FOXP3 infiltrating lymphocyte density and PD-L1 expression in operable non-small cell lung carcinoma

Running title: FOXP3 and PD-L1 in NSCLC

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ABSTRACT

Purpose/Aim: Regulatory FOXP3+ T-cells control the cytotoxic activity of effector cells and may have an essential role in the development of immune tolerance in cancer patients. Programmed death ligand 1 PD-L1, expressed on cancer cell membranes also blocks the cytotoxic activity of PD1+ cytotoxic lymphocytes.

Materials and Methods: We assessed the immunohistochemical detection of these immune-tolerance related markers in a series of 98 non-small cell lung carcinomas (NSCLC) treated with surgery. The Tumor Infiltration Lymphocyte TIL density (mean number per x400 optical field) and the percentage of FOXP3+ TILs were assessed.

Results: PD-L1 expression was directly linked with the TIL density (p=0.01) and with the extent of infiltration with FOXP3+ TILs, named as the FIL-score (p=0.01). FIL-score was significantly higher in stage I disease (p=0.04). IL6 expression was linked with high TIL-score. A low TIL-score, characterizing immune deficient tumors defined a significantly poorer prognosis subgroup of patients (p=0.03). Stratification of these tumors according to the FIL-score showed that FOXP3 expression by TILs correlated with an even a poorer prognosis in univariate (p=0.007; median survival 14 vs. 44 months, respectively) and in multivariate analysis (p=0.01, hazard ratio 4.3).

Conclusion: Tumor stroma infiltration by FOXP3+ Tregs is an early event in the progression of NSCLC. Low lymphocytic infiltration defines poor prognosis, which becomes worse when the small numbers of infiltrating lymphocytes characterizing these tumors contain FOXP3+ Tregs. Furthermore, the direct association of FOXP3+ Treg infiltration density with PD-L1 expression by cancer cells implies a co-ordinated immune-suppressive activity in NSCLC.

Key-words: non-small cell lung cancer, FOXP3, PD-L1, IL6, prognosis

INTRODUCTION

Despite the long history of cancer immunotherapy, it was only recently that randomized trials using immune check-point inhibitors firmly established immunotherapy as a valuable therapy for cancer patients **[1].** The programmed death ligand 1 and programmed death 1 (PD-L1/PD-1) pathway is one of the best understood and validated in clinical practice **[2]**, with several monoclonal antibodies being approved for the treatment of non-small cell lung cancer (NSCLC) and other tumors **[3,4]**. The PD-L1 ligand expressed on the cancer cell membranes binds to the PD-1 receptor on effector CD8 T-cells, blocking their cytotoxic activity. This pathway has an essential physiological role in preventing T-cell mediated inflammatory response to infection and, also, a crucial pathway to avoid autoimmunity **[5].**

Regulatory CD4+/CD25+ T-cells control the cytotoxic activity of effector cells. Expression of FOXP3 is essential for the development of natural $CD4^{+}CD25^{+}$ Tregs in humans **[6],** although this marker also characterizes a functionally suppressive regulatory population independently of CD25 expression **[7].** It has been previously shown that the FOXP3 lymphocyte infiltration density relates to poor prognosis in many cancer types, including breast cancer **[8,9].** IL6 has been suggested to act as a attracting chemokine for regulatory T-cells **[10,11].**

In the current study, we assessed in parallel immunohistochemical markers of two pathways of cytotoxic T-cell activity blockage, i.e., the expression of PD-L1 and IL6 by cancer cells and of FOXP3 by tumor infiltrating lymphocytes (TILs), in a series of operable NSCLC patients.

MATERIALS AND METHODS

Tissue samples from the primary non-small cell lung carcinomas (NSCLC), from 98 patients treated with surgery, were retrieved from the archives of Pathology, Democritus University of Thrace. The age of patients ranged from 32-81years (median 68). Twelve of them were female. Forty-six patients were of UICC stage I, 22 of stage II and 30 of stage III. Fifty-eight cases were of the squamous histology, 22 were adenocarcinomas, and 18 were undifferentiated large cell carcinomas. The time of follow-up ranged from 26-112 months, median 46 months. Survival analysis was based on disease-specific events. Ethical approval was obtained from the Internal Scientific Committee and the Ethic Research Committee of the University Hospital of Alexandroupolis (study approval number ES11-26-11-18). The study was conducted according to the criteria set by the declaration of Helsinki.

Immunohistochemistry

Immunohistochemistry was performed on 3μm thick formalin-fixed paraffin-embedded tissue sections. The slides were then deparaffinized using xylene and rehydrated through graded ethanol solutions to water. A microwave oven was used for antigen retrieval, using Dako EnVision FLEX Target Retrieval Solution (pH 6,0). Slides were incubated thrice at 97^{o}C for 5 min. A polymer detection method was employed using the UltraVision Quanto Detection System (ThermoFisher Scientific). Nonspecific background staining was blocked by preincubation with UltraVision Protein Block. Before incubation with the primary antibodies. The rabbit monoclonal PD-L1 antibody (clone CAL10, Biocare Medical, CA, USA) was used at a dilution of 1:100 and 60min incubation at room temperature. For FOXP3^{+} Treg detection, we used

'in house' undiluted hybridoma supernatant from the well validated murine monoclonal antibody 236A/E7 that is widely used for FOXP3 detection in routinely fixed tissues **[8].** Afterwards, tissue sections were washed in buffer for 2x6min. The UltraVision Hydrogen Peroxide Block was applied for 10min, to neutralize endogenous peroxide activity. The slides were washed with buffer 2x6min and then incubated with Primary Antibody Amplifier Quanto for 10min, which is suitable for both mouse and rabbit primary antibodies. After 2x6min buffer washing, HRP Polymer Quanto was applied, and the slides were incubated for another 10min. Thorough buffer washing was performed (3x6min) and tissue sections were incubated with DAB Quanto Chromogen for 6min. Slides were washed in buffer, counterstained with hematoxylin QS (Vector H-3404), dehydrated through graded ethanol solutions as well as xylene, and mounted in synthetic resin.

The API3171AA (clone CAL10, Biocare Medical, CA, USA) antibody used for the detection of PD-L1 is a well-validated antibody, raised against a peptide corresponding to a region within human PD-L1. In our hands, this gives excellent immunostaining results with clear predominant PD-L1 membrane and cytoplasmic staining. Karnik et al. validated the above antibody against the Ventana SP263 and the Dako 22C3 PD-L1 antibodies in various human carcinomas, showing a general agreement with the Ventana and consistently higher expression compared to the Dako antibody **[12].** All antibodies had shown the same associations with clinic-pathological parameters studied.

Assessment of PD-L1 expression by cancer cells

The percentage of cancer cells with strong membrane/cytoplasmic expression was recorded in the entire tissue section, in x200 optical fields, and the mean value was

used to score each case. Cases were grouped in 3 categories: negative (<1% or lack of reactivity), low (expression in 1-9% of cancer cells), and high (expression in ≥10% of cells). There is an overall consensus that reactivity in less than 1% of cancer cells should be considered as negative, but the cut-off point to characterize a case of low vs. high positive reactivity varies among studies **[13]**.

Assessment of TIL-score

Tumor-infiltrating lymphocytes were assessed in the FOXP3 immunostaining slides. The number of hematoxylin stained TILs was assessed in the entire tissue section, in x40 optical fields, and the mean value defined the final score for each case. Four different *TIL-score* categories were initially defined subjectively for scoring (minimal, low, medium and high). Counting the absolute numbers of lymphocytes per optical field, it was noted that : *TIL-score* 1 (or minimal) defined cases with 1-10 lymphocytes/o.f., 2 (or low) 10-70 lymphocytes/o.f., 3 (or medium) 70-150 lymphocytes/o.f. and, 4 (or high) >150 lymphocytes/o.f..

Assessment of 'FOXP3 / TIL' lymphocytic ratio (FOXP3-ratio)

The percentage of FOXP3 expressing lymphocytes among the TILs present was assessed in the entire tissue section, in x40 optical fields, and the mean score was used to define the FOXP3-ratio for each case

Assessment of the extent of FOXP3+ lymphocyte infiltration (FIL-score)

The FOXP3-ratio provides only the % of TILs expressing FOXP3 and does not reveal the extent of FOXP3 lymphocytic infiltration in the tissue, which also depends

upon the extent of TIL presence. This was assessed as the FIL-score, which is the product of '*TIL-score'* x '*FOXP3-ratio*'.

Assessment of IL6 expression by cancer cells

The percentage of cancer cells with cytoplasmic IL6 expression was recorded in all optical fields and the mean value was used to define the mean % of positive cells.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5.0 package. The chi-square or the Fisher's exact t-test was used to compare categorical variables as appropriate. The Kaplan-Meier survival curves were used to assess the impact of assessed variables on the disease-specific overall survival of patients. A Cox's proportional hazard regression model to compare the categorical variables, including the stage (I vs. II vs. 3), the TIL-score (1 vs. 2,3,4) and the FIL-score (0 vs. 1,2) and using backward elimination, was used to assess the effect of the parameters on the death events. A p-value of <0.05 was used for significance.

RESULTS

PD-L1 expression in cancer cells

The expression of PD-L1 was membranous, always accompanied by a strong cytoplasmic expression in cells with membrane reactivity (**Figure 1a**). Lack of reactivity was noted in 53/98 (54.1%) cases (negative). Twenty-two out of 98 (22.4%)

cases had expression in 1-9% (low expression) and, 23/98 (23.5%) in 10-90% of cancer cells (high expression).

TIL and FIL-scores

The TIL-score obtained was as follows: TIL-score 1 (20/98 cases; 20.4%), 2 (36/98 cases; 36.7%), 3 (26/98 cases; 26.6%) and 4 (16/98; 16.3%).

FOXP-3 stained a varying percentage of TILs (**Figure 1b**). The FOXP3-ratio ranged from 0-50% (median 1%). In 47/98 cases there was no evidence of FOXP3+ lymphocytic infiltration (zero ratio '0'). In 24/98 cases this ranged from 1-9% (low-ratio '1') and in 27/98 cases from 10-50% (high ratio '2').

The FIL-score (*TIL-score'* x '*FOXP3-ratio*) ranged from 0-1 (median 0.02). Out of 98 cases 47 (47.9%) had a FIL-score equal to 0 (zero '0'), 35 (35.7%) had a score of 0.02-0.2 (low '1') and 16 (16.3%) a score of 0.21-1 (high '2').

Association with PD-L1

In categorical analysis, PD-L1 expression was directly linked with TIL-score (p=0.01) and FIL-score (p=0.01), while a marginal direct association was found with FOXP3-ratio (p=0.06); **Table 1**. Linear regression analysis of the % of PD-L1+ lymphocytes recorded in each case with the FIL-score showed a significant direct association (p=0.04, r=0.20), which however showed a low r-score. Nevertheless, a significant association was also confirmed in a continuous variable analysis of FIL-score vs. PD-L1 used as the categorical variable (**Figure 2a,b**).

Association with histopathological variables

Analysis according to histology type, age and gender did not reveal any statistically significant association with PD-L1 or FOXP3 parameters (data not shown). FIL-score was significantly higher in stage I disease compared to II/III (high FIL-score in 12/46 (26%) stage I cases vs. 4/52 (7.7%); p=0.04.

Association with IL6 cancer cell expression

The mean % of cancer cells expressing of IL6 per case, ranged from 0-80% (median 0%). Expression in more than 10% of cancer cells was noted in 11/98 cases (ranging from 10-80%). These were considered as positive for IL6 expression. Eight out of 42 cases with TIL-score 2,3 had high IL6 vs. 3/56 of cases with low TIL-score 1,2 (p=0.03). No statistical association was noted with FOXP3 parameters.

Survival analysis

Kaplan-Meier disease-specific overall survival analysis showed a trend for cases with high PD-L1 expression to have a poorer prognosis, but the difference did not reach significance (p=0.10). There was no association of FOXP3-ratio or FIL-score with prognosis. Low TIL-score of 1, characterizing tumors with 'immune desertification', defined a significantly poorer prognosis patient subgroup (p=0.03; median survival 23 months vs. undefined; **Figure 3a**). Of interest, stratification of these tumors according to FOXP3-ratio or FIL-score showed that FOXP3 expression by TILs defined an even poorer prognosis group of patients (p=0.007; median survival 14 vs. 44 months; **Figure 3b**). IL6 expression by cancer cells was not related with prognosis. In a multivariate model including stage, histology, PD-L1 status and FOXP3-ratio, in

patients with TIL-score 1, the FOXP3-ratio was independently linked with death events (p=0.01, hazard ratio 4.3).

In a multivariate model including stage, TIL-score and FIL-score (or FOXP3-ratio), stage and TIL-score revealed as independent prognostic variables of death events (p=0.0001, Hazard ratio 1.99 and p=0.01, Hazard ratio 0.66, respectively)

DISCUSSION

Regulatory T-cells (Tregs) are a subset of CD4+ T-cells with immunosuppressive function, whose normal role is preserving immune tolerance and preventing the development of autoimmune conditions **[14].** Circulating Tregs represent 5% of the total CD4+ lymphocytic population, while Tregs are increased in the bloodstream of cancer patients **[15**]. Cytotoxic T-cells recognize tumor-related antigens and drive immunological cancer cell death, but as many antigens expressed on cancer cells are identified by autologous T-cells as normal self-antigens, Tregs suppress cytotoxic T-cell antitumor activity **[16,17].** High blood Treg counts relate with aggressive lung cancer behavior, with pleural extension and lymphovascular invasion **[18].**

FOXP3 (forkhead box P3) is the most specific marker of regulatory CD4+ lymphocytes, as mutations of the FOXP3 gene deplete Tregs leading to severe autoimmune diseases **[19].** Tumor infiltration by FOXP3+ lymphocytes is, therefore, expected to counteract anti-tumor cytotoxic T-cell activity and promote growth and metastasis. Indeed, several clinicopathological studies have shown that FOXP3 expression by TILs is linked with poor prognosis in patients with non-small cell lung cancer **[20-25].** Bonano et al. also found an association of FOXP3+ Tregs with better

prognosis in small cell lung cancer **[26].** Our study did not find an overall association of FOXP3 Treg density with the prognosis of patients. Tumors with very low lymphocytic infiltration (TIL-score 1) had a significantly poorer prognosis, and of interest, when such tumors showed FOXP3 Tregs, this defined a rather devastating postoperative outcome.

Another finding brought forward in the current study, is that extensive FOXP3+ lymphocytic infiltration is more frequently noted in early stages of the disease. This may indicate that FOXP3 immunosuppressive activity has a more important role at early stages of non-small cell lung carcinogenesis, while after a certain point other mechanisms become more critical to driving tumor progression and clinical outcome. Ishibashi et al have reported such an inverse association of FOXP3+ lymphocytic infiltration with tumor dimensions **[27].** In support to this hypothesis, a recent experimental study in melanoma oncogenesis showed that activation of BRAF and loss of PTEN, an essential step for the transformation of melanocytes to melanoma cells, leads to early and preferential accumulation of FOXP3+ Tregs in tumor-induced skin sites **[28].** Moreover, increasing accumulation of FOXP3+ lymphocytes in early stages of colon cancer carcinogenesis has been also reported **[29].** During the cervical metaplasia-dysplasia-cancer sequence, plasmacytoid dendritic cells are recruited and induce differentiation of CD4+ lymphocytes to FOXP3+ Tregs **[30].** The critical role of FOXP3+ lymphocytic infiltration in early stages of carcinogenesis has been also reported in a study on rat tongue carcinogenesis induced by 4-nitroquinoline-1-oxide (4NQO), where increasing density of FOXP3+ Tregs in lymph nodes was noted in the sequence of progression from normal mucosa to dysplasia and cancer **[31].**

An additional interesting observation in the current study was the direct association of the density of FOXP3+ TILs with another immune suppressive pathway

defined by the PD-L1 overexpression by cancer cells. This association has also been reported by a very recent study by Silva et al. **[32].** PD-L1 is involved in CD4+ lymphocyte activation, although this interaction looks quite complicated, and contradictory results have been published **[33].** Of interest, DiDomenico et al. showed that PD-L1 induces Treg cell expansion through PD-1 ligation **[34].** A direct effect of cancer cell PD-L1 on CD4 cell maturation to Tregs may underlie this positive association of PD-L1 expression and intense FOXP3+ TIL density in non-small cell lung cancer.

It is concluded that infiltration of non-small cell lung cancer by FOXP3+ Tregs is an early event in the progression of these tumors. NSCLCs with poor lymphocytic infiltration have a poor prognosis, which becomes worse when the low numbers of infiltrating lymphocytes characterizing these tumors contains FOXP3+ Tregs. Furthermore, the direct association of FOXP3+ Treg infiltration density with PD-L1 expression by cancer cells implies an orchestrated immune-suppressive activity, mediated by both the tumor and its microenvironment, in a subgroup of NSCLC. The implications of this study will need to be prospectively tested, in that the most immunosuppressed patients may be the ones to benefit from PD-L1 inhibitors.

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FIGURE LEGENDS

Figure 1

Typical immunohistochemical images of a squamous cell lung carcinoma with high membrane/cytoplasmic PD-L1 expression (a1 x200, a2 x600), high TIL-score of 4 with intense FOXP3+ lymphocytic expression (b1 x200, b2 x600) and low TIL-score of 1 with intense FOXP3+ lymphocytic expression (c1 x200, c2 x600).

Figure 2

Correlation between FIL-score and PD-L1 expression by cancer cells: (a) linear regression analysis of continuous variables and, (b) group analysis according to PD-L1 expression.

Figure 3

Kaplan-Meier overall disease specific survival analysis: (a) stratification according to TIL-score and, (b) stratification according to FOXP3-ratio in the TIL-score 1 cases, (c) stratification according to FOXP3-ratio in the TIL-score 2,3,4 cases.