# Association of p53/p21 expression and cigarette smoking with tumor progression and poor prognosis in non-small cell lung cancer patients

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Received July 14, 2014; Accepted September 16, 2014

DOI: 10.3892/or.2014.3538

**Abstract.** Non-small cell lung cancer (NSCLC) accounts for approximately 80-85% of all lung cancer cases. Cigarette smoking is the number one risk factor which is attributed to more than four out of five cases of lung cancers. The prognostic impact of cell cycle regulation-associated tumor suppressors including p53 and p21 for NSCLC is still controversial. In the present study, we examined p53 and p21 expression using immunoblotting in tumor and adjacent non-cancerous tissues from NSCLC patients. Moreover, tissue microarrays (TMAs) including 150 specimens was used to examine p53 and p21 expression by immunohistochemical staining (IHC). The association between p53/p21 and various clinicopathological characteristics was evaluated. Kaplan-Meier overall survival was used to analyze the association between p53/p21 expression and prognosis of NSCLC patients, as well as the association of cigarette smoking with p53/p21 expression and prognosis. The results of the immunoblotting showed that expression of p53 and p21 in tumor tissues was significantly higher than that in the matched adjacent non-cancerous tissues (P<0.001 and P<0.05, respectively). The IHC results showed that 50.67% of the cases had high expression of p21; however, the percentage of patients having high expression of p53 was 31.3%. Univariate and Cox regression models were used to evaluate the factors related to prognosis with p53 and p21 expression. Multivariate analysis indicated that p53 expression was an independent prognostic factor for NSCLC (P=0.005), while p21 could not serve as an independent prognostic factor (P=0.123). In addition, smoking history was closely related to lung cancer risk (P=0.041), but could not be an independent assessment factor (P=0.740). In this study, we further demonstrated the association of p53/p21 expression and cigarette smoking. Our results suggest that cigarette smoking and overexpression of p53 or p21 are associated with poor prognosis. The combination of p53/p21 expression and smoking history may be a useful biomarker for tumor progression and prognosis of NSCLC patients.

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*Key words:* non-small cell lung cancer, p21, p53, western blotting, immunohistochemistry, prognosis

## Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide, accounting for 1.37 million deaths annually (1-3). Of all lung cancer cases, ~85-90% are non-small cell lung cancers (NSCLC). Lung cancer is a multistep process in which the activation of oncogenes and inactivation of tumor-suppressor genes play a critical role in the process of malignant transformation. Smoking and occupational asbestos exposure also interact with other genetic susceptibility factors to synergistically enhance lung cancer risk (4-7). Cigarette smoking (both active and passive inhalation) is the leading cause of multiple tumor types, and there is a broad consensus that cigarette smoking increases the risk of lung cancer in the general population (8-12). Due to the special physiological function, there is no malignancy more closely linked with smoking than lung cancer. In China, the incidence and mortality rates of lung cancer have increased rapidly (4), which may be attributed to the dramatic increase in the cigarette smoking rate during the past 2 decades; a peak in lung cancer incidence is still expected. Among adult Chinese, ~2/3 individuals are smokers, representing 1/3 of all smokers worldwide.

Despite recent advances in early diagnosis/screening and development of novel treatment strategies, the overall survival rate of lung cancer patients remains low (2). The identification of reliable new biomarkers and better understanding of the tumorgenesis of NSCLC, as well as the development of more efficient therapeutic targets will improve the prognosis of NSCLC patients.

The *p53* gene, is the most frequently mutated tumor suppressor in all cancers. Structural alteration has been found in more than 50% of human tumors. Wild-type p53 protein remains difficult to detect by IHC, because of a very short half-life; however, the mutated type of p53, which is encoded by the *p53* gene with missense mutations has (but not always) a prolonged half-life, and is easily detected by IHC (12). p21 (also known as $p21^{WAF1/CIP1}$) is a member of the Cip/Kip family of cyclin-dependent kinase inhibitors, and the expression of p21 is tightly regulated at the transcriptional and post-transcriptional levels (13). p21 protein can bind to and inhibit the activity of cyclin-CDK2 or cyclin-CDK4 complexes, which could participate as a regulator of cell cycle progression at the G1 phase. Previous studies have shown that p21 is regulated by both p53-dependent and p53-independent mechanisms, but the p53-dependent p21 pathway plays a critical role in cell cycle arrest and prevents cellular DNA synthesis in response to DNA damage (14,15). p21 can interact with proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory factor, and act as a regulatory role in S phase DNA replication and DNA damage repair (16). On the other hand, p21 was reported to be specifically cleaved by CASP3-like caspase, which leads to activation of CDK2, and then activates downstream caspase leading to programmed cell death (9).

To our knowledge, there are few studies that have focused on the relationship between both p53/p21 expression and cigarette smoking and lung cancer. We hypothesized that both p53/p21 expression in combination with smoking history may have a close relationship with lung cancer progression, as well as prognosis. In the present study, we analyzed the expression of p53 and p21 in tumor and adjacent non-cancerous tissues obtained from 50 NSCLC patients by performing western blot analysis. We further investigated the association between p53/p21 expression as well as smoking with NSCLC parameters by conducting IHC in tissue microarrays (TMAs), including the survival status of NSCLC patients.

## Materials and methods

*Reagents and antibodies*. Protease inhibitor cocktail tablet was obtained from Roche Applied Science (Indianapolis, IN, USA). Polyclonal anti-p21 Waf1/Cip1 was obtained from Proteintech (Wuhan, China); monoclonal anti-p53, anti-actin and horseradish peroxidase-conjugated secondary antibodies were all from Abmart (Shanghai, China). Pierce BCA protein assay kit and ECL reagent were purchased from Thermo Scientific (Waltham, MA, USA). Biotinylated goat anti-rabbit IgG and the streptavidin-biotin complex were purchased from Boster (Wuhan, China)

*Patient specimens and tissue microarrays*. A total of 50 paired human non-small lung cancer and adjacent non-cancerous tissues were obtained from the Department of Cardiothoracic Surgery, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China, from March 2010 to November 2013. The tissues were frozen immediately in liquid nitrogen after surgery and stored at -80˚C for subsequent extraction of RNA and tissue lysate preparation. NSCLC TMAs were constructed with established routine methods by experienced pathologists (R.W. and K.H.). TMAs contained a total of 150 formalin-fixed, paraffin-embedded tissue samples from NSCLC patients collected between 2005 and 2011. None of the patients included in the present study received chemotherapy or/and radiation therapy prior to surgery. Written informed consent for experimental use of the specimens was obtained from all patients, and the study was approved by the Board and Ethics Committee of Wenzhou Medical University, China. All of the patients were clinically and pathologically confirmed to have NSCLC. The tumor tissues were classified according to the TNM system guidelines of the American Joint Committee on Cancer (AJCC)/Union Internationale Contre Cancer (UICC), and the histologic classification of the tumors was based on the World Health Organization criteria (WHO).

*Protein extraction from tissue samples and immunoblotting*. The tissue samples were retrieved at -80˚C, washed 3 times with ice-cold PBS and homogenized using a homogenizer (Kinematica AG, Luzern, Switzerland) in 1.5 ml tissue RIPA lysis buffer [50 mMTris-HCl (pH 7.4), 1.0% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl], containing protease inhibitor cocktail tablet, 1 mM NaF and 1 mM $Na_3VO_4$. Tissue homogenates were centrifuged at 13,000 rpm for 25 min at 4˚C, and the supernatants were collected in clean microcentrifuge tubes on ice.

The protein concentrations of the tissue homogenates were determined using the Pierce BCA protein assay kit. Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. Blots were incubated with the appropriate primary antibodies overnight at 4˚C and horseradish peroxidase-conjugated secondary antibodies for 1 h followed by detection with the enhanced chemiluminescence (ECL) detection system according to the manufacturer's instructions. The optical density was quantified using the National Institutes of Health ImageJ software.

*Immunohistochemistry*. Immunohistochemistry (IHC) was performed as described previously (17,18). Briefly, sections were deparaffinized in xylene and then gradually hydrated using a graded alcohol series followed by blocking endogenous peroxidase activity by 0.5% $H_2O_2$ in methanol for 60 min at room temperature. Nonspecific binding was blocked by 5% BSA. Following antigen retrieval, the sections were incubated overnight at 4˚C with rabbit polyclonal anti-p21 (dilution 1:100) and mouse monoclonal anti-p53 (dilution 1:100). After washing with ice-cold PBS, the TMA was incubated with biotinylated secondary antibody for 30 min, followed by further incubation with the streptavidin-horseradish peroxidase for 20 min at room temperature. Finally, 3,3'-diaminobenzidine (DAB) was applied to visualize the signals of the TMA staining and lightly counterstained with Mayer hematoxylin. For the negative control staining, PBS was used to replace the primary antibody and no staining was observed. A brown particle in the tissue was considered as positive labeling. The staining

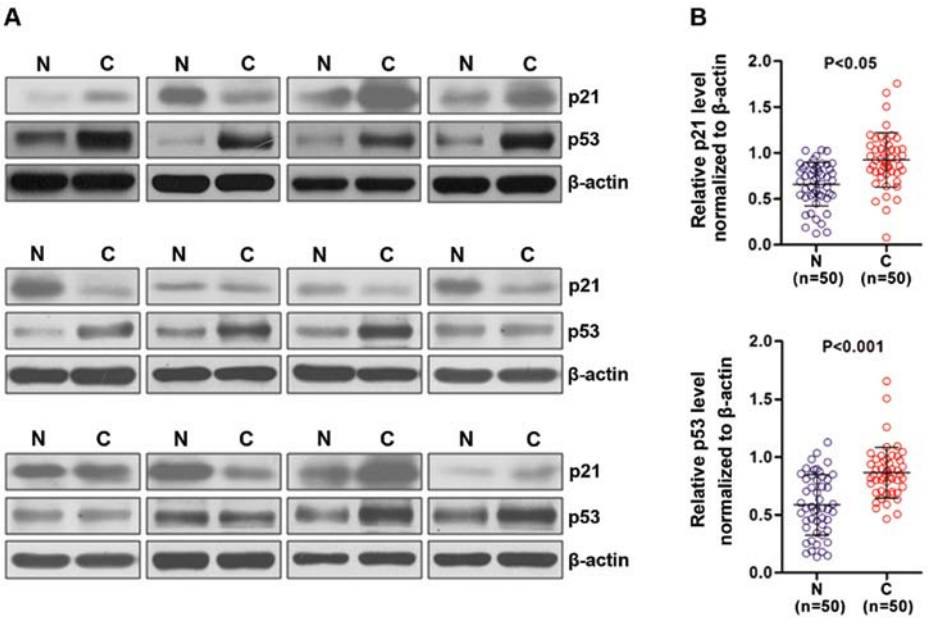


Figure 1. p53 and p21 protein expression in NSCLC patient tissues. (A) Western blot analysis of p53 and p21 protein expression in NSCLC tumor tissues (C) and matched non-cancerous tissues (N). (B) The quantitative results of western blotting showed that p53 and p21 protein expression was increased in the tumor tissues compared to levels in the matched non-cancerous tissues of NSCLC patients (n=50, P<0.001 and P<0.05, respectively).

was evaluated independently by two pathologists (K.H. and R.W.) without knowledge of the clinicopathological data and p53/p21 expression. An average value of two independent scores was presented in the present study.

An immunoreactivity scoring system was applied as described elsewhere (19,20). The percent of p53/p21-positive cells was scored according to four grades (percentage scores): <10% (1), 10-50% (2), 51-80% (3) and >80% (4). The intensity of staining was divided into four grades (intensity scores): no staining (0), weakly stained (1), moderately stained (2) and strongly stained (3). The overall p21 and p53 immunostaining score was calculated using the percentage score x intensity score. Overall scores 0-6 were defined as low p21 or p53 expression, and scores >6 were high p21 or p53 expression.

*Statistical analysis*. The optical density of the immunoblotting signals was quantified by National Institutes of Health ImageJ software. All statistical analyses were performed using SPSS 15.0 software (Chicago, IL, USA). The Student's t-test was used to analyze the relationship between the p21/p53 protein expression levels in NSCLC. The $\chi^2$ test was performed to evaluate the significance of the IHC results for the association between p53/p21 expression in IHC and clinicopathological parameters. The overall survival was calculated according to Kaplan-Meier method and compared with the log-rank test. Univariate and multivariate analyses were based on Cox regression model. This model was used to identify which independent factors jointly had significant effects on survival. Differences were considered statistically significant at P<0.05.

## Results

*Overexpression of p21 and p53 protein in NSCLC tumor tissues*. To investigate the role of p53 and p21 in NSCLC progression, we examined the p21 and p53 protein expression in 50 paired tumor tissues and adjacent non-cancerous tissues (2 cm away from the cancer tissues) by western blotting. Our results showed that p53 and p21 protein expression levels were significantly higher in the NSCLC tumor tissues than these levels in the matched adjacent non-cancerous tissues. Representative immunoblotting images are shown in Fig. 1A. Additionally, the quantitative results of p53 and p21 expression are shown in Fig. 1B (n=50; P<0.001 and P<0.05, respectively).

*Association of p53 and p21 expression with clinicopathological parameters of NSCLC*. To further confirm the expression pattern of p53 and p21 in NSCLC tissues, IHC staining analysis was conducted in TMA containing 150 archived paraffin-embedded NSCLC tissues. The representative IHC images of p53 and p21 in non-cancerous and cancer tissues of different TNM stage are shown in Fig. 2A. We found that p21 and p53 protein levels were predominantly localized in the nucleus. According to the scoring system, we divided the 150 NSCLC cases into two groups: high-expression and low-expression. In total, 50.67% (76 of 150) showed high p21 expression within the nucleus (Table I), whereas high p53 expression was detected in 31.3% (47 of 150) of the NSCLC cases (Table II). As summarized in Table I, p21 expression was significantly associated with gender (P=0.004), smoking history (P=0.006), T stage (P=0.014) and TNM stage (P=0.001), but not with alcohol history, age, tumor grade and LN metastases. We next analyzed the relationship between p53 expression and clinicopathological characteristics. As documented in Table II, our results showed that p53 expression was significantly associated with gender (P=0.013), age (P=0.026), smoking history (P=0.021), tumor grade (P=0.012), TNM stage (P=0.023) and LN metastases (P=0.014). However, no significant relationship was found between p53 expression and variables such as alcohol history and T stage (P=0.853 and P=0.502, respectively).

Table I. Correlation between p21 expression and various clinicopathological factors of the NSCLC patients.

| Variables | Total (n=150) | p21 protein expression Low (n=74) | High (n=76) | P-value |
|---|---|---|---|---|
| Gender | | | | **0.004** |
| Male | 100 | 41 | 59 | |
| Female | 50 | 33 | 17 | |
| Alcohol history | | | | 0.640 |
| Yes | 40 | 21 | 19 | |
| No | 110 | 53 | 57 | |
| Age (years) | | | | 0.634 |
| <61 | 68 | 35 | 33 | |
| ≥61 | 82 | 39 | 43 | |
| Smoking history | | | | **0.006** |
| Yes | 78 | 30 | 48 | |
| No | 72 | 44 | 28 | |
| Grade | | | | 0.302 |
| G1 | 16 | 8 | 8 | |
| G2 | 91 | 49 | 42 | |
| G3 | 43 | 17 | 26 | |
| T stage | | | | **0.014** |
| T1 | 11 | 10 | 1 | |
| T2 | 126 | 59 | 67 | |
| T3-T4 | 13 | 5 | 8 | |
| LN metastasis (N) | | | | 0.090 |
| N0 | 89 | 49 | 40 | |
| N≥1 | 61 | 25 | 36 | |
| TNM stage | | | | **0.001** |
| I | 65 | 43 | 22 | |
| II | 40 | 13 | 27 | |
| III-IV | 45 | 18 | 27 | |

The $\chi^2$ test was used to analyze the relationship between p21 expression and clinicopathological characteristics.

Table II. Correlation between p53 expression and various clinicopathological factors of the NSCLC patients.

| Variables | No. (n=150) | p53 protein expression Low (n=103) | High (n=47) | P-value |
|---|---|---|---|---|
| Gender | | | | **0.013** |
| Male | 100 | 62 | 38 | |
| Female | 50 | 41 | 9 | |
| Alcohol history | | | | 0.853 |
| Yes | 40 | 27 | 13 | |
| No | 110 | 76 | 34 | |
| Age (years) | | | | **0.026** |
| <61 | 68 | 53 | 15 | |
| ≥61 | 82 | 50 | 32 | |
| Smoking history | | | | **0.021** |
| Yes | 78 | 47 | 31 | |
| No | 72 | 56 | 16 | |
| Grade | | | | **0.012** |
| G1 | 16 | 13 | 3 | |
| G2 | 91 | 68 | 23 | |
| G3 | 43 | 22 | 21 | |
| T stage | | | | 0.502 |
| T1-T2 | 137 | 93 | 44 | |
| T3-T4 | 13 | 10 | 3 | |
| LN metastasis (N) | | | | **0.014** |
| N0 | 89 | 68 | 21 | |
| N≥1 | 61 | 35 | 26 | |
| TNM stage | | | | **0.023** |
| I-II | 105 | 78 | 27 | |
| III-IV | 45 | 25 | 20 | |

The $\chi^2$ test was used to analyze the relationship between p53 expression and clinicopathological characteristics.

*Survival analysis*. To understand the prognostic value of p53/p21 for NSCLC, Kaplan-Meier analysis with a log-rank test was performed to evaluate the association between p53/p21 expression and overall survival. A total of 150 NSCLC patients who had adequate follow-up data were used for survival analysis. The log-rank test results showed that patients with high p53 expression were associated with poor overall survival when compared to those with low p53 expression (Fig. 2B, P<0.001). Similarly, the high p21 expression group also showed decreased overall survival compared with the low p21 expression group (Fig. 2C, P=0.0068). Moreover, we proceeded to analyze the relationship between NSCLC patient survival status with combinations of p53/p21 and found a strikingly reverse correlation with overall survival (Fig. 3A, P=0.0002). Particularly, in the low p53 expression group ($p53^{low}$), the high p21 expression patients had a lower survival rates compared to the low p21 expression group. While in the group of patients with high p53 expression ($p53^{high}$), the overall survival was independent of p21 expression. Collectively, these results suggest that the aberrant overexpression of p53 and p21 may potentially contribute to NSCLC carcinogenesis and tumor progression.

*Cigarette smoking may play a marked role in NSCLC progression*. As known, cigarette smoking plays critical roles in lung cancer carcinogenesis. We first analyzed the role of cigarette smoking in NSCLC patient overall survival. Consistently, NSCLC patients who had a smoking history exhibited obviously poor overall survival than those without a smoking history (Fig. 3B, P=0.0379). As our statistical results showed (Fig. 3C), p53 and p21 expression were both significantly positively associated with smoking history. Thus, we proposed that cigarette smoking together with upregulation of p53 and

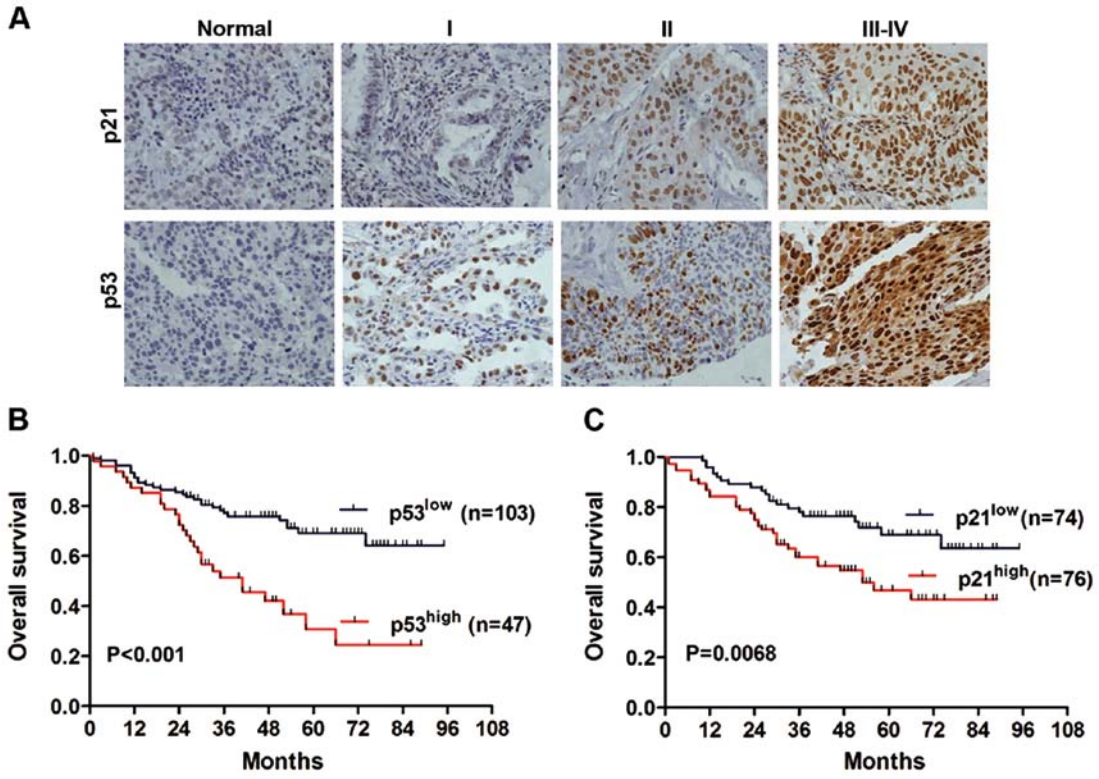


Figure 2. p53 and p21 are favorable prognostic factors for NSCLC progression and patient survival. (A) Representative p21 and p53 IHC staining images of NSCLC sections (magnification, 400x). The nuclei of lung cancer carcinoma cells were clearly stained. p21 and p53 protein were expressed in TNM stage of I-IV NSCLC tumor tissue, as well as weakly expressed in adjacent non-cancerous tissues (normal). (B and C) Kaplan-Meier overall survival curves according to p21 and p53 expression levels in NSCLC (n=150, P=0.0068 and P<0.001, respectively). The P-value was determined by a two-sided log-rank test.

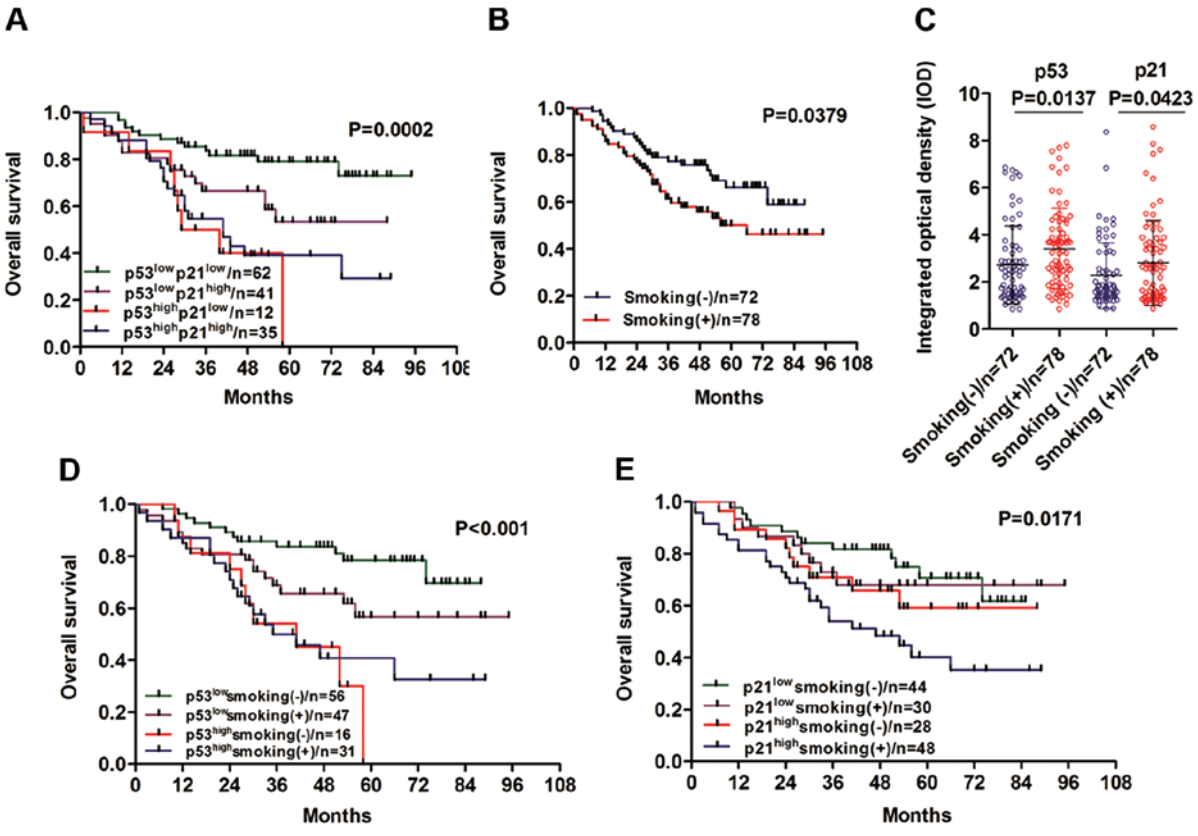


Figure 3. The combination of p53 and p21 expression or cigarette smoking is a favorable prognostic factor for NSCLC cancer patient survival. (A) Kaplan-Meier curves of overall survival according to the combination of p53 and p21 expression levels (P=0.0002). (B) Kaplan-Meier curves of overall survival of NSCLC patients according to cigarette smoking history (P=0.0379). (C) p53 and p21 protein expression were significantly associated with smoking history. (D and E) Kaplan-Meier curves of overall survival according to the combination of cigarette smoking and p53 or p21 (P<0.001 and P=0.0171, respectively).

Table III. Univariate analysis to identify factors influencing the overall survival of NSCLC patients[a].

| | Univariate analysis | | |
|---|---|---|---|
| Variables | RR | 95% CI | P-value |
| p21 | 2.618 | 1.495-4.586 | 0.001 |
| p53 | 2.809 | 1.661-4.752 | <0.001 |
| Age | 1.029 | 0.988-1.061 | 0.065 |
| Gender | 1.694 | 0.938-3.061 | 0.081 |
| Smoking | 1.745 | 1.022-2.979 | 0.041 |
| Alcohol | 0.912 | 0.490-1.699 | 0.772 |
| Grade | 1.971 | 1.241-3.131 | 0.004 |
| T stage | 7.009 | 3.781-12.933 | <0.001 |
| LN metastasis | 1.719 | 1.020-2.896 | 0.042 |
| Clinical stage | 2.061 | 1.492-2.847 | <0.001 |

[a]Cox's proportional hazards model was used to identify the factors that had a significant influence on survival. Statistical significance was set at P<0.05. RR, relative risk; CI, confidence interval; LN, lymph node.

p21 may contribute to NSCLC carcinogenesis. To validate this hypothesis, we quantified the corresponding integrated optical density (IOD) value of IHC images by Image-Pro Plus 6.0. We divided the p53/p21 expression into two groups based on smoking status. Interestingly, we found a notable increase in p53 expression in the smoking (+) group compared with that in the smoking (-) group (Fig. 3C, P=0.0137). Similarly, p21 protein expression was also significantly higher in the smoking (+) group (Fig. 3C, P=0.0423). Moreover, we examined the effect of the combination of smoking and p53 on overall survival of NSCLC patients. A marked negative correlation was observed in overall survival (Fig. 3D, P<0.001). In detail, the low p53 expression ($p53^{low}$)/smoking (+) group exhibited worse outcomes compared with the low p53 expression ($p53^{low}$)/smoking (-) group; whereas no statistically significant difference was found in the high p53 expression ($p53^{high}$) group, whether or not the patients had a smoking history. We further analyzed the overall survival with the combination of smoking and p21. A negative correlation was found by Kaplan-Meier analysis (Fig. 3E, P=0.0171). Finally, a significant decrease in survival rates was found in the smoking (+) and high p21 expression group ($p21^{high}$). Taken together, our results indicate that cigarette smoking may play a critical role in promoting NSCLC progression via modulation of p53 and p21 protein expression.

*Assessment of NSCLC prognostic factors*. To determine the factors which affect the overall survival of NSCLC patients, univariate and multivariate analyses using a Cox regression hazards model were conducted to evaluate the impact of p53/p21 expression and clinicopathological factors on survival status in 150 NSCLC patients. As the univariate analysis results show in Table III, p21 and p53 expression levels (P=0.001 and P<0.001, respectively), smoking history (P=0.041), tumor grade (P=0.004), T stage (P<0.001), lymph node (LN) metastasis (P=0.041) and TNM stage (P<0.001), but not patient age (P=0.065), gender (P=0.081) and alcohol history (P=0.772) were significant prognostic factors for overall survival of NSCLC. Next, as summarized in Table IV, our data showed that p53 expression (P=0.005) was an independent prognostic factor for NSCLC, but not p21 (P=0.123).

We performed Kaplan-Meier analysis to determine the effects of p53/p21 expression on overall survival of NSCLC patients in various tumor grades, and histological grade was a potential prognostic factor in NSCLC patients (Fig. 4A, P=0.0036). We further proceeded to analyze p53/p21 expression in well differentiated (G1), moderately differentiated (G2) and poorly differentiated (G3) tumor tissues. Notably, we found an obviously lower survival rate in the $p53^{high}$ group compared to that in the $p53^{low}$ group for G1/G2 NSCLC patients (Fig. 4B, P=0.0012). Similarly, we found that overall survival of the $p21^{high}$ group was worse than that of the $p21^{low}$ group for G1/G2 NSCLC patients (Fig. 4D, P=0.0123).

Table IV. Multivariate analysis to identify factors influencing the overall survival of NSCLC patients[a].

| | p21 Multivariate analysis | | | p53 Multivariate analysis | | |
|---|---|---|---|---|---|---|
| Variables | RR | 95% CI | P-value | RR | 95% CI | P-value |
| Expression level | 1.603 | 0.880-2.919 | 0.123 | 2.305 | 1.294-4.105 | 0.005 |
| Age | 1.026 | 0.991-1.062 | 0.148 | 1.024 | 0.989-1.059 | 0.181 |
| Gender | 1.239 | 0.481-3.194 | 0.657 | 1.293 | 0.500-3.344 | 0.597 |
| Smoking | 1.204 | 0.504-2.874 | 0.676 | 1.157 | 0.490-2.731 | 0.740 |
| Alcohol | 1.017 | 0.498-2.080 | 0.962 | 1.072 | 0.531-2.166 | 0.846 |
| Grade | 1.026 | 0.598-1.761 | 0.927 | 0.903 | 0.527-1.545 | 0.709 |
| T stage | 3.969 | 1.860-8.470 | <0.001 | 4.516 | 2.088-9.771 | <0.001 |
| LN metastasis | 0.432 | 0.178-1.051 | 0.064 | 0.033 | 0.133-0.835 | 0.019 |
| Clinical stage | 2.479 | 1.385-4.438 | 0.002 | 2.738 | 1.526-4.911 | 0.001 |

[a]Cox's proportional hazards model was used to identify the factors that had a significant influence on survival. Statistical significance was set at P<0.05. RR, relative risk; CI, confidence interval; LN, lymph node.

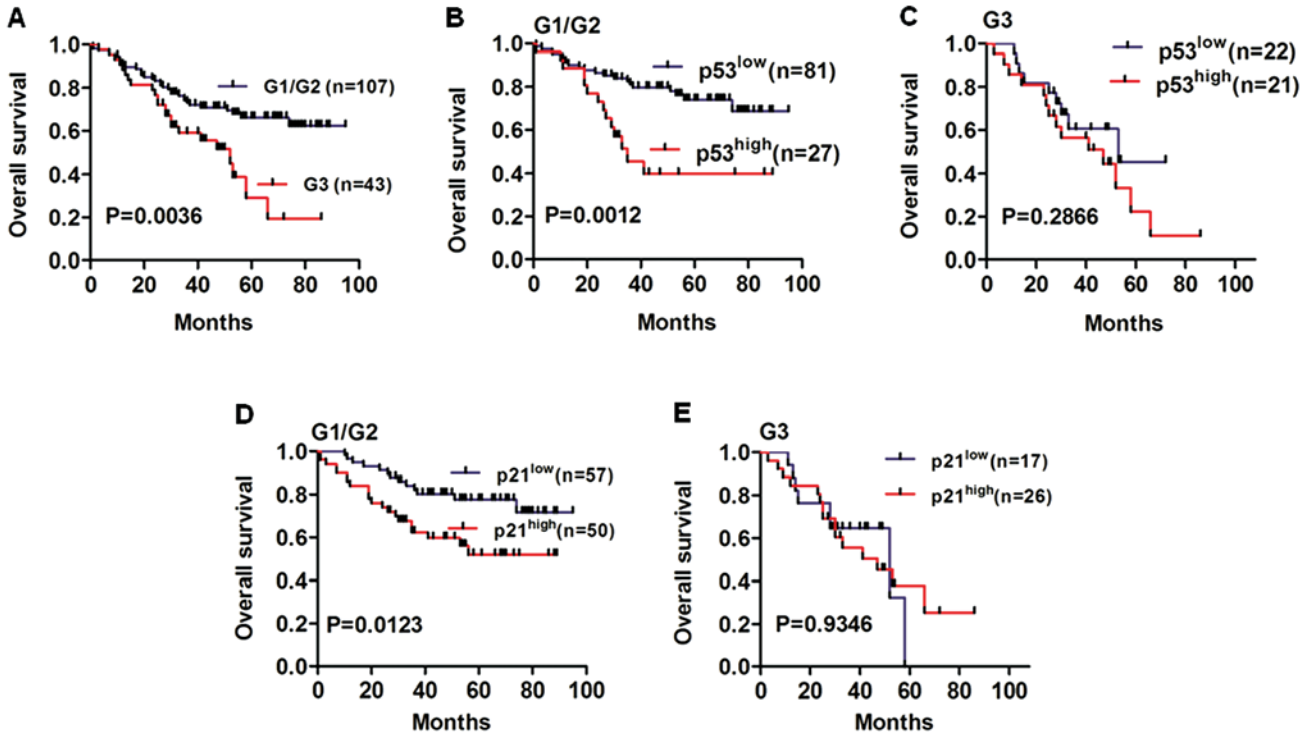


Figure 4. Overall survival curves of NSCLC cancer patients according to pathological grade (G1, G2 and G3), p53 or p21 expression in G1-2 and G3. (A) Kaplan-Meier curves of overall survival according to the pathological grade (P=0.0036). (B and C) Kaplan-Meier curves of overall survival according to p53 expression level in groups G1-2 (P=0.0012) and G3 (P=0.2866). (D and E) Kaplan-Meier curves of overall survival according to p21 expression levels in group G1-2 (P=0.0123) and group 3 (P=0.9346).

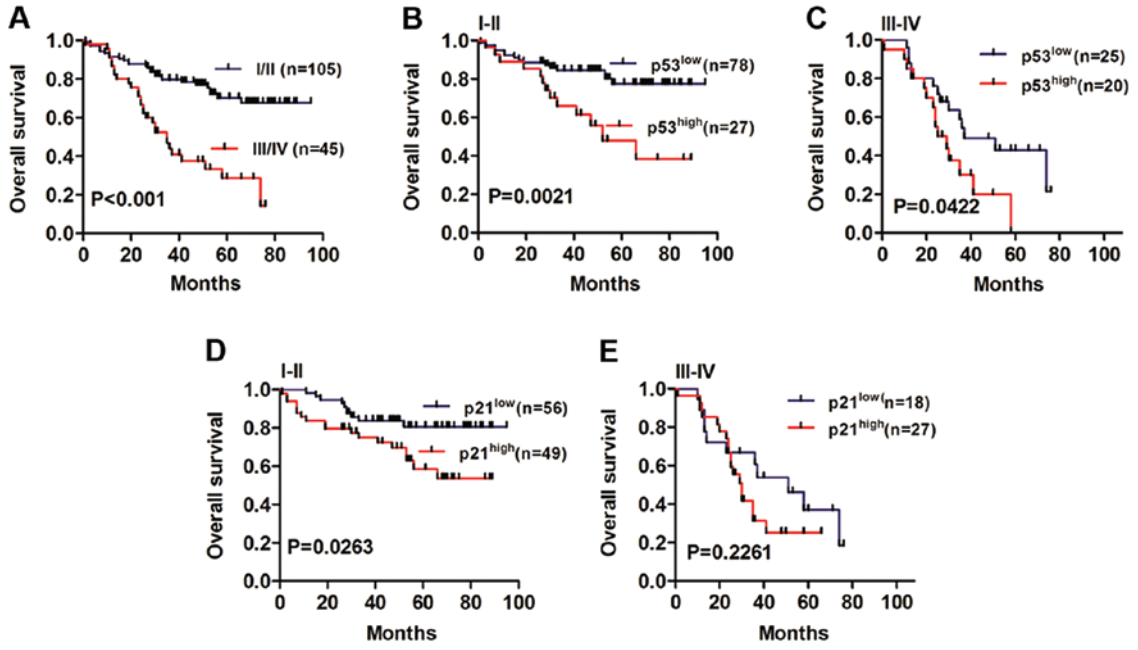


Figure 5. Overall survival curves of NSCLC cancer patients according to the TNM stage, and p53 or p21 expression in TNM stage I-II and III. (A) Kaplan-Meier curves of overall survival according to the TNM stage (P<0.001). (B and C) Kaplan-Meier curves of overall survival according to p53 expression level in clinical stage I-II and III-IV patients (P=0.0021 and P=0.0422, respectively). (D and E) Kaplan-Meier curves of overall survival according to p21 expression level in clinical stage I-II and III-IV patients (P=0.0263 and P=0.2261, respectively).

However, no statistically significant difference was found in the overall survival of G3 NSCLC patients according to p53 or p21 expression levels (Fig. 4C and E, P=0.2866 and P=0.9346, respectively).

Next, we analyzed overall survival in different TNM-stage NSCLC patients (Fig. 5A, P<0.001). Notably, the NSCLC patients with high-expression of p53 showed worse outcomes in both stages I/II (Fig. 5B, P=0.0021) compared to the the

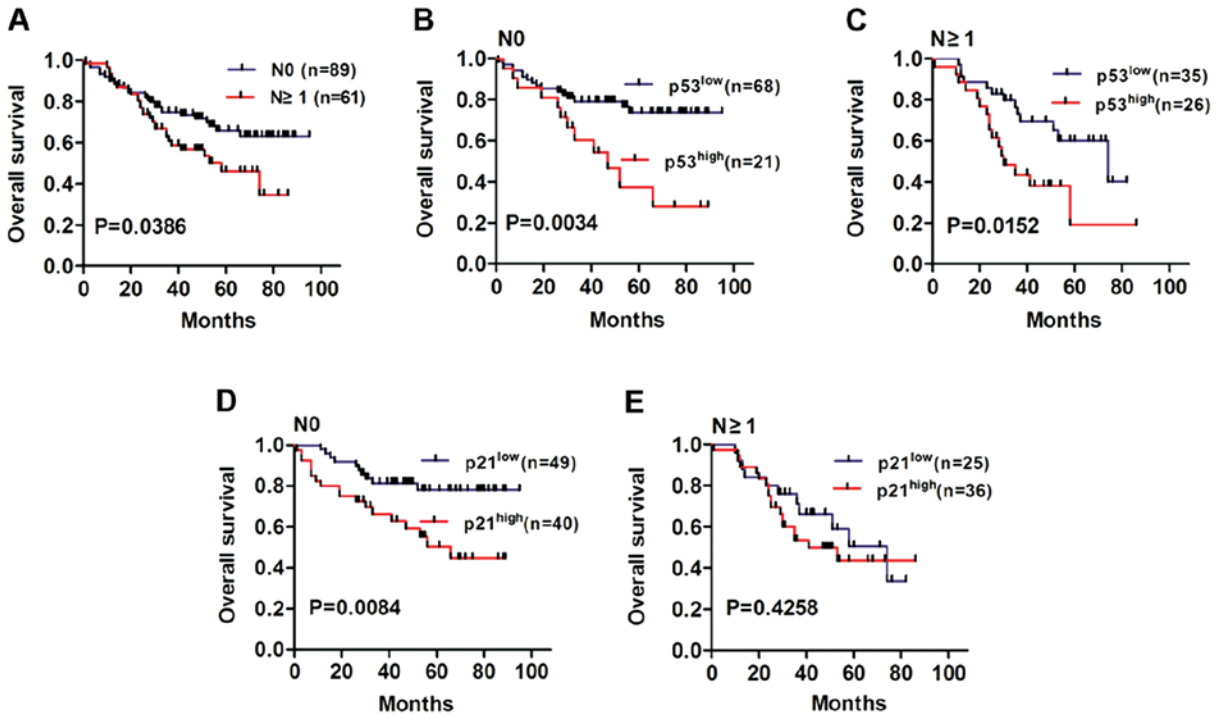


Figure 6. Overall survival curves of NSCLC cancer patients according to the LN metastasis stage, and p53 or p21 expression in LN metastasis stage N0 and N≥1. (A) Kaplan-Meier curves of overall survival according to the LN metastasis stage (P=0.0386). (B and C) Kaplan-Meier curves of overall survival according to p53 expression level in LN metastasis stage N0 and N≥1 patients (P=0.0034 and P=0.0152, respectively). (D and E) Kaplan-Meier curves of overall survival according to p21 expression level in LN metastasis stage N0 and N≥1 patients (P=0.0084 and P=0.4258, respectively).

stage III subgroup (Fig. 5C, P=0.0422). Moreover, the $p21^{high}$ group had lower overall survival rates than the $p21^{low}$ group in I/II stage NSCLC patients (Fig. 5D, P=0.0263). In patients diagnosed with III stage, no significant difference was found for the $p21^{high}$ group and $p21^{low}$ group (Fig. 5E, P=0.2261).

Finally, we examined the relationship between p53/p21 expression and LN metastases (Fig. 6A, P=0.0386). Our results showed that the overall survival of NSCLC patients with high p53 expression was worse than patients with low p53 expression in both N0 (Fig. 6B, P=0.0034) and N≥1 stage groups (Fig. 6C, P=0.0152). In addition, the overall survival of NSCLC patients with high p21 expression was much worse than patients with low p21 expression in the N0 stage group (Fig. 6D, P=0.0084). However, no statistically significant difference in overall survival was observed in the N≥1 stage group regardless of p21 expression (Fig. 6E, P=0.4258). Taken together, these findings indicate that p53/p21 expression may play a potential role in NSCLC progression and correlate with the outcome of NSCLC patients.

## Discussion

p53 is a well-known tumor suppressor encoded by the *p53* gene located on chromosome 17p13, which is composed of 393 amino acids and is a member of a highly conserved family containing at least another two genes, p63 and p73. p53 is closely related to many human cancers and is the most frequently mutated tumor-suppressor gene in human cancers. The mutation or loss of the *p53* gene can be identified in more than 50% of all human cancers (21,22), which results not only in the loss of tumor-suppressor function, but also leads to the gain of novel cancer-related functions that contribute to tumorigenesis. Under normal conditions, p53 protein levels in cells are maintained at very low levels due to extremely short half-life, and p53 is largely inactive, unless the cells are activated by signals from DNA damage and a number of other cellular stress factors, including hypoxia and nucleotide deprivation (15,23). In response to DNA damage and cellular stresses, the p53 protein level is upregulated, which leads to cell cycle arrest, DNA repair or apoptosis. Hence, p53 plays a critical role in the inhibition of cancer cell malignancy. In contrast to wild-type p53, the mutant protein is stably expressed, which leads to the immunohistochemical staining detection of mutant p53 with high levels of prognostic significance.

p21 protein mediates p53-dependent G1 growth arrest and acts as a tumor suppressor. However, it has been reported that p21 is upregulated in a variety of human cancers including breast, cervical, prostate and esophageal carcinoma (23). The upregulation of p21 is associated with tumor grade, clinical stage, invasiveness and aggressiveness. Moreover, p21 overexpression may also predict the poor prognosis of human cancers. Thus, the role of p21 as a tumor suppressor is still controversial and the association with tumor progression remains to be further understood.

In the present study, we performed western blotting using frozen tissues which were snap-frozen in liquid nitrogen immediately after surgical resection and stored at -80˚C. Our data showed that both p53 and p21 protein levels were upregulated in tumor tissues. Similar results were also reported by Zhang *et al* (17) for hepatocellular carcinomas.

Additionally, we examined the p53 and p21 protein expression pattern with IHC analysis, and our results confirmed that the tumor tissues of NSCLC patients had strong p53 and p21 protein staining, whereas there was no or weak staining

Table V. Ratio of $p53^{high}/p53^{low}$ in the p21 expression and smoking combination groups.

| Group | | p53 expression | | |
|---|---|---|---|---|
| | | Low (n=103) | High (n=47) | $p53^{high}/p53^{low}$ |
| $p21^{low}$ smoking (-) | n=44 | 36 | 8 | 0.22 |
| $p21^{low}$ smoking (+) | n=30 | 26 | 4 | 0.15 |
| $p21^{high}$ smoking (-) | n=28 | 20 | 8 | 0.40 |
| $p21^{high}$ smoking (+) | n=48 | 21 | 27 | **1.29** |

in the matched adjacent non-cancerous tissues. These results revealed that overexpression of p53 and p21 in NSCLC patient tissues plays an important role in the progression of NSCLC. The upregulation of p53 was significantly associated with gender, age, smoking history, tumor grade, T stage and TNM stage of NSCLC patients. In addition, upregulation of p21 was associated with gender, smoking history, T stage and TNM stage; however, there was no association between p21 expression and age or tumor grade of the NSCLC patients.

Kaplan-Meier survival analysis showed that high expression levels of both p53 and p21 were associated with poor overall survival of patients with NSCLC. In cases with low p53 expression, the high expression of p21 promoted tumor progression and led to poor survival. However, once p53 was mutated and with stable high expression at the protein level, p21 protein had no more effects on overall survival.

Among the 150 NSCLC patients included in this study, we found that cigarette smoking led to poor overall survival for NSCLC patients compared with never-smokers, which is consistent with most previous reports on other cancers (24-26). While the p53 protein level is low, cigarette smoking is significantly associated with overall survival; however, when p53 is overexpressed, cigarette smoking is no longer associated with overall survival of NSCLC patients, since overexpression of p53 dominates the effects on tumor progression. We further found that cigarette smoking did not exhibit a significant effect on overall survival while p21 protein expression was low. Contrary to p53, our data showed that cigarette smoking was significantly associated with overall survival for NSCLC patients with high p21 protein expression levels. This could be attributed to the difference in the p53 protein expression level in the p21 high expression group (Table V). The ratio of $p53^{high}/p53^{low}$ was low (0.40) in the group of $p21^{high}$/smoking (-) (n=28), whereas the $p53^{high}/p53^{low}$ ratio was high (1.29) in the group of $p21^{high}$/smoking (+) (n=48). These results suggest that the significant difference in overall survival for p21 high expression NSCLC patients with or without smoking history was due to the difference in p53 protein expression level.

Our results showed that tumor grade, TNM stage and LN metastasis were all significantly associated with prognosis and could serve as an independent outcome biomarker for NSCLC patients. In patients with low tumor grade (G1/G2), both high p53 and p21 expression led to poor overall survival. On the contrary, neither p53 or p21 expression had an impact on overall survival in the high tumor grade (G3) NSCLC patients. This indicated that the p53 or p21 protein expression level had no further effects on tumor progression in NSCLC patients with high tumor grade. The expression of p53 protein was significantly associated with TNM stage and LN stage. In addition, high expression of p21 was significantly associated with the overall survival of NSCLC patients in TNM stage I-II or LN stage N0; however, there was no difference for stage III-IV patients. These results further suggest that p21 protein expression had less effect on tumor progression in NSCLC patients who were in late TNM or N stage. Moreover, the univariate analysis showed that overexpression of both p53 and p21 was associated with survival status of NSCLC patients. However, the multivariate analysis showed that p53 but not p21 could be an independent prognostic factor for NSCLC, which indicates that p53 plays a more decisive role in the progression of NSCLC.

In conclusion, our findings suggest that cigarette smoking and p53/p21 overexpression are associated with the poor prognosis of NSCLC patients. Although the incidence of NSCLC is a multifactor process, p53/p21 plays an important role in cell cycle regulation. Better understanding of this cell cycle regulatory system involved in NSCLC development is necessary for understanding tumor biological behavior, seeking reliable diagnostic markers and providing new therapeutic strategies. The combination of p53/p21 expression and cigarette smoking history could be a useful clinical biomarker for tumor progression and prognosis of NSCLC patients.

## Acknowledgements

This study was partially supported by the National Basic Research Program of China (no. 2013CB531702), the National Natural Science Foundation of China (nos. 31070710 and 31171345), Zhejiang Qianjiang Talent Project B Grant (no. 2010R10045) and the Scientific Research Foundation for the Returned Overseas Chinese Scholar, State Education Ministry to B.L., Natural Science Foundation of Zhejiang Province (no. Y2110097) and Wenzhou Science and Technology Bureau grant (no. H20100064) to Y.L.

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