Association of FOXP3 Expression with Non-small Cell Lung Cancer

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Abstract. Background/Aim: Lung cancer is rarely cured by current therapeutic approaches. Although numerous studies have implicated FOXP3 positive regulatory T-cells in cancer pathogenesis, the role of FOXP3 in lung cancer pathogenesis remains unknown. Materials and Methods: Using immunohistochemistry FOXP3 expression was determined in 44 NSCLC tissue specimens, 20 samples from adjacent non-neoplastic lung parenchyma and 5 normal lung tissue specimens. Results: FOXP3 immunostaining was always nuclear in both tumor and non-neoplastic adjacent tissues. FOXP3 was also detected at lower levels in normal bronchial epithelium. Moreover, FOXP3 expression in cancer cells correlated with lymphocytic FOXP3-immunopositivity and the presence of lymph node metastasis. FOXP3 lymphocytic expression was also negatively associated with the age of the patients. Conclusion: FOXP3 is overexpressed in NSCLC cells and tumor-infiltrating lymphocytes. This study provides evidence that lymphocytic FOXP3 expression may be age related and that tumor FOXP3 expression is correlated with lymph node metastasis.

At the beginning of the 20th century, William Osler stated that “primary tumors of the lung are rare” (1). Lung cancer is today the most common cause of cancer-related death in both sexes (30% in males, 26% in females) in the United States and worldwide (2, 3). Lung cancer can be clinically divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Approximately 85% of lung malignant tumors are NSCLC (squamous cell carcinoma, adenocarcinoma and large cell carcinoma) and about 15% of them are SCLC (3). The current therapeutic approaches, which include surgical resection, platinum-based doublet chemotherapy, radiotherapy and targeted therapies, rarely cure the disease and the overall survival rate is about 15% (4).

Understanding the molecular mechanisms which are implicated in lung carcinogenesis will change the current therapeutic approach towards lung cancer. In recent decades, much research effort has been spent to delineate the pathobiology of malignant epithelial lung tumors, albeit with little impact on disease prognosis. Although a wide spectrum of genes and proteins has been studied, the role of FOXP3 in lung cancer remains unexplored.

FOXP3 belongs to a family of evolutionarily conserved transcriptional regulators (forkhead box proteins) defined by a winged helix DNA-binding domain (5). FOXP3 acts as a master regulator of the development and function of regulatory T-cells, in particular the CD4+CD25+ thymus-derived subset (natural Tregs), which have a critical role in the transfer of immune tolerance and in the formation of an immunosuppressive tumor microenvironment (6).

Until recently, the role of FOXP3 was thought to be restricted to the T-cell lineage (7). A recent study in mice showed immunohistochemically and by real-time PCR that FoxP3 is expressed in breast, lung and prostate epithelial cells but not in liver, heart and intestine (8). FOXP3 mRNA was also detected in human cancer (pancreas, colon, breast, lung, melanoma) and leukemia cell lines using conventional reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR, while FOXP3 protein expression in the same cell lines was appraised by immunocytochemistry and flow cytometry (9, 10).
addition, FOXP3 expression changes in human pancreatic, breast, melanoma and prostate cancer have been confirmed by immunohistochemistry (7, 10-13).

In this work, by immunohistochemistry, we investigated whether FOXP3 is expressed in invasive NSCLC and whether its expression correlates with tumor clinicopathological parameters.

Materials and Methods

Tissue specimens. This study was carried out according to the principles and after the approval of the Committee on Research and Ethics and the Scientific Committee of the University Hospital of Patras, Greece. The study comprised 44 formalin-fixed paraffin-embedded (FFPE) NSCLC samples and 20 FFPE samples of adjacent non-neoplastic lung parenchyma from 44 patients with NSCLC. All cases were surgically managed at the University Hospital of Patras between 2005 and 2008 and all the samples were retrieved from the archives of the Laboratory of Pathology of the University Hospital of Patras. In addition, 5 FFPE normal tissue specimens were obtained at autopsy from lungs of never-smokers.

Clinicopathological information was obtained from the pathology reports and is shown in Table I. Twenty-three samples were squamous cell carcinomas, 19 were adenocarcinomas and 2 were large cell carcinomas. The majority of patients were men (91%), and patients had a mean age of 64 years (range 40-84 years). Two point four percent of the patients had grade 1 tumors, 59.5% grade 2 and 38.1% grade 3. The pathological stage of the tumor was defined according to the primary pathology reports. In our study, samples were almost equally distributed between stages I, II and III. Lymph node involvement was known for 41 patients; 56% of them had lymph node infiltration.

Immunohistochemical analysis. Representative FFPE tissue sections (4 μm-thick) were used for immunohistochemistry. Tissue sections were first deparaffinized in xylene and rehydrated in a series of graded alcohols. The sections were then pre-treated in a microwave oven and peroxidase activity was blocked with 1% hydrogen peroxide for 20 min, followed by incubation with an appropriate protein-blocking solution. Sections were subsequently incubated with a human-reacting mouse monoclonal antibody against FOXP3 (clone: 236/E7 diluted ×50; Abcam, Cambridge, UK). The Envision Detection System kit (DAKO, Denmark) was used for visualization of bound primary antibody, with diaminobenzidine (DAB) as a chromogen (which yielded brown reaction products). Sections were counterstained with Harris’ hematoxylin solution, dehydrated and mounted. To test for specificity, the procedure was repeated in consecutive sections substituting the anti-FOXP3 antibody with protein-blocking solution.

Evaluation of immunohistochemistry. All slides were assessed by one pathologist (H.P.) and one investigator (F.D.) independently and blinded to the case. The histological type and tumor grade were confirmed according to the 2004 WHO classification of lung tumors (14). Cases with staining in >10% of cells were considered positive. Immunohistochemical reactivity was graded on a scale of 0–3 according to the intensity of the staining and the percentage of immunopositive cells as follows: 0: no staining or <10% positive cells; 1: weak staining in >10% of cells or moderate staining in 10-70% of cells; 2: moderate staining in >70% of cells or strong staining in 10-70% of cells; 3: strong staining in >70% of cells. FOXP3 expression in cancer cells was categorized into three groups (high vs. median vs. low) using as a cut-off point the 33rd and 66th percentiles (15). The intensity and distribution of FOXP3 signal were the parameters used to estimate FOXP3 expression. The total score for each slide was the sum of the intensity and distribution (between 0 and 6). Per tissue section, the more representative tissue areas were selected using low-power fields (magnification ×40). Microphotographs were obtained using a Nikon DXM 1200C digital camera mounted on a Nikon Eclipse 80i microscope and ACT-1C software (Nikon Instruments Inc., Melville, NY, USA).

Statistical analysis. Statistical analysis was performed with the Statistical Package for Social Sciences version 17 (SPSS, Chicago, IL, USA). Correlation of FOXP3 protein expression (immunohistochemical scores) with clinicopathological parameters of the tumors was evaluated with Kruskal-Wallis or Mann-Whitney tests for ordinal variables and $\chi^2$ test for nominal variables. Survival rates were estimated using the Kaplan-Meier method and were then compared with the log-rank test. For all comparisons, statistical significance was defined as $p<0.05$.
**Results**

**Nuclear staining for FOXP3 in tumor cells and tumor-associated lymphocytes.** FOXP3 immunostaining was demonstrated in all tumor, non-neoplastic tumor-adjacent tissues and in the 5 control specimens (Figures 1 and 2). FOXP3 staining had exclusively nuclear localization in both tumor and lymphocytic cells. No differences were observed in the staining pattern between lung cancer subtypes (Figure 1). However, nuclear FOXP3 staining in normal bronchial epithelium in tumor-adjacent samples and the 5 control specimens was weaker than in NSCLC specimens (Figure 2). FOXP3 expression in cancer cells and adjacent non-neoplastic and normal lung parenchyma. FOXP3 protein levels were significantly higher in cancer cells than in non-neoplastic adjacent tissue (p<0.001) (Figures 1 and 2, Table II). FOXP3 expression in cancer cells did not correlate with the age or sex of the patients, nor with tumor primary site, histologic type, stage, grade, the maximum diameter of the tumor or the two-year survival (Table II).

**Correlation of FOXP3 expression in cancer cells with the presence of lymph node metastasis and lymphocytic immunopositivity.** The immunohistochemical expression levels of FOXP3 in cancer cells were associated with lymph node metastasis in a statistically significant manner (p=0.03). Tumors with high FOXP3 immunostaining scores were significantly correlated with nodal metastasis in comparison with tumors with lower FOXP3 immunostaining scores. In addition, FOXP3 immunostaining levels in cancer cells correlated with FOXP3 expression in infiltrating lymphocytes (p<0.001) (Table II).

**Association of FOXP3 expression levels in lymphocytes with the age of the patients.** FOXP3 expression levels in infiltrating lymphocytes were associated with the age of the patients. FOXP3 expression in the lymphocytes situated in the vicinity of tumor tissue and in non-neoplastic adjacent tissues was similar (p=0.586) in all patients, but FOXP3 expression levels in lymphocytes were

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**Table II. FOXP3 protein expression and clinicopathological parameters.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N</th>
<th>FOXP3 protein expression score</th>
<th>Cancer cells, n (%)</th>
<th>p-Value</th>
<th>Lymphocytes, n (%)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0-3 4 5 6</td>
<td></td>
<td>0-3 4 5 6</td>
<td></td>
</tr>
<tr>
<td>Tissue</td>
<td>Neoplastic</td>
<td></td>
<td>44 (0) (2.44) (17.38) (25.56)</td>
<td>1(2.3)</td>
<td>8(18.2)</td>
<td>34(77.3)</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>40 (0) (2.5) (15.37) (23.57)</td>
<td>0.890</td>
<td>1(2.5)</td>
<td>7(17.5)</td>
<td>31(77.5)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>4 (0) (0) (2.5) (2.50) (2.50)</td>
<td>0(0)</td>
<td>1(25.0)</td>
<td>3(75.0)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Primary site</td>
<td>Left lung</td>
<td>22 (0) (1.45) (7.31) (14.63)</td>
<td>0.396</td>
<td>1(4.5)</td>
<td>5(22.8)</td>
<td>15(68.2)</td>
</tr>
<tr>
<td></td>
<td>Right lung</td>
<td>22 (0) (1.45) (10.45) (11.50)</td>
<td>0(0)</td>
<td>3(13.6)</td>
<td>19(86.4)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Stage</td>
<td>I</td>
<td>13 (0) (0.0) (9.69) (4.30)</td>
<td>0.069</td>
<td>0(0)</td>
<td>3(13.6)</td>
<td>10(76.9)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>14 (0) (1.71) (4.28) (6.43)</td>
<td>1(7.2)</td>
<td>2(14.3)</td>
<td>11(78.5)</td>
<td>0(0)</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>13 (0) (0.0) (3.23) (10.70)</td>
<td>0(0)</td>
<td>2(15.4)</td>
<td>10(76.9)</td>
<td>1(7.7)</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>Positive</td>
<td>23 (0) (1.43) (6.26) (16.67)</td>
<td>0.030</td>
<td>1(4.3)</td>
<td>4(17.4)</td>
<td>17(73.9)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>18 (0) (1.55) (11.61) (6.33)</td>
<td>0(0)</td>
<td>4(22.2)</td>
<td>14(77.8)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Max. diameter</td>
<td>&lt;Median</td>
<td>18 (0) (1.55) (8.44) (9.50)</td>
<td>0.517</td>
<td>0(0)</td>
<td>6(33.3)</td>
<td>11(61.1)</td>
</tr>
<tr>
<td></td>
<td>&gt;Median</td>
<td>25 (0) (1.40) (9.36) (15.60)</td>
<td>0(0)</td>
<td>2(8.0)</td>
<td>22(88.0)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Age</td>
<td>&lt;Median</td>
<td>20 (0) (1.50) (5.25) (14.70)</td>
<td>1(5.0)</td>
<td>0(0)</td>
<td>19(95.0)</td>
<td>1(5.0)</td>
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<tr>
<td></td>
<td>&gt;Median</td>
<td>24 (0) (1.42) (12.50) (11.45)</td>
<td>1(4.2)</td>
<td>8(33.3)</td>
<td>15(62.5)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Histology</td>
<td>Squamous</td>
<td>23 (0) (1.44) (10.43) (12.52)</td>
<td>0.446</td>
<td>0(0)</td>
<td>4(17.4)</td>
<td>19(82.6)</td>
</tr>
<tr>
<td></td>
<td>Adenocarcinoma</td>
<td>19 (0) (1.53) (7.36) (11.57)</td>
<td>1(5.3)</td>
<td>4(21.0)</td>
<td>13(68.4)</td>
<td>1(5.3)</td>
</tr>
<tr>
<td></td>
<td>Large cell</td>
<td>2 (0) (0) (0) (2) (1) (1) (2)</td>
<td>0(0)</td>
<td>0(0)</td>
<td>1(50.0)</td>
<td>9(100)</td>
</tr>
<tr>
<td>Smoking</td>
<td>&lt;95py</td>
<td>9 (0) (0) (4) (4.44) (5.56)</td>
<td>0.265</td>
<td>0(0)</td>
<td>0(0)</td>
<td>9(100)</td>
</tr>
<tr>
<td></td>
<td>&gt;95py</td>
<td>10 (0) (0) (0) (2) (20.0) (8.80)</td>
<td>0(0)</td>
<td>2(20.0)</td>
<td>8(80.0)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Relapse</td>
<td>No</td>
<td>16 (0) (1.625) (6.375) (9.562)</td>
<td>0.440</td>
<td>1(6.2)</td>
<td>3(18.8)</td>
<td>12(75.0)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>10 (0) (0.00) (3.30) (7.00)</td>
<td>0(0)</td>
<td>2(20.0)</td>
<td>8(80.0)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Two-year survival</td>
<td>Dead</td>
<td>18 (0) (1.55) (7.38) (10.56)</td>
<td>0.879</td>
<td>1(5.5)</td>
<td>4(22.2)</td>
<td>13(72.3)</td>
</tr>
<tr>
<td></td>
<td>Alive</td>
<td>14 (0) (0) (7) (7) (7) (7) (7)</td>
<td>0(0)</td>
<td>4(28.6)</td>
<td>10(71.4)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Differentiation</td>
<td>Well</td>
<td>1 (0) (1) (0) (0) (0) (0) (0)</td>
<td>0.064</td>
<td>0(0)</td>
<td>1(100)</td>
<td>0(0)</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>25 (0) (1.4) (12.48) (12.48)</td>
<td>1(4.0)</td>
<td>4(16.0)</td>
<td>19(76.0)</td>
<td>1(4.0)</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>16 (0) (0) (4.25) (12.75) (0)</td>
<td>0(0)</td>
<td>3(18.8)</td>
<td>13(81.2)</td>
<td>0(0)</td>
</tr>
</tbody>
</table>

py: Pack-years.
Figure 1. a: Strong nuclear immunopositivity of FOXP3 protein in the majority of tumor cells of an invasive lung squamous cell carcinoma (×40). b: Representative histologic section of lung adenocarcinoma showing strong diffuse nuclear immunopositivity for FOXP3 (×40). c: Strong nuclear FOXP3 expression in a representative section of human lung large cell carcinoma (×40). d: Lymphocytic infiltrates in lung adenocarcinoma with strong nuclear immunopositivity for FOXP3 (×40).
significantly higher in patients under 64 years old ($p=0.002$). These patients had lymphocytic FOXP3 staining of 3 or 4, which were the lowest scores observed (Table II). FOXP3 lymphocytic immunopositivity did not correlate with the sex of the patients, tumor primary site, histological type, stage, grade, maximum diameter of tumor, smoking, disease relapse within two years, two-year survival or the presence of positive lymph nodes (Table II). The age of 64 years was used as a cut-off point as it was the median age of the patients.

**Discussion**

In this study, we found that the FOXP3 transcription factor is expressed in the nucleus of human bronchial epithelial cells and lymphocytes of normal lungs. The expression of FOXP3 was similar in both normal tissues adjacent to tumor and normal tissues from healthy lungs, taken by autopsy from persons who had never smoked and who had never been affected by cancer. Thus, it can be assumed that FOXP3 expression in normal tissues adjacent to the tumor is not
smoke-induced. Moreover, this finding confirms that FOXP3 protein expression is not confined to T-cells (7-10). This is the first report of FOXP3 expression in human lung epithelial cells and agrees with the findings of Chen et al. who had shown that Foxp3 is expressed in lung epithelial cells in mice (8). Furthermore, FOXP3 immunohistochemical expression was significantly stronger in lung cancer cells than in the adjacent non-neoplastic lung tissue (p<0.001). Ishibashi et al. had reported that FOXP3 mRNA levels in NSCLC are significantly higher in tumor tissue samples than in normal tissues, acknowledging Tregs as the only cell population that expresses FOXP3 protein in the tumor microenvironment, while ignoring tumor cell FOXP3 expression (16). In more recent studies, Ebert et al. and Karanikas et al. documented FOXP3 expression in lung cancer cell lines (7, 9).

Additionally, our study shows that FOXP3 expression levels in lymphocytes of the tumor microenvironment are age-related. It is possible that this finding reflects the phenomenon of immunosenescence and the changes of immune responses with age (17). The lower levels of FOXP3 protein in lymphocytes in patients over 64 years old may accompany the recently described low-grade inflammation due to a disequilibrium of the immune response with aging (18).

The role of FOXP3 in carcinogenesis is intriguing. It seems unlikely that FOXP3 expression influences carcinogenesis and cancer progression in a uniform manner, but, rather, that it has a cancer type-dependent function.

In breast cancer, FOXP3 is often down-regulated compared to normal breast epithelium. FOXP3 normally binds and represses HER-2/ErbB2 promoter and therefore, loss of FOXP3 function leads to enhanced HER-2 expression, which, in turn, is directly linked to breast cancer progression (12). However, only a percentage of HER-2-positive breast tumors are FOXP3-negative (19, 20). Moreover, FOXP3 has been recently reported as a novel transcriptional repressor for the breast cancer oncogene S-phase kinase-associated protein 2 (SKP2), which is a component of the E3 ubiquitin ligase SKP1-Cul1-Fbox complex (13). SKP2 expression is increased in nearly 50% of breast tumors (13). Alternatively, FOXP3-expressing melanoma or pancreatic cells may have Treg-like activity, thus suppressing effector T-cell activity (7).

Previous to this work, to our knowledge, no data existed relating FOXP3 expression in the lung epithelium with lung pathobiology. Having the knowledge that there is no central function of HER-2 in lung cancer, we can suppose that the FOXP3 HER-2 mechanism, is not functional in lung tumorigenesis (21). Therefore, it is likely that FOXP3 may affect other pathways involved in lung carcinogenesis. SKP2 gene amplification has been identified in NSCLC and so FOXP3 may participate in lung carcinogenesis interacting with and repressing the SKP2 promoter (13, 22). SKP2, which was found to be overexpressed in NSCLC, has a critical role in regulating the G1-S checkpoint, participating in ubiquitination and proteolysis of the cell cycle regulatory protein p27 (22, 23).

In addition, Zhu et al. have observed that SKP2 overexpression combined with RAS mutations exerts an independent adverse prognostic impact in NSCLC patients (22).

In addition, factors in the tumor microenvironment, such as prostaglandin E2 (PGE2), may induce FOXP3 expression in lung cancer cells: Cyclooxygenase (COX)-2 is reported to be constitutively overexpressed in human NSCLC (24). Because of constitutive COX-2 overexpression, the tumor environment is a rich source of PGE2, which in turn induces FOXP3 expression (25, 26). Amano and colleagues recently reported that the EP3 receptor, which is one of the four cognate receptors via which PGE2 exerts its cellular effects, is required for lung cancer angiogenesis and tumor growth (27). It is known that high concentrations of PGE2 in the tumor environment increase tumor progression, inhibit apoptosis and enhance the immunosuppressive tumor network (24).

Another point that is remarkable is the correlation of FOXP3 expression in cancer cells with regional lymph node metastasis. Mansfield et al. recently showed that in breast cancer patients there were more FOXP3-positive T-cells in sentinel lymph nodes with metastasis than in tumor-free lymph nodes (28). Although there is no analogous information regarding lung cancer, this observation taken together with our results may suggest that FOXP3 protein endows cancer cells with Treg properties. However, further studies are required to show this.

It is possible that in NSCLC, tumor microenvironment factors induce FOXP3 expression in both cancer cells and lymphocytes. These factors may include PGE2 (23-25), and/or transforming growth factor-β (TGF-β), which are known to induce CD4+CD25+ T-cells to express FOXP3 (29). It has also been shown that pancreatic adenocarcinomas secreting TGF-β can attract Tregs, functioning as an immune escape mechanism (10). In addition, it is known that Tregs in NSCLC and other solid tumors secrete TGF-β (30). It is therefore possible that TGF-β modulates the expression of FOXP3 in both cancer cells and lymphocytes.

In conclusion, our findings demonstrate that FOXP3 protein is normally expressed in bronchial epithelium but it is overexpressed in NSCLC cells and tumor-infiltrating lymphocytes. Our data also indicate that lymphocytic FOXP3 expression is an age-related factor, an observation that may reflect a different role of FOXP3 in different age groups. Importantly, this study provides evidence that cancer cell FOXP3 expression is correlated with lymph node metastasis but further studies are needed to confirm our findings.

Acknowledgements

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References


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