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Inactivation of STAT3 by shRNA Suppresses Cell Growth and Migration in EBV-positive Nasopharyngeal Carcinoma Cells

[STAT3'ün shRNA ile İnaktivasyonu EBV-Pozitif Nazofarengiyal Karsinoma Hücrelerinde Hücre Büyümesi ve Migrasyonunu Baskılar]*

Jiali Wang[1],
Yan Zhang[1],
Jianjun Xiong[1],
Xian ping Shi[1],
Guang Yang[2],
Zhenyu Zhu[2]

[1]Department of Biochemistry and Molecular Biology, Zhongshan Medical College of Sun Yat-sen University, 2 Zhongshan Road, Guangzhou 510080, China
[2]DaAn Gene Co., Ltd.of Sun Yat-sen University, Xiangshan Road, Guangzhou, China

Yazışma Adresi
[Correspondence Address]

Zhenyu Zhu

DaAn Gene Co., Ltd.of Sun Yat-sen University, Xiangshan Road, Guangzhou, China
Tel: 86-20-87334631
E-mail:zhenyuzhu_11@yahoo.cn

Translated by
*[Çevirmen]

Ebru Bodur

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ABSTRACT

Rationale: Signal transducer and activator of transcription 3 (STAT3) is confirmed to be constitutively activated in many human cancers, and plays an important role in tumorigenesis, and accumulating evidence has shown that STAT3 may play an important role in the development of nasopharyngeal carcinoma.

Objective: determination of the STAT3 activation in EBV-positive nasopharyngeal carcinoma cell, and clarifying the role of STAT3 signalling in cell growth and migration using RNA interference (RNAi).

Methods: we constructed a short hairpin RNA (shRNA) expression vector specific for STAT3 and detected the expression of STAT3 by RT-PCR and Western blot. The cell cycle and apoptosis was analyzed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltertrazolium bromide (MTT) and Hoechst 33342.

Findings: we demonstrated that STAT3 is constitutively activated in EBV-positive nasopharyngeal carcinoma cell lines, and demonstrated that blockade of STAT3 activation induced apoptosis and inhibited migration of EBV-positive nasopharyngeal carcinoma cells. We also analyzed that knockdown of STAT3 can decrease the expression of STAT3-targeted genes including bcl-2, surviving both at mRNA and protein levels.

Conclusions: taken together, we suggested that targeting STAT3 signalling using RNAi technique may serve as a novel therapeutic strategy for treatment of EBV-positive nasopharyngeal carcinoma.

Keywords: Signal transducer and activator of transcription 3 (STAT3), RNA interference, CNE-2, bcl-2, survivin

ÖZET

Amaç: Sinyal uyarıcı ve transkripsiyon aktivatörü 3'ün (STAT3) insanda gözlenen bir çok kanser türünde sürekli etkinliğe sahip olarak tümör gelişiminde önemli rolü bilinmektedir. Son zamanlarda artan bulgular STAT3'ün nazofarengiyal karsinoma gelişiminde de önem taşıdığını göstermektedir. Bu çalışmada Eppstein-Barr virus (EBV) pozitif nazofarengiyal karsinoma hücresinde STAT3 etkinliğinin belirlenmesi ve STAT3 sinyalinin hücre büyümesi ve göçünde oynadığı rol RNA interferansı (RNAi) kullanılarak aydınlatılmaya çalışılmıştır.

Yöntem ve gereçler: STAT3'e özgü kısa saç tokası RNA (shRNA) ekspresyon vektörü geliştirilerek STAT3 ifadesi RT-PCR ve Western Blot ile saptanmıştır. Hücre döngüsü ve apoptoz by 3-(4, 5-dimetiltiyazol-2-yl)-2, 5-difeniltetrazolium bromür (MTT) and Hoechst 33342 kullanılrak incelenmiştir.

Bulgular: STAT3 etkinliğinin EBV pozitif nazofarengiyal karsinoma hücre hatlarında uyarılardan bağımsız halde sürekli olarak aktif olduğunu ve STAT3 aktivasyonunu durdurulmasının apoptoza yol açarak EBV-pozitif karsinoma hücrelerinin göçünü engellediğini gösterdik. Ayrıca STAT3 ifadesinin RNAi aracılı azaltımı ile STAT3 ile hedeflenen bcl-2 ve survivin gibi genlerin hem mRNA hem de protein seviyesinde ifadelerinin azaldığını gözlemledik.

Sonuç: Tüm bu gözlemler ele alınarak RNAi aracılı STAT3 sinyal yolağının hedeflenmesi ile EBV-pozitif karsinoma tedavisinde yeni bir tedavi startejisi geliştirilebileceğini önermekteyiz.

Anahtar kelimeler: Sinyal iletim ve transkripsiyon aktivatörü 3 (STAT3), RNA interferansı, CNE-2, bcl-2, survivin

Introduction

Nasopharyngeal carcinoma (NPC) is a human epithelial tumour with high prevalence in southern China and Southeast Asia [1]. Epstein-Barr virus (EBV) is a ubiquitous human herpes virus related to epithelial infection resulting in the development of NPC. Almost 100% of NPCs are associated with EBV. Because the nasopharynx is poorly accessible and NPC is radiosensitive, radiotherapy is the usual treatment. Current management by curative radiation therapy, with or without adjuvant chemotherapy, attains a five-year survival rate of around 65% [2], indicating that novel therapeutic strategies to combat this disease are urgently needed.

Signal transducer and activator of transcription 3 (STAT3) protein is a member of a family of latent cytoplasmic transcription factors that transfer signals from cell surface to the nucleus on activation by various cytokines (e.g.interleukin-6,IL-6) and growth factors (e.g. epidermal growth factor, EGF) .Under normal physiological conditions, STAT3 dimerizes and translocates to the nucleus to induce expression of critical genes which are essential in normal physiological cellular events, including cell proliferation, survival and development, and the activation of STAT3 protein is rapid and transient [3, 4]. Constitutively activated STAT3 is found in a wide variety of human tumours including multiple myelomas, breast cancer, lung cancer, leukaemia, gastric cancer, lymphomas, ovarian cancer, prostate carcinoma, and head and neck tumours [5-12], specifically, the observations that STAT3 is frequently aberrantly activated in EBV-associated human cancers, provide a background for exploring its role in the development and progression of NPC.

Accumulating evidence demonstrates two mechanisms by which STAT3 participates in tumorigenesis. Since numerous studies have shown that tumorigenesis is the result of breaking balance between cell cycle progression and apoptosis [13], one route for STAT3 is through up-regulation of genes encoding apoptosis inhibitors (survivin and bcl-xl), cell-cycle regulators (cyclinsD1/D2 and *c*-myc). The other route is promoting migration based on numbers of observations which unambiguously acclaim a critical role for STAT3 in cell motility [14- 17].

A relatively new technique using RNA interference (RNAi) provides a novel approach of experimental inhibition of gene expression.[18] Small interference RNAs (siRNA) are short oligonucleotides of 19-21 nucleotides in length that can be used in vitro to specifically inhibit gene expression. However, to date no studies have investigated the effect of inhibition of STAT3 gene expression by siRNA in nasopharyngeal carcinoma cells.

Here we demonstrate that STAT3 is constitutively activated in EBV-positive nasopharyngeal carcinoma cell lines, and clarified the role of STAT3 signalling in cell growth and migration using RNAi.

Materials and Methods

Plasmid vectors and reagents

The oligonucleotides designed from the website, sense and antisense strands of STAT3 (GenBank: accession no. **NM003150**)-specific or scramble were synthesized and annealed. The STAT3-specific sequence, psiRNA-STAT3 was:5'-*GATCC*GAATCACGCCTTCTACGGATTCAAGAGATCTGTAGAAGGCGTGATTCTTTTTGGAA*A*-3', 5'-*AGCTT*TTCCAAAAAGAATCACGCCTTCTACAGATCTCTTGAATCTGTAGAAGGCGTGATTC*G*-3' and the scramble sequence was 5'-*GATCC*CAACAACCTGAACAACATGTTCAAGAGACATGTTGTTCAGCTGCTGCTTTTTGGAAAA*A*-3',5'-*AGCTT*TTCCAAAAACAACAACCTGAACAACATGTCTCTTGAACATGTTGTTCAGCTGCTGC*G*-3'.Then the annealed DNA oligonucleotides were inserted into the pSilencer2.0-U6 siRNA expression vector(Ambion Inc,Austin,TX) which was digested with BamH *I*and Hind*III* (underlined). So, the hairpin RNA (shRNA) was expressed under the control of the U6 promoter. The pEGFP-C1 (BD) was kept in the laboratory. Cytokine, recombinant human IL-6 (rhIL-6), was purchased from Bio source Int (Camarillo, CA).

Cell culture and transfection

Cell lines including CNE-1, CNE-2 and SUNE-1 derived from NPC tissues were studied, however, CNE-1, well-differentiated, is EBV-negative, and CNE-2, SUNE-1, poorly-differentiated, are EBV-positive.

The human prostate cancer cell line, PC3M, the human lung adenocarcinoma cell line, A549, all the cell lines were cultured in Dullbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 at 37°C. The transfections were carried out by the lipofectamine 2000 (Invitrogen,USA) as described by the manufacturer. In order to observe the efficiency of the transfection, pEGFP-C1 was co-transfected with psiRNA-STAT3 or scramble vector into cells.

RT-PCR analyses

Total RNA that was extracted from cell samples with Trizol (Invitrogen) was reverse-transcribed at 42°C 30min. The resultant cDNA was subjected to PCR amplification using primers for full-STAT3 (5'-atggcccaatggaatcagctac-3' and 5'-tcacatgggggaggtagcgcac-3'), latent membrane protein-1 (LMP-1, 5'-ctaacacaaacacacgctttctac-3' and 5'-gagagcaataatgagcaggatc-3') and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 5'-cgggaaactgtggcgtgat-3' and 5'-caaaggtggaggagtgggt-3') genes. The reaction system was:2μl cDNA template,1μl each of forward primer (10umol/L) and reverse primer (10umol/L), 2μl dNTP (10umol/L), 0.5μl La Taq DNA Polymerase (TaKaRa), 5μl La Taq buffer, and ddH_2O up to 50μl. The cycling conditions were as follows: 94°C for 10 min, followed by 30 cycles at 94°C 30s, 55°C 40s,

72°C 30s, and 72°C for 10 min. The resultant products were separated by gel electrophoresis through a 1.2% agarose gel and stained with ethidium bromide.

Western blot analyses

Cell extracts were prepared and resolved on a 10% SDS-PAGE and samples were transferred onto PVDF membranes (Millipore, Bedford ,MA) and incubated with specific antibodies against STAT3, Bcl-2, Survivin and pTry-STAT3(Santa Cruz, CA) and anti-β-actin antibody (Sigma, USA). The blots were visualized by enhanced chemiluminescence detection system (Amersham). The expression of β-actin was used as a normalization control for protein loading.

MTT assay

CNE-2 cells were seeded in 96-well plates. After 24h transfection, the cells were incubated in growth medium without serum in the presence of rhIL-6. Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT, Sigma). The absorbance values at 570nm (A_{570}) were determined on a multiwell plate reader.

Hoechst 33342 staining

CNE-2 cells grown in 24-well plates were treated as above described. After being washed with PBS three times, cells were stained with 1μM Hoechst 33342 in the dark for 30min at 37°C. The fluorescence images of stained cells were observed under inverted fluorescence microscope (Olympus, Japan).

Cell migration assay

Assay of cell migration into a wound area was performed as previously described [19]. Cells treated as above described were grown to subconfluency on collagen (type-1)-coated culture dishes for 24h. A wound track was then introduced by scraping the cell monolayer with a yellow pipette tip. Then all the cell groups starved for serum were cultured for a further 24h before observed.

Statistical analysis

Statistical analysis was performed using SPSS software (SPSS for windows package release 11.0, SPSS, Chicago). Data were expressed as mean±SD.

Results

STAT3 was constitutively activated in EBV-positive nasopharyngeal carcinoma cell lines

EBV infection in NPC is classified as latency typeⅡin which only a limited set of latent gene expression, e.g. LMP-1, can be detected. RT-PCR analysis revealed that cell lines, CNE-2 and SUNE-1 which expressed LMP-1, were EBV-positive (Fig.1A), whereas cell line CNE-1 is EBV-negative. To assess STAT3 expression in EBV-positive nasopharyngeal carcinoma cells, both the NPC-derived EBV-negative cell line CNE-1 and the EBV-positive cell lines CNE-2 and SUNE-1were used. The human prostate cancer cell line, PC3M, and the human lung adenocarcinoma cell line, A549 were used as positive control. RT-PCR analysis revealed that EBV-positive cell lines were constitutively expressed STAT3 (Fig.1B). As shown in Fig.1C, Western blotting was performed using anti-STAT3 antibody and anti-phospho-specific STAT3 (Tyr705) antibody. STAT3 was constitutively phosphorylated on Tyr705 in EBV-positive cell lines except EBV-negative cell line.

STAT3 siRNA specifically inhibited STAT3 expression in CNE-2 cells

Since STAT3 was only constitutively activated in EBV-positive NPC cell lines, we attempted to determine whether the synthetic STAT3 siRNA could inhibit the expression of STAT3 gene in CNE-2 cells. Treatment of CNE-2 cells with psiRNA-STAT3 resulted in a significant decrease of STAT3 expression at both mRNA (Fig.2A) and protein (Fig.2B) levels compared to the untreated CNE-2 cells and the scramble vector-treated CNE-2 cells, respectively. In the STAT3 siRNA CNE-2 cells, Tyr-STAT3 expression was significantly reduced, indicating that STAT3 siRNA also inhibited the activity of STAT3.

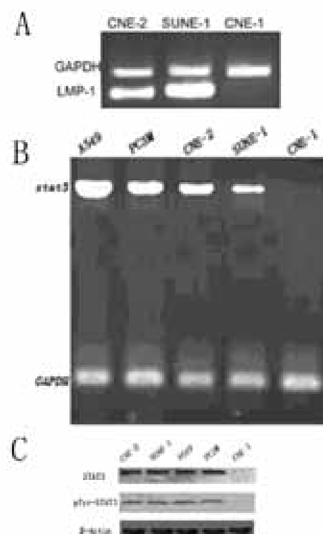

Fig.1. STAT3 was constitutively activated in EBV-positive nasopharyngeal carcinoma cell lines.
A. LMP-1 expression in NPC cell lines. RT-PCR analysis. B. STAT3 expression in various cell lines. All analyses were performed by the RT-PCR assay. C. Western blot analysis of STAT3 and pTyr-STAT3 protein expression in sorts of cell lines.

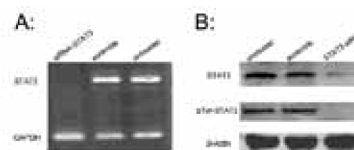

Fig.2 Effect of STAT3 siRNA on STAT3 expression in CNE-2 cells.
A. STAT3 mRNA levels of CNE-2 cells transfected with psiRNA-STAT3 or scramble vector or untreated were determined by RT-PCR.
B. Effect of STAT3 protein expression .Western blots were performed using either antibodies against phosphor-specific STAT3 (Tyr-705) or total STAT3 with whole cell extracts isolated from CNE-2 cells transfected with psiRNA-STAT3 or scramble vector.

STAT3 siRNA inhibited cell growth and induced apoptosis in vitro

As previous studies demonstrated that IL-6 can induce the constitutive activation of STAT3 in cancer cells [19], we next determined the effect of exogenous IL-6 on STAT3 activation in STAT3 siRNA CNE-2 cells, which did not constitutively express STAT3. To examine whether synthetic STAT3 siRNA had an inhibitory effect on CNE-2 cells growths, we accomplished determination of cell proliferation with MTT assay. Table 1 shows that, compared with control groups, treatment of CNE-2 cells with psiRNA-STAT3 led to significant inhibition of CNE-2 cells, and the IL-6 treated STAT3 siRNA CNE-2 cells were only slightly inhibited on proliferation.

Hoechst 33342 was adopted for determination of apoptosis in CNE-2 cells. As shown in Fig.3A, it revealed that a significant higher percentage of apoptotic nuclei were observed in psiRNA-STAT3 treated CNE-2 cells than in control groups. Consistent with this observation, Western blot analysis of CNE-2 cell extracts showed an obvious reduction expression of Bcl-2 and Survivin (Fig.3B) in psiRNA-STAT3 treated cells. These data both indicated that STAT3 siRNA led to a rapid induction of apoptosis.

STAT3 siRNA exhibited an impaired migration in CNE-2 cells

We next sought to identify that STAT3 could influence cell motility in CNE-2 cells. As shown in Fig.3C, we observed that the expression of STAT3 siRNA had an inhibitory effect on the ability of CNE-2 cells to migrate into a wound track. This is consistent with previous reports of STAT3 function in regulating cell motility in cancer cells [16, 20]. We also observed that treated with exogenous IL-6, the deficiency in STAT3 siRNA cells migration into a wound track was partially reversed.

Table 1 Inhibitory effect of STAT3 siRNA on the growth of CNE-2 cells

Group	A_{570}nm	Inhibition rate(%)
Untreated	0.79±0.05	0.0
scramble vector	0.75±0.04	5.06
STAT3 siRNA	0.43±0.02	45.57[a]
STAT3 siRNA +IL-6	0.69±0.03	12.66[b]

After transfected with psiRNA-STAT3 or scramble vector for 24h, the viability of CNE-2 cells was determined by MTT assay. Cell growth inhibition rate was calculated according to the following formula: inhibition rate (%) = $[(A_{570}c-A_{570}e)/A_{570}c]\times100\%$ $A_{570}c$:$A_{570}c$ in control group; $A_{570}e$:$A_{570}c$ in experimental group. Results are an average of quadruplicate experiments and statistical significance was determined compared to the control groups: [a], significantly different at 95% confidence level. [b], not significant at 95%confidence level.

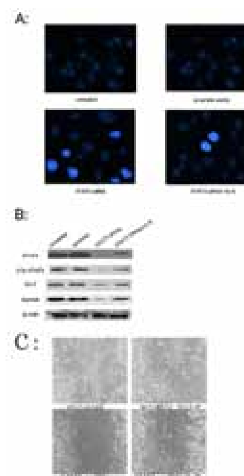

Fig.3. Inactivation of STAT3 expression inhibited proliferation and migration of CNE-2 cells and down-regulated STAT3-targeted genes. After having been transfected with psiRNA-STAT3 or empty vector for 24h. In rhIL-6 treated groups, 20ng/ml IL-6 were incubated for 16h after 24h transfected cells were starved of serum for 4h.A. Cells were stained with Hoechst 33342 dye to visualize apoptotic nuclei (magnification 400×).B. The whole cell extracts were isolated, Bcl-2 and Survivin were then determined by Western blotting, compared with the expression of STAT3 and p-Tyr STAT3. C. Cells were seeded at confluence and subjected to scratch wounding. Wound closure was evaluated and recorded after 24h.The fields in between the grey lines indicate the wound track (magnification 400×).

Discussion

STAT3 is activated by a number of cytokines and growth factors and has diverse functions during embryogenesis and early development [21], and the activation of STAT3 is rapid and transient [6]. Accumulating evidence shows that abnormal activation of STAT3 signalling plays a critical role in oncogenesis [7-15] and constitutive activation of STAT3 was found to be required for de novo epithelial carcinogenesis, by maintaining the survival of DNA-damaged stem cells and mediating the proliferation necessary for clonal expansion of initiated cells during tumour promotion[22- 25]. Disrupting STAT3 signalling in tumours reduced cell viability, caused cell growth arrest and induced apoptosis, so targeting STAT3 protein for therapeutic intervention in cancer may be a potent strategy.

There have been contradictory conclusions about whether STAT3 constitutively activated in NPC in previous studies. Some demonstrated STAT3 was strongly expressed in cancer cells of NPC patients [26, 27], but others acclaimed that STAT3 was negative in some NPC patients [26, 28]. The discrepancy may be explained by the different type of EBV infection of nasopharyngeal epithelial. Since it was reported that EBV infection resulted in the activation of STAT3 signal cascade in

human nasopharyngeal epithelial cells [30], we decided to investigate the expression of STAT3 in EBV-positive NPC cell lines and its role in tumorigenesis.

LMP-1 is essential for the EBV-induced transformation of primary B lymphocytes. The oncogenic properties of LMP-1 are associated with stimulation of DNA synthesis, stimulation of the transcription of anti-apoptotic genes, and suppression of cellular senescence. Expression of LMP-1 itself was shown to be sufficient to induce a higher level of STAT3 expression. In our study, we found that STAT3 is constitutively activated in CNE-2 and SUNE-1 NPC cell lines which are EBV-positive, but is negative in CNE-1 NPC cell line which is EBV-negative (Fig.1). Hence, we chose CNE-2 cells for study. To inhibit the expression of STAT3, we transfected psiRNA-STAT3 into CNE-2 cells. Through RT-PCR and Western blot assays, it was shown that STAT3 siRNA could specifically inhibit STAT3 expression in CNE-2 cells compared to the control groups.

As previous studies demonstrated that interleukin-6 (IL-6) promoted survival and proliferation of tumour cells through phosphorylation of STAT3 [20], we next investigated the cell growth in STAT3 siRNA CNE-2 cells which were treated with exogenous IL-6 or not. Our results showed that STAT3 siRNA efficiently reduced cell growth in vitro, and significantly induced apoptosis, however, the rhIL-6 treated STAT3 siRNA CNE-2 cells had slight change with no significance, indicating that exogenous IL-6 could partially rescue the deficiency function through promoting activation and phosphorylation of STAT3. As shown in Fig.3, these results suggested that efficient inhibition of STAT3 could suppress cell growth, induce apoptosis, and exogenous stimulating factors can partially attenuate the effect. We are the first to report that siRNA can specifically inhibit STAT3 in EBV-positive NPC cell lines.

One mechanism by which STAT3 participates in tumorigenesis is by inducing proliferation and inhibiting apoptosis through controlling the targeted-genes. In NPC cells, the apoptosis process may be interfered by multiple genetic changes. Over expression of bcl-2 and inactivation of p53 pathway is believed to be the major mechanisms for the reduction in apoptosis in cancers. The correlation between p53 expression and histological type, stage, age and sex distributions of NPC was tested in previous studies. After statistical analysis, no significant difference was demonstrated [29]. Since the association between NPC and p53 is not clear, in this study we just analyzed the expression of Bcl-2. Survivin expression is detected in a number of cancers, and is implicated in cell cycle regulation and inhibition of apoptosis, recent investigations also have highlighted survivin as a downstream target of STAT3 signalling [8, 30]. We found that both anti-apoptotic genes bcl-2 and survivin were down-regulated in STAT3 siRNA treated CNE-2 cells. The other mechanism for STAT3 is inducing migration. From our study, we observed that STAT3 siRNA treated CNE-2 cells had little ability to migrate into a wound track, indicating the critical role of STAT3 in invasion of EBV-positive NPC. It was used to believe that the mechanisms by which STAT3 imparted its function had been attributed almost to its tyrosine phosphorylation-mediated transcription. Some evidence has also been presented for non-tyrosine-phosphorylated STAT3 as a transcriptional regulator [31]. Recently, Ng et al [17] provided evidence that non-tyrosine-phosphorylated, nontranscriptional STAT3 can stabilize the polymerization of microtubules by disrupting stathmin-tubulin interactions. The mechanism remains to be fully explored. Further studies are also needed to clarify the cause of constitutive activation of STAT3 signalling in EBV-positive NPC cells.

Here, we have identified the role of STAT3 in EBV-positive NPC cell lines. Considering that STAT3 is constitutively activated in EBV-positive NPC cell lines, our results suggest that specifically inhibiting STAT3 expression could inhibit cell proliferation, induce cell apoptosis and migration. It is the first time that RNAi is used to inhibit activation of STAT3 to suppress cell growth and migration in EBV-positive NPC cell lines. Therefore, it provides a novel therapeutic strategy for EBV-positive NPC by targeting STAT3.

Acknowledgments

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