-actin were scanned and the intensity was determined by optical density (O.D.) measurements. Data expressed as folds vs. control were the mean ±S.E. and analyzed by bars with standard error. *p<0.05.

cant increase in apoptosis indexes (AI), respectively 31.20±2.45% ($p<0.05$) and 29.40±1.72% ($p<0.05$), compared with control-shRNA treated group and PBS treated group, respectively 6.10±1.93% and 6.60±1.52%. There was no significant difference in AI between transient and stable transfection group (Fig. 4).

Anti-proliferative effect of survivin-shRNA treatment. Cell proliferation was measured by counting the number of viable cells using trypan blue staining. Because the silencing effect of survivin expression by transient transfection may not be last over 72 hours, we just evaluated the proliferative status of Raji cells stably treated with survivin-shRNA, control-shRNA and PBS reagent. Cell numbers of survivin-shRNA stably transfected group, Control-shRNA stably transfected group and PBS treated group were determined on days 1–7. The average values for each group expressed as mean ±S.E. were determined to compose the growth curve. From cell growth curves (Fig. 5), survivin-shRNA stable treatment resulted in a marked inhibition of cell proliferation over this 7-day period. Cell growth was not influenced significantly by treatment with Control-shRNA and PBS reagent.

A **Apoptosis index of Raji cells with different treatments**

Raji cells	Apoptosis index (AI) by flow cytometry analysis
PBS treated	6.60 ± 1.52%
Control-shRNA treated	6.10 ± 1.93%
Survivin-shRNA treated (48 h)	31.20 ± 2.45%
Survivin-shRNA treated (stable)	29.40 ± 1.72%

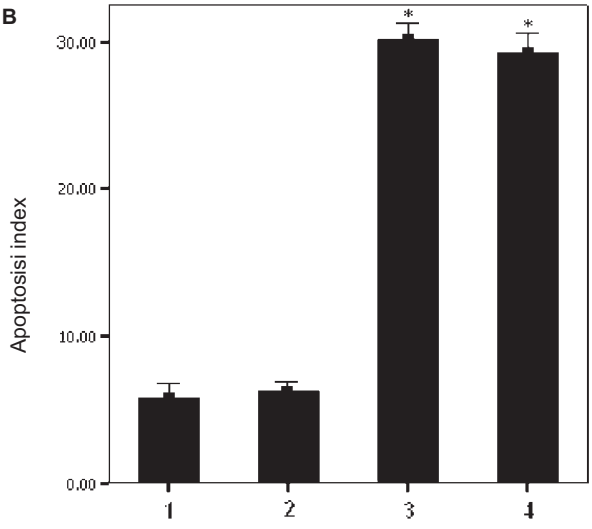

Figure 4. Effect of survivin-shRNA on cell apoptosis in Raji cells. A. Apoptosis index (AI) of Raji cells with different treatments was expressed as mean ±S.E. B. Data expressed as mean ±S.E. was analyzed by bars with standard error. *$p<0.05$. 1 – PBS treated cells. 2 – control-shRNA treated cells. 3 – survivin-shRNA treated cells (48 h). 4 – survivin-shRNA treated cells (stable).

Discussion

Regulation of cell apoptosis and growth is critical for normal embryonic development and homeostasis in adult tissues. In human cancers, malignant cells possess defects in apoptotic cell death, with the consequence of increased resistance to cell death, which plays a crucial role in tumorigenesis. Therefore, promotion of spontaneous apoptotic cell death of cancer cells by alternative ways may lead to tumor growth inhibition and represent a significant approach to anticancer therapy.

Survivin remains an attractive target for cancer gene therapy because it is present in most malignant cells but undetectable in most normal cell. Thus, the differential expression of survivin inhibitors may have relative specificity for malignant cells. Nowadays, alternative strategies have been developed for triggering apoptosis in cancer and other human disease by counteracting survivin expression in tumor cells, with the aim to inhibit cell growth through an increase in spontaneous apoptosis as well as to enhance cell sensitivity to apoptosis-inducing agents. It was reported that using various survivin molecular antagonists, such as antisense oligonucleotides [17, 18], dominant-negative mutants [19], ham-

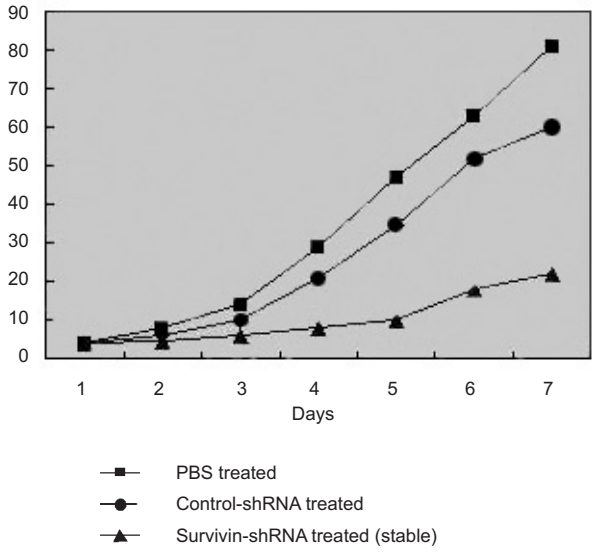

Figure 5. Effect of survivin-shRNA on cell proliferation in Raji cells. Growth curves of Raji cells stably transfected with survivin-shRNA and control-shRNA, as well as PBS treated cells. Survivin-shRNA stably treated treatment resulted in a marked inhibition of cell proliferation over this 7-day period. Cell growth was not influenced significantly by treatment with control-shRNA and PBS reagent. (Because the silencing effect of survivin expression by transient transfection may not be last over 72 hour, here we just evaluated the proliferative status of Raji cells stably treated with survivin-shRNA, control-shRNA and PBS reagent in a 1–7 day period.)

merhead ribozymes [20], cyclin-dependent kinase inhibitors [21] and siRNA [22] can trigger cell apoptosis, induce cell division defects, reduce tumor growth potential and also sensitize tumor cells to chemotherapeutic drugs and x-irradiation.

In this study, we used vector-based shRNA technique and constructed the recombinant plasmid expressing survivin-shRNA to transfect Burkitt's lymphoma Raji cells. Based on the results of RT-PCR and western blotting, we confirmed that the specific survivin-shRNA designed and used in this study successfully reduced the expression of survivin gene, with significant effects on inducing apoptosis of both transitorily and stably transfected Raji cells, as well as inhibiting the proliferation of stably transfected cells. In several reports, survivin targeted siRNA/shRNA was introduced into human cancer cell lines which highly express survivin, including hepatocellular carcinoma cell line SMMC-7721 [23], Hela cell line [24], colon tumor cell line HCT116 [25], esophageal squamous cell carcinoma cell line KYSE510 [26], melanoma cell lines [27] and a panel of human sarcoma cell lines [28, 29]. The results demonstrated that survivin siRNA/shRNA markedly inhibited the expression of survivin mRNA and its corresponding protein product, increased cell apoptosis and inhibited the growth of transfected cells. Our results were consistent with these findings. However, most studies only presented the data of transient transfection (24–48 h). In our study, both transient (48 h) and stable transfection (over 30 days) with survivin-shRNA in Raji cells were studied and compared. In selected cells with stably integrated shRNA expression vector we did not observe dramatic reduction in the amount of survivin mRNA and protein expression compared with transient transfection, and no significant differences on survivin mRNA or protein expression were detected between transient and stable transfection. This finding suggested that the plasmid vector-based shRNA system can constitute a promising method to achieve persistent knockdown of survivin mRNA and its corresponding protein product in human NHL cells, together with the persistent effects on inducing cell apoptosis and growth inhibition.

On the other hand, some evidences revealed that inhibition of survivin gene can strongly inhibit the growth of tumor cells, not only because it can inhibit apoptosis of tumor cells, but also it plays a crucial role in tumor angiogenesis [30]. A recent study showed that transfection of endothelial cells with survivin specific siRNA induced a significant increase of apoptotic rate, a dose-dependent inhibition of migration on vitronectin and a decrease in capillary formation [31]. These findings indicated that repression of survivin gene could lead to hypersensitivity to anti-cancer treatment, not only through direct interference with the apoptotic pathways in tumor cells but also by intervention of apoptosis of newly formed tumor vasculature.

In this study, we used p*Silencer*^{TM} 4.1-CMVneo plasmid as the vector for RNAi. Unlike many commonly used vectors of U6 or H1 promoters (pol III promoters) used in most RNAi studies [32, 33], the p*Silencer* 4.1-CMVneo vectors carry a modified RNA polymeraseII-type CMV promoter (human cytomegalovirus immediate-early promoter), an optimized SV40 polyadenylation signal and an SV40 promoter which expresses one of three antibiotic resistance genes (hygromycin, neomycin, or puromycin) for stable transfections requiring long-term antibiotic selection. For siRNA or shRNA expression, the pol II-type CMV promoter has some advantages over pol III promoters such as U6 or H1. For example, pol II can tolerate strings of 4 or more U's within the siRNA or shRNA sequence, unlike pol III that will terminate transcription after incorporation of a stretch of U's. Furthermore, CMV promoter does not interfere with other transcription events, such as expression of the antibiotic resistance gene, making it easier to perform long-term gene silencing studies. In fact, it is possible to produce cell lines with reduced levels of GAPDH mRNA and protein lasting over 8 months using the p*Silencer* 4.1-CMV vector system [34]. Our results also showed an evidence that pCMV vector expressing survivin-shRNA could effectively and significantly reduce the expression of survivin mRNA and protein in Raji cells. Nevertheless, further investigations remain to be done to validate a both safe and efficient RNAi approach before it is considered to be clinical application.

In summary, this study demonstrated that using vector-based shRNA significantly suppresses the expression of survivin mRNA and protein in Raji cells, with a marked increase of apoptosis and growth inhibition of transfected cells. These findings suggest that survivin gene can be regarded as an ideal target for cancer genetic therapy in NHL. Furthermore, the application of shRNA expressing vector approach could be a powerful tool for functional analysis of genes and genetic therapy of cancers.

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