The Expressions of pAkt and PTEN in Lung Cancer Patients
24 Hours After the Cisplatin-Based Chemotherapy: A Prospective Pilot Study

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ABSTRACT
Akt/PKB is a protooncogen while PTEN is a tumor suppressor gene. Their expressions are of immense importance in the development of lung cancer. However, little is known about their relations to anti-cancer treatments. Therefore, we aimed to elucidate how are these parameters affected by the treatment.
Expression of phosphorylated Akt (pAkt) and PTEN have been analysed on tissues of 32 patients (stage III and IV) with lung cancer. In addition, the expression of these variables in 14 out of 32 patients have furtherly been analysed in terms of their response to cisplatin-based chemotherapy in vivo. Prior to and 24 h after the treatment, tumor tissues were obtained via broncoscopy and then evaluated immunohistochemically by indirect streptavidin-biotin peroxidase method.
Immunoreactivity for pAkt was detected in 29 of 32 cases (91%), pAkt was observed to localize in the nucleus of positively stained cells. However, PTEN expression was found in 27 of 32 cases (84%). In contrast to the localization of pAkt that is nucleus, PTEN was however localized in the cytoplasm of positively stained cells. pAkt and PTEN expression levels of 14 post-chemotherapy patients were compared to those before chemotherapy. There was no statistically significant differences (p>0.05).
Although these results do not imply any possible roles of pAkt or PTEN in the late stage lung cancer patients as a biomarker for the prediction of early response to treatment in vivo, this conclusion needs to be analyzed further at later time points in a larger cohort.

Keywords: Lung Cancer, pAkt, PTEN, Cisplatin, Chemotherapy
INTRODUCTION

Cancer cells are known to escape normal growth control mechanisms as a consequence of activating mutations and increased expression of one or more cellular protooncogenes and inactivating mutations and decreased expression of one or more tumor suppressor genes. Most oncogene and tumor suppressor gene products are components of signal transduction pathway that control cell cycle entry or exit, promote differentiation, sense DNA damage and initiate repair mechanisms, and regulate cell death programs. Several oncogenes and tumor suppressor genes belong to the same signaling pathway.1 The understanding of the signaling pathway involved may help us find predictive factors for tumor aggressiveness and therapy resistance. The growth factor receptors utilize several signaling pathways, such as the ras/mitogen-activated protein kinase pathway that is important for mitogenic stimulation. Other receptor signals are transmitted by the PI3K/Akt pathway.2

Akt, known as protein kinase B, represent a subfamily of the serine/threonine protein kinases. By a variety of stimuli, Akt is activated by phosphorylation at Thr-308/309 in the kinase activation loop and Ser-473/474 in the carboxyl-terminal tail.3 Recent studies have shown the roles of Akt implicated in tumorigenesis.4 Activated Akt induces survival and suppresses apoptosis induced by a variety of stimuli, including growth factor withdrawal and anoikis in numerous cell types.5 Akt inhibits apoptosis by phosphorylating the Bcl-2 family member pro-apoptotic BAD and by preventing the release of cytochrome c from mitochondria.6 The activation of this pathway has proven important for cell survival, and inhibitors have been shown to facilitate apoptosis and to sensitize cells to cytotoxic drugs in experimental studies.2 Proteins that participate in this signaling might therefore be good candidates for predicting the result of therapy. Overexpression of Akt therefore may contribute to tumor development and progression.

PTEN (phosphatase and tensin homolog deleted on chromosome ten) tumor suppressor gene plays an important role in the modulation of the PI3K (phosphatidylinositol 3-kinase), which is involved in cell proliferation and survival.6 PTEN antagonizes PI3K activity and negatively regulates its downstream-target, Akt7 and frequently mutated or deleted in a large number of human cancer.8 An earlier report documented that Akt is constitutively active in the cell lines arising form NSCLC and the Akt activation promotes cancer survival and resistance to chemotherapy and radiation.9

A rational approach to understanding cancer pathogenesis and developing novel, mechanism-based therapies requires identifying the components of these signaling pathways and determining how mu-
tations in oncogenes and tumor suppressor genes disrupt them. \(^\text{10}\) Akt and PTEN expressions are of immense importance in lung cancer. However, little is known about their relation to anti-cancer treatments. In fact, there is no information at all about how their expressions are changed in vivo after the treatment.

In this study, we therefore investigated the expressions of pAkt and PTEN in lung cancer patients before and after the treatment with cisplatin-based chemotherapy and then prospectively analyzed their relations to the response to treatment in vivo. To our knowledge, this is the first report implying that cisplatin-based chemotherapy increases the levels of PTEN in vivo although it did not reach the statistically significant level. However, a larger sample size is required to analyze it better.

**MATERIAL AND METHODS**

**Patient selection and treatment**

Patients with previously untreated lung carcinomas were included in the study. All patients involved were in the inoperative stage (stage IIIB, IV) and had bronchial vegetative lesions. The study population consisted of 32 patients with lung cancer. The patients who had additional malignancy, pneumonia, severe dispnea, infection, interstitial lung disease were not included in the study. Performance status of patients was determined according to the Eastern Cooperative Oncology Group (ECOG) criteria. Patients had an ECOG performance status \(\leq 2\); had an adequate marrow, renal and hepatic function (WBC count \(\geq 4 \times 10^3/L\), platelet count \(\geq 100 \times 10^3/L\), and bilirubin, serum creatinine, and transaminase levels within the normal range); had no previous history or electrocardiographic evidence of abnormal cardiac function and comorbidity contraindicating the administration of the chemotherapeutic agents. The patients were treated with cisplatin-based chemotherapy (cisplatin and vinorelbine/gemcitabine for non small cell lung cancer, cisplatin and etoposide for small cell lung cancer). Cisplatin was applied at the dose of 70 mg/m\(^2\) on 1st day only. Vinorelbine (30 mg/m\(^2\)) or gemcitabine (1250 mg/m\(^2\)) was applied on both 1st day and 7th day. Etoposide (120 mg/m\(^2\)) was applied on 1st, 2nd, and 3rd days.

The response to treatment was evaluated according to the criteria of the Lung Diseases and Tuberculosis Department of Uludag University Medical School by referring to the computerised tomography (CT) scan after the application of 3 cycles of chemotherapy. According to the CT scan results: responses were classified as progressive disease if the tumor volume increased by more than 25\% or patient died; as regression if the tumor volume decreases by more than 50\%; as stable disease if the tumor volume remains the same. The ethical committee of Uludag University approved the study. An informed consent was obtained from all the subjects.

**Assessment of pAkt and PTEN expressions**

The analyses have been performed on tumor tissues obtained prior to and 24 h after the treatment. Tumor tissues were fixed in PBS-buffered 10\% formalin and paraffin embedded for immunohistochemical processing. For immunohistochemical staining, the usual indirect streptavidin-biotin peroxidase method was employed as follows. Formalin-fixed paraffin-embedded tissue sections (5 \(\mu m\)) were deparaffinized in xylene and rehydrated in a graded series of ethanol. For staining of pAkt and PTEN, the sections were placed in a citrate buffer solution and then microwaved at 400 W for the antigen retrieval for 25 minutes. The sections were allowed to cool in the solution for 20 minutes at room temperature, rinsed in distilled water, and transferred to a bath of phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked with 10 min incubation in 3 \% H\(_2\)O\(_2\) in distilled water. Then, tissues were incubated at 37\^\circ C for 45 min in a moist chamber with primary antibodies (dilution: 1:500). Sections were incubated with biotinylated goat anti-rabbit IgG secondary antibody (Dako ABC kit) for 25 minutes, and they were incubated with streptavidin-horseradish peroxidase for 25 minutes, following this step, sections were incubated in the 3-3 diaminobenzidin (DAB) chromogen until the specific regions were stained brown, and the sections were counterstained for 30 s with hematoxylin. The primary antibodies used rabbit polyclonal phospho-Akt [ser473] antibody (Santa Cruz Biotech, Santa Cruz, CA) at a dilution of 1:500 and rabbit polyclonal PTEN (Santa Cruz Biotech, Santa Cruz, CA) at a dilution of 1:300. For
positive control for each of the antibodies, sections previously validated by us to be strongly positive were used. Negative controls for each of the antibodies were performed using PBS instead of primary antibodies to exclude nonspecific staining.

**Semi-quantitative scoring of protein expressions**

Scoring has been made by taking into account both the intensity of staining and the distribution (extent) of positively staining cells. Therefore, the expression of the proteins was evaluated by a semi-quantitative scoring system, according to a recently-published report by Xu. The intensity of staining was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The extent of staining was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%) according to the percentage of cells staining positive for each protein. The sum of the intensity and extent scores was calculated to estimate the final staining score (0-7). Tissues having a final staining score >2 were considered to be positive for each protein. Final staining score of 2-3 was considered + (weak expression), 4-5 was ++ (moderate expression), and 6-7 was +++ (strong expression). Tissues having a final staining score <2 were considered to be negative. In the text and the tables; the final scores of negative, +, ++, and +++ stainings were shown as 0, 1, 2, and 3, respectively. All slides were coded so that two independent investigators were blinded to staining for each slide.

**Statistical analysis:** The statistical analysis was performed using SPSS 11.0 version for windows program (SPSS Inc., Chicago, IL, USA). Mann-Whitney U test was used for the comparison of the distributions of continuous variables between groups. Within-group analysis was evaluated with Wilcoxon Signed Rank’s test. Correlations of the analytic variables were investigated with Spearman’s correlation analysis. A value of p<0.05 was considered statistically significant.

**RESULTS**

In this study, expressions of pAkt and PTEN have been analyzed on tumor tissues of 32 patients with lung cancer. In addition, the expression of these variables in 14 out of 32 patients have further been analyzed in terms of their response to cisplatin–based anti-cancer treatments.

In the lung cancer samples analyzed, immunoreactivity for pAkt was detected in 29 of 32 cases (91%). pAkt was observed to localize in the nucleus of positively stained cells as shown in Figure 1. Regarding the semi-quantitative values of the immunostaining, the strong, moderate, weak, and negative expressions were observed in 12 (38%), 15 (47%), 2 (6%), and 3 (9%) of lung cancer patients, respectively. The relevant descriptive statistics are shown in Table 1A and 1B. The majority of tumors (85%) expressed moderate to strong expressions of pAkt.

**Figure 1.** pAkt positively-stained cells displayed nuclear staining. Immunohistochemistry was performed as explained in the Materials and Methods. (Mag. X200)

**Figure 2.** PTEN-positively-stained cells displayed cytoplasmic staining. Immunohistochemistry was performed as explained in the Materials and Methods. (Mag. X200)
PTEN expression was found in 27 of 32 cases (84%). In contrast to the localization of pAkt that is nucleus, PTEN was however localized in the cytoplasm of positively stained cells (Figure 2).

According to the evaluation of positively stained cancer cells for PTEN, the final scores were found to be strong, moderate, weak, and negative expressions in 9 (28%), 13 (40%), 5 (16%), and 5 (16%) of lung cancer patients, respectively. For the PTEN, the majority of tumors (68%) expressed moderate to strong expressions, quite similar to those observed for the pAkt. There was a statistically significant correlation between pAkt and PTEN levels before the chemotherapy (Spearman’s correlation, r= 0.718, p= 0.000).

Patients were also evaluated in terms of the expressions of pAkt and PTEN after the chemotherapy. 14 post-chemotherapy patients were available in order to evaluate the difference between the levels before and after the chemotherapy. In the evaluation, the expression levels of pAkt were found to be strong, moderate, and negative in 4 (28%), 7 (50%), and 3 (21%) of the post-chemotherapy patients, respectively. In the same 14 patients, the expression levels of PTEN was found to be strong, moderate, weak, and negative in 4 (28%), 4 (28%), and 2 (14%), and 4 (28%), respectively. The relevant descriptive statistics are shown in Table 2.

Taking into account the pre- and post-chemotherapy values of PTEN in 14 patients, the strong expression was present in 14% (n= 2) of the patients before the chemotherapy. However, it has doubled to 28% (n= 4) after the chemotherapy. In parallel with this change, the expression levels of 6 patients were in the trend of increase after the chemotherapy as appeared in Table 2.

In addition, in these 14 evaluable patients, the correlation between pAkt and PTEN levels was found to be weaker after the chemotherapy (Spearman’s correlation, r= 0.616, p= 0.019), compared to that prior to the chemotherapy (Spearman’s correlation r= 0.850, p= 0.000). Thus, this correlation has, to some extent, been disturbed after the chemotherapy although it remained statistically significant.

PTEN and pAkt values of 14 post-chemotherapy patients were compared to those values before chemotherapy. There was no statistically significant differences (Wilcoxon Sign Rank’s, z= 0.317, 30

### Table 1A.
The descriptive data of final staining scores (expression levels) of pAkt and PTEN, according to the diagnosis.

<table>
<thead>
<tr>
<th></th>
<th>NSCLC pAkt</th>
<th>SCLC pAkt</th>
<th>NSCLC PTEN</th>
<th>SCLC PTEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (±S.D.)</td>
<td>2.1 (±0.97)</td>
<td>2.2 (±0.44)</td>
<td>1.85 (±1.02)</td>
<td>1.6 (±1.14)</td>
</tr>
<tr>
<td>(min-max)</td>
<td>0-3</td>
<td>0-3</td>
<td>0-3</td>
<td>0-3</td>
</tr>
<tr>
<td>Median</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

NSCLC: nonsmall cell lung cancer, SCLC: small cell lung cancer

### Table 1B.
The descriptive data of final staining scores (expression levels) of pAkt and PTEN, according to the stage of the disease

<table>
<thead>
<tr>
<th></th>
<th>Stage III pAkt</th>
<th>Stage IV pAkt</th>
<th>Stage III PTEN</th>
<th>Stage IV PTEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (±S.D.)</td>
<td>2.1 (±1.06)</td>
<td>2.07 (±0.64)</td>
<td>1.73 (±1.06)</td>
<td>1.92 (±0.95)</td>
</tr>
<tr>
<td>(min-max)</td>
<td>0-3</td>
<td>0-3</td>
<td>0-3</td>
<td>0-3</td>
</tr>
<tr>
<td>Median</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
Out of 32 patients, 11 were progressive, 17 regressive, and 4 stable disease. The progressive and regressive patients were compared in terms of their pAkt and PTEN values. It was found that there were no differences between these two groups (Mann-Whitney U, z = 0.295, p > 0.05). Out of 32 patients, 11 were alive, and 21 were dead at the time of evaluation. The relevant descriptive statistics of alive and dead patients are shown in Table 3. It has been found no significant difference in terms of pAkt and PTEN expressions between the dead and alive groups (Mann-Whitney U test, z = 0.573, p > 0.05). In 11 alive patients, there was statistically significant correlation between pAkt and PTEN values (Spearman’s correlation, r = 0.736, p = 0.010). There was similar correlation between the same variables in 21 dead patients (Spearman’s correlation, r = 0.739, p = 0.000).

**DISCUSSION**

Most chemotherapeutic agents activate apoptotic cell death pathways. Preclinical and clinical studies have shown that apoptosis significantly increases 24 h after chemotherapy administration. Therefore, the evaluation of apoptosis pathways-related proteins (e.g. pAkt and PTEN) might be a rational approach to elucidate the efficacy of specific or novel therapies as early as 24 h after the chemotherapy. The present study therefore had the aims: to determine the expressions of pAkt and PTEN before cisplatin-based chemotherapy in late stage lung cancer patients, to determine whether there is any correlation between the expressions of pAkt and PTEN before and after the therapy, and to analyse their possible relations to both the early response to therapy and the overall survival.

In our study, considerable numbers of lung cancer patients (91%) cases showed pAkt immunoreactivity in tumor cells. This result was in accordance with the study (in press) by Tsurutani et al in which they found that 95% of all the lung cancer patients had Akt expression. The pAkt expression was reported to be observed both in cytoplasm and nucleus according to the previous reports but we observed in nucleus only. This could be due to the fact that different antibodies or cells might cause different stainings depending on the cross reactivities with other proteins in the cells.

There is accumulating evidence that deregulation of apoptosis may be directly involved in the development and progression of human cancers. Also, apoptosis is the predominant mechanism by which cancer cells die following chemotherapy and radiation therapy. Activated Akt is a well-established

<table>
<thead>
<tr>
<th>Before treatment pAkt</th>
<th>After treatment pAkt</th>
<th>Before treatment PTEN</th>
<th>After treatment PTEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (±S.D.)</td>
<td>1.71 (±1.13)</td>
<td>1.85 (±1.09)</td>
<td>1.42 (±1.08)</td>
</tr>
<tr>
<td>(min-max)</td>
<td>0-3</td>
<td>0-3</td>
<td>0-3</td>
</tr>
<tr>
<td>Median</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

**Table 3.** The descriptive data of alive and dead patients

<table>
<thead>
<tr>
<th>pAkt, Alive (n= 11)</th>
<th>pAkt, Dead (n= 21)</th>
<th>PTEN, Alive (n= 11)</th>
<th>PTEN, Dead (n= 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (±S.D.)</td>
<td>143.8 (±63.44)</td>
<td>127.8 (±89.7)</td>
<td>100.9 (±58.8)</td>
</tr>
<tr>
<td>(min-max)</td>
<td>60-240</td>
<td>0-270</td>
<td>0-180</td>
</tr>
<tr>
<td>Median</td>
<td>130</td>
<td>140</td>
<td>110</td>
</tr>
</tbody>
</table>
survival factor, exerting anti-apoptotic activity by preventing release of cytochrome c from mitochondria and inactivating forkhead transcription factors known to induce expression of pro-apoptotic factors such as Fas ligand. Moreover, Akt activates IκB kinase, a positive regulator of NF-κB, which results in transcription of anti-apoptotic genes. Thus, it is conceivable that inhibition of the Akt pathway could prevent cancer cells from surviving after therapy. Therefore, targeting Akt pathway by any means (e.g., siRNAs in combination with cytotoxic regimens) could be a rational approach for the better treatment of lung cancer patients. For this aim, patients should be sub-grouped according to their Akt expressions otherwise there is a risk of getting disappointing results. In fact, targeted therapies resulted in some failures in advanced lung cancer patients and the reasons underlying these failures were thought to be, not always fully understood, the lack of careful patient selection (sub-grouping). However, we do not think the sub-grouping of patients in terms of their expression levels of Akt could be much useful in order to achieve better response rates or overall survival rates. The reason for this is the fact that a significant portion (85%) of the patients has moderate to strong levels of expressions, according to our study. In fact, it was recently reported that there was not found any association with Akt and the survival.

In our study, we found a positive correlation between pAkt and PTEN expressions although a negative correlation was also reported. In contrast to these two different findings, it was also reported that there was no correlation between Akt and PTEN. It seems that there is no proper consensus on this debate. In fact, there are more debates on the role of Akt in lung cancer (e.g., its impact on survival or its role as a prognostic factor). One recent study suggests that pAkt was a significant independent favorable prognostic factor (p = 0.004) in NSCLC, while another recent study reported that pAkt was a poor prognostic factor for all stages of NSCLC. In the latter study, it was reported that these conflicting results were due to the fact that only one site of phosphorylation of Akt had so far been evaluated in clinical non-small cell lung cancer specimens although two different sites together were of importance in the activation. Moreover, the immunolocalization of pAkt also seems to have an impact on the prognosis. Therefore, the different findings on the correlations as well as on survival/prognosis might be explained by these matters (two site-activation and immunolocalization) as well as by the characteristics of our patients whom of all were only at the late stage (stage III or IV). In our study, the initial (before chemotherapy) correlation was found to retain even after the chemotherapy although it was disturbed to some extent. This disturbance is highly possible due to the effect of chemotherapy on the expressions of both proteins. We would even expect higher levels of disturbances. In our study, the finding of no statistically significant change in the expression levels of either pAkt or PTEN after the chemotherapy could also be due to the fact that only the patients with advanced stage were involved in the study. Therefore, we are currently in preparation of recruiting early stage of patients to test this hypothesis as another project. The other important point is that we evaluated the cancer tissues 24 h after the chemotherapy in order to look at the relatively early effects of the chemotherapy. However, it might also be interesting to observe the late effects of the chemotherapy (after the 3 cycles of the chemotherapy, for example).

In conclusion, although there has not been a statistically significant change in the expression levels of pAkt and PTEN 24 h after the chemotherapy, the results tend to imply that PTEN is induced by the chemotherapy in vivo. The unique property of this study is to be performed on cancer tissues removed from patients followed by chemotherapy. However, recruitment of the patients into the study just following the chemotherapy was a hard task, which limited the number of the patients involved. With regard to this limitation, our results should therefore be confirmed by a larger sample size at later time points in order to better elucidate the roles of pAkt and PTEN in the response to treatment in lung cancer patients.

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