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Prognostic Relevance of the Relative Presence of CD4, CD8 and CD20 Expressing Tumor Infiltrating Lymphocytes in Operable Non-small Cell Lung Cancer Patients

ALEXANDRA GIATROMANOLAKI[1], IOANNIS ANESTOPOULOS[1,2,3], MIHALIS I. PANAYIOTIDIS[2,3], ACHILLEAS MITRAKAS[1,4], AGLAIA PAPPA[5] and MICHAEL I. KOUKOURAKIS[4]

[1]*Department of Pathology, Medical School, Democritus University of Thrace, Alexandroupolis, Greece;*
[2]*Department of Cancer Genetics, Therapeutics & Ultrastructural Pathology, The Cyprus Institute of Neurology & Genetics, Nicosia, Cyprus;*
[3]*School of Molecular Medicine, The Cyprus Institute of Neurology & Genetics, Nicosia, Cyprus;*
[4]*Department of Radiotherapy/Oncology, Medical School, Democritus University of Thrace, Alexandroupolis, Greece;*
[5]*Department of Molecular Biology & Genetics, Democritus University of Thrace, Alexandroupolis, Greece*

Abstract. *Background/Aim: Non-small cell lung cancer (NSCLC) is one of the most lethal tumors. Given the failure of conventional therapeutic strategies, immunotherapy has emerged as a promising treatment modality that may improve the survival of patients with operable and advanced disease. Patients and Methods: We examined the relative presence of CD20+ B-cells, CD8+ cytotoxic, and CD4+ helper/regulatory T-cells in the tumor-infiltrating lymphocyte (TIL)-population in a series of surgically-treated NSCLCs, and assessed their role as prognostic indicators after surgery. Results: A high percent of CD4+ and CD8+ TILs in the tumor stroma was linked with poor (p=0.003) and good prognosis (p=0.01), respectively. High CD4/CD8 ratio defined a significantly worst prognosis [median survival 22 months vs. undefined, p=0.0002, hazard ratio (HR) 0.3 vs. 3.0]. Statistically significant results were also noted when the analysis was focused on the invading tumor front. In a multivariate model, the CD4/CD8-ratio assessed in the tumor stroma and the stage of disease were independent prognostic variables (p=0.0001, HR=4.1 and p=0.001, HR=1.5, respectively). Conclusion: The balance between CD4+ and CD8+ lymphocytes infiltrating the tumor stroma is a crucial factor defining anti-tumor immune surveillance, has strong prognostic value, and may be tested as a predictive biomarker for immunotherapy in operable NSCLC.*

Correspondence to: Alexandra Giatromanolaki, MD, Department of Pathology, Democritus University of Thrace, Alexandroupolis 68100, Greece. Fax: + 30 2551030353, e-mail: agiatrom@med.duth.gr

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Non-small cell lung cancer (NSCLC) is one of the most common tumors in humans with high lethality, as half of the patients die within a year from diagnosis and the 5-year survival is below 25% (1). Early operable stages of the disease have a better prognosis; still, the 5-year survival drops from 60% down to 33% if the disease has spread to the lymph nodes. The addition of radiotherapy and chemotherapy may increase the 5-year survival by only 5% (2).

The importance of the immune system in controlling the disease has been recently revealed in randomized trials focusing on the value of immune checkpoint inhibitors (ICIs) in advanced disease, as 25% percent of patients with high PD-L1 expression reach 5-year survival (3). The addition of anti-PD-L1 immunotherapy after chemo-radiotherapy for locally advanced tumors tripled the median progression-free survival (4). Anti-PD-1/PD-L1 immunotherapy blocks the binding of PD-L1 expressed by tumor cells on the PD-1 receptor of T-cells, preventing activation of the PD-1/PD-L1 immunosuppressive pathway, thus providing the window for cytotoxic T-cells to exert their anti-tumor efficacy (5). Accumulation, however, of regulatory lymphocytes and monocytes blocks cytotoxic T-cell activity, bypassing the beneficial effect of immunotherapy.

Studying the subtypes of tumor-infiltrating lymphocytes (TILs) provides an immunological profile of the anti-tumor immune response at the time of operation. Intense infiltration of the tumor stroma with lymphocytes is a well-recognized feature that relates to favorable prognosis in many human malignancies, including NSCLC (6). However, the balance between regulatory and cytotoxic T-cells may differ among tumors, which may shift the nature of immune response towards tumor tolerance and tumor aggressiveness.

Profiling of TILs to assess the prevalence of the regulatory or the cytotoxic component may change the prognostic value of TIL density. In the current study, we examined the relative presence of CD20+ B-cells, CD8+ cytotoxic and CD4+ helper/regulatory T-cells in the TIL-population, in a series of NSCLCs, and assessed their role as a prognostic indicator after surgery.

Patients and Methods

The study was performed on a series of 98 surgically resected NSCLC paraffin-embedded tissues. All patients were treated with surgery alone. The age of patients ranged from 32-82 years (median 68 years). According to the Union for International Cancer Control (UICC) staging system, there were 46 patients with stage I, 22 with stage II, and 30 with stage III disease. Histological diagnosis showed squamous histology in 58 cases, adenocarcinoma in 22, and undifferentiated large cell carcinoma in 18 cases. The follow-up of patients ranged from 26-112 months (median 46 months). Ethical approval was obtained from the local Scientific Committee and the Ethics Research Committee (study approval number ES11-26-11-18). The study was conducted according to the criteria set by the declaration of Helsinki.

Immunohistochemistry. We performed immunohistochemistry for the detection of CD20 (B-cells) (7), CD4 cells (helper T-cells that regulate innate immune response and are also the main source of regulatory T-cells) (8), and CD8 (the main source of cytotoxic T-cells) (9). We used the 4B12 mouse monoclonal antibody (DAKO, Copenhagen, Denmark) for the detection of CD4 (dilution 1:60, 60 min incubation), the C8/144B mouse monoclonal antibody (DAKO) for the detection of CD8 (dilution 1:70, 60 min incubation), and the L26 mouse monoclonal antibody (DAKO) for the detection of CD20 (dilution 1:200, 60 min incubation).

Immunohistochemistry was performed in parallel (sequentially cut) tumor sections. Three μm thick tissue sections from formalin-fixed, paraffin-embedded (FFPE) material were cut and mounted on positively charged slides. Following deparaffinization and rehydration, heat-induced epitope retrieval was achieved by heat by placing the slides in a microwave oven using Dako EnVision FLEX Target Retrieval Solution at pH 9.0. Subsequently, we performed immunohistochemistry with the DAKO EnVision FLEX detection system. Briefly, following incubation with the primary antibodies, endogenous peroxidase activity was quenched using EnVision FLEX Peroxidase-blocking reagent, followed by incubation with the EnVision FLEX+ Mouse Linker for 15 min. After TBS washing, the EnVision FLEX HRP detection reagent was applied for 30 min. Subsequently, tissue sections were incubated with EnVision FLEX DAB+ Chromogen for 6 min. Slides were stained with hematoxylin QS (Vector H-3404, Burlingame, CA, USA).

The percentage of CD+ lymphocyte subtypes among the entire immune cell population (as recognized by hematoxylin) was assessed subjectively in all x200 optical fields by two experienced pathologists. The % of CD4, CD8, and CD20 positive lymphocytes were recorded simultaneously for each case by examining the three stained slides sequentially. The assessment was performed separately in the invading tumor front and in the inner stroma areas. The mean score obtained for the three stainings was normalized to 100% and used to score each case. Correlation analysis between the results obtained by the two observers (inter-observer variability) was highly significant ($p<0.001$, r=0.91). Discrepancies were resolved on the conference microscope.

Statistical analysis. The GraphPad Prism 7.0 (San Diego, CA, USA) package was used to perform statistical analysis and produce graphs. The Fisher's exact *t*-test was applied to compare groups of categorical variables. The paired or the unpaired two-tailed *t*-tests were used to compare groups of continuous variables. Linear regression analysis was used to assess correlations between continuous variables. Kaplan–Meier survival curves were produced to evaluate the impact of CD-related variables on the overall disease-specific survival. A Cox proportional hazard model was used for multivariate analysis. A *p*-value of <0.05 was considered for significance.

Results

TIL composition. Figure 1 shows typical immunohistochemical images of CD4, CD8, and CD20 staining, with low and high lymphocytic density. The relative percentage of CD20+, CD4+, and CD8+ TILs, as well as the ratio CD4+/CD8+, is shown in Table I. The normalized median % values were 10, 40, and 40 in the tumor stroma and 20, 40, and 30 in the invading tumor front, respectively. The median CD4/CD8 ratio was 1 and 1,29 in the tumor stroma and the invading front, respectively. The percentage of CD20+ TILS was higher in the invading tumor front ($p=0.002$), while the % of CD8+ TILs was higher in the tumor stroma ($p=0.002$) Figure 2. The % of CD4+ cells and the CD4/CD8 ratio were similar in the two tumor areas.

TIL composition and histopathological variables. The relative percentage of CD20, CD4, and CD8 TILs did not differ between stage I and stage 2/3 tumors, neither in the tumor stroma (median values 10 vs.15, 40 *vs*. 40, 40 *vs*. 40, respectively) nor in the invading tumor front (median values 20 *vs*. 20, 40 *vs*. 40 and 30 *vs*. 30, respectively). The same applied for the CD4/CD8 ratio (median values 1 *vs*. 1.25, and 1.29 *vs*. 1.29 in the tumor stroma and invading tumor front, respectively.

Regarding the association between TIL-subtypes and histological types of tumors, adenocarcinomas showed a marginal statistical association with increased CD20+, decreased CD8+ lymphocyte infiltration, and increased CD4/CD8 ratio in the invading front ($p>0.06$). There was no association between TIL-related parameters of the tumor stroma and histopathological variables.

Stroma TIL composition and survival. Two groups of patients per CD were considered using the median value reported in Table I (≤median *vs*. >median) as a cut-off point. High percentage of CD4+ TILs in the tumor stroma was linked with poor prognosis ($p=0.003$), while high percentage of CD8+ TILs was linked with a good prognosis ($p=0.01$). High CD4/CD8 ratio defined a significantly worse prognosis (median survival 22 months *vs*. undefined, $p=0.0002$, HR 0.3 *vs*. 3.0) Figure 3. Statistically significant results, although of lesser magnitude, were also noted when the analysis was

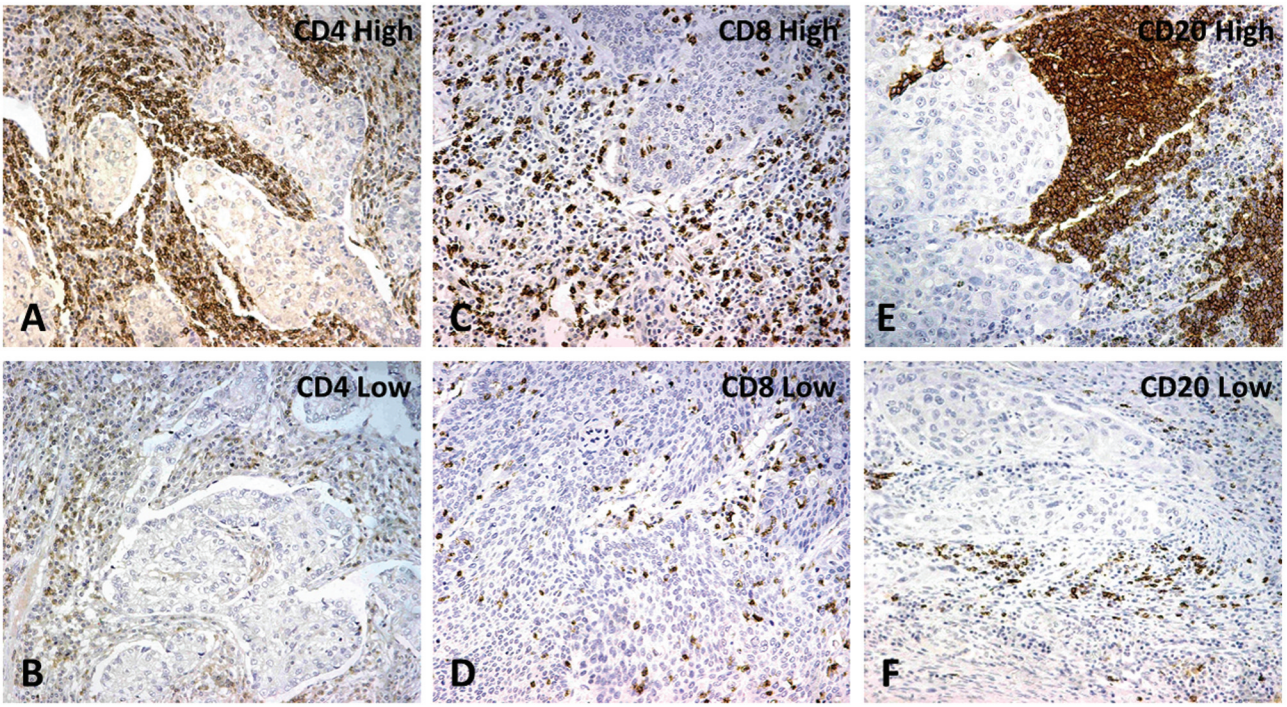

Figure 1. *Typical immunohistochemical images of CD4, CD8 and CD20 immunostaining: A: high CD4+ lymphocytic density, B: low CD4+ lymphocytic density; C: high CD8+ lymphocytic density, D: low CD8+ lymphocytic density; E: high CD20+ lymphocytic density, F: low CD20+ lymphocytic density.*

Table I. *Distribution of the percentage of CD20, CD4 and CD8 lymphocytes and of the CD4/CD8-ratio in a series of 98 non-small cell lung carcinomas.*

	CD20% stroma	CD4% stroma	CD8% stroma	CD4/CD8 stroma	CD20% IF	CD4% IF	CD8% IF	CD4/CD8 IF
Minimum	0	10	10	0.13	5	10	10	0.13
25% Percentile	10	30	30	0.5	10	30	25	0.6
Median	10	40	40	1	20	40	30	1.50
75% Percentile	20	60	60	2	30	50	50	2
Maximum	70	80	80	8	60	70	80	6
Mean	18.57	40.61	40.71	1.52	23.01	40.56	35.82	1.64
Std. Deviation	12.41	17.55	18.36	1.513	13.16	15.48	16.82	1.4

Stroma: Stroma in the inner tumor areas; IF: invading front.

performed for the invading tumor front (Figure 4). In a multivariate model including the stage, CD4/CD8-ratio in the invading front and in the stroma, stage, and CD4/CD-ratio in the stroma were independent prognostic variables (p=0.0001, HR=4.1, and p=0.001, HR=1.5, respectively).

Discussion

The anti-tumor immune response is followed by infiltration of tumors with lymphocytes (TILs), and the magnitude of this effect varies among tumors, even of the same histology. Several studies have suggested that a high density of TILs is linked with better survival in operable NSCLC (10-14). In a previous study, we also found that intense infiltration of tumor stroma by lymphocytes defines a better prognosis, independently of the stage of disease (15). The composition of TILs, however, varies among tumors, and the prevalence of specific lymphocyte subtypes may differentially define the TIL-prognostic relevance. In the current study, we investigated the prognostic relevance of the relative presence

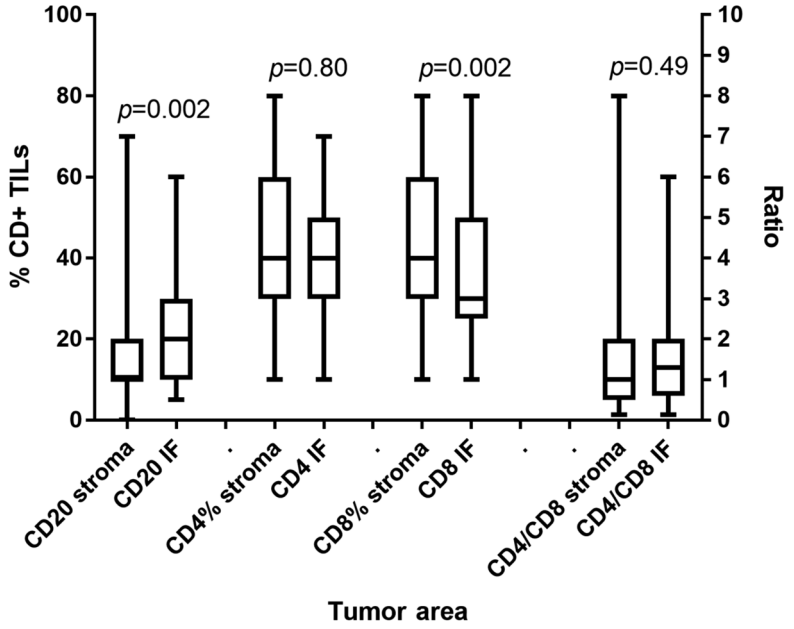

Figure 2. *The percentage of CD4, CD8, CD20 expressing tumor-infiltrating lymphocytes (TILs), and the CD4/CD8-ratio in the inner tumor stroma and the invading tumor front (IF).*

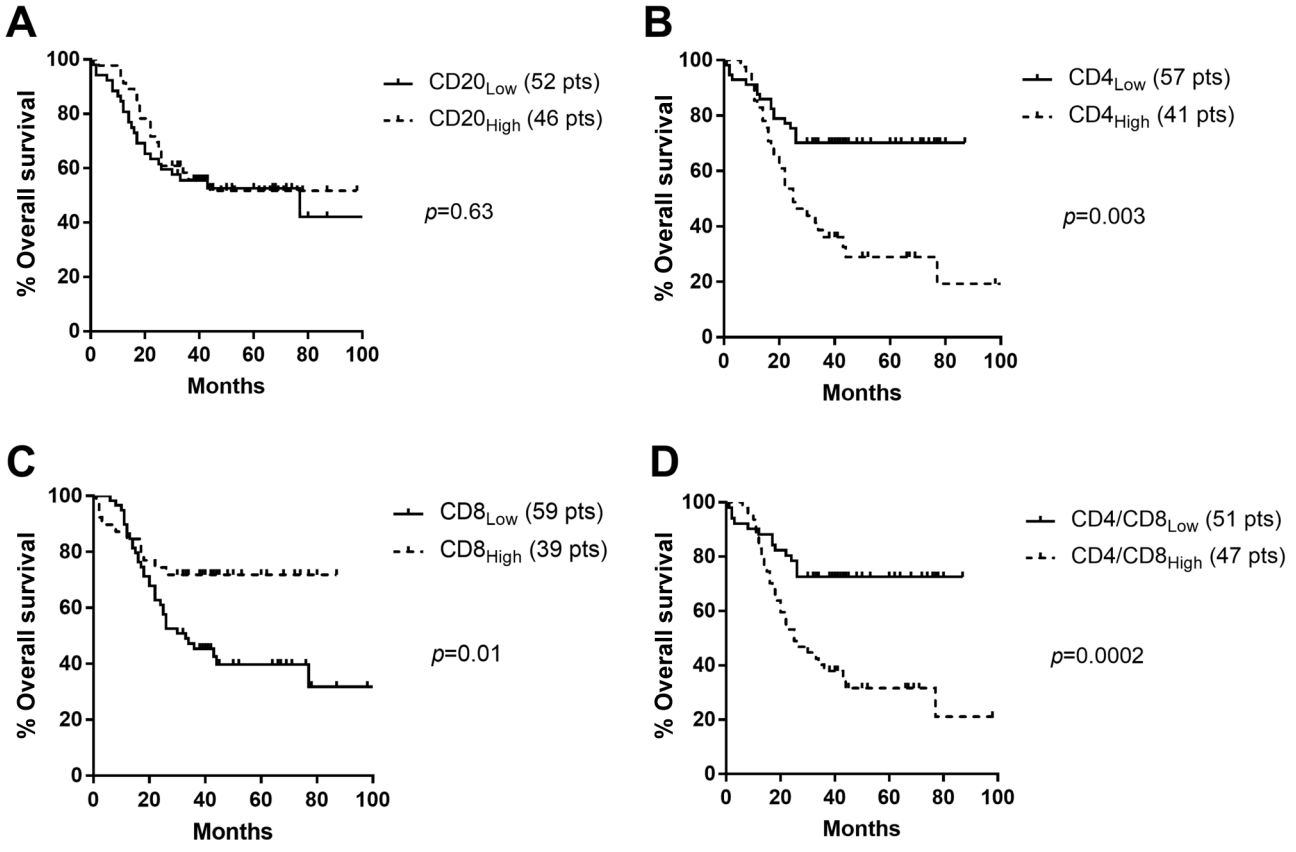

Figure 3. *Kaplan–Meier overall disease-specific survival curves stratified for the CD+ lymphocyte density in the tumor stroma: (A) CD20+, (B) CD4+, (C) CD8+, and (D) CD4/CD8-ratio.*

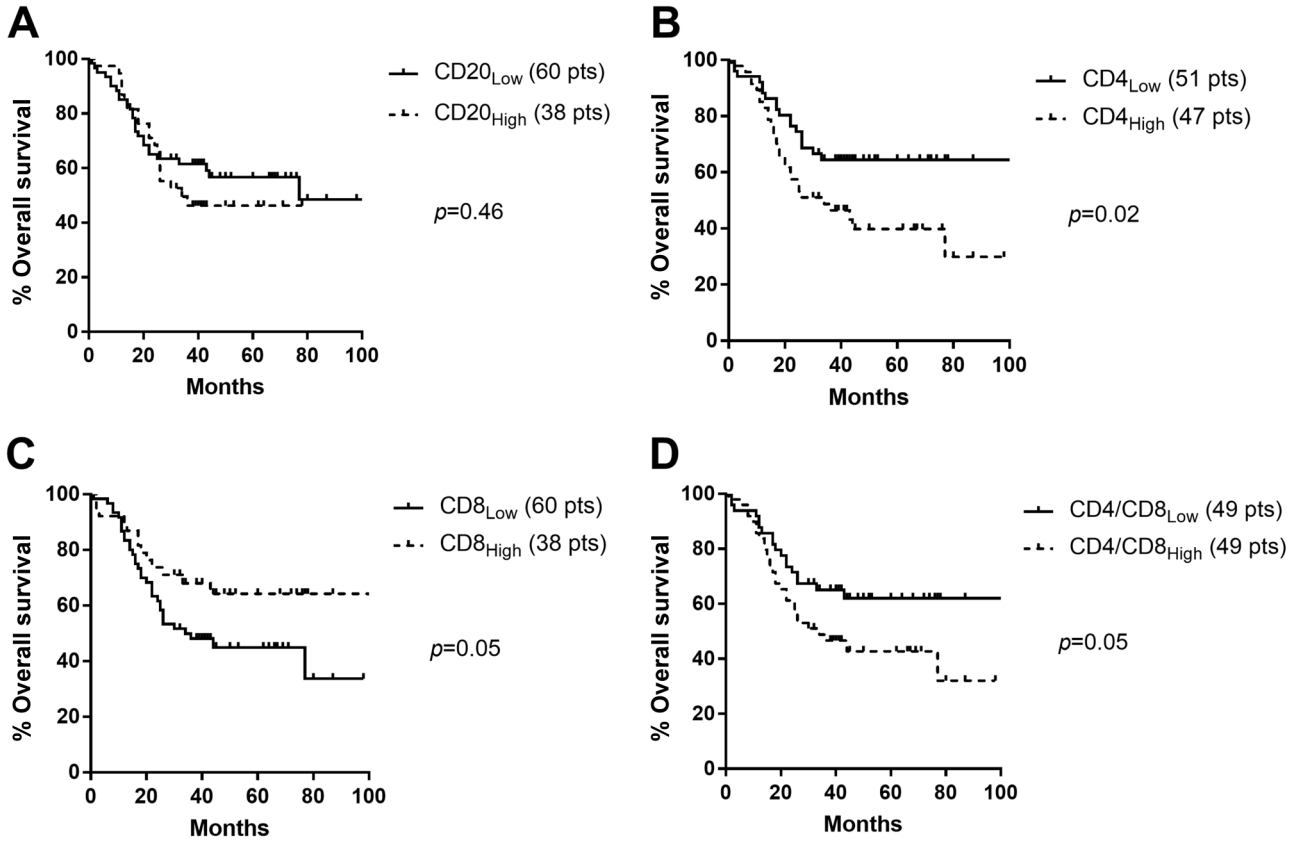

Figure 4. *Kaplan–Meier overall disease-specific survival curves stratified for the CD+ lymphocyte density in the invading tumor front: (A) CD20+, (B) CD4+, (C) CD8+, and (D) CD4/CD8-ratio.*

of three main lymphocyte types, namely the CD20+ B-cells, the CD4+ helper T-cells that can differentiate to regulatory T-cells, and the CD8+ cytotoxic T-cells (16).

Cytotoxic T-cells expressing CD8 are considered the main anti-tumor armory of the body. CD8 serves as a co-receptor for the T-cell receptor (TCR), enabling the interaction of cytotoxic T-cells with cancer cells presenting foreign peptides through HLAs. It is, therefore, expected that high CD8+ TILs in the tumor stroma should parallel intense cytotoxic anti-tumor response, which eventually suppresses growth and installation of metastasis (17). Indeed, in our study, the prevalence of the CD8+ lymphocytes in the stroma and the invading tumor front defined a favorable prognosis after surgery. This finding has also been highlighted in previously reported studies. Among 13 studies included in a meta-analysis by Chem *et al*. (18), eight observed a positive association of CD8+ TILs with a good prognosis. An exception is a study by O'Callaghan *et al*., who found an adverse prognostic effect of CD8+ counts in the tumor stroma. However, CD8+ counts in the tumor nests defined a better postoperative outcome (19).

CD4+ lymphocytes are multifunctional cells that can differentiate towards a helper phenotype that assists CD8+ cytotoxic T-cells or towards regulatory T-cells that suppress cytotoxic cells (20). Their presence in the tumor environment may have a dual role, suppressive or supportive of anti-tumor immunity, depending upon microenvironmental or systemic conditions that affect this balance (21). In the current study, we noted that the intense presence of CD4+ TILs in the tumor periphery and especially in the inner tumor stroma was directly linked to poor postoperative prognosis in NSCLC. This finding reveals a contrasting effect on survival between the CD4 and CD8 lymphocytes, suggestive of an immune balance that often shifts towards immune suppression in the tumor microenvironment. This was further confirmed by the strong association of a high CD4/CD8 ratio with a poor prognosis. It seems that the prevalence of CD4+ cells abrogate the cytotoxic activity of CD8+ cytotoxic T-cells. These findings are in accordance with a previous study by Ohtaki *et al*., where CD4+ TILs were an independent adverse prognostic variable in NSCLC (22). A study by Hiroaka *et al*. has shown that although the separate

consideration of CD4+ and CD8+ lymphocyte counts did not have an impact on the prognosis of NSCLC, simultaneous high expression defined a better outcome (23). This, however, may be an indirect effect of total TIL infiltration, a well-defined variable of good prognosis. The prognostic relevance of the CD4/CD8 ratio has not been reported in that study. Schulz *et al*. found that a low CD4/CD8 ratio was linked with high-grade histology, but the authors did not find an association of CD4+ TILs with prognosis (24). A study by Hasegawa *et al*., did not confirm any association of CD4+ TILs with prognosis. At the same time, flow-cytometry analysis of peripheral blood circulating lymphocytes, showed that patients with a higher frequency of regulatory CD4+/FOXP3+ lymphocytes related with poor outcomes (25). A study by Meng *et al*. did not confirm an association of CD4+ TILs in the stroma with prognosis, although CD8+ TILs were linked with better outcomes (26). In contrast to ours and others' findings, Wakabayashi *et al*. found that dense infiltration by CD4+ (and not by CD8+) lymphocytes was linked marginally with a good prognosis (27).

The role of CD20+ B-cells as a component of TILs has not been thoroughly examined. Overall, the B-cell role in solid tumors' growth and clinical behavior is controversial (28). In our study, CD20+ TIL density did not have any prognostic relevance, whether this was assessed in the invading tumor front or inner stroma areas. In a study by Kadota *et al*., low CD20+ TIL counts combined with high neutrophil tumor infiltration were related to poor prognosis in squamous cell lung cancer (29). An association of high CD138+ plasma cells with a good prognosis has been also reported (30). It may be that the role of B-cells in tumors is complex and dependent on multiple microenvironmental conditions and other immune cell balances.

In conclusion, the balance between CD4+ and CD8+ lymphocytes infiltrating the tumor stroma is a crucial factor defining the control of the immune system over tumor growth and metastasis. Assessment of the CD4/CD8-ratio using the herein proposed methodology emerges as a potent prognostic tool. Moreover, the CD4/CD8-ratio identifies a subset of early-stage operable patients that suffer from an immunosuppressive imbalance, correction of which with immunotherapy would contribute to the postoperative eradication of the disease and the improvement of curability of this highly lethal disease.

Conflicts of Interest

There are no conflicts of interest to report regarding this study.

Authors' Contributions

Alexandra Giatromanolaki: Conception, design, analysis, diagnosis, data interpretation, writing of the article; Achilleas Mitrakas: Immunohistochemistry, data acquisition, analysis, writing of the article; Ioannis Anestopoulos: Immunohistochemistry, data acquisition, analysis, writing of the article; Aglaia Papa: Data interpretation, writing of the article; Mihalis I. Panayiotidis: Data interpretation, writing of the article; Michael I. Koukourakis: Conception, design, analysis, data interpretation, writing of the article.

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