Int J Clin Exp Med 2014;7(8):2096-2099
www.ijcem.com /ISSN:1940-5901/IJCEM0001036

Original Article

Suppression of EGFR-STAT3 signaling inhibits tumorigenesis in a lung cancer cell line

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Received June 7, 2014; Accepted July 11, 2014; Epub August 15, 2014; Published August 30, 2014

Abstract: Overactive epidermal growth factor receptor (EGFR) signaling often underlies the rapid expansion of cancerous tissue. EGFR signaling is mediated by transcription factor signal transducer and activator of transcription 3, or STAT3. This study sought to investigate the effects of altered EGFR/STAT3 signal transduction on lung cancer cells in vitro. Lung cancer cells from the cell line A549 were divided into test and control groups. Test group cells were treated with an EGFR monoclonal antibody, Nimotuzumab, while control cells received no treatment. EGFR and STAT3 protein expression, cell apoptosis rate, cell proliferation, cell invasion, and cell division were analyzed and compared. Compared to cells in the control group, lung cancer cells treated with Nimotuzumab showed slowed proliferation rates, accelerated apoptosis, decreased invasion, and arrested cell division ($P < 0.05$). In conclusion, altered EGFR/STAT3 signaling results in significant changes in the biology of lung cancer cells.

Keywords: EGFR/STAT3, signal transduction, lung cancer

Introduction

Epidermal growth factor receptor (EGFR) is a multifunctional membrane glycoprotein found in a variety of tissue types. Studies have shown that overexpression of EGFR can lead to excessive cell growth and malignancy [1, 2]. Signal transducers and activators of transcription (STATs) are transcription factors activated by peptide ligands-such as cytokines and growth factors-including EGF. Recent studies provide evidence that constitutively activated STAT proteins are present in many tumor cells and cancer tissues, wherein STAT3 is the most active transcription factor [3]. Through its role as a nuclear transcription factor, STAT3 can become phosphorylated by tyrosine or serine kinases under the control of cytokines or growth factors, and then interact with the SH2 domain of signaling proteins to form homo- or heterodimers. These homo- or heterodimers then enter the nucleus and bind to a target DNA segment to regulate the transcription of genes affect cell proliferation, transformation, and apoptosis [4]. In addition, through the JAK-STAT pathway, EGFR-mediated signals can be transduced into the nuclei to promote metabolism, proliferation, and migration of cells invasculogenesis [5, 6]. In this study, A549 lung cancer cells were treated with an anti-EGFR monoclonal antibody to investigate the feasibility of such a treatment for patients with lung cancer.

Materials and methods

Experimental cell line

Human lung adenocarcinoma cell line A549 (Shanghai Institute for Biological Sciences Cell Bank, Chinese Academy of Sciences) was cultured in RPMI1640 medium containing 10% calf serum, with added penicillin (100 kU/L) and streptomycin (0.1 g/L), placed at 37°C, cultured in a 5% CO_2 incubator (Forma, Waltham, MA, USA), and digested with 2.5 g/L trypsin for passage.

A549 cells were seeded in 24-well plates ($3X10^4$ cells per well) for 72 hours. In the experimental group, the medium contained 50 μg/mL anti-EGFR monoclonal antibodies (Nimotuzumab, Baitai Biological Pharmaceutical Co., Ltd.), while control cells were treated with regular medium.

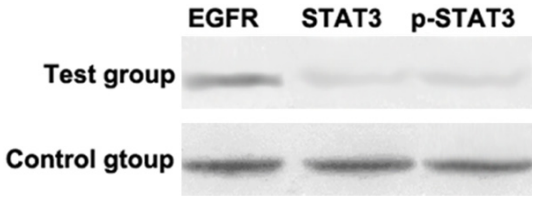

Figure 1. EGFR, STAT3, p-STAT3 protein expression.

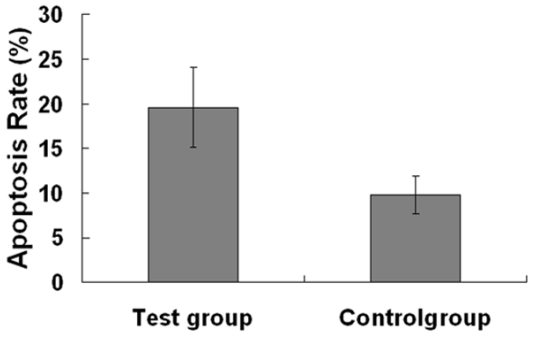

Figure 2. Cell apoptosis rate.

Expression of EGFR and STAT3

After 72 hours of incubation, protein was extracted from A549 cells and its concentrations was detected by using the Bradford method. 100 μg of protein was separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes using semidry electroblotting. Membranes were blocked overnight with TBST containing 5% nonfat milk powder at 4°C in a refrigerator. Membranes were subsequently incubated with primary antibodies (with EGFR antibody diluted at 1:500, STAT3 antibody at 1:1000, and p-STAT3 antibody at 1:1000 (Maixin Biotechnology Development Col. Ltd, Fuzhou, China) and secondary antibodies [biotin-labeled goat anti-mouse IgG (H+L), 1:1000]. Staining was developed with DAB (3,3'-diaminobenzidine, Sigma) or developed and fixed by ECL (Enhanced chemiluminescence, Invitrogen) after being washed.

Apoptosis detection

A549 cells cultured for 72 hours were trypsinized, centrifuged at 1000 rpm for 5 minutes, and washed with PBS. A buffer solution was added to suspend the cells and adjust their concentration to $2x10^5$ to $5x10^5$ cells/mL. 195 μL of the suspension was added to 5 μL of Annexin V-FITC reagent (Bender Med Systems in Vienna, Austria), and the mixture was incubated for 10 minutes in a dark chamber. Cells were then washed and suspended using 195 μL of binding solution, then 10 μL of 20 μg/mL propidium iodide (PI, Sigma) was added. The number of apoptotic cells was measured by a flow cytometer (BD in Franklin Lakes, USA).

Detection of cell proliferation

A549 cells that had been cultured for 72 hours were added to 20 L of 10 g/L MTT and cultured again for 4 hours. The culture solution was removed and treated with 200 μL of DMSO, then shaken and dissolved for 10minutes. Finally, a microplate reader (Thermo Fisher Scientific Inc., USA) was used to measure the absorbance value (A value) to calculate the inhibition ratio [Inhibition ratio = (A value of the negative control-A value of the experimental group) / A value of the negative control x 100%].

Cell invasion capacity

Matrigel (Becton Dickinson and Company) stored at -20°C was placed at 4°C to thaw, and then diluted with serum-free RPMI 1640 medium at 1:8. Next, the dilution (60 μL per pore) was evenly laid on the inner surface of polycarbonate membranes (8 μm in pore size) of a Transwell chamber, and dried at 37°C for 4 hours to form a mock matrix layer. After the layer was formed, the cells were digested and prepared into a $3x10^5$/mL cell suspension using RPMI1640 medium containing 10% BSA. 200 μL of the suspension from each group was added to the upper part of the Transwell chamber, and 600 μL of RPMI1640 medium containing 10% FBS was added to the lower part. Each group contained 3 pores. After conventional culture for 24 hours, the cells on the inner surface of polycarbonate membranes were wiped with a cotton swab. Then the membranes were washed and dried, and stained with 0.1% crystal violet for 30 minutes. Next, cells on the outer surface of polycarbonate membranes were observed with a microscope, and the number of penetrating cells in 5 fields of vision was counted randomly and used to represent the in vitro invasion capacity of lung adenocarcinoma A549 cells.

Cell cycle analysis

A549 cells cultured for 72 hours were fixed with 70% ethanol, stained with propidium iodide (PI), and filtered through a 180-mesh nylon net. A flow cytometer (BD, USA) was used to analyze

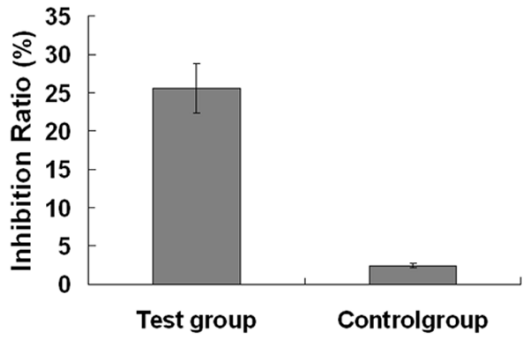

Figure 3. Cell proliferation.

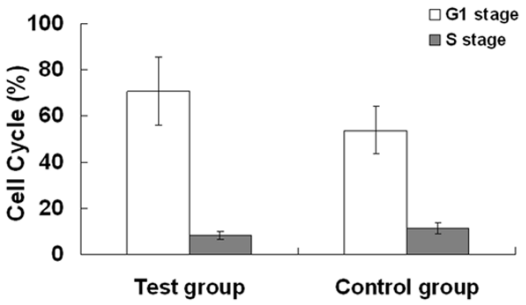

Figure 4. Percentage of cells in G1 and S phases.

the cell cycle; Cell Quest software (BD, USA) was used to obtain the data.

Statistical methods

SPSS17.0 (Statistical Program for Social Sciences, SPSS Inc, Chicago, USA) was used to perform statistical analysis. Independent samples t-test was used to compare data from the two groups. The test was two-tailed, with α=0.05 denoting a significance level, and $P < 0.05$ indicating that the difference was statistically significant.

Results

Western blot analysis showed that EGFR, STAT3, and p-STAT3 were all expressed in the control group, while in the experimental group, EGFR was expressed, but STAT3 and p-STAT3 were not (**Figure 1**). Flow cytometry showed that the apoptosis rate in the experimental group was (19.6±4.5)%, while the apoptosis rate in the control group was (9.8±2.1)%. The difference between the two groups was statistically significant (P < 0.05, **Figure 2**). Transwell chamber analysis showed that the inhibition rate in the experimental group was (25.6±3.4)%, while the inhibition rate in the control group was (2.4±0.3)%. The difference was statistically significant (P < 0.05, **Figure 3**). MTT detection showed that the number of cells that penetrated through the polycarbonate membranes in the experimental group was (96.2±19.5), versus (32.4±6.4) in the control group. The difference was statistically significant (P < 0.05). Flow cytometry showed that in the experimental group, cells in theG1 and S phases accounted for (70.6±14.7)% and (8.2±1.7)% of cells, respectively. In the control group, cells in the G1 and S phases accounted for (53.8±10.3)% and (11.3±2.6)% of cells, respectively. The differences in the proportions of cells in G1 and S phases between groups were statistically significant (P < 0.05, **Figure 4**).

Discussion

In recent years, the incidence of lung cancer has risen steadily and now ranks among the most common tumors across Europe, North America, and China. The global death rate due to lung cancer exceeds 1 million, and every year, the number of new cases increases by 1.2 million [7]. There are two types of lung cancers: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), wherein NSCLC accounts for approximately 85% of cases, and is further divided into squamous cell carcinoma, adenocarcinoma, large cell carcinoma, and several other types of carcinoma [8]. Adenocarcinoma has recently become the most commonly diagnosed type of lung cancer [9]. The treatment for lung cancer is primarily based on the stage of the cancer and the physical condition of patient. Surgical resection is the main treatment for patients with early-stage (stages I and II) lung cancer [10]. However, many patients present with locally advanced (stage III) and metastatic (stage IV) lung cancer. For these patients, chemotherapy is the preferred treatment [11]. Despite its success, chemotherapy carries major toxicity, especially for elderly patients and patients with poor physical state, whose overall 5-year survival rate is lower than 20% [12]. Therefore, additional treatment options are needed to effectively treat lung cancer. Recent research has focused on molecularly targeted drugs with the potential to suppress cancer cell growth.

EGFR is overexpressed in most NSCLCs and is an important target in lung cancer treatment. EGFR is a member of the EFR-related family of

tyrosine kinase receptors, whose ligand binding can promote the formation of dimers, which then activate tyrosine kinases in the EGFR cytoplasmic domain and enhance downstream signals affecting gene transcription. This process involves the Ras-Raf-MAPK, PI3K-AKT, and STAT pathways. Activation of the EGFR signal transduction pathway can promote metabolism, proliferation, and migration of cells and vasculogenesis, while inhibiting apoptosis [13-15]. Anti-EGFR monoclonal antibody (Nimotuzumab) is a humanized monoclonal antibody that binds to epitopes in the extracellular domain III of EGFR and competitively inhibits ligand binding to EGFR. This inactivates the receptors and blocks the EGFR-mediated downstream signal transduction pathway and associated cytological effects, thus inhibiting the proliferation of tumor cells, promoting the apoptosis of tumor cells, inhibiting tumor vasculogenesis, and inhibiting the infiltration and metastasis of tumor cells [16, 17].

This study shows that, after treatment with anti-EGFR monoclonal antibodies, lung cancer cells did not express STAT3 or p-STAT3 and showed slowed proliferation, accelerated apoptosis, weakened invasiveness, and arrested cell cycles. These results implicate a role for EGFR-STAT3 signaling in the proliferation of lung cancer cells, and suggest the use of anti-EGFR monoclonal antibodies in the clinical treatment of lung cancer.

Disclosure of conflict of interest

None.

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References

[1] Prigent SA, Lemoine NR. The type 1 (EGFR-related) family of growth factor receptors and their ligands. Prog Growth Factor Res 1992; 4: 1-24.

[2] Nicholson RI, Gee JM, Harper ME. EGFR and cancer prognosis. Eur J Cancer 2001; 37: 9-15.

[3] Hoey T, Schindler U. STAT structure and function in signaling. Curr Opin Genet Dev 1998; 8: 582-587.

[4] Giraud AS, Menheniott TR, Judd LM. Targeting STAT3 in gastric cancer. Expert Opin Ther Targets 2012; 16: 889-901.

[5] Chan KS, Carbajal S, Kiguchi K, Clifford J, Sano S, Digiovanni J. Epidermal growth factor receptor-mediated activation of Stat3 during multistage skin carcinogenesis. Cancer Res 2004; 64: 2382-2389.

[6] Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. Epidermal growth factor receptor: mechanisms of activation and signaling. Exp Cell Res 2003; 284: 31-53.

[7] Juergens RA, Brahmer JR. Adjuvant treatment in non-small cell lung cancer: Where are we now? J Natl Compr Canc Netw 2006; 4: 595-600.

[8] Brambilla E, Travis WD, Colby TV, Corrin B, Shimosato Y. The New World Health Organization Classification of lung tumors. Eur Respir J 2001; 18: 1059-1068.

[9] Smith RA, Glynn TJ. Epidemiology of lung cancer. Radiol Clin North Am 2000; 38: 453-470.

[10] Miller JD, Gorenstein LA, Patterson GA. Staging: the key to rational management of lung cancer. Ann Thorac Surg 1992; 53: 170-178.

[11] Fossella FV, DeVore R, Kerr RN, Crawford J, Natale RR, Dunphy F, Kalman L, Miller V, Lee JS, Moore M, Gandara D, Karp D, Vokes E, Kris M, Kim Y, Gamza F, Hammershaimb L. Randomized phase III trial of docetaxel versus vinorelbine or ifosfamide in patients with advanced non-small-cell lung cancer previously treated with platinum-containing chemotherapy regimens. The TAX 320 Non-Small Cell Lung Cancer Study Group. J Clin Oncol 2000; 18: 2354-2362.

[12] Herrera B, van Dinther M, Ten DP, Inman GJ. Autocrine bonemorphogenetic protein-9 signals through activin receptor-likekinase-2/Smad1/Smad4 to promote ovarian cancer cell proliferation. Cancer Res 2009; 69: 9254-9262.

[13] Scagliotti GV, Selvaggi G, Novello S, Hirsch FR. The biology of epidermal growth factor receptor in lung cancer. Clin Cancer Res 2004; 10: 4227-4232.

[14] Ford AC, Grandis JR. Targeting epidermal growth factor receptor in head and neck cancer. Head Neek 2003; 25: 67-73.

[15] Arteaga CL. Epidermal growth factor receptor dependence in human tumors: more than just expression? Onclogist 2002; 7: 31-39.

[16] Jean GW, Shah SR. Epidermal growth factor receptor monoclonal antibodies for the treatment of metastatic colorectal cancer. Pharmacotherapy 2008; 28: 742-754.

[17] Tiseo M, Loprevite M, Ardizzoni A. Epidermal growth factor receptor inhibitors: a new prospective in the treatment of lung cancer. Curr Med Chem Anticancer Agents 2004; 4: 139-148.