SPANDIDOS PUBLICATIONS

STAT3-decoy oligodeoxynucleotide inhibits the growth of human lung cancer via down-regulating its target genes

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Received December 28, 2006; Accepted March 6, 2007

Abstract. Double-stranded decoy oligodeoxynucleotide (ODN) is a promising approach for inhibiting gene transcription. Signal transducer and activator of transcription (STAT) 3, a potent transcription factor, is usually constitutively activated in a variety of malignancies, and considered as an attractive drug target. In this study, it was noted that STAT3 was over-activated in human lung cancer cells, and STAT3-decoy ODN, which was high-efficiently transfected into nucleus of cancer cells, significantly inhibited the proliferation of PG cells by inducing apoptosis or cell cycle arrest. The transcription levels of mcl-1, cyclin D1, bcl-xl and survivin were significantly decreased by 64.4, 56.1, 72.8% ($P<0.01$) and 31.8% ($P<0.05$), respectively; and the synthesis levels of bcl-xl and cyclin D1 in PG cells showed 64.5% ($P<0.01$) and 28.6% ($P<0.05$) decrease, respectively. Our study demonstrated that decoy-ODN targeting at activated STAT3 may potentially be used as an anti-lung cancer therapeutic approach.

Introduction

With the delineation of important signal transduction cascades, it has become clear that the STAT proteins may play important roles in regulating physiologic cell growth, differentiation, apoptosis, angiogenesis and immune responses in carcinogenesis (1,2). The family of STATs includes seven proteins (STATs 1, 2, 3, 4, 5a, 5b, and 6), which transduce extracellular signals to the nucleus and directly regulate the target genes' transcription, among which STAT3 is the most attractive (3,4). Gene knockout studies have demonstrated that STAT3 null mice are embryonic lethal (5). Some cytokines and growth factors can bind to their receptors and activate tyrosine kinases, which can phosphorylate the tyrosine residue (705) of STAT3 (6,7). Upon activation, STAT3 dimerizes and translocates to the nucleus, where it binds to STAT3-specific DNA response elements and initiates their transcription (4).

In normal cells, STAT3 is activated transiently. However, in numerous primary cancers or cancer-derived cell lines, STAT3 shows constantly activated status (8-18), which is considered to contribute to oncogenesis by up-regulating expression of apoptosis or cell cycle related genes, such as bcl-xl, bcl-2, c-myc, cyclin D1, survivin and mcl-1 (9,10,18,19). Blocking STAT3 signaling is often sufficient to induce growth arrest and apoptosis in many different tumors (10-14,18,20). Therefore, the association of STAT3 activation and tumor progression suggest that STAT3 may be an attractive molecular target for cancer therapy.

Decoy ODN which has high affinity for target transcription factors (TFs) was used as a promising approach to inhibit the action of TFs and has been used as a research tool for investigating the role of TF activity (21). This approach is based on the competition between the endogenous cis-elements within the regulatory regions of target genes and exogenously added molecules mimicking the specific cis-elements (22). This strategy has been successfully used for inhibiting STAT3 and STAT6 activity, which abrogates head and neck cancer cell growth and restricts IL-4-driven Th2 cell activity (23-25). Clinical application of decoy ODN against E2F was approved by the United States Food and Drug Administration (FDA), demonstrating the potential values of the decoy approach.

In the present study, we demonstrate that STAT3 is constantly activated and critical for the proliferation of human lung cancer cells (PG). STAT3-decoy ODN can efficiently block STAT3 signaling and inhibit proliferation by significantly decreased expression of bcl-xl and cyclin D1 at transcription and translation levels, suggesting its potential application in the treatment of human lung cancer.

Materials and methods

Cell lines. The human pulmonary giant cell carcinoma cell line, PG (obtained from ATCC, USA) was maintained in RPMI-1640 medium (Gibco/BRL) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin at 37˚C in 5% CO_2.

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Abbreviation: STAT, signal transducer and activator of transcription; ODN, oligodeoxynucleotide; ds, double-stranded; IL, interleukin

Key words: oligodeoxynucleotide, decoy, lung cancer, STAT3

Antibodies and reagents. Anti-STAT3, anti-phospho-specific STAT3 (Tyr705, Ser727), anti-bcl-xl, anti-cyclin D1, anti-ß-actin antibodies and horseradish peroxidase-conjugated second antibody were purchased from Cell Signaling Technology (New England BioLabs Inc.). Jakus kinase inhibitor, AG490, was purchased from Calbiochem (Darmstadt, Germany).

STAT3-decoy and scramble ODN. Sense and antisense strands of STAT3-decoy or scramble ODNs were synthesized using phosphorothioate chemistry by Expedite™ Nucleic Acid Synthesis System (Takara Biotechnology, Dalian). The STAT3-decoy ODN sequence was 5'-CATTTCCCGTAA ATC-3', 3'-GTAAAGGGCATTTAG-5' and the scramble ODN sequence was 5'-CATCTTGCCAATATC-3', 3'-GTAG AACGGTTATAG-5'. The sense and antisense strands were annealed and purified by HPLC.

Western blotting. As described previously (26), PG cells were lysed in lysing buffer [20 mmol/l Tris-HCl (pH 7.4), 150 mmol/ l NaCl, 1 mmol/l Na_3VO_4, 1 mmol/l EDTA, 1 mmol/ l EGTA, 1 mmol/l PMSF, 50 mmol/l NaF and 1% NP-40]. The whole cell extracts (30 μg/lane) were separated by SDS-PAGE on 8% gels and transferred to nitrocellulose membrane. After blocking with 5% non-fat milk in TBST, membranes were incubated with anti-STAT3, anti-phospho-specific STAT3, anti-bcl-xl, anti-cyclin D1 or anti-ß-actin at a dilution of 1:1000 overnight at 4˚C and then washed 3 times with TBST, incubated with secondary antibodies conjugated with horseradish peroxidase. After extensive washing with TBST, membranes were detected with an enhanced chemiluminescence system (Pierce, Rockford, IL). The bands were examined by densitometry using AlphaEaseFC software (Version 4.0.0, Alpha Innotech Corp.) with normalization of each band to their corresponding loading control.

Electrophoretic mobility shift assay (EMSA). Nuclear extract was prepared using high-salt buffer. EMSA was performed using a ^{32}P-radiolabeled ODN probe containing the high-affinity sis-inducible element (hSIE, sense strand: 5'-CATTT CCCGTAAATC-3'). Labeled probe was mixed with 5 μg of nuclear extracts in 1X binding buffer (12 mmol/l HEPES, pH 7.5, 60 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l EDTA, 0.5 mmol/l DTT, 1 mmol/l PMSF, 12% glycerol, 50 ng/μl poly-dI:dC) and incubated at room temperature for 20 min. The DNA-protein complexes were then resolved by 5% non-denaturing PAGE, and detected by autoradiography. Competitor assay was performed using a 200-fold molar excess of unlabeled ODN which was incubated with nuclear extract for 15 min prior to addition of labeled probe.

Transfection of ODN. Transient transfections were carried out using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA). According to the manufacturer's instructions, PG cells were seeded at a density of $7x10^3$ cells/well (400 μl) in 48-well plates (Costar, Corning, NY) 24 h before transfection. After being washed with PBS, cells were transfected with Lipofectamine 2000/vehicle (TE), Lipofectamine 2000/ decoy ODN, Lipofectamine 2000/scramble ODN, or not transfected. The final concentration of ODN was 50, 25 and 12.5 nmol/l.

Flow cytometry and fluorescent microscope. The decoy or scramble ODN labeled with FITC at 5' of ODNs (Takara Biotechnology) was transfected into PG cells. After 6 h, these cells were detached, washed with PBS extensively and then the transfection efficiency was determined by flow cytometry (FACSCalibur, BD Biosciences, USA). For ODN location analysis, cells transfected with FITC-ODN were washed, fixed and permeabilized. Then the nuclei were stained with DAPI (Jingmei Biotechnologies, P.R. China) for 10 min at room temperature and detected under fluorescent microscope, and the photos were treated with Photoshop software.

Cell proliferation assay. For AG490 inhibiting proliferation assays, PG cells were seeded at a density of $1x10^4$ cells/well in 48-well plates. After 24 h, the cells were incubated in growth medium in the presence of AG490 at 25 and 50 μmol/l or vehicle (0.05 and 0.1% DMSO) for 24, 48 and 72 h. For decoy ODN inhibiting proliferation assays, PG cells were plated and transfected as described above. Cell number was determined by counting with a hemocytometer using trypan blue exclusion. Above 90% viability was regarded as no toxicity.

Semi-quantitative reverse transcription-PCR (RT-PCR) assay. RT-PCR was performed as previously described (26). Briefly, after being transfected with decoy ODN for 24 h, cells were harvested and TRIzol (Invitrogen, Carlsbad, CA) was used to extract total-RNA. Then, according to the manufacturer's instructions, 2 μg of RNA per sample was reverse-transcribed using M-MLV Reverse Transcriptase (Invitrogen). For PCR, 1 μl cDNA was subsequently amplified with the corresponding gene-specific primers which were synthesized by Shanghai Genecore Biotechnologies (Shanghai, P.R. China) (Table I). Temperature conditions and product sizes were shown in Table I. The expression of ß-actin was used as an internal control. The PCR products were electrophoresed on 2% agarose gels and photographed using AlphaEaseFC. The bands were examined by densitometry using AlphaEaseFC software with normalization of each band to its corresponding loading control.

Statistical analysis. All of the values are presented as the mean ± SD for three or more individual experiments. SPSS software (version 10.0, SPSS Inc.) was used to test for significance. P-values <0.05 were considered statistically significant.

Results

Constitutive activation of STAT3 was critical for proliferation of human lung cancer cells. In order to examine whether STAT3 is persistently activated in human lung cancer cells, the whole cell lysates from human pulmonary giant cell carcinoma line PG were processed for Western blotting with anit-STAT3 and anti-phospho-specific STAT3 (Y705, S727) antibodies. As shown in Fig. 1A, STAT3 was constitutively phosphorylated on Tyr705 and Ser727 in PG cells. Moreover, the STAT3 shift bands (^{32}P-labelled STAT3 probe binding with the activated STAT3) were observed to be completely blocked by a 200-fold cold probe using the EMSA method (Fig. 1B), indicating the active function of STAT3 in these cells. The Jak2 specific

T-PCR primers used for STAT3 target genes.

Genes	Primer sequences	Temperature (˚C)	Product size (bp)
ß-actin	5'-CTCCTTAATGTCACGCACGATTT-3' 5'-GTGGGGCGCCCCAGGCACCA-3'	56	539
Cyclin D1	5'-GAGACCATCCCCCTGACGGC-3' 5'-CTCTTCCTCCTCCTCGGCGGC-3'	58	484
bcl-xl	5'-GGAAAGCGTAGACAAGGAGATGC-3' 5'-GGTGGGAGGGTAGAGTGGATGGT-3'	55	236
c-myc	5'-GGTCTTCCCCTACCCTCTCAACGA-3' 5'-GGCAGCAGGATAGTCCTTCCGAGT-3'	55	386
mcl-1	5'-GTGGTGGTGGTGGTTGGTTA-3' 5'-CGGCAGTCGCTGGAGATTAT-3'	54.5	575
Survivin	5'-GCATGGGTGCCCCGACGTTG-3' 5'-GCTCCGGCCAGAGGCCTCAA-3'	55	446
Cyclin E	5'-ATACAGACCCACAGAGACAG-3' 5'-TGCCATCCACAGAAATACTT-3'	54.5	301

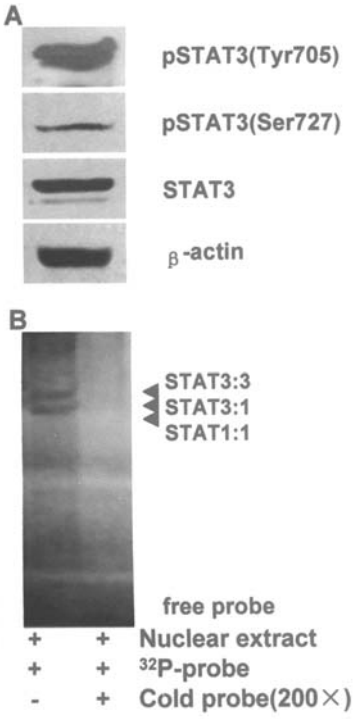

Figure 1. Constitutive activation of STAT3 correlated with proliferation in human lung cancer cells. (A) The STAT3 is constantly activated in PG cells. Whole cell lysates were examined by Western blotting using anti-STAT3, anti-phospho-specific STAT3 (Y705, S727) or ß-actin antibodies. (B) Nuclear extracts were prepared and then EMSA analysis was performed using ^{32}P-radio-labeled hSIE probe. A specific cold (isotope-free) probe was used as a competitor at 200-fold concentration of the labeled probe.

inhibitor AG490 can dose-dependently inhibit the proliferation of PG cells, suggesting the critical role of STAT3 in their growth (data not shown).

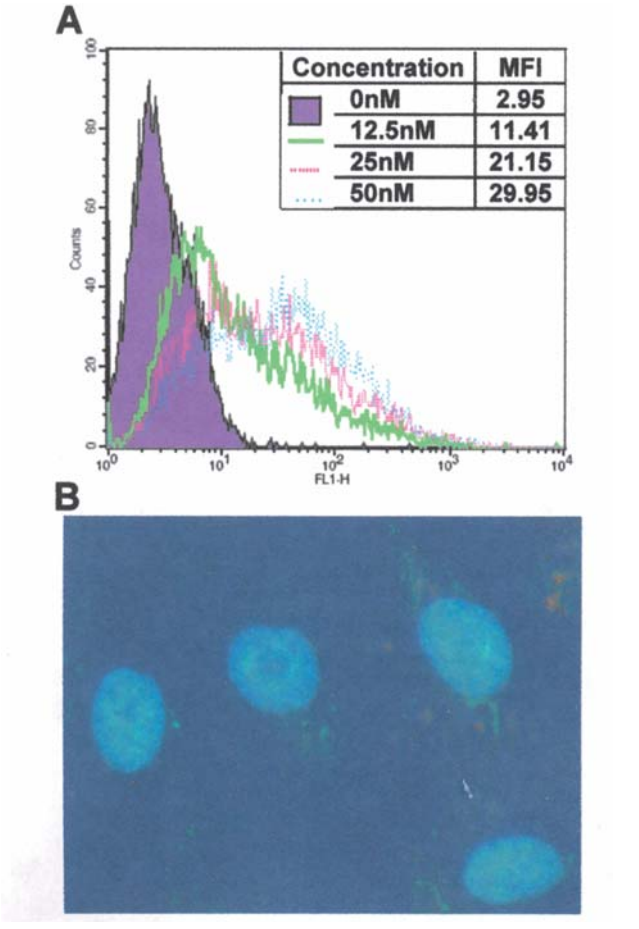

Figure 2. The efficient transfection of STAT3-decoy ODN into PG cells. (A) PG cells were transfected with FITC labeled STAT3-decoy ODN followed by flow cytometry assay. (B) After transfection, the nuclei were stained with DAPI, fluorescent microscopy was used to observe the nucleus location ODN. STAT3-decoy ODN (green) and nuclei (blue) were overlaid with their contrast image (original magnification x400).

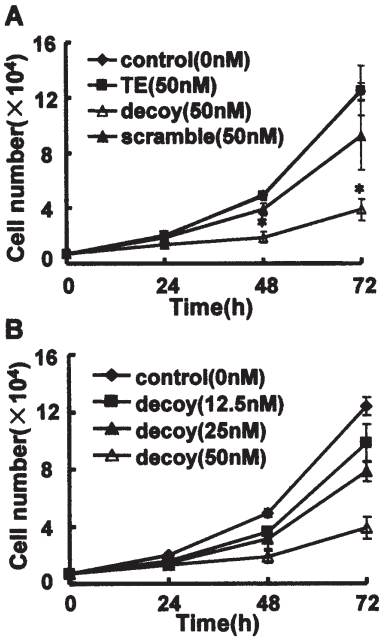

Figure 3. STAT3-decoy ODN inhibited proliferation of human lung cancer cells. (A) PG cells were treated with STAT3-decoy ODN, STAT3 scramble ODN or vehicle control (TE) at 50 nmol/l for different times. (B) STAT3-decoy ODN dose-dependently inhibited the proliferation of PG cells. PG cells were treated with STAT3-decoy ODN at 12.5, 25 and 50 nmol/l. Statistical significance was determined as $^{*}P<0.01$ compared to control.

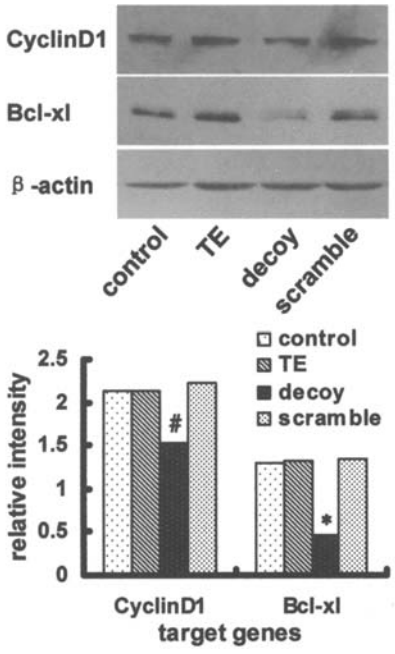

Figure 5. Decrease of cyclin D1 and bcl-xl synthesis after treatment with STAT3-decoy ODN. PG cells were transfected with STAT3-decoy ODN for 24 h and the whole-cell extracts were obtained. Bcl-x1 and cyclin D1 were then examined by Western blotting. The histogram presents the relative expression levels of bcl-xl and cyclin D1. Statistical significance was determined as $^{*}P<0.01$ and $^{\#}P<0.05$ compared to other groups.

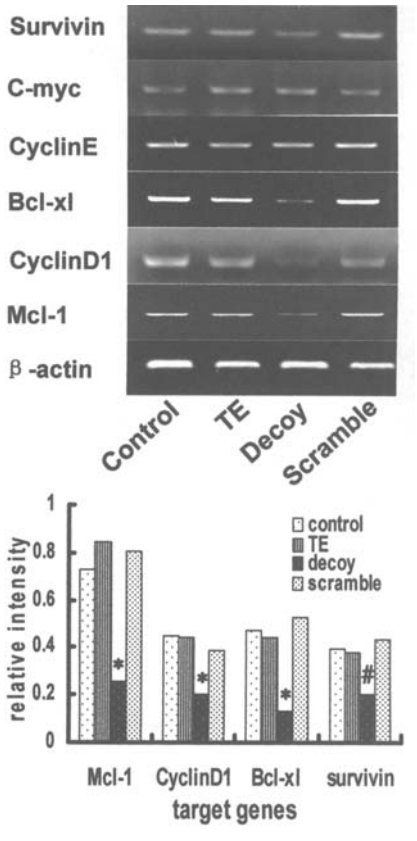

Figure 4. Down-regulation of STAT3-targeted gene expression by STAT3-decoy ODN. After being transfected with STAT3-decoy ODN for 24 h, PG cells were harvested and mRNA levels of STAT3-targeting genes were detected using RT-PCR method. The histogram presents the relative expression level of each gene after normalization to its corresponding internal control. Statistical significance was determined as $^{*}P<0.01$ and $^{\#}P<0.05$ compared to other groups.

STAT3-decoy ODN was efficiently transfected into cells and located in the nucleus. Firstly, the efficiency of decoy ODN incorporation into PG cells was examined by flow cytometry using FITC-labeled decoy ODN. The results showed that the decoy ODN was efficiently transfected into PG cells with a dose-dependent manner after incubating for 6 h (Fig. 2A). Secondly, the sub-cellular localization of decoy ODN was determined by fluorescent microscopy. As shown in Fig. 2B, most decoy ODN was located in the nucleus.

STAT3-decoy ODN inhibited proliferation of lung cancer cells. To examine the effects of STAT3-decoy ODN on growth of cancer cells, cells were transfected with STAT3-decoy ODN or scramble control ODN. The results demonstrated that the growth of PG cells was dose-dependently inhibited by STAT3-decoy ODN with maximum inhibition rate at 69% ($P<0.01$), however, STAT3 scramble ODN treatment did not influence cell growth (Fig. 3).

STAT3-targeted cell cycling and anti-apoptotic genes were down-regulated by decoy ODN. As indicated in Fig. 4, the transcription levels of mcl-1, cyclin D1, bcl-xl and survivin in PG cells were decreased by 64.4%, 56.1%, 72.8% ($P<0.01$) and 31.8% ($P<0.05$), respectively, but the transcription levels of cyclin E and c-myc did not show obvious changes. By contrast, scramble ODN showed no effect on the transcription levels of the cancer cells. It was also observed that the expression levels of bcl-xl and cyclin D1 in PG cells showed a 64.5% ($P<0.01$) and 28.6% ($P<0.05$) decrease, respectively (Fig. 5). These data demonstrated that STAT3-decoy ODN influences the proliferation of human lung cancer cells via blocking the expression of STAT3-targeted genes.

There are several strategies for intervening STAT3 activity, such as antisense ODNs, dominant negative mutants or siRNA. Moreover, decoy ODNs have been proposed as a useful approach to block the action of transcription factors (22). The ds decoy ODNs against E2F, NF-κB, AP-1, ERE, Ets and STAT6 have been widely used as a research tool of gene function, and large preclinical researches have been practiced to evaluate their potential to treat diseases (27-32,25). There are several attractive advantages of the decoy method over other approaches. As a small DNA molecule, decoy ODN can easily be delivered to specific tissues and transfected into cells, and directly abrogate the activated TFs other than all status TFs. On the other hand, the synthesis, storage and transportation of decoy ODNs are economic and easier due to its stability (24).

The decoy ODN approach was approved for clinical application by FDA in 1996. The phase I/II clinical trial study of E2F-decoy demonstrated its safety, efficacy and feasibility (33), although it was not as effective as in previous phase III clinical trial. Another phase I/II pilot trial of E2F-decoy assessed its effectiveness. Based on the preclinical promising results, a clinical trial using NF-κB-decoy ODN was also started in Japan, and positive results of phase I/II trial for the treatment of atopic dermatitis were achieved in 2006. All the preclinical and clinical studies demonstrated the potential values of the decoy approach.

Unlike in normal cells and tissues, constitutively activated STAT3 has been observed in a wide variety of human cancer cell lines and primary tumors. As demonstrated in the present study, STAT3 was constitutively activated in human lung cancer cell lines (Fig. 1), and up-regulated the genes which improve cell growth and provide protection against apoptosis (Figs. 4 and 5), and the blockade of STAT3 signaling using STAT3-decoy ODN down-regulated target genes such as cyclin D1 and bcl-xl at transcription and translation levels (Figs. 4 and 5). These results suggest that STAT3 may be an ideal target for lung cancer therapy and STAT3-decoy ODN may be a good therapeutic candidate as a small bio-molecule.

Acknowledgements

This study was supported by Natural Science Foundation of China (no. 30628014; no. 30571696) and National 973 Science Program by Ministry of Science and Technology of China (no. 2004CB518807)

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