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Foxp3 and IL17 expression in tumor infiltrating lymphocytes (TIL) and tumor cells – correlated or independent factors?

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Abstract

Tumor infiltrating lymphocytes (TIL), as a microenvironment component were studied in various epithelial tumors, with contradictory results. Recent data about regulatory T-cells (Treg) revealed new explanations for pro- and anti-tumor implications of TIL. Tregs immunoprofile was recently completed with Foxp3 expression. A T-cell fraction (Th) is producing cytokine IL17 and is now considered acting in tumor progression. Our study aimed to analyze immunohistochemically (IHC) Foxp3+ and IL17 expression in resected lung adenocarcinomas, since they could become possible targets in the antitumor immunotherapy. The studied material was represented by paraffin-embedded tumor fragments from 59 patients with TIL identified on HE staining. The antibodies used were Foxp3 and IL17. The statistical analysis used logistical regression on SPSS19 software (Chicago, IL, USA). TIL was usually mild or scarce. A positive statistic correlation resulted between the amounts of TIL in peritumoral and intratumoral location but without correlation to histopathological grading. Foxp3 and IL17 were present in TIL lymphocytes, tumor cells and fibroblasts; IL17 was expressed also in periendothelial cells (PEC). Foxp3 positivity was significantly correlated for lymphocytes/tumor cells, lymphocytes/fibroblasts and tumor cells/fibroblasts, suggesting their concerted action. Tumor cells and lymphocytes Foxp3 expression was inversely correlated with the amount of TIL. Between lymphocytic Foxp3 and PEC IL17, we found a weak negative correlation. The TIL had a quite positive correlation with PEC IL17. In these conditions, Foxp3 could be a mediator of the tumor cells inhibitory aggression upon the immune system and could be used as a molecular target for biological antitumor therapy.

Keywords: lung adenocarcinoma, Foxp3, IL-17, immunohistochemistry.

Introduction

A detailed analysis of the complex interactions between genetically altered tumor cells and their adjacent microenvironment (the tumor stroma) is essential to understand the various mechanisms involved in tumor growth and metastatic spreading.

The stromal cells (lymphocytes, fibroblasts, macrophages) and extracellular matrix involvement in the tumor progression was the base of the reason why the tumor stroma has to be considered as a potential therapeutic target, used even in personalized antitumor therapies. Useful therapeutic strategies can be developed, to disrupt tumor–stroma complex interactions, targeting elements of the tumor microenvironment or the signaling pathways of the tumor cells activated because of the stromal implications. Different cancer types may require distinct contributions from the microenvironment to determine malignant progression [1]. The tumor infiltrating lymphocytes (TIL) were studied in various epithelial tumors (squamous carcinoma, urothelial carcinoma, gastrointestinal tract adenocarcinoma) in the last 20 years, but the results were contradictory since some reports showed a benefic role of TIL and other pointed out on their adverse prognostic action. Recent data regarding the regulatory T-cells (Treg) identified and studied in the thymus [2] offered a new explanation of pro and antitumor implications of TIL. Tregs, a subpopulation of T-lymphocytes with an immunophenotype CD4+CD25+, have a potent immunomodulatory capacity, especially in the maintenance of immunological tolerance, controlling both of CD4+ and CD8+ T-cells response [3] and immunoregulation [4]. Their immunoprofile was recently completed with Foxp3 expression [4–6], a member of the transcription factors family (forkhead box protein 3), implicated in regulation, activation and differentiation of T-cells [5]. The initial opinion, that FoxP3 is specific to the regulatory T-cells, was contradicted by further demonstrations, revealing that it also can be temporally expressed in antigen activated T-cells, without Treg function, and even in non-lymphoid or tumor cells [7].

In the last years, research was focused also on the concerted role of T-lymphocytes and cytokines, as interleukins, in cancer. It was found that a fraction of T-helper (Th) lymphocytes (Th17) is synthesizing IL17 [8]. Th17 lymphocytes and IL17 involved in inflammation and autoimmunity [9], are now considered as active factors in both pro and antitumor processes [10], having the capability to promote the development of cancer-

initiating cells [11] and to suppress tumor specific CD8+ T-cells [12]. Experimentally, it was shown an interrelation between Foxp3 positive Tregs and IL17 producing Th17 [13]. Understanding their double role in the tumor microenvironment and the processes that regulate their pro- and anti-tumor activities, can offer a support for identification of new prognostic factors and the development of more efficient antitumor immunotherapies [14].

Recent data point out Foxp3 expression in T-lymphocytes and in the tumor cells of pancreatic carcinoma, malignant melanoma or hepatocellular carcinoma, where both FoxP3 mRNA and FoxP3 protein were identified [15], the high number of Treg permitting to cancer cells to evade the immune surveillance [16] and to manipulate the immune response.

The accumulation of large amounts of Foxp3 positive Treg cells in other human cancers (gastric, ovarian, mammary, melanoma) is generally associated with a poor prognosis, because of their capacity to inhibit antitumor immunity [17–23]. An exception is represented by the colorectal carcinoma, where Foxp3 positive Treg cell high tumor infiltration is associated with a favorable prognosis, [24, 25], especially in patients undergoing chemo- or chemo-immunotherapy [26]. Similar observations were reported for extranodal NK/T-cell lymphoma [27] and diffuse large B-cell lymphoma [28].

In non-small cell lung cancer (NSCLC), the Foxp3 expression in T-lymphocytes and tumor cells was correlated with the presence of lymph node metastasis [29]. The lung adenocarcinoma is a no smoking associated tumor, with an increasing incidence, an overall advanced stage at the moment of the diagnosis and an unpredictable prognosis. Histological typing alone does not offer satisfactory prognostic elements. A study addressing the presence of Foxp3+ Treg cells showed their larger number in adenocarcinoma as compared with squamous carcinoma, and an increased number in metastatic lymph nodes associated with a decrease of natural killer cells amounts [30]. Different signaling ways and their factors as COX2 activating Treg were correlated with a worse prognostic [31], or with recurrence in NSCLC [32].

The mechanisms by which Treg suppress the antitumoral action of immune cells in epithelial tumors are incompletely known. The major part of the studies regarding immunosuppressive elements as Treg cells in NSCLC pointed on only the Foxp3 expression in lymphocytes without considering other parameters as tumor cells, histological type or activator factors as interleukins.

Our study aimed to analyze immunohistochemically the Foxp3+ expression of Tregs and tumor cells in correlation with the presence of IL17 on resected lung adenocarcinomas, since they could represent a possible target in the antitumor immunotherapy.

Materials and Methods

The studied material was represented by tumor fragments from 59 lung adenocarcinomas selected from a total number of 80 consecutive cases archived at "Victor Babeş" National Institute of Pathology, Bucharest, Romania, as paraffin blocks. The cases selection was made considering the lymphoid infiltrate presence inside or around the tumor. The histopathologic type was established based on the presence of gland-like/pseudotubular structures with or without compact areas and/or extension in the alveolar cavities. The presence of mucinous structures was also noted. The tumor infiltrating lymphocytes (TIL) were assessed in the peri- and intratumoral areas, and the amount of infiltrate was scored as following: 0 – absent, 1 – low, 2 – mild, 3 – strong.

After the histopathologic appreciation of the intratumoral (IT) and peritumoral (PT) inflammatory infiltrates, the immunohistochemical analysis was accomplished on 5 μm-thick sections, on poly-L-Lysine coated slides. The immunohistochemical (IHC) technique used was an indirect bistadial one, based on polymerized Dextran conjugated with secondary antibody and horseradish peroxidase (DAKO, EnVision), using overnight primary antibody incubation and DAB saline solution with 0.03% hydrogen peroxide as substrate. Appropriate positive and negative controls were used.

The following antibodies were used: FoxP3 polyclonal (Abcam, Cambridge, UK, dilution 1:200) and IL17 (polyclonal, Santa Cruz, CA, USA, 1:50). Previous immunohistochemical stainings for CD4, CD45RO (UCHL1 clone), CD20 and CD25 were available for the studied cases.

Statistic analysis of data was performed using logistical regression on SPSS19 software (Chicago, IL, USA). *P*-values <0.05 were considered as statistically significant.

Results

The pulmonary adenocarcinomas presented a heterogeneous histopathological aspect, most of cases being mixed forms of bronchiolo-alveolar adenocarcinoma with areas of papillary adenocarcinoma or poorly differentiated adenocarcinoma/solid adenocarcinoma, sometimes with mucinous zones.

The studied group included 59 cases comprising 43 male and 16 female patients. The mean age was 60 years.

Histologically, the adenocarcinomas were comprised in the three prognostic subtypes: G1 – lepidic non-mucinous, with favorable prognostic, G2 – papillary and acinar, with moderate prognostic, and G3 – solid zones and/or micropapillary, pleomorphic nuclei, with poor prognostic. In the mixed types, the less differentiated one was considered. A histological typing was established for all the 59 cases: G2 – 24 (40.67%) cases, G3 – 22 (37.28%) cases, and G1 – 13 (22.03%) cases. The number and sex of patients, histological grading and the score of total tumor infiltrating lymphocytes (TIL) (both peritumoral and intratumoral) is presented in Table 1.

Four cases with a score 0 served as controls. Thirty cases out of 59 (50.84%) had a total TIL score of 1 and 25 (42.37%) had a score of 2–3. The TIL score *vs.* G histological grade was as following: from a total of 13 G1 cases, one had a score 0; six had score 1 and six had score 2–3; from a total of 24 G2 cases, three had a score 0, 10 had a score 1 and the other 11 had a score 2–3; from the 22 G3 cases, 11 cases had a score 1 and 11 a score 2–3.

Table 1 – ***Grading, sex and TIL repartition on number of cases***

TIL Score	No. of cases																	
	M									*F*								
	PT			*IT*			*PT+IT*			*PT*			*IT*			*PT+IT*		
	G1	*G2*	*G3*	*G1*	*G2*	*G3*	*G1*	*G2*	*G3*	*G1*	*G2*	*G3*	*G1*	*G2*	*G3*	*G1*	*G2*	*G3*
0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	1	0
1	0	0	1	4	4	5	1	1	4	0	0	1	1	1	1	0	4	2
2	0	0	1	1	0	0	2	2	6	0	0	0	0	0	0	0	3	0
3	0	0	0	0	1	0	3	3	1	0	0	0	0	1	0	0	1	0
Total	0	0	2	5	5	5	7	8	11	0	0	1	1	2	1	0	9	2

G – Histological grade; F – Female; M – Male; G1–G3 – Histological degree, 0–3 – Score for peritumoral (PT) and intratumoral (IT) TIL.

Between the amount of TIL in peritumoral (PT) and intratumoral (IT) location it was found a positive statistic correlation (p=0.01, r=0.438). We observed that the PT lymphoid infiltrate alone was present in three cases, all having G3 grade; from the 19 cases with only IT lymphoid infiltrate, six cases were G1 and 13 cases were G2–G3 histological grade. We analyzed also the cases with PT+IT lymphoid infiltrate; from 37 cases, seven cases were G1 and 30 were G2–G3 histological grade.

Foxp3 had a heterogeneous expression in different histological types of lung adenocarcinoma. In some cases, the reactions were intense and diffuse in both the tumor cells and in the peri- and intra-tumoral lymphocytes (Figures 1 and 2). The intracellular localization was nuclear in lymphocytes and both nuclear and cytoplasmic in tumor cells. Besides the tumor cells reaction, Foxp3 was expressed in peritumoral normal bronchi in some cases, even if the bronchi did not present preneoplastic lesions. In poor differentiated forms and in cases with predominantly papillary structure, the reaction was more intense in the tumor cells as compared to the lymphocytes (Figure 3). In the major part of the cases, also fibroblasts were positive for Foxp3.

Analyzing the IL17 expression, we observed a positive staining not only in tumor cells and lymphocytes, but also in stromal fibroblasts (Figure 4). As a general observation, IL17 presented a weaker reaction in the tumor cells comparative with the micromedium; it stained intensely the micromedium lymphocytes, plasma cells and fibroblasts and was weaker in the tumor cells, even though in a diffuse manner.

The CD25 positive lymphocytes were only isolated in the lymphoid infiltrate, not quantifiable.

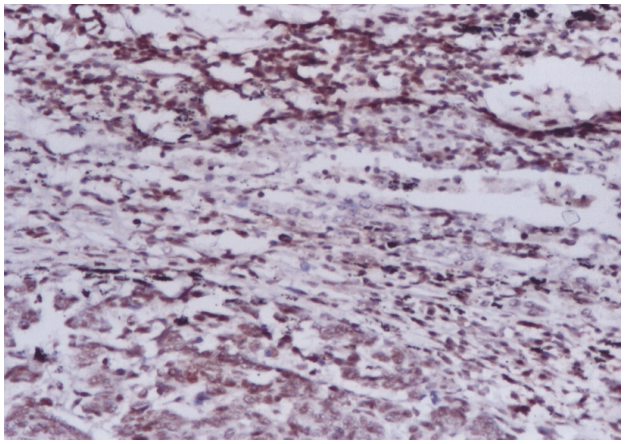

Figure 1 – ***Lung adenocarcinoma moderately differentiated. IHC stain for Foxp3 with strong expression in tumor cells and TIL lymphocytes, ob. 10×.***

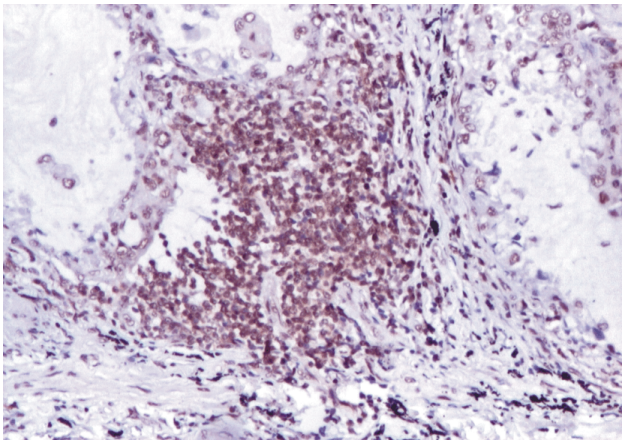

Figure 2 – ***Lung adenocarcinoma. IHC intense staining for Foxp3 in TIL lymphocytes, ob. 20×.***

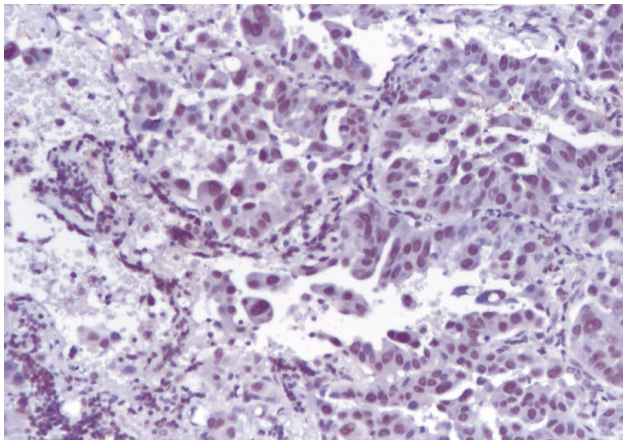

Figure 3 – ***Pulmonary adenocarcinoma with papillary zones. IHC staining for Foxp3 expression in tumor cells, ob. 10×.***

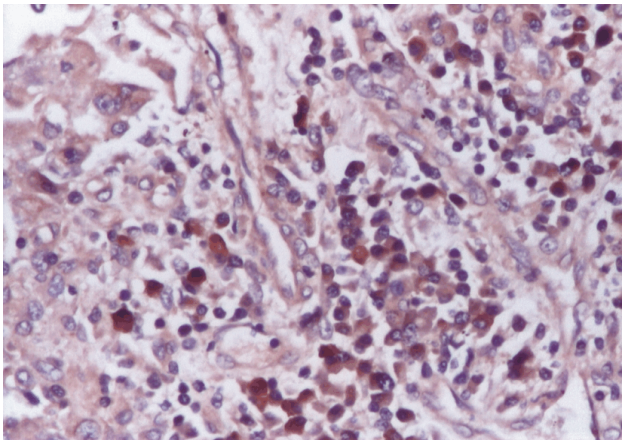

Figure 4 – ***Lung adenocarcinoma. IHC staining for IL17 with expression in tumor cells and in stromal cells, ob. 20×.***

Case statistic processing is represented in Table 2 and the obtained correlations table was depicted in Table 3.

Table 2 – ***Case statistic processing of lung adenocarcinoma (SPSS19 software, Chicago, IL, USA)***

	Cases					
	Valid		Missing		Total	
	N	%	N	%	N	%
IL17_tum	52	88.1	7	11.9	59	100
IL17_Ly	52	88.1	7	11.9	59	100
IL17_PEC	52	88.1	7	11.9	59	100
Fox_p3_tum	52	88.1	7	11.9	59	100
Fox_p3__Ly	52	88.1	7	11.9	59	100
Fox_p3__Fb	52	88.1	7	11.9	59	100
inflam_intraTu	52	88.1	7	11.9	59	100
infl_periT	52	88.1	7	11.9	59	100

At statistical analysis, we found some correlations between the analyzed biomarkers. Always the positivity for Foxp3 was significantly correlated for lymphocytes and tumor cells (p=0.000, r=0.841), lymphocytes and fibroblasts (p=0.000, r=0.537), and tumor cells and fibroblasts (p=0.000, r=0.602). The Foxp3 expression in tumor cells and lymphocytes was inversely correlated with the amount of intratumor lymphoid infiltration (TIL) (p=0.000, r=-0.494/-0.436) as depicted in the Figure 5.

Between lymphocytic Foxp3 and periendothelial cells (PEC) IL17 we found a week negative correlation (p=0.002, r=-0.421). The peritumoral lymphocytic infiltration had a quite positive correlation with the PEC IL17 (p=0.02, r=0.406).

Table 3 – ***Statistic correlations of the immunohistochemical results***

		Correlations						
		IL17_Ly	IL17_PEC	Fox_p3_tum	Fox_p3__Ly	Fox_p3__Fb	IT_TIL	PT
IL17_Ly	Pearson correlation	1	,081	,178	,205	,260	,021	,083
	Sig. (2-tailed)		,560	,206	,140	,061	,878	,550
	N	54	54	52	53	53	54	54
IL17_PEC	Pearson correlation	,081	1	-,319*	-,421**	-,149	,410**	,406**
	Sig. (2-tailed)	,560		,021	,002	,288	,002	,002
	N	54	54	52	53	53	54	54
Fox_p3_tum	Pearson correlation	,178	-,319*	1	,841**	,602**	-,494**	-,170
	Sig. (2-tailed)	,206	,021		,000	,000	,000	,206
	N	52	52	57	57	57	57	57
Fox_p3__Ly	Pearson correlation	,205	-,421**	,841**	1	,537**	-,436**	-,163
	Sig. (2-tailed)	,140	,002	,000		,000	,001	,222
	N	53	53	57	58	58	58	58
Fox_p3__Fb	Pearson correlation	,260	-,149	,602**	,537**	1	-,330*	-,067
	Sig. (2-tailed)	,061	,288	,000	,000		,011	,615
	N	53	53	57	58	58	58	58
inflam_intraTu	Pearson correlation	,021	,410**	-,494**	-,436**	-,330*	1	,438**
	Sig. (2-tailed)	,878	,002	,000	,001	,011		,001
	N	54	54	57	58	58	59	59
infl_periT	Pearson correlation	,083	,406**	-,170	-,163	-,067	,438**	1
	Sig. (2-tailed)	,550	,002	,206	,222	,615	,001	
	N	54	54	57	58	58	59	59

* – Correlation is significant at the 0.05 level (2-tailed); ** – Correlation is significant at the 0.01 level (2-tailed).

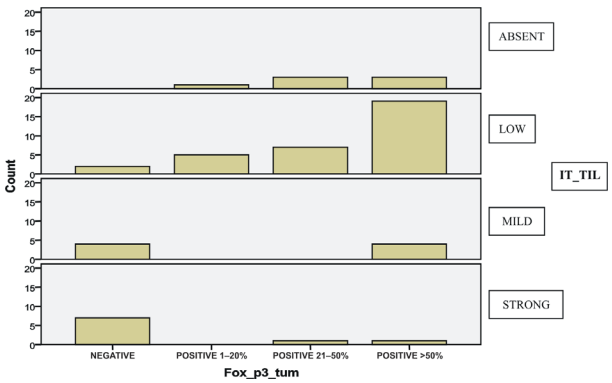

Figure 5 – ***Graph of the correlation between Foxp3 and TIL.***

Discussion

We selected our cases based on the histological type (adenocarcinoma) and the presence of a lymphoid infiltrate (TIL) which was scored semiquantitatively. The four cases with a TIL score 0 were used as controls. The percentage of G2–G3 cases was different if reported to a total score of TIL or at a splitting of TIL in PT, IT and PT+IT. As the number of G2–G3 grade cases is clearly higher in association with PT+IT lymphoid infiltrate (30 G2–G3 *vs.* seven G1), it could suggest that in lung adenocarcinoma TIL would not act as a favorable outcome factor. We also obtained a positive statistic correlation between the amount of peritumor and intratumor lymphocytic infiltration (p=0.01, r=0.438).

Regarding the TIL presence in various types of adenocarcinomas, we observed in 19 out of 46 G2 and G3 pulmonary adenocarcinomas cases a rich TIL scored 2–3. This is a small number if we consider that *bronchus associated lymphoid infiltrate in the lung* (BALT) is an active participant in all immune phenomena of lung tissues. However, it is known that in humans the BALT is only inducible (iBALT) by different factors and is not normally present. As Treg can attenuate the iBALT expansion [33], this can explain why a rich lymphoid infiltrate appeared only in a reduced number of cases. The repartition of TIL was variable in peritumoral, intratumoral or both locations and the amount of lymphocytes was not correlated with the histological grade of adenocarcinoma.

Our IHC study of Foxp3 expression (Treg cells

biomarker) generated unexpected results. We observed an extensive staining for Foxp3 in a high number of lymphocytes (until a maximum of 50% positive cells) since CD25 was positive only in few cells; we can suppose like other authors that not only Treg cells express Foxp3 [7]. The tumor cells Foxp3 expression was very high (until 95% in some cases), but we did not found a correlation with the histological grade of tumors.

The statistically strong positive correlation of Foxp3 expression in both lymphocytes and tumor cells (p=0.000, r=0.841) of our cases was in accord with some observations in the literature [7, 15]; also, the percentage of positive cells is similar [34]. More than 50% Foxp3 positive tumor cells in invasive breast cancer [35] were found and their presence was correlated with the tumor stage, invasion, size and vascularisation [36]; it was also observed a strong correlation between the Foxp3 expression and a worse overall survival or distant metastases-free survival [37].

Our observation seems to be sustained by recent demonstrations, revealing that Foxp3 can also be temporarily expressed in antigen activated T-cells, without Treg function, and even in non-lymphoid or tumor cells [34], contradicting the initial opinion, that Foxp3 is specific to regulatory T-cells [5].

A lower but significant correlation was found also between the Foxp3 expression in tumor cells and fibroblasts (r=0.602) and lymphocytes and fibroblasts (r=0.537). This fact suggesting a concomitant implication in the lung adenocarcinoma biology is supported by recent observations regarding the tumor-associated fibroblasts participation in the tumor progression [38].

In our study, Foxp3 expression in tumor cells and lymphocytes was inversely correlated with the amount of intratumor lymphoid infiltration (TIL). This fact is in accord with recent observations sustaining that Foxp3 expressing cells would determine the decrease of the active antitumor lymphocytes and they could also induce immunosuppressive functions to the regulatory T-lymphocytes [7]. The stronger Foxp3 expression we observed in tumor cells and intratumor fibroblasts than in lymphocytes could also explain their immunosuppressive action.

FoxP3 gene expression, codifying a protein involved in the immune response, is higher in tumor CD4+CD25+ Treg cells than in normal tissue ones [7]. This type of Treg cells is named induced Treg cells as they are converted from CD4+CD25- T-cells in CD4+CD25+ FoxP3+ by TGF-beta and IL2 [39] and these Treg cells can act as Th17 cells inducers [40]. The concerted action of TGF-beta and IL9 also induces IL17-producing cells [41]. We did not identify the induced Tregs in the studied tumors. We found in some moderately differentiated adenocarcinoma Foxp3 positivity in the normal bronchial epithelium nearby the tumor, suggesting a possible aberrant expression related to the tumor progression.

We found a stronger positivity for IL17 in lymphocytes and fibroblasts than in tumor cells, but their simultaneous staining could suggest a participation in the tumor-stromal events. Between lymphocytic Foxp3 and peri-endothelial cells (PEC) IL17, we found a week negative correlation (p=0.002, r=-0.421), suggesting that they are acting antagonistically in the immune phenomena of the tumor microenvironment. The weakly positive correlation between peritumoral lymphocytic infiltration and the PEC IL17 could be interpreted as an implication of TIL in the inflammatory/tumoral type of cytokines production as it was observed by some authors [42].

The positivity of IL17 not only in the tumor cells and lymphocytes but also in the stromal fibroblasts could agree the possibility of a role of these tumor associated fibroblasts in inhibiting the antitumor immune response. Fibroblasts with aberrant IL17 expression were observed even in systemic lupus erythematosus, an autoimmune disease with a similar mechanism to the rheumatoid arthritis. Understanding their double role in the tumor microenvironment and the processes, which regulate their pro- and anti-tumor activities, could offer a support for identifying new prognostic factors and the development of more efficient antitumor immunotherapies as was suggested by some authors [14].

Conclusions

In our series, the peri- and intra-tumoral lymphoid infiltrate was usually mild or scarce. Our findings suggest that in lung adenocarcinoma TIL did not act as a favorable outcome factor. Foxp3 expression was well correlated for tumor cells/fibroblasts and lymphocytes/fibroblasts suggesting a concerted action in the tumor progression. In these conditions, Foxp3 could be a mediator of the tumor cells inhibitory aggression upon the immune system, avoiding its antitumor function. Foxp3 expressing cells would determine the decrease of number of the active antitumor lymphocytes and they could also induce immunosuppressive functions to the regulatory T-lymphocytes. Considering tumor cells Foxp3 expression as illustrating a possible action of antitumor immune response annihilation, the marker can be used as a molecular target for biological antitumor therapy.

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