Original Article
c-Myc silencing impedes cell proliferation and enhances cytotoxicity of cisplatin in non-small cell lung cancer

Xiang Liu[1*], Chen Wu[2*], Yanhu Wu[1], Yihu Tang[1], Jin Du[1]

[1]Department of Cardiothoracic Surgery, The First Affiliated Hospital with Nanjing Medical University, Nanjing 210029, China; [2]Department of Oncology and Tumor Biological Treatment, The Third Affiliated Hospital of Soochow University, Changzhou 213003, China. *Equal contributors.

Abstract: We investigated the expression of c-Myc in non-small cell lung cancer (NSCLC) tissues and explored the effect of c-Myc silencing on cell proliferation and cisplatin cytotoxicity. mRNA expression levels of c-Myc in 84 NSCLC tissue samples and corresponding para-cancerous tissues were detected by real-time PCR. c-Myc small interfering RNA (siRNA) was transfected into NSCLC H1299 cells by Lipofectamine 2000. Real-time PCR was carried out to validate the transfection efficiency. Cell Counting Kit-8 was used to evaluate cell proliferation. Cisplatin toxicity experiments using CCK-8 assays showed cytotoxicity of cisplatin plus c-Myc gene silencing in NSCLC cells. The c-Myc mRNA level in NSCLC tissues was significantly higher than that in corresponding para-cancerous tissues (P < 0.05). c-Myc siRNA was successfully transfected into H1299 cells. The proliferation rates of H1299 cells after c-Myc silencing at 24, 48 and 72 h were lower than those of control groups (P < 0.05). The combination of c-Myc silencing and cisplatin had the most cytotoxic effect on H1299 cells compared with individual and control groups (P < 0.05). c-Myc may act as an oncogene in NSCLC and could be a potential target for NSCLC gene therapy.

Keywords: c-Myc, non-small cell lung cancer, cell proliferation, cisplatin

Introduction

Lung cancer is the most common cancer and the leading cause of cancer mortality worldwide for both men and women. At diagnosis, most patients will be at a late stage and have a very low 5-year relative survival rate, even with current standard therapies [1]. More than 80% of lung cancer cases are non-small cell lung carcinoma (NSCLC) that includes adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and NSCLC that cannot be classified further [2]. Treatment of NSCLC depends on the tumor stage and the patient's overall condition. Patients with early stage disease are generally treated with curative intent by surgery, chemotherapy, radiation therapy, or a combined modality approach. Unfortunately, lung cancer is usually metastatic at the time of diagnosis, and thus requires systemic therapy rather than local.

Much attention has been paid to gene therapy of NSCLC in recent years. Realization, analysis, and integration of various genes related to the development of NSCLC are the foundation for NSCLC gene therapy. The c-Myc gene is an important member of the myc gene family, which is related to the development of a variety of tumor types. Amplification or overexpression of the c-Myc gene is found in stomach cancer, breast cancer, colon cancer, cervical cancer, Hodgkin's disease, and head tumors [3, 4]. In recent years, some studies have found abnormal expression of the c-Myc gene in NSCLC [5, 6]. Our study further confirmed abnormal expression of the c-Myc gene in NSCLC and explored the possible prospects of c-Myc gene silencing for NSCLC gene therapy.

Materials and methods

Patients and tissue samples

The study was performed using 84 randomly selected NSCLC patients (59 adenocarcinoma and 25 squamous cell carcinoma). The patients were treated at The First Affiliated Hospital with

Table 1. Relationship of c-Myc expression and clinical characteristics of NSCLC patients

Characteristics	Number	Relative mRNA level ($2^{-\Delta\Delta Ct}$)	P value
Age (year)			
≤ 50	13	10.66±6.180	0.6933
> 50	71	12.63±1.816	
Sex			
Male	67	12.53±2.199	0.8236
Female	17	11.52±1.907	
Histology			
A[1]	25	11.87±4.156	0.8692
S[2]	59	12.52±1.874	
Clinical stage			
I	50	13.17±2.601	0.8532
II	26	11.08±2.917	
III	8	11.13±1.848	
IV	0	0	
Metastasis			
Yes	30	10.88±3.490	0.551
No	54	13.13±2.021	

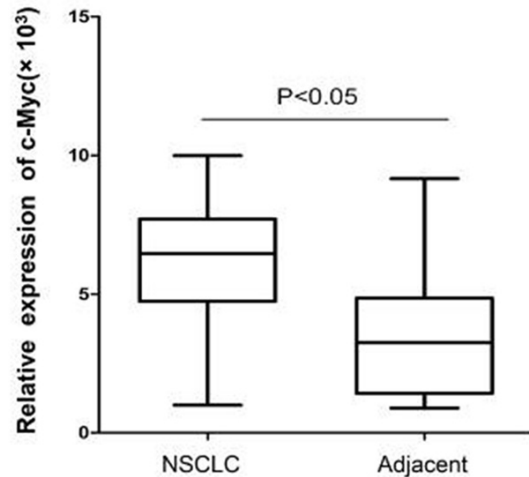Note: 1, adenocarcinoma; 2, squamous cell carcinoma.

Figure 1. mRNA levels of c-Myc in NSCLC and paracancerous tissues. c-Myc mRNA levels were analyzed in NSCLC specimens and corresponding adjacent samples from 84 patients by qRT-PCR. Significantly higher c-Myc RNA levels were found in tumor specimens than in adjacent areas (P < 0.05).

Nanjing Medical University between December 2011 and June 2012. All specimens were collected under a protocol approved by the Human Ethics Committee of Nanjing Medical University. Each patient participated after providing informed consent. Most patients were men (79%). A total of 168 tissue specimens were included in the study with one tumor sample and one corresponding adjacent sample (5 cm or more from the cancer tissue edge and pathologically confirmed as normal lung tissue) from each patient. All specimens were immediately snap frozen in liquid nitrogen and stored at -80°C until RNA extraction. The tumors were histologically classified by two professional pathologists according to the World Health Organization classification (**Table 1**).

Real-time polymerase chain reaction (PCR)

RNA was obtained from snap-frozen tissues and NSCLC cell lines using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. RNA concentrations and qualities were examined by a Beckman Coulter DU800 spectrophotometer (Beckman, Brea, CA, USA). cDNA was synthesized with a Primescript™ RT reagent kit (TaKaRa, Japan). Total RNA (12 µL) mixed with 8 µL Primescript buffer and 20 µL

DEPC-treated water was incubated at 37°C for 15 min and then 85°C for 5 s. cDNA was stored at 4°C until use.

The ABI Prism7900 Sequence Detector System (ABI, USA) and $2^{-\Delta\Delta Ct}$ method were employed to determine the relative levels of mRNAs in tumor and adjacent tissues. Quantitative real-time PCR (qRT-PCR) analysis of c-Myc and β-actin was performed with SYBR Premix ExTaq™ (TaKaRa) according to the manufacturer's instructions. PCR was performed in a final volume of 20 µl using 10 µl of 2× Premix buffer, 0.4 µl of each primer, 2 µl of sample, and 7.2 µl distilled water. The PCR conditions were 50°C for 5 min, 95°C for 10 min and then 45 cycles at 95°C for 15 sec and 60°C for 2 min. The following primers were used: c-Myc forward primer, 5'-GGCTCCTGGCAAAAGGTCA-3' and c-Myc reverse primer, 5'-AGTTGTGCTGATGTGTGGAGA-3'; β-actin forward primer, 5'-GTGCCCATCT-ACGAGGGGTATGC-3' and β-actin reverse primer, 5'-GGTACATGGTGGTGCCGCCAGACA-3'. Fluorescence detection was performed at the end of the extension phase. To discriminate specific and nonspecific cDNA products, a melting curve was obtained at the end of each run.

Cell culture and small interfering RNA (siRNA) transfection

The H1299 cell line (American Type Culture Collection) was employed in the present study.
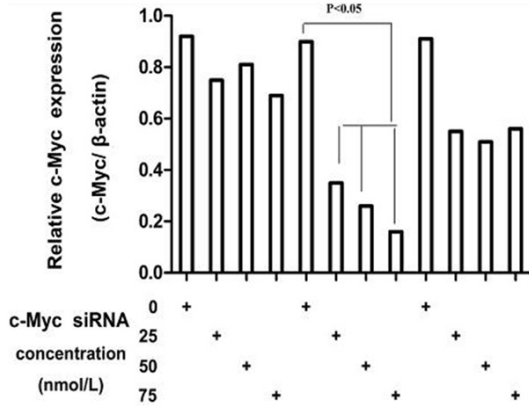
Figure 2. qRT-PCR analysis of c-Myc mRNA down-regulation by c-Myc siRNA in H1299 cells. H1299 cells were successfully transfected with c-Myc siRNA. After 48 h, expression of c-Myc RNA in H1299 cells was significantly inhibited and proportional to the concentration of c-Myc siRNA. The inhibition disappeared after about 72 h.

Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) in a humidified incubator at 37°C with 5% CO_2. For c-Myc silencing, H1299 cells were seeded into 6-well plates ($1.5×10^5$ cells/well) and incubated for 24 h. The H1299 cells were then transfected with 25, 50 and 75 nmol/L Signal Silenc™ c-Myc siRNA (#6341, Cell Signaling Technology, USA) or Signal Silenc™ control siRNA (#6568, Cell Signaling Technology) using Lipofectamine (lip) 2000 reagent (Invitrogen, USA) following the manufacturers' protocols. After 6 h of incubation, the siRNA and Lipofectamine 2000 solutions were gently mixed and added to the cells. The cells were incubated in fresh complete medium until analysis. Non-specific control siRNA was used as a negative control. To evaluate the effects of siRNA on gene silencing, transfections ($5×10^5$ cells/well) were performed in 6-well plates for 24-72 h, and then mRNA expression was determined by qRT-PCR.

Cell proliferation assay

H1299 cells were seeded in 96-well plates ($2×10^3$ cells/well) and transfected with 50 nmol/L c-Myc siRNA. The cells were divided into five groups including blank, control, lip, control siRNA, and c-Myc siRNA. Cell viability was assessed by Cell Counting Kit-8 (CCK-8) assays (Beyotime Institute of Biotechnology, China). Cells were subjected to CCK-8 assays before transfection and at 0, 24, 36, 48, 60 and 72 h after transfection. After 4 h of incubation in CCK-8 solution, the optical density (OD) of each well was read on a spectrophotometer (Thermo, Pittsburgh, PA, USA) at 450 nm (A450). The following formula was used to analyze the results: cell proliferation rate = (the average value in the experimental group - that in the control group)/that in the control group × 100%. Three independent experiments were performed in quintuplicate.

Drug toxicity test

H1299 cells were seeded in 96-well plates ($4×10^3$ cells/well). After 24 h, the cells were transfected with 50 nmol/L c-Myc siRNA and/or treated with 5 µg/ml cisplatin (cis). Cells were divided into six groups including blank+cis, control, control+cis, lip+cis, control siRNA+cis, and c-Myc siRNA+cis. Cell viability was assessed by CCK-8 assays as described above.

Statistical analysis

A paired t-test was employed to analyze the expression levels of c-Myc mRNA. One-way analysis of variance was applied to the results of cell proliferation and cisplatin cytotoxicity experiments. All analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation (SD). P < 0.05 was considered as statistically significant.

Results

c-Myc mRNA expression in NSCLC tissues is higher than that in corresponding adjacent lung tissues

c-Myc mRNA levels were analyzed in NSCLC specimens and corresponding adjacent samples from 84 patients by qRT-PCR. Significantly higher c-Myc mRNA levels were detected in tumor specimens than in adjacent areas (P < 0.05) (**Figure 1**; **Table 1**). Moreover, the of c-Myc mRNA expression in NSCLC tissues ($6.28×10^3$) was 1.77-fold higher than that in adjacent tissues ($3.54×10^3$) and had no correlation to sex, age, histological type, clinical stage, or lymph node metastasis.

Transfection of c-Myc siRNA into NSCLC H1299 cells

H1299 cells were successfully transfected with c-Myc siRNA (**Figure 2**). After 48 h, expression
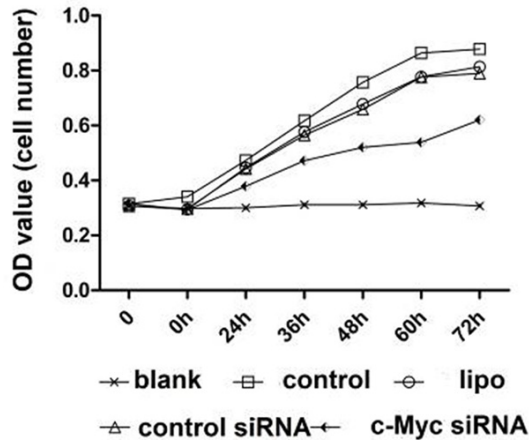
Figure 3. Effect of c-Myc silencing on H1299 cell proliferation. The proliferation rates of H1299 cells after transfection with c-Myc siRNA at 48 and 72 h were significantly lower than those of cells without transfection ($P < 0.05$). There were no significant differences in control, lip, or control siRNA groups at each time point.
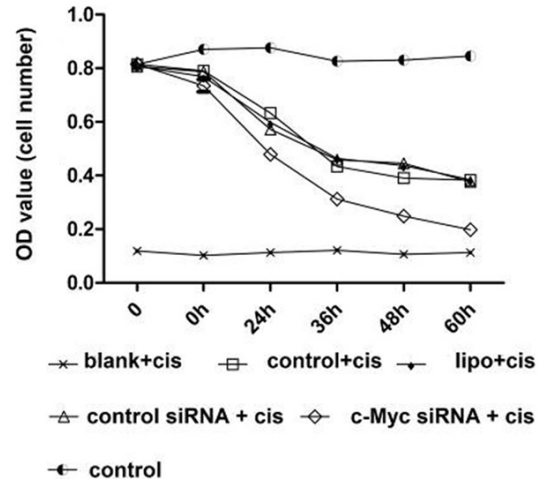

Figure 4. Effect of c-Myc silencing on cisplatin cytotoxicity. Results of drug toxicity testing showed that the cytotoxicity of cisplatin and c-Myc siRNA+cisplatin groups was significantly higher than that of the control group at 60 h after c-Myc siRNA transfection ($P < 0.05$). Cytotoxicity of the c-Myc siRNA+cisplatin group was significantly higher than that of the cisplatin group ($P < 0.05$).

of c-Myc mRNA in H1299 cells was significantly inhibited and proportional to the concentration of c-Myc siRNA. The inhibition disappeared after about 72 h.

C-Myc silencing suppresses the proliferation of H1299 cells

The proliferation rates of H1299 cells after transfection with c-Myc siRNA for 48 and 72 h were significantly lower than those of cells without transfection ($P < 0.05$) (**Figure 3**). There were no significant differences in control, lip, and control siRNA groups at each time point.

Combined c-Myc silencing and cisplatin treatment have the most cytotoxic effect on H1299 cells

Results of drug toxicity testing showed that the cytotoxicity of cisplatin and c-Myc siRNA+cisplatin groups was significantly higher than that of the control group at 60 h after c-Myc siRNA transfection ($P < 0.05$) (**Figure 4**). Cytotoxicity of the c-Myc siRNA+cisplatin group was significantly higher than that of the cisplatin group ($P < 0.05$).

Discussion

Although the c-Myc gene has been investigated in recent studies, its specific function in NSCLC

remains unclear. In this study, we found that the mRNA level of c-Myc in NSCLC specimens was markedly higher than that in adjacent tissues. c-Myc gene silencing significantly suppressed the proliferation of H1299 cells in vitro. The effect of c-Myc gene silencing was comparable to that of cisplatin. In particular, c-Myc silencing combined with cisplatin had the strongest cytotoxicity. Therefore, c-Myc might play an important oncogenic role in NSCLC and may be a new target for NSCLC gene therapy.

With the development of molecular genetics and gene transfer technology, gene therapy to change the genetic structure of cells for therapeutic purposes has made significant progress and gradually proceeded to clinical application. The occurrence and development of NSCLC are a multistep process that involves multiple genes, including activating mutations and inactivation of tumor suppressor genes. Oncogenes are involved in initiation, development, and prognosis of each tumor stage [7, 8]. The genetic characteristics of NSCLC has been reviewed previously, and more than 50 genomic regions are frequently added or deleted in human lung cancer [9].

However, so far, the vast majority of gene therapy strategies for cancer, including NSCLC,

remain in the conception and laboratory stages. Few gene therapies have been tested in clinical trials. Strategies for gene therapy have mainly included replacement of tumor suppressor genes, antisense therapy, immunotherapy approaches, and suicide gene therapy [10].

Replacement of tumor suppressor genes involves the use of a gene therapy vector to encode a tumor suppressor gene that is absent or mutated in the cancer. The mechanisms include mutation, deletion, methylation, or their combination. Theoretically, gene therapy for NSCLC by replacement of a tumor suppressor would lead to inhibition of tumor growth or increase tumor cell mortality in vivo [10]. Currently, the tumor suppressor gene p53 is the most widely studied, and its mutation is at the highest frequency among genetic alterations in lung cancer. p53 gene mutations are detected in 45-75% of NSCLC cases [11, 12].

A wide variety of molecules can promote tumor growth, and reducing these growth factors can significantly inhibit the growth of tumor cells. It is rational to use anti-sense therapy for NSCLC. For example, experience with drugs targeting vascular endothelial growth factor (VEGF) and epidermal growth factor receptor is summarized elsewhere [13, 14]. In recent years, research of the molecular biology of tumors has made great progress. Researchers can design and synthesize certain materials for the development of tumor pathways that regulate tumor cell growth, proliferation, foreign invasion, and angiogenesis [15].

Tumor cells can synthesize certain proteins that can enhance the innate immune system to recognize and kill the tumor cells, which is the theoretical basis of immunotherapy [16]. The mechanism of suicide gene therapy is that genes encoding certain enzymes are transfected into tumor cells to increase sensitivity to drug treatment [17].

The c-Myc gene is an important member of the MYC gene family. MYC proto-oncogenes include N-Myc, c-Myc, and L-Myc [18]. The c-Myc gene is a transposal gene that is not only regulated by a variety of substances, but also promotes cell division and decreases cell mortality, leading to cell longevity [19, 20]. The protein function of c-Myc located at chromosome 8q24 is related to the regulation of various processes, including tumor cell growth, proliferation, differentiation, and apoptosis. Overexpression of c-Myc protein promotes tumorigenesis [21].

In recent years, an increasing number of researchers are becoming concerned about "driver mutations" in cancer therapy, because the success of targeted cancer therapy depends on the presence of driver mutations. Epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) are two important driver mutations in lung cancer therapy [22]. EGFR is the positive choice and causes tumorigenesis. It can provide advantages in terms of the growth, proliferation, and transformation of cells, and drives normal cells toward cancer cells [23]. Recently, some findings have suggested that c-Myc might be involved in the progression of early stage lung adenocarcinoma, especially in conjunction with EGFR and ALK pathways [24].

High expression of the c-Myc gene is closely related to increases in the density of vascular and lymphatic vessels. The ability of c-Myc to induce angiogenesis has been observed previously in lung and other tumor models. MYC has been shown to induce angiogenesis in conjunction with hypoxia inducible factor-1α via VEGF [25]. Tumor tissue hypoxia, necrosis, and expression of the c-Myc gene can promote the expression of VEGF [26]. High expression of VEGF can promote tumor tissue angiogenesis and the expression of other vascular growth factors, which plays an important role in the occurrence, development, and prognosis of a variety of tumors including lung cancer [27].

Despite recent advances in NSCLC treatment, the clinical outcomes of these patients are still far from satisfactory. For example, cisplatin is active against many types of tumors and is toxic to cochlear hair cells, renal convoluted tubule cells, dorsal root ganglion neurons, and gastrointestinal enterochromaffin cells, but is not toxic to most rapidly dividing normal tissues. In conclusion, it is very important to develop more effective NSCLC treatments with fewer side effects, and the c-Myc gene is a suitable target of study for such purposes.

Acknowledgements

and the Health Department of Jiangsu Province project (H201406).

Address correspondence to: Dr. Yanhu Wu, Department of Cardiothoracic Surgery, The First Affiliated Hospital with Nanjing Medical University, 300 Guangzhou Road, Nanjing 210029, China. Tel: +86-25-68136935; Fax: +86-25-83724440; E-mail: wuyanhu@njmu.edu.cn

References

[1] Siegel RL, Sahar L, Portier KM, Ward EM, Jemal A. Cancer death rates in US congressional districts. CA Cancer J Clin 2015; 65: 339-44.

[2] Farhat FS, Houhou W. Targeted therapies in non-small cell lung carcinoma: what have we achieved so far? Ther Adv Med Oncol 2013; 5: 249-70.

[3] Delgado MD, León J. Myc Roles in Hematopoiesis and Leukemia. Genes Cancer 2010; 1: 605-616.

[4] Cavalheiro GR, Matos-Rodrigues GE, Gomes AL, Rodrigues PM, Martins RA. c-Myc regulates cell proliferation during lens development. PLoS One 2014; 9: e87182.

[5] Rapp UR, Korn C, Ceteci F, Karreman C, Luetkenhaus K, Serafin V, Zanucco E, Castro I, Potapenko T. MYC is a metastasis gene for non-small-cell lung cancer. PLoS One 2009; 4: e6029.

[6] Krysan K, Kusko R, Grogan T, O'Hearn J, Reckamp KL, Walser TC, Garon EB, Lenburg ME, Sharma S, Spira AE, Elashoff D, Dubinett SM. PGE2-driven expression of c-Myc and on-comiR-17-92 contributes to apoptosis resistance in NSCLC. Mol Cancer Res 2014; 12: 765-74.

[7] Shtivelman E, Hensing T, Simon GR, Dennis PA, Otterson GA, Bueno R, Salgia R. Molecular pathways and therapeutic targets in lung cancer. Oncotarget 2014; 5: 1392-433.

[8] Black RC, Khurshid H. NSCLC: An Update of Driver Mutations, Their Role in Pathogenesis and Clinical Significance. R I Med J (2013) 2015; 98: 25-8.

[9] Govindan R, Ding L, Griffith M, Subramanian J, Dees ND, Kanchi KL, Maher CA, Fulton R, Fulton L, Wallis J, Chen K, Walker J, McDonald S, Bose R, Ornitz D, Xiong D, You M, Dooling DJ, Watson M, Mardis ER, Wilson RK. Genomic landscape of non-small cell lung cancer in smokers and never-smokers. Cell 2012; 150: 1121-34.

[10] Vachani A, Moon E, Wakeam E, Haas AR, Sterman DH, Albelda SM. Gene therapy for lung neoplasms. Clin Chest Med 2011; 32: 865-85.

[11] Bian C, Li Z, Xu Y, Wang J, Xu L, Shen H. Clinical outcome and expression of mutant P53, P16, and Smad4 in lung adenocarcinoma: a prospective study. World J Surg Oncol 2015; 13: 128.

[12] Ali A, Bhatti MZ, Shah AS, Duong HQ, Alkreathy HM, Mohammad SF, Khan RA, Ahmad A. Tumor-suppressive p53 Signaling Empowers Metastatic Inhibitor KLF17-dependent Transcription to Overcome Tumorigenesis in Non-small Cell Lung Cancer. J Biol Chem 2015; 290: 21336-51.

[13] Matam K, Goud I, Lakshmi M AM, Ravi V, Sridhar K, Vijayanand PR, Chakravarthy S, Prasad S, Tabassum SN, Shaik NA, Syed R, Alharbi KK, Khan IA. Correlation between EGFR Gene Mutations and Lung Cancer: a Hospital-Based Study. Asian Pac J Cancer Prev 2015; 16: 7071-6.

[14] Krupnova EV, Shapetska MN, Mikhalenko EP, Chebotaryova NV, Shchayuk AN, Pissarchik SN, Prokhorov AV. Role of vascular endothelial growth factor in non-small cell lung cancer pathogenesis. Exp Oncol 2015; 37: 213-7.

[15] Reungwetwattana T, Dy GK. Targeted therapies in development for non-small cell lung cancer. J Carcinog 2013; 12: 22.

[16] Fridlender ZG, Sun J, Singhal S, Kapoor V, Cheng G, Suzuki E, Albelda SM. Chemotherapy delivered after viral immunogene therapy augments antitumor efficacy via multiple immune-mediated mechanisms. Mol Ther 2010; 18: 1947-1959.

[17] Zarogoulidis P, Darwiche K, Sakkas A, Yarmus L, Huang H, Li Q, Freitag L, Zarogoulidis K, Malecki M. Suicide Gene Therapy for Cancer - Current Strategies. J Genet Syndr Gene Ther 2013; 16849.

[18] Zhang L, Hou Y, Ashktorab H, Gao L, Xu Y, Wu K, Zhai J, Zhang L. The impact of C-MYC gene expression on gastric cancer cell. Mol Cell Biochem 2010; 344: 125-135.

[19] Perez EA, Jenkins RB, Dueck AC, Wiktor AE, Bedroske PP, Anderson SK, Ketterling RP, Sukov WR, Kanehira K, Chen B, Geiger XJ, Andorfer CA, McCullough AE, Davidson NE, Martino S, Sledge GW, Kaufman PA, Kutteh LA, Gralow JR, Harris LN, Ingle JN, Lingle WL, Reinholz MM. C-MYC alterations and association with patient outcome in earlystage HER2-positive breast cancer from the north central cancer treatment group N9831 adjuvant trastuzumab trial. J Clin Oncol 2011; 29: 651-659.

[20] Li Z, Owonikoko TK, Sun SY, Ramalingam SS, Doetsch PW, Xiao ZQ, Khuri FR, Curran WJ, Deng X. c-Myc suppression of DNA dou-

blestrand break repair. Neoplasia 2012; 14: 1190-1202.

[21] Lin CY, Lovén J, Rahl PB, Paranal RM, Burge CB, Bradner JE, Lee TI, Young RA. Transcriptional amplification in tumor cells with elevated c-Myc. Cell 2012; 151: 56-67.

[22] Pao W, Girard N. New driver mutations in non-small-cell lung cancer. Lancet Oncol 2011; 12: 175-180.

[23] Antonicelli A, Cafarotti S, Indini A, Galli A, Russo A, Cesario A, Lococo FM, Russo P, Mainini AF, Bonifati LG, Nosotti M, Santambrogio L, Margaritora S, Granone PM, Dutly AE. EGFR-Targeted Therapy for Non-Small Cell Lung Cancer: Focus on EGFR Oncogenic Mutation. Int J Med Sci 2013; 10: 320-330.

[24] Seo AN, Yang JM, Kim H, Jheon S, Kim K, Lee CT, Jin Y, Yun S, Chung JH, Paik JH. Clinicopathologic and prognostic significance of c-MYC copy number gain in lung adenocarcinomas. Br J Cancer 2014; 110: 2688-99.

[25] Lee JG, Wu R. Erlotinib-Cisplatin Combination Inhibits Growth and Angiogenesis through c-MYC and HIF-1α in EGFR-Mutated Lung Cancer In Vitro and In Vivo. Neoplasia 2015; 17: 190-200.

[26] Otrock Z, Hatoum H, Musallam K, Awada A and Shamseddine A. Is VEGF a predictive biomarker to anti-angiogenic therapy? Crit Rev Oncol Hematol 2011; 79: 103-111.

[27] Baudino TA, McKay C, Pendeville-Samain H, Nilsson JA, Maclean KH, White EL, Davis AC, Ihle JN, Cleveland JL. c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression. Genes Dev 2002; 16: 2530-2543.