The Prognostic Value of p53 Expression and Ki-67 Proliferation Index in the Post Resection Lung Adenocarcinoma Cases

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Abstract: Lung cancer is the leading cause of cancer-related deaths and there is a debate over the effects of p53 expression and Ki-67 proliferation index on survival in cases with lung adenocarcinoma. In the present study, p53 gene expression and Ki-67 proliferation index were studies in pathological specimens of 26 cases who underwent anatomical resection and mediastinal lymph node dissection due to lung adenocarcinoma between January 2002 and December 2007. The results of immunohistochemical staining were compared with the parameters including tumor stage, lymphovascular invasion, lymph node involvement, pleural invasion, and survival. There was a significant negative relationship between p53 expression and survival using non-parametric analysis methods (for Kendall's tau-b test, r=-0.505; for Spearman's rho test, r=-0.536). It was found that increased p53 expression unfavorably affected survival. In addition, there was a significant relationship between p53 expression and pleural invasion using non-parametric analysis methods; pleural invasion increased with increasing p53 expression (for Kendall's tau-b test, r=0.505; for Spearman's rho test, r=0.536). However, there was no significant relationship between p53 expression and tumor stage, lymphovascular invasion, and lymph node involvement. There are a positive significant relationship between Ki-67 proliferation index and survival, an increasing proliferation index was associated with increasing survival expression (for Kendall's tau-b test, r=0.553; for Spearman's rho test, r=0.588). All non-survivors in the study group had a Ki-67 proliferation index less than 10%. Ki-67 proliferation index was not associated with tumor stage, lymphovascular invasion, pleural invasion, and lymph node involvement. Although increased Ki-67 proliferation index was not shown to have a negative effect on survival in patients with lung adenocarcinoma who underwent lung resection, patients with a low Ki-67 proliferation index were found to have a shorter survival. Our study results suggest that p53 expression and Ki-67 proliferation index can be a prognostic marker and guide the prediction of survival in patients with lung adenocarcinoma after resection.
Keywords: Lung Cancer, Adenocarcinoma, Resection, p53 expression, Ki-67 Proliferation Index, Survive, Recurrence, Disease-free.

INTRODUCTION

Lung cancer is a worldwide problem and the leading cause of cancer-related mortality around the world [1]. It is the leading cause of death from cancer in men and women, accounting for approximately 26% of all cancer deaths in the United States [2]. While smoking is the leading cause of lung cancer, genetics also play a key role [1,3].

Lung cancer consists of two main subtypes, including small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), and the latter accounts for approximately 85% of diagnosed lung malignancies [2,4]. Non-small cell lung cancer has several subtypes such as adenocarcinoma, squamous-cell carcinoma, and large-cell carcinoma. In addition, adenocarcinoma is the most common subtype and accounting for about 40% of all NSCLC cases, followed by squamous-cell carcinoma, which accounts for about 25-30%.

Genetic alterations play a central role in the carcinogenesis of lung cancer, mutation of the p53

gene, which is a tumor suppressor gene located on chromosome 17p, is one of the most common genetic abnormalities in lung cancers [5]. The genetic abnormality in this gene has been reported in approximately 90% in SCLC and more than 50% in NSCLC cases [5, 6]. The mutation of this gene can be detected using immunohistochemical staining methods and it is expressed as p53 expression.

Antigen Ki-67, also known as MKI67, is a nuclear protein which is encoded by the MKI67 gene in humans, and the expression of this protein is strongly associated with cellular proliferation [7]. The Ki-67 protein is present during all active phases of the cell cycle; however, it is absent in the resting cells, which makes it an excellent marker to evaluate the growth fraction of the cellular activity [7]. Therefore, Ki-67 is a protein in cell nucleus which increases as they prepare to divide into new cells. The Ki-67 production in cells preparing to divide can be detected using immunohistochemical staining methods and it is known as Ki-67 expression or Ki-67 proliferative index.

There is a debate in the literature over the relation of p53 expression and Ki-67 proliferation index with survival in patients with NSCLC. Several studies have associated p53 gene mutation to aggressive tumor behavior and poor prognosis, whereas others have found no such association. In contrast, few studies demonstrated that p53 gene mutation was present in more than 50% of tumor cells and was an independent prognostic factor for improved outcomes. Similarly, there are some reports of shorter survival in patients with high Ki-67 proliferation index, while some authors suggest that there is no relationship between survival and Ki-67 proliferation index.

In the present study, we aimed to evaluate the relationship between p53 expression and Ki-67 proliferation index and tumor stage, lymph node involvement, pleural invasion, lymphovascular invasion, and survival in patients with NSCLC.

MATERIAL AND METHODS
Patient Selection

Twenty-six patients, who underwent complete anatomical resection and mediastinal lymph node dissection due to a lung adenocarcinoma at Hacettepe University, Department of Thoracic Surgery, Ankara, Turkey between January 2002 and December 2007, were included in the study. The preoperative examinations were carried out for each patient and the resectable cases were operated on. At least one year follow-up, survival data were recorded for all patients.

Data including disease stage, lymph node involvement, lymphovascular invasion, and pleural invasion were recorded for each patient using the postoperative pathological reports. The p53 expression and Ki-67 proliferation index were examined using the paraffin blocks of the pathological specimens.

The study was approved by the local Ethics Committee (LUT-09/5-25) and a written informed consent was obtained from each patient. The study was conducted in accordance with the principles of Declaration of Helsinki. Survival data of the patients were obtained from the patients or their relatives by contacting over the telephone numbers recorded in the hospital automation system.

Preparation of pathology slides

Tissue microarray, paraffin block and slides pertaining to the patients were available in the archive of the department of pathology. Immunohistochemical studies were performed to evaluate p53 expression and Ki-67 proliferation index in sections prepared from the tumor tissues. Immunohistochemical studies were conducted using 5-μm-thick sections of 10% formalin-fixed tissue microarray and paraffin blocks. Antigen retrieval procedure and avidin-biotin peroxidase method were used for all sections. The DO-7 mouse, monoclonal antibody (Neomarkers, Fremont, CA, USA) and SP-6 mouse, monoclonal antibody (Neomarkers, Fremont, CA, USA) were used for p53 and Ki-67 proteins, respectively.

Examination of pathology slides

Immunohistochemical findings were evaluated using the Zeiss Axioskop 2 microscope. Nuclear staining for p53 and Ki-67 was considered to be positive in immunohistochemical studies. Tumor samples were examined in five groups during evaluation of p53 expression. Staining in less than half of the tumor cells was considered weak staining, while staining in more than half of the tumor cells was defined as strong staining. Focal staining was defined as staining of tumor cells in a particular focus in the same tissue, while diffuse staining was defined as uniform staining of all tumor cells. Thus, five subgroups for p53 expression were created as follows:
- Tumors showing no staining for p53.
- Tumors with focal and weak staining.
- Tumors with focal and strong staining.
- Tumors with diffuse and weak staining.
- Tumors with diffuse and strong staining.

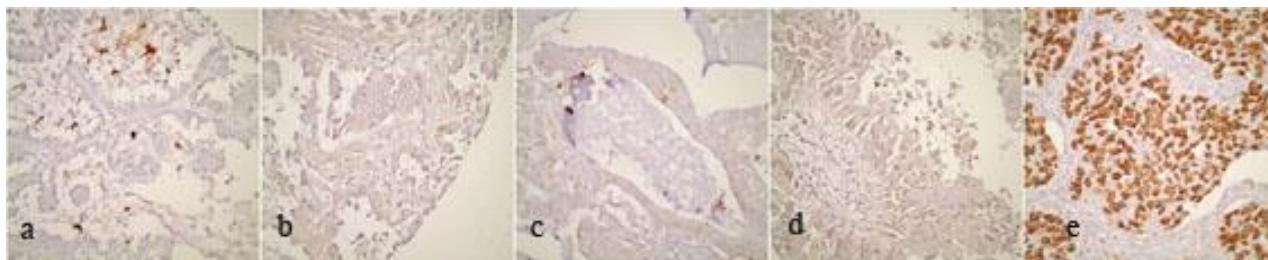The samples according to the p53 staining patterns are shown in Figure 1.

Fig-1: According to p53 staining pattern; (a) tumor tissue with negative staining, (b) focal and weak staining, (c) focal and strong staining, (d) diffuse and weak staining, (e) diffuse and strong staining (light microscopy, X400 magnification).

For the evaluation of the Ki-67 proliferation index in tissue microarrays, tumor cells were evaluated using a similar method. Tumor cells were divided into four groups according to the intensity of staining as follows:

- Tumor cells showing Ki-67 reactivity less than 10%.
- Tumor cells showing Ki-67 reactivity between 10% and 50%.

- Tumor cells showing Ki-67 reactivity between 50% and 90%.
- Tumor cells showing Ki-67 reactivity more than 90%.

The Ki-67 staining patterns of the samples are shown in Figure 2.
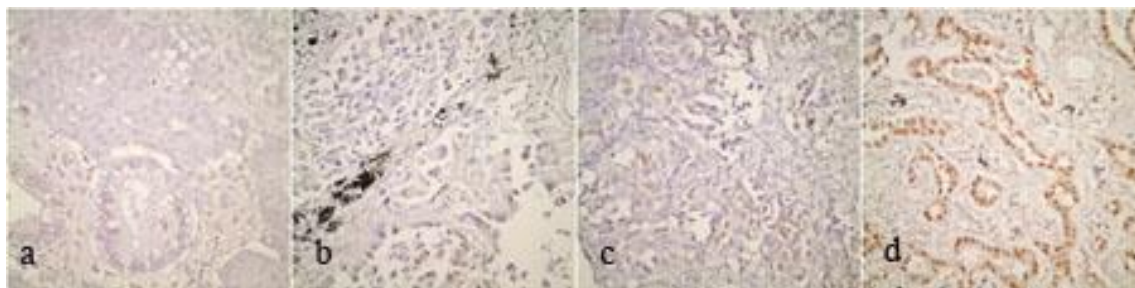

Fig-2: According to Ki-67 staining pattern; tumor tissues showing Ki-67 reactivity (a) less than 10%, (b) between 10% and 50%, (c): between 50% and 90%, and (d) higher than 90% (light microscopy, X400 magnification)

STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS version 11.5 software (SPSS Inc., Chicago, IL, USA). Non-parametric analysis methods were used, as the number of subjects to retrieve data from was not sufficient for the use of parametric analysis methods. Non-parametric Kendall's tau-b and Spearman's rho tests were used for the statistical analysis [8]. The "r" values at 0.05 alpha level in the Kendall's tau-b and Spearman's rho tests evaluating the relationship between parameters.

RESULTS

Of a total of 26 patients included in the study, 18 (69.2%) were males and eight (30.8%) were females. The mean age was 56.3 ± 10.4 (range: 35 to 74) years. At the time of the study, 18 patients (69.2%) were alive and eight patients (30.8%) were dead. According to the staging after the pathological examination of the resected specimens; 16 patients (61.5%) had Stage I disease, two patients (7.7%) had Stage II disease, and eight patients (30.8%) had Stage III disease. Seventeen patients (65.4%) had N0, one patient (3.8%) had N1, and eight patients (30.8%) had N2 disease. The disease stage was confirmed by pathological examination of the resected specimens in all cases. Seventeen patients (65.4%) had lymphovascular invasion, whereas, nine patients (34.6%) had no lymphovascular invasion. Twelve patients (46.2%) had pleural invasion and 14 patients (53.8%) had no pleural invasion.

The Relationship between p53 Expression and Tumor Stage, Lymphovascular Invasion, Lymph Node Involvement, Pleural Invasion, and Survival

Of the study patients, 11 (42.3%) showed no nuclear staining for p53 (no p53 expression). Other cases showed variable degrees of p53 expression. Of the patients with nuclear staining for p53, one (3.8%) showed focal and weak staining, two (7.7%) showed focal and strong staining, four (15.4%) showed diffuse and weak staining, and eight (30.8%) showed diffuse and strong staining. The p53 expression patterns of the cases are shown in Table 1.

Table-1: Distribution of cases according to p53 nuclear staining pattern

p-53 nuclear staining pattern	n	%
No staining for p53	11	42.3
Focal and weak staining	1	3.8
Focal and strong staining	2	7.7
Diffuse and weak staining	4	15.4
Diffuse and strong staining	8	30.8
Total	26	100.0

When cases showing variable degrees of p53 gene expression were divided into groups according to focal, diffuse, weak, and strong staining patterns, the number of the cases in certain groups was not sufficient to perform statistical analysis. Therefore, 15 cases showing variable degrees of p53 expression were included in a single group to compare them with 11 cases showing no p53 expression.

When the cases were evaluated in terms of survival, seven of 11 cases (63.6%) with no p53 expression survived, whereas four patients (36.4%) were dead. Eleven of 15 cases (73.3%) showing p53 gene expression was survivors, whereas four cases (26.7%) were non-survivors. All four non-survivor patients showed diffuse and strong staining pattern. The distribution of cases according to p53 expression and survival is shown in Table 2.

Table-2: Survival characteristics of cases according to p53 expression

p53 expression	Survive (n/%)	Exitus (n/%)	Total
Showing no p53 expression	7 (63.7%)	4 (36.3%)	11
Showing p53 expression	11 (73.3%)	4* (26.7%)	15
Total	18	8	26

* All four non-survivor patients showed diffuse and strong staining pattern.

The relationship between p53 gene expression and survival was investigated and a significant relationship was found. There was a significant negative relationship between p53 gene expression and survival at an alpha level of 0.05 both using Kendall's tau-b test (r=-0.505) and Spearman's rho test (r=-0.536). In 15 patients showing positive p53 expression, survival decreased with increasing intensity of staining and it was found that p53 expression negatively affected survival.

The relationship between p53 expression and tumor stage, lymphovascular invasion, lymph node involvement, and pleural invasion was investigated in these 15 patients, and a statistical analysis using the Kendall's tau-b test and Spearman's rho test showed no significant relationship between p53 expression and tumor stage, lymphovascular invasion, and lymph node involvement. However, statistical analysis using both tests showed a significant relationship between p53 expression and pleural invasion. There was a significant positive relationship between p53 gene expression and pleural invasion at an alpha level of 0.05 both using Kendall's tau-b test (r=0.505) and Spearman's rho test (r=0.536). In other words, the probability of pleural invasion increases with increasing p53 expression. The r values at 0.05 alpha levels in the Kendall's tau-b and Spearman's rho test evaluating the relationship between p53 gene expression and survival, tumor stage, lymphovascular invasion, lymph node involvement, and pleural invasion are presented in Table 3.

Table-3: The r values at 0.05 alpha levels in the Kendall's tau-b and Spearman's rho test in comparison of cases with p53 gene expression in terms of survival, tumor stage, lymphovascular invasion, lymph node involvement, and pleural invasion

Non-parametric analysis methods	p53 expression vs. survive	p53 expression vs. tumor stage	p53 expression vs. lymphovascular invasion	p53 expression vs. lymph node involvement	p53 expression vs. pleural invasion
Kendall's tau-b	-0.505*	-0.71	0.180	-0.019	0.505*
Spearman's rho	-0.536*	-0.74	0.191	-0.029	0.536*

* Statistically significant parameters.

The Relationship between Ki-67 Proliferation Index and Tumor Stage, Lymphovascular Invasion, Lymph Node Involvement, Pleural Invasion, and Survival

Tumor cells were divided into four groups according to the intensity of staining for Ki-67: tumors showing Ki-67 reactivity less than 10%, tumors showing Ki-67 reactivity between 10% and 50%, tumors showing Ki-67 reactivity between 50% and 90%, and tumors showing Ki-67 reactivity more than 90%. Accordingly, 14 of the patients included in the study (53.8%) showed Ki-67 reactivity less than 10%

and Ki-67 proliferation index was expressed as less than 10%. Three cases (11.5%) showed Ki-67 reactivity between 10% and 50%, two cases (7.7%) showed Ki-67 reactivity between 50% and 90%, and seven cases (26.9%) showed Ki-67 reactivity more than 90% (Table 4).

Table-4: Distribution of cases according to Ki-67 nuclear staining pattern

Ki-67 Proliferation Index	n	%
Showing Ki-67 reactivity less than 10%	14	53.8
Showing Ki-67 reactivity between 10% and 50%	3	11.5
Showing Ki-67 reactivity between 50% and 90%	2	7.7
Showing Ki-67 reactivity more than 90%	7	26.9
Total	26	100.0

Mortality was seen in eight of 26 patients. These non-survivor patients were further analyzed in terms of their Ki-67 reactivity. Accordingly, all non-survivor patients showed nuclear Ki-67 reactivity less than 10%. Survival characteristics of the cases according to Ki-67 proliferation index are shown in Table 5.

Table-5: Survival characteristics of cases according to Ki-67 proliferation index

Ki-67 Proliferation Index	Survive (n/%)	Exitus (n/%)	Total
Showing Ki-67 reactivity less than 10%	6 (42.9%)	8 (57.1%)	14
Showing Ki-67 reactivity between 10% and 50%	3 (100%)	0 (0%)	3
Showing Ki-67 reactivity between 50% and 90%	2 (100%)	0 (0%)	2
Showing Ki-67 reactivity more than 90%	7 (100%)	0 (0%)	7
Total	18	8	26

The relationship between the Ki-67 proliferation index and survival was investigated. There was a significant positive relationship between the Ki-67 proliferation index and survival at an alpha level of 0.01 using both Kendall's tau-b test (r=0.553) and Spearman's rho test (r=0.588). In other words, survival increased with increasing Ki-67 proliferation index. Also, the relationship between the Ki-67 proliferation index and tumor stage, lymphovascular invasion, pleural invasion, and lymph node involvement was investigated. Accordingly, there was no statistically significant relationship between the Ki-67 proliferation index and aforementioned parameters. The r values at 0.01 alpha levels in Kendall's tau-b and Spearman's rho test evaluating the relationship between the Ki-67 proliferation index and survival, tumor stage, lymphovascular invasion, lymph node involvement, and pleural invasion are presented in Table 6.

Table-6: The r values at 0.01 alpha level in Kendall's tau-b and Spearman's rho test in comparison of cases according to Ki-67 proliferation index in terms of survival, tumor stage, lymphovascular invasion, lymph node involvement, and pleural invasion

Non-parametric analysis methods	Ki-67 vs. survive	Ki-67 vs. tumor stage	Ki-67 vs. lymphovascular invasion	Ki-67 vs. lymph node involvement	Ki-67 vs. pleural invasion
Kendall's tau-b	0.553*	0.102	0.011	-0.065	0.075
Spearman's rho	0.588*	0.113	0.012	-0.067	0.079

* Statistically significant parameters

DISCUSSION

Gene p53, also named as TP53, is a tumor suppressor gene and it regulates the cellular response to a variety of cellular stress signals by inducing cell cycle arrest, senescence, and apoptosis [9]. The genetic alterations in the p53 pathway include mutations, polymorphisms, and over-expression [9]. The p53 gene remains the most frequently altered gene in all human cancers, and genetic alterations are associated with cancer risk, therapy resistance, and poor prognosis in several tumor types including lung adenocarcinomas [10]. The genetic alterations of the p53 gene occur very frequently in lung carcinomas (more than 90% of SCLCs and more than 50% of NSCLCs), and they play an important role in the oncogenic transformation of lung epithelial cells to lung carcinoma progression [11].

There is a debate in the literature over as how the alterations in this gene and associated increase in p53 expression affect survival in patients with a lung adenocarcinoma. In review of the literature, studies reporting that p53 expression negatively affects prognosis and survival in patients with NSCLC predominate [6-16]. However, there are also studies suggesting no relationship between p53 expression and survival in patients with NSCLC [11, 17, 18]. Interestingly, there are a few studies reporting a relationship between p53 expression and long-term

survival in patients with a lung adenocarcinoma [19]. Deben *et al*. investigated p53 expression and its association with clinical outcomes in patients with a lung adenocarcinoma and authors concluded that p53 expression was a negative predictor of the outcome in patients with NSCLC and adenocarcinoma [9, 10]. Similarly, a meta-analysis by Gu *et al*. reported that p53 mutation was associated with a poor clinical outcome in patients with NSCLC [12]. Bian *et al*. examined 120 patients with a lung adenocarcinoma and 76 (63.3%) of them had p53 expression and this expression was significantly associated with shorter overall survival [13].

Apart from papers reporting a negative impact of p53 expression on prognosis and survival of patients with lung adenocarcinoma, there are also studies reporting that cases showing p53 expression have a poor response to chemotherapy [5, 20]. In the analysis of Kawasaki et al. p53 expression was reported to be associated with poor prognosis and chemo-resistance in patients with NSCLC showing p53 expression [5]. In the study by Rusch *et al*. cases with p53 expression showed a higher rate of resistance to cisplatin-based chemotherapy [20].

Although poorer prognosis, shorter survival and higher rate of resistance to chemotherapy have been reported in patients with a lung adenocarcinoma showing p53 expression, there are also studies reporting no relationship between p53 expression and survival. In an analyses published by Smardova *et al*. the authors found no statistically significant differences in overall and disease-free survival in relation to the p53 expression [11]. Similarly, Kosaka *et al*. reported that p53 gene mutations were not independently associated with the prognosis in the surgically treated lung adenocarcinoma cases [18]. In contrast to these articles, Lee *et al*. reported an optimistic relationship between p53 expressions and survival [19]. According to their study, the patients in the high p53 expression group survived longer than low expression group; therefore, p53 positivity in more than 50% of tumor cells was an independent prognostic factor for improved outcomes [19].

In evaluating the relationship between p53 expression and survival in the present study, a significant negative relationship was found using the non-parametric analysis methods. In other words, p53 expression negatively affects survival and survival decreased with increasing intensity of staining. The results on p53 gene expression are consisted with the literature data. In addition, there was a significant relationship between p53expression and pleural invasion using non-parametric analysis methods: pleural invasion increased with increasing p53 expression. However, there was no significant relationship between the p53expression and tumor stage, lymphovascular invasion, and lymph node involvement.

Ki-67 is a cellular nuclear protein regulating the cell cycle and this nuclear antigen indicates tumor proliferation [21, 22]. Ki-67 is one of the most extensive cell proliferation markers and expressed at all stages of the cell cycle [23]. The expression of this protein is found to be highly associated with tumor development, metastasis, and prognosis [24]. According to Yamashita *et al*. Ki-67 expression of tumors in the segmentectomy patients was associated with disease-free survival [25]. The patients with Ki-67 proliferation index less than 5% also showed improved disease-free survival compared to those with an index of more than 5% [25].

The relationship between the Ki-67 proliferation index and survival is another controversial subject in the literature. There are several studies reporting no relationship between the Ki-67 proliferation index and risk of recurrence and survival [22, 26]. In the study by D'Amico *et al*. increased Ki-67 proliferation index was not found to be associated with the risk of recurrence in NSCLC [22]. In another study by D'Amico et al. no relationship was reported between the Ki-67 proliferation index and survival in Stage I NSCLC [26]. However, there are studies reporting a negative relationship between the Ki-67 proliferation index and survival in patients with a lung adenocarcinoma [21, 27, 28, 29, 30]. In a meta-analysis by Martin *et al*. Ki-67 expression was found to be a poor prognostic factor in the lung cancer and these patients had shorter survival [21]. In a similar meta-analysis published by Wen *et al*. increased Ki-67 proliferation index was reported to be associated with decreased survival in patients with NSCLC [28].

Along with its value in predicting disease-free survival and overall survival, the Ki-67 proliferation index is also valuable in predicting the risk of recurrence. In the study by Woo *et al*. the Ki-67 proliferation index more than 10% was found to be a poor prognostic factor in patients with Stage I lung adenocarcinoma and it increased the risk of recurrence [29]. Among the cases included in the study by Woo *et al.* the risk of tumor recurrence was 11.4% in patients with Ki-67 proliferation index more than 10%, while the risk was 2.2% in patients with Ki-67 proliferation index less than 10% (p<0,0001) [29]. In the study by Oka *et al*. Ki-67 was reported to be a useful predictive marker in cases with NSCLC undergoing resection and that it could be used to predict the probability of recurrence in the post-operative period [30]. In addition, there are reports suggesting that the Ki-67 proliferation index is a better prognostic marker than the histological subtype in cases with lung carcinoma [31]. In the study by Soomra *et al*. patients with a lung carcinoma and with a Ki-67 proliferation index less than 5% were reported to have a significantly longer survival and Ki-

67 was a better prognostic marker than the histological subtype [31].

The present study found a significant positive relationship between the Ki-67 proliferation index and survival which is inconsistent with the common literature data. In other words, survival was found to increase with increasing Ki-67 proliferation index. All non-survivors in the study group had a Ki-67 proliferation index less than 10%. There are a vast number of factor affecting long-term survival in patients with a lung adenocarcinoma. Along with the success of surgery and post-operative care, tumor stage, involvement of the lymph nodes, presence of undetected distant metastasis and post-operative adjuvant therapy are the leading factors. Inconsistent with the literature, the finding that all non-survivors in the present study had a Ki-67 proliferation index less than 10% may be related with small number of subjects in the study group. In addition, our findings may be also associated with factors such as adjuvant therapy application and palliative care included in the long-term follow-up of cases with lung cancer and affecting survival. Other than these findings, the present study found no relationship between p53 expression and Ki-67 proliferation index and tumor stage, lymphovascular invasion, and lymph node involvement, and there was only a relationship between p53 expression and pleural invasion.

The limitation of the present study was that there were a small number of subjects in the study group. In addition there was no knowledge about adjuvant therapy applications to patients with operated NSCLC. On the other hand, palliative care is an important subject on long-term follow-up. However, no information was available about palliative care of the lung carcinoma patients.

CONCLUSION

Survival was negatively affected with increasing intensity of staining in patients showing p53 expression. Although increased Ki-67 proliferation index was not shown to have a negative effect on survival, the patients with a low Ki-67 proliferation index were found to have a shorter survival. However, considering the fact that survival in cases with lung cancer is related with many other factors, further studies evaluating multiple parameters including those affecting survival are required to more accurately interpret the current findings.

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