Original Article

Effect of Survivin-siRNA on Drug Sensitivity of Osteosarcoma Cell Line MG-63

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CLC number: R73-36 Document code: A Article ID: 1000-9604(2010)01-0068-05
DOI: 10.1007/s11670-010-0068-x

ABSTRACT

Objective: Survivin is one of the apoptosis inhibitor genes and is rarely expressed in adult tissues. However, survivin expression has been detected in various human cancers and correlations have been recognized between the level of expression of this gene in tumors and prognosis. In this study, we investigated the effect of Survivin-siRNA on the drug sensitivity of osteosarcoma cell line MG-63.

Methods: Two siRNAs (Survivin-siRNA1, Survivin-siRNA2) specifically targeting Survivin gene were chemically synthesized and transfected into MG-63 cells. The Survivin mRNA level was detected by reverse transcription-polymerase chain reaction (RT-PCR). The survivin protein expression and cell apoptosis rate were analyzed by flow cytometry (FCM). The 50% inhibition concentration (IC_{50}) of cisplatin (DDP) and adriamycin (ADM) on MG-63 cells was determined by MTT method.

Results: Two short siRNA targeting survivin down-regulated the transcription of survivin gene dramatically and elevated apoptosis rate. They increased the drug sensitivity of MG-63 cells to ADM by five-fold and to DDP by nine-fold.

Conclusion: Validated Survivin specific siRNA can effectively inhibit Survivin expression in survivin-overexpressing osteosarcoma MG-63 cell line and enhance the drug sensitivity of MG-63 cell line to ADM and DDP. Short survivin-siRNA mediated gene silencing may be a useful therapeutic strategy for osteosarcoma. These results suggest that survivin might be helpful for diagnosis of osteosarcoma and survivin siRNA combined with adriamycin or cisplatin may be a feasible strategy to enhance the effects of chemotherapy in patients with osteosarcoma.

Key words: Survivin; siRNA; RNAi; Osteosarcoma; Drug sensitivity

INTRODUCTION

Osteosarcoma is associated with high morbidity rates in young adults and adolescents. Current treatment protocols for osteosarcoma include wide surgical resection of the primary lesion and multidrug chemotherapy. The progress of treatment for osteosarcoma in recent years is largely due to the clinical application of neoadjuvant determined chemotherapy. Clinically, prognosis is postoperatively by the response to chemotherapy. However, tumor cell resistance to chemotherapeutic drugs is the main reason for the failure of chemotherapy. One possible solution lies in the strategy of improving the chemosensitivity and decreasing drug resistance of cancer cells. Research has shown that doxorubicin (adriamycin, ADM) and Cisplatin (diamminedichloroplantinum, DDP) are the most effective anti-osteosarcoma drugs [1].

Survivin is a member of the inhibitor of apoptosis protein (IAP) family and over-expression

Received 2009−06−24; **Accepted** 2009−11−18
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of survivin inhibits apoptosis, increases survival rate of tumor cells and promotes tumor progression and resistance to anti-cancer drugs[2–5]. Therefore, inhibiting the expression of Survivin gene may improve osteosarcoma cell sensitivity to chemotherapeutic drugs. Several laboratories have demonstrated that antisense strategies could successfully inhibit survivin expression, causing apoptosis and sensitization to anticancer drugs in some tumor lines including mesothelioma and lung adenocarcinoma[6, 7]. In this study, we used synthetic, ready to- use siRNA targeting survivin to silence survivin gene expression in MG-63 cells and tested its feasibility to induce apoptosis and to increase chemosensitivity of MG-63 cells to doxorubicin and Cisplatin.

MATERIALS AND METHODS

Drug and Chemicals

Cisplatin (diamminedichloroplantinum, DDP) and doxorubicin (adriamycin, ADM) were from Pfizer. siRNA corresponding to Survivin and nonsilencing control siRNAs were designed and synthesized by Ambion Company. MEM/NEAA and 10% fetal bovine serum were from Hyclone. Opti-MEM Medium and LipofectAMINE 2000 Reagent were from Gibco. Survivin monoclonal antibody was from Chemicon and PI, MTT and RNase from Sigma. Customed PCR regeant kit was purchased from Beijing BODA Biological Company. PCR primers were made by Shanghai Sangong Biotechnology Co., LTD. Apoptosis detection kit was obtained from Beijing Biosea Biotechnology Co., LTD.

Cell Lines

The human osteosarcoma MG-63 cell line was obtained from Basic Medical Sciences Cell Bank of Peiking Union Medical College. The cells were routinely maintained in RPMI1640 with 10% fetal bovine serum in a well humidified atmosphere of 5% CO_2 at 37°C.

Experiment Groups and Transfection

Experiment included four groups: survivin-siRNA1 group, survivin-siRNA2 group, untreated control group and nonsilencing siRNA group. The day before transfections, human osteosarcoma MG-63 cells were inoculated in a 6-well plate at 2×10^5 cells per well. When they were 40%–60% confluence, positive survivin-siRNA1, survivin-siRNA2 and negative siRNA (nonsilencing control siRNAs) composition were transfected to them according to the manufacturer's protocol. Cultures were evaluated for survivin expression and biologic effects 24 h or 48 h after transfection.

RT-PCR Analysis of Survivin mRNA Expression

Cells were harvested by trypsinization 48 h after transfection and total RNA was extracted with Trizol reagent and subjected to RT-PCR. The PCR products were analyzed by electrophoresis on 2 % agarose gels. The amplified products were visualized and photographed under ultraviolet light, scanned and analyzed by GIS system. The primers used for survivin amplification were 5′-GG-ACCACCGCATCTCTACAT-3′ (upstream) and 5′-GCACTTTCTTCGCAGTTTCC-3′ (downstream), with an amplification fragment of 491 bp. The primers used for GAPDH amplification were 5′-GAGTCAACGGATTTGGTCGT-3′ (upstream) and 5′-TTCTAGACGGCAGGTCAGG-3′ (down-stream), with an amplification fragment of 731 bp.

Detection of Survivin Protein by FCM

Cells were seeded in 6-well plates, 48 h after transfection, both floating and attached cells (use trypsin) were collected and all the following steps were performed on ice. The cells were washed twice with 1×PBS, and then incubated with survivin monoclone antibody (1:40) for 30 min at 4°C. After washing twice again they were stained with FITC-conjugated rabbit anti-mouse antibody for 30 min in the dark. The homologue mouse IgG was used as negative control. Survivin protein expression was analyzed on a FACScan flow cytometry.

Cell Apoptosis Assay

Cells were seeded in 6-well plates, 48 h after transfection, the cells were collected, washed twice with PBS, and fixed over night at -20°C in 70% ethanol. After washing twice with PBS, cells were incubated with 5 μg/ml propidium iodide and 50 μg/ml RNase-A in PBS for 30 min at 37°C. Flow activated cell sorter analysis was carried out using FACScan flow cytometry (Becton Dickinson, MountainView, USA) with Cell Quest software. A total of 10,000 cells were measured per sample. The sub-G_1–G_0 cell fraction was considered as representative of apoptotic cells.

Treatments with Chemotherapeutic Agents and Measurement of Cell Viability

Growth inhibition of MG-63 cells was determined by the colorimetric MTT cell viability/proliferation assay. Briefly, cells were seeded in 96-well plates with 5000 cells per well. Forty-four hours after transfection, the chemotherapeutics were added in varying concentrations to each well. Cells were incubated for 72 h and MTT (5 mg/ml) was added to the wells (100 μl/well) at the end of experimental period. After a 4 h incubation at 37°C, medium was removed from the wells, and dimethylsulfoxide was added to each well (100 μl/well). The plates were agitated at room temperature for 5 min. The absorbency was detected at 570 nm with a Bio-Rad mode l550-microplate Reader (Bio-Rad, Hercules, USA). The percentage of dead (or growth inhibited) cells was normalized to untreated controls. All of the experiments were carried out at least three times in triplicate.

Statistical Analysis

SPSS 10.0 software was used to perform statistical analysis. ANOVA test was used for comparison of variance among several groups. $P<0.01$ or $P<0.05$ was considered significant.

RESULTS

Expression of Survivin Was Blocked Efficiently by RNAi

The expression of Survivin was examined by RT-PCR and flow cytometry 48 h after siRNA transfection. We found that survivin specific siRNA efficiently blocked survivin expression both at mRNA and protein level. And the efficiency reached more than 95% at protein level in MG-63 cells (Figure 1, 2).

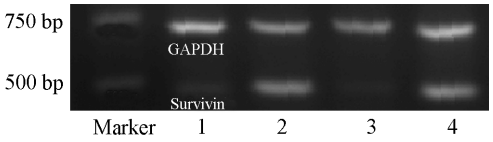

Figure 1. Effect of survivin-siRNA on the expression of survivin mRNA in MG-63 cells. 1: Survivin-siRNA$_1$ group; 2: Untreated control group; 3: Survivin-siRNA$_2$ group; 4: Nonsilencing siRNA group.

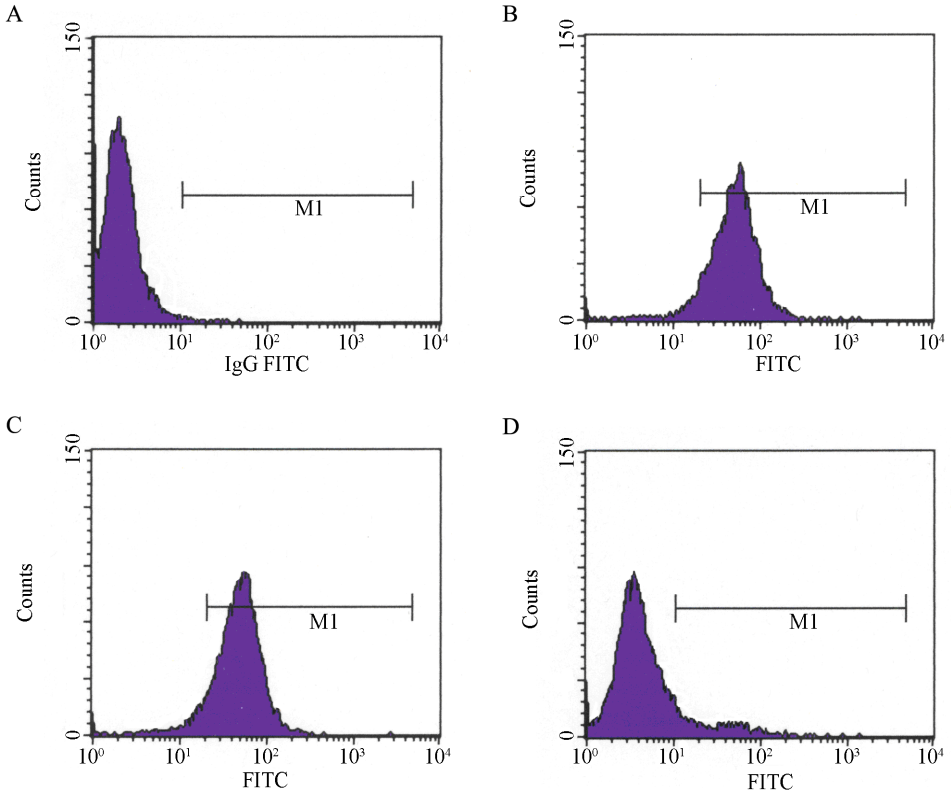

Figure 2. Effect of survivin-siRNA on survivin protein level in MG-63 cells. A: Mouse IgG negative control; B: Untreated control group; C: Nonsilencing siRNA group; D: Survivin-siRNA$_1$ group.

Effect of RNAi on Apoptosis

To explore whether survivin downregulation by RNAi could induce apoptosis, we performed flow cytometry 48 h after transfection in 6-well plates. The number of apoptotic cells was increased in survivin-siRNA1 group and survivin-siRNA2 group as compared with other groups. In quantitative terms, the percentages of apoptotic cells in survivin-siRNA1 group and survivin-siRNA2 group were (23.7±1.5)% and (25.8±2.3)%, respectively. The corresponding values in untreated control group and nonsilencing siRNA group were (5.2±0.3)% and (7.6±0.7)%, respectively. There was significant difference between survivin-siRNA groups and the two control groups (P<0.01) (Figure 3).

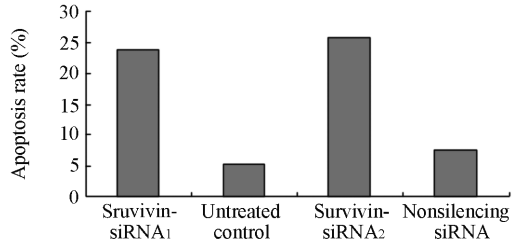

Figure 3. Effect of survivin-siRNA on apoptosis of MG-63 cells.

Survivin Downregulation Sensitizes MG-63 Cells to Chemotherapeutics

To investigate whether downregulation of survivin expression has the potential to sensitize MG-63 cells to chemotherapy, a combination treatment of Survivin specific siRNA with anticancer drugs was performed. Twenty-four hours after transfection with siRNA, the cells were treated with cisplatin and doxorubicin at scaled concentrations for 72 h. The IC_{50} was determined by MTT assay. The results demonstrated that the cells exposed to survivin siRNA in the presence of cisplatin and doxorubicin showed a significant decrease of IC_{50} compared with untreated control group and nonsilencing siRNA group. The IC_{50} of survivin siRNA + ADM group, nonsilencing siRNA + ADM group and untreated control + ADM group were (0.26±0.11) μg/ml, (1.32±0.14) μg/ml and (1.36±0.28) μg/ml respectively. There were statistically significant differences among them (P<0.01). Survivin-siRNA increased the sensitivity of osteosarcoma cells to adriamycin by 5-fold. Similarly, the IC_{50} of survivin siRNA + DDP group, nonsilencing siRNA + DDP group and untreated control + DDP group were (0.98±0.07) μg/ml, (7.68±1.45) μg/ml and (8.74±2.56) μg/ml respectively. There were statistically significant differences among them (P<0.01). Survivin-siRNA increased the sensitivity of osteosarcoma cells to DDP by 9-fold. The nonsilencing siRNA had either no effect or only a minimal effect.

DISCUSSION

Osteosarcoma is the most frequent malignant bone tumor with a peak incidence in the second and third decade of life. Chemotherapeutic agents based on cisplatin and doxorubicin play an important role in osteosarcoma treatment. But intrinsic chemoresistance profiles in osteosarcoma has been a very difficult problem disturbing clinics. Therefore, development of novel chemotherapeutic strategies is necessary to improve the outcome for patients with osteosarcoma.

Inhibitor of apoptosis proteins are a family of caspase inhibitors with eight members in human, among which survivin has garnered the most attention as potential therapeutic targets. Survivin mRNA has been detected in various human malignant neoplasms, such as gastric cancer, breast cancer, lung cancer and osteosarcoma but not in adult human tissues. Thus survivin has been thought to promote tumor progression[8]. Survivin has anti-apoptosis effects by suppression of caspase-3 and caspase-7 at downstream of caspase apoptosis pathways. Survivin is expressed specifically at the G_2/M stage of cell cycle, participating in the modulation of cell division by binding specifically to canaliculus protein of spindle during the period of cell mitosis[9]. Asanuma[10] demonstrated that survivin acted as an inducible radioresistance factor and enhanced radioresistance in pancreatic cancer cells. Ikeguchi[11] found that the cell expression rates of survivin mRNA after 48 h treatment with 0.1 and 1 mg/ml of CDDP were 2 to 6 fold higher than that of untreated gastric cancer cells (MKN-45), and the relative cell expression level of survivin protein after 24 h treatment with 0.1 or 1 mg/ml of CDDP was 3 to 6.5 fold higher, indicating that survivin expression may correlate with the chemo-resistance of malignant cells.

RNAi technology is a potent new technology. RNA interference (RNAi) is a double stranded RNA which acts as a signal to promote degradation of mRNA with sequence identity[12, 13]. Williams showed that siRNA against survivin inhibited colon

tumor cells growth and increased apoptotic rate[14].

In this study, validated survivin specific siRNA was transfected into survivin high expression cells, MG-63 and survivin could be downregulated effectively both at mRNA and protein levels. Since survivin was the most powerful inhibitor of apoptosis of IAPs, we speculated that apoptosis would be increased following siRNA transfection and we did find that survivin downregulation could increase apoptosis in MG-63 cells. We further investigated the effect of survivin-siRNA on the drug sensitivity of osteosarcoma MG-63 cell line. The results showed that validated survivin specific siRNA enhanced the drug sensitivity of MG-63 cells to ADM by 5-fold and to cisplatin by 9-fold.

In summary, we showed that survivin specific siRNA mediated efficient and selective inhibition of survivin expression in osteosarcoma MG-63 cells which overexpress survivin. Treatment with survivin siRNA in combination with cisplatin and doxorubicin further showed enhanced chemo-sensitivity. These results strongly suggest that survivin may serve as a marker for the diagnosis of osteosarcoma and survivin siRNA combined with cisplatin, and doxorubicin may be a feasible and complementary strategy to enhance the effects of chemotherapy in patients with osteosarcoma.

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