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CD4/CD8 co-expression shows independent prognostic impact in resected non-small cell lung cancer patients treated with adjuvant radiotherapy

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ABSTRACT

Background: Though traditionally regarded as immunosuppressive, radiotherapy may also stimulate immune cells and facilitate an anti-tumor immune response. We therefore aimed to explore the prognostic significance of immune cell markers in non-small cell lung cancer (NSCLC) patients treated with postoperative radiotherapy (PORT).
Methods: In addition to demographic and clinicopathological information, tumor tissue samples were collected and tissue microarrays (TMAs) were constructed from 55 patients with stage I-IIIA NSCLC who received PORT. Tumor and stromal expression of CD1a+, CD3+, CD4+, CD8+, CD20+, CD56+, CD68+, CD117+ and CD138+ cells, as well as M-CSF and CSF-1R, was assessed by immunohistochemistry.
Results: In univariate analysis, high co-expression of CD4+ and CD8+ T lymphocytes as well as high expression of CD1a+ dendritic cells in the tumor stroma correlated with improved disease-specific survival (DSS). In multivariate analysis patients with stromal ↓CD4/↓CD8 expression had a hazard ratio of 21.1 (CI95% 3.9–115.6, $P < 0.001$) when compared to those with ↑CD4/↑CD8 expression.
Conclusions: Stromal ↓CD4/↓CD8 expression was an independent negative prognostic factor for survival in NSCLC patients receiving PORT, indicating a highly detrimental prognosis.

1. Introduction

Lung cancer remains the leading cause of cancer-related mortality in the western world, and is projected to account for 28% of all cancer deaths in the United States in 2012 [1]. Non-small cell lung cancer (NSCLC) represents 80–85% of all lung cancers, and surgical resection of early stage disease presents the best opportunity for long term survival [2]. Despite extensive research efforts, the prognosis of NSCLC patients, even with complete surgical resection, remains disappointing [3]. Immunotherapy has shown potential impact in the treatment NSCLC, and clinical studies on the significance of immunological markers are warranted [4].

The immune system can be divided into two compartments, the innate and the adaptive immune systems. The innate system consists of dendritic cells (DCs), natural killer (NK) cells, NK T cells, macrophages, neutrophils, basophils and eosinophils, and is the body's first line of defense against pathogens. B cells, CD4+ T helper cells and CD8+ cytotoxic T cells, express a diverse set of somatically generated antigen-specific receptors, thereby enabling the highly specific adaptive immune response [5].

Tumor-promoting inflammation mediated by cells of the innate immune system is recognized as an enabling characteristic of cancer development, and the tumor's ability of avoiding immune destruction is recognized as an emerging hallmark of cancer [6]. Innate cells such as macrophages, mast cells and neutrophils contribute to tumor angiogenesis, and tumor infiltration by such cells often correlates with a poor prognosis [6,7]. In contrast, an abundance of infiltrating lymphocytes often correlates with a favorable prognosis [7].

While cell death by damage to tumor DNA is thought to be the main mode of action of radiotherapy, evidence suggests that, it in addition mobilizes tumor specific immunity and stimulates an anti-tumor response [8,9]. Hence, radiotherapy can improve the effect of immunotherapy in cancer treatment [10]. Recent studies have also shown that the efficacy of high dose radiotherapy depends on the presence of CD8+ T cells [11,12]. We previously reported

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on the prognostic impact of both innate and adaptive immune cell markers in NSCLC [13–15]. In addition, we have shown that angiogenic markers have prognostic impact in surgically resected NSCLC patients receiving postoperative radiotherapy (PORT) [16]. To the best of our knowledge, no studies have explored the prognostic significance of immune cell markers in this group of patients. In light of the link between radiotherapy and tumor specific immune responses, we aimed to explore if in situ immunity had an impact on survival in NSCLC patients treated with PORT.

2. Materials and methods

2.1. Patients

Patients surgically resected for NSCLC stage I-IIIA at the University Hospital of Northern Norway and Nordland Central Hospital from 1990 through 2004 were identified in this retrospective study. In total, 371 patients from the hospital databases were registered. Of these, sixty-three patients received radiotherapy within 12 weeks postoperatively, with a cumulative radiation dose of ≥50 Gy. Eight patients were excluded due to: Preoperative chemotherapy ($n = 3$), other malignancy within 5 years prior to NSCLC diagnosis ($n = 3$) or inadequate paraffin-embedded surgical specimens ($n = 2$). A total of 55 patients were thereby included in the study. Adjuvant chemotherapy had not been introduced in Norway during this period (1990–2004). Clinicopathologic and demographic data were collected retrospectively. This study includes follow up data as of January 2011. Patients were staged according to the revised 7th edition of UICC TNM classification of lung cancer [17], and histologically graded and subtyped according to the World Health Organization guidelines [18]. The Norwegian Data Inspectorate and The Regional Committee for Medical and Health Research Ethics approved the study.

2.2. Microarray construction

All specimens were examined by two pathologists (S.Al-S and K.Al-S). The most representative paraffin blocks were selected and two areas of viable tumor cells (neoplastic epithelium) and two from the central tumor-surrounding stroma were chosen and marked on the donor blocks. The tissue microarrays were assembled using a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD, USA). The detailed methodology has been reported previously [19]. Using a 0.6 mm-diameter stylet, cores from two separate predefined neoplastic epithelial areas and two stromal areas were transferred to recipient blocks. To include all core samples, a total of eight tissue array blocks were constructed. Multiple 4-μm sections were cut with a Micron microtome (HM355S) and stained with specific antibodies for immunohistochemical analysis. Both normal lung tissues localized distant to the primary tumor and one slide with normal lung tissue sample from 20 patients without a diagnosis of cancer were used as controls.

2.3. Immunohistochemistry

The following antibodies from Ventana Medical (Tucson, Ariz, USA) were used in this study: CD20 (clone L26), CD8 (clone 1A5), CD68 (clone KP1), CD138 (clone B-A38), CD1a, CD3 (clone PS1), CD117 (clone anti-C Kit, 9.7) and CD138 (clone B-A38). All Ventana antibodies were prediluted from the manufacturer. In addition CD4 (clone 1F6, Novocastra Laboratories Ltd. Newcastle upon Tyne, UK, dilution 1:5), M-CSF (Santa Cruz Biotechnology, Santa Cruz, CA, USA, dilution 1:5) and CSF-1R (clone H-300, Santa Cruz Biotechnology, dilution 1:25) were used. The detailed immunohistochemical procedures have been published previously [13–15]. For each antibody, including negative staining controls, all staining was done in a single experiment. As negative staining controls, the primary antibodies were replaced with the primary antibody diluents.

2.4. Scoring of Immunohistochemistry

Tissue sections were scored by light microscopy for degree of infiltration of the specified immune cells.

The CD8+ cells were scored as low if ≤5% or as high if >5% of the whole surface area of the epithelial compartments were infiltrated, and was scored as low if ≤50% or high if >50% of the total nucleated surface area of the stromal compartments were infiltrated. CD4+ cells were scored as high if representing ≥5% or ≥25% of the total nucleated cells in the epithelial and stromal compartments, respectively. Few CD4+ and CD8+ T cells (0 to <5% of the total nucleated cells) were observed in the interstitial tissue of the nonneoplastic controls.

CD1a+ cells were scored as low if absent or if representing <1% of the nucleated cells and high otherwise, in both epithelial and stromal compartments. Intraepithelial CD68+ cells were scored as low if absent or representing <1% of the nucleated cells and high otherwise, while the more abundant stromal CD68+ cells were scored as low if they represented <25% of the total nucleated cells and high otherwise. CD56+ cells were scored as present (high score) or absent (low score) in both epithelial and stromal compartments. The intensity of M-CSF and CSF-1R in both epithelial and stromal compartments were scored as follows: 0 = negative; 1 = weak; 2 = intermediate and 3 = strong. The cell density of the stroma was scored as the ratio of positive cells compared to the surface area of the extracellular matrix in the following manner: 1 = low density (<25% cell/matrix ratio); 2 = intermediate density (25–50%) and 3 = high density (>50%). High expression in the tumor epithelium was defined as a score ≥1.5 for both M-CSF and CSF-1R. Expression in the stroma was calculated by adding density score to intensity score prior to categorizing into low and high expression. High expression was defined as >3.5 for M-CSF and >3 for CSF-1R.

CD3+ cells were scored as low if they represented <1% of the nucleated cells in the epithelial cores and high otherwise, and as high if representing >50% of nucleated cells in the stroma and low otherwise. CD138+ cells were scored as high if representing >5% of the nucleated cells in the epithelial compartment or >25% in the stromal compartment, and as low otherwise. As CDD138+ cells also stain epithelial cells themselves the staining intensity in the epithelial compartment was scored in the following manner: 0 = negative; 1 = weak; 2 = intermediate and 3 = strong. High expression was defined as a score >1. CD117+ cells were extremely rare in the epithelial compartments and sparse in the stromal compartment, they were therefore scored as present (high score) or absent (low score) and only in the stromal compartment.

All samples were anonymized and independently scored by two pathologists (S.A.S and K.A.S). In case of disagreement, the slides were re-examined and the observers reached a consensus. When assessing one marker in a given core, both observers were blinded to the scores of the other markers as well as to the patient's outcome. The interobserver scoring agreement between the two pathologists was tested on the current material previously [20], with a mean correlation coefficient of 0.95 (range 0.93–0.98).

2.5. Statistical methods

All statistical analyses were carried out using the statistical package IBM SPSS, version 20 (SPSS Inc., Chicago, IL, USA). Univariate analysis of the association between marker expression and survival was done using the Kaplan–Meier method and the statistical significance of differences between survival curves was assessed

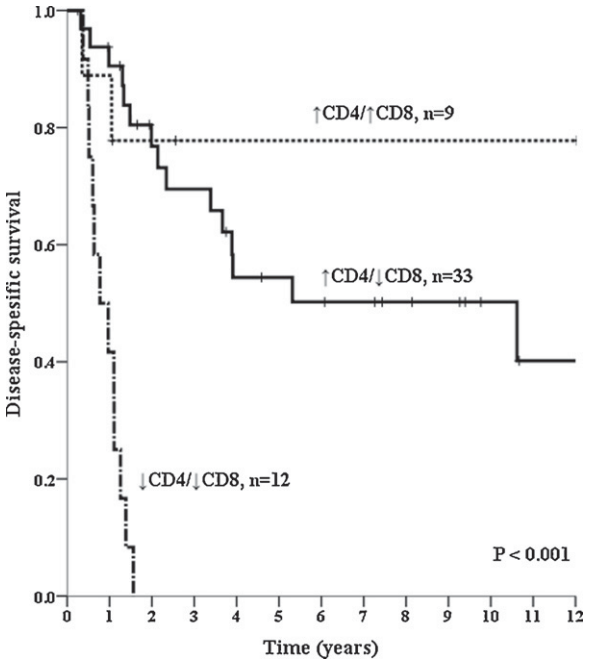

Fig. 1. Disease-specific survival curves according to the co-expression of stromal CD4 and CD8 in 54 NSCLC patients administered post-operative radiotherapy.

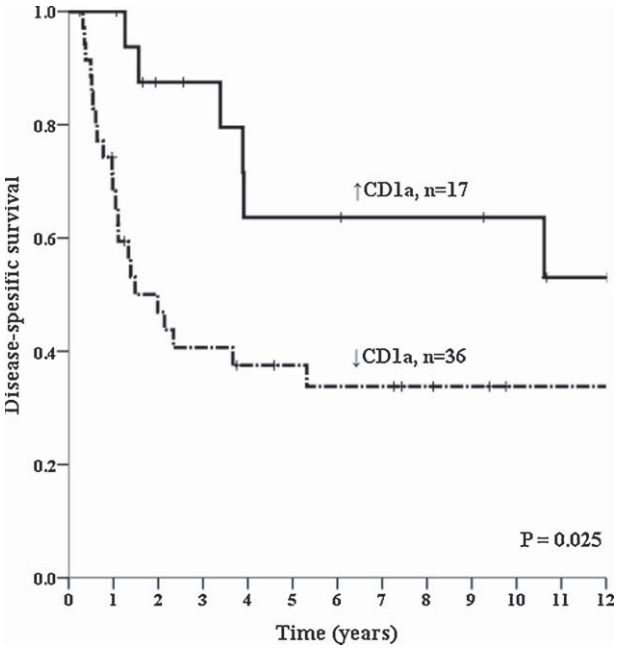

Fig. 2. Disease-specific survival curves according to the expression of stromal CD1a in 53 NSCLC patients administered post-operative radiotherapy.

by the log-rank test. The disease-specific survival (DSS) was determined from the date of surgery to the time of lung cancer death. Only variables of significant value from the univariate analysis were entered into the multivariate analysis, using the Cox proportional hazards model. Probability for stepwise entry and removal was set at 0.05 and 0.10. The significance level was set at $P < 0.05$.

3. Results

3.1. Clinicopathologic variables

Demographic, clinical and histopathology variable are presented in Table 1. The median survival time for all 55 patients was 24 months (range 3–197). The 5-year DSS was 44% and the 10-year DSS was 42%. Median patient age was 65 years (range 39–76) and the majority of patients were men (69%). The NSCLC tumors were comprised of 33 squamous cell carcinomas, 16 adenocarcinomas and 6 large cell carcinomas.

In univariate analysis, weight loss >10% ($P = 0.029$), histology ($P = 0.048$), poor tumor cell differentiation ($P = 0.026$) and nodal metastasis ($P = 0.010$) were significant prognostic variables (Table 1). The association between molecular marker expression and disease-specific survival data is presented in Table 2. The co-expression of CD4 and CD8 was a strong significant prognostic factor (Fig. 1 and Table 2), as was stromal CD4 expression (Table 2). In addition, patients with high stromal expression of CD1a had a significantly better DSS than those with a low expression (Fig. 2).

3.2. Multivariate analysis

None of the clinicopathologic variables emerged significant in multivariate analysis, while the hazard ratio was 21.2 (CI95% 4.5–120.4, $P < 0.001$) for the ↓CD4/↓CD8 combination and 1.8 (CI95% 0.4–8.4, $P = 0.430$) for other CD4/CD8 combinations, when compared to the reference group ↑CD4/↑CD8 (Table 3). Low CD1a had a hazard ration of 2.5 (CI95% 0.97–6.2, $P = 0.058$) when compared to high expression.

4. Discussion

We present the first study examining the prognostic impact of immune cell marker expression in surgically resected NSCLC patients treated with adjuvant radiotherapy. Our main finding is that the stromal co-expression of CD4+ and CD8+ T lymphocytes is a strong and independent prognostic factor in this group. Patients with ↓CD4+/CD8+↓ expression seem to have remarkably poor prognosis and will therefore most likely have a very limited benefit of adjuvant radiotherapy. The 5-year survival rate for patients with ↑CD4+↑CD8+ expression (16%, $n = 9$) was 78%, whereas ↓CD4+↓CD8+ patients (22%, $n = 12$) had median survival rate of only 9 months, with none surviving longer than 19 months from the time of diagnosis. The observed hazard ratio of 21.1 between ↓CD4+↓CD8+ and ↑CD4+↑CD8+ indicates a substantial and independent impact on DSS. However, due to the small number of patients the results have to be interpreted cautiously.

Hiraoka et al. have previously shown that there is a synergistic effect of simultaneous high CD4+ and CD8+ T-cell expression on survival in NSCLC [21], while we previously showed that stromal expression of CD4 and CD8 both are independent prognostic factors in NSCLC [13]. The high hazard ratio observed in our subgroup of patients indicates that CD4/CD8 expression has higher prognostic significance in PORT treated patients, and may suggest a link between stromal in situ immunity and radiotherapy response.

Results from cell lines and murine models reveal close interplay between the immune system and the effects of radiotherapy. Radiotherapy may enhance expression of tumor-associated antigens, facilitate immune-mediated targeting of the tumor stroma and diminish the activity of regulatory T-cells. [4]. However, our results suggest that radiotherapy alone does not up-regulate the immune response sufficiently to inhibit tumor growth in ↓CD4/↓CD8 patients. In a murine model of melanoma, Lee et al. observed that the therapeutic effect of radiotherapy was dependent on CD8+ T-cells, since tumors of CD8 depleted mice became radioresistant [11]. Gupta et al. recently described how CD8+ T-cells are crucial for the effect of local high-dose radiotherapy, whereas CD4+ T-cells and macrophages were not [12]. Low stromal CD4/CD8 may indicate an insufficient level of these cells for a successful

Table 1
Prognostic clinicopathologic variables as predictors of disease-specific survival in 55 NSCLC-patients receiving adjuvant postoperative radiotherapy.

Characteristic	Patients (*n*)	Patients (%)	Median survival (months)	5-Year survival (%)	*P*
Age					0.471
≤65 years	31	56	44	42	
>65 years	24	44	41	48	
Sex					0.433
Female	17	31	64	53	
Male	38	69	26	41	
Smoking					0.491
Never	1	2	NR	100	
Current	31	56	41	40	
Former	23	42	47	47	
Performance status					0.159
ECOG 0	28	51	47	50	
ECOG 1	23	42	26	36	
ECOG 2	4	7	NR	67	
Weight loss					**0.029**
<10%	49	89	47	47	
>10%	6	11	8	20	
Histology					**0.048**
Squamous cell carcinoma	33	60	NR	61	
Adenocarcinoma	16	29	21	19	
Large cell carcinoma	6	11	18	17	
Differentiation					**0.026**
Poor	27	49	18	21	
Moderate	21	38	127	65	
Well	7	13	NR	63	
Surgical procedure					0.795
Lobectomy	29	53	47	43	
Pneumonectomy	26	47	18	45	
Pathological stage					0.084
I	7	13	NR	83	
II	20	36	NR	51	
III	28	51	21	30	
Tumor status					0.923
1	7	13	44	40	
2	32	58	26	44	
3	16	29	47	45	
Nodal status					**0.010**
0	14	25	NR	75	
1	19	35	41	50	
2	22	40	19	21	
Surgical margins					0.174
Free	38	69	21	37	
Not free	17	31	NR	60	
Vascular infiltration					0.146
No	42	76	64	51	
Yes	13	24	26	21	
Clinican reason for PORT					0.063
Insufficient margin or tumor cells in resection margin	18	33	NR	65	
N1	14	25.5	16	50	
N2	20	36	19	22	
Local recurrence	3	5.5	NR	67	
Fractioning regime					0.460
$2.8 \times 15 = 42$ Gy	29	53	19	41	
$2 \times 30 = 60$ Gy	21	38	47	48	
$2 \times 25–29 = 50–59$ Gy	5	9	24	40	

NR: not reached; PORT: postoperative radiotherapy; NCSLC: non-small cell lung cancer.
Bold values indicate $p < 0.05$

"boosting" of the radiotherapy effect. Stimulating the immune response via immunotherapy could therefore possibly augment the responsiveness to radiotherapy in those individuals lacking concurrent high CD4/CD8 levels in the tumor stroma.

Experimental data suggest that radiotherapy and immunotherapy may have additive and synergistic effects. Reits et al. showed that radiotherapy prior to adoptive treatment with cytotoxic T-cells greatly enhanced the efficacy of the immunotherapy [10]. Takeshima et al. observed that local tumor irradiation augmented the therapeutic effect of Th1 cell therapy, accompanied by induction of cytotoxic T-lymphocytes in the tumor draining lymph nodes and tumor mass [22]. In a murine model of Lewis Lung Carcinoma, Yokouchi et al. reported greater efficacy when combining radiotherapy with an agonistic monoclonal antibody to αOX40 (CD134), which augments T-cell expansion and survival, when compared to either single treatment given separately [23]. Similar results were presented by Gough et al., with a significant portion of long-term tumor-free survivors [24]. Combining CTLA-4 blockade with radiation, Demaria et al. were able to induce an immune-mediated inhibition of metastases in a mouse model of breast cancer [25]. Similarly, Dewan et al. were able to induce an abscopal effect by combining fractionated radiotherapy with an anti-CTLA-4 antibody [26]. Adjuvant immunotherapy has shown encouraging results in NSCLC [27], but few trials have looked at combining immunotherapy with radiotherapy. As the above presented pre-clinical studies indicate, this treatment combination may be an interesting approach for resected NSCLC patients.

Table 2
Prognostic molecular variables as predictors of disease-specific survival in 55 NSCLC-patients receiving adjuvant postoperative radiotherapy.

Marker expression	Patients (*n*)	Patients (%)	Median survival (months)	5-Year survival (%)	*P*
CD4					
Tumor					0.799
Low	40	73	47	40	
High	14	25	44	49	
Missing	1	2			
Stroma					**<0.001**
Low	12	22	9	0	
High	42	76	NR	59	
Missing	1	2			
CD8					
Tumor					0.525
Low	41	74.5	41	45	
High	13	23.5	47	45	
Missing	1	2			
Stroma					0.072
Low	45	82	41	39	
High	9	16	NR	78	
Missing	1	2			
CD4/CD8					
Stroma					**<0.001**
↑CD4+/↑CD8+	9	16	NR	78	
Other CD4+/CD8+ combination	33	60	127	54	
↓CD4+/↓CD8+	12	22	9	0	
Missing	1	2			
Tumor					0.476
↑CD4+/↑CD8+	6	11	NR	63	
Other CD4+/CD8+ combination	15	27	26	31	
↓CD4+/↓CD8+	33	60	47	47	
Missing	1	2			
CD20					
Tumor					0.059
Low	40	73	26	40	
High	14	25	NR	61	
Missing	1	2			
Stroma					0.419
Low	10	18	16	34	
High	44	80	47	47	
Missing	1	2			
CD68					
Tumor					0.661
Low	23	42	19	45	
High	31	56	47	45	
Missing	1	2			
Stroma					0.414
Low	38	69	47	45	
High	16	29	44	48	
Missing	1	2			
CD56					
Tumor					0.316
Low	52	94	47	47	
High	2	4	18	0	
Missing	1	2			
Stroma					0.108
Low	49	89	41	41	
High	5	9	NR	80	
Missing	1	2			
CD1a					
Tumor					0.499
Low	32	58	28	39	
High	22	40	64	54	
Missing	1	2			
Stroma					**0.025**
Low	36	65	24	38	
High	17	31	NR	64	
Missing	2	4			
M-CSF					
Tumor					0.939
Low	15	27	16	47	
High	38	69	46	44	
Missing	2	4			
Stroma					0.843
Low	28	51	47	45	
High	24	44	41	48	
Missing	3	5			

Table 2 (*Continued*)

Marker expression	Patients (*n*)	Patients (%)	Median survival (months)	5-Year survival (%)	*P*
CSF-1R					
Tumor					0.215
Low	21	38	16	34	
High	22	40	127	55	
Missing	12	22			
Stroma					0.701
Low	26	47	47	42	
High	27	49	41	48	
Missing	2	4			
CD3					
Tumor					0.619
Low	38	69	41	41	
High	16	29	64	55	
Missing	1	2			
Stroma					0.212
Low	42	76	41	41	
High	12	22	NR	57	
Missing	1	2			
CD138					
Tumor					0.292
Low	24	43.5	19	35	
High	29	52.5	64	54	
Missing	2	4			
Stroma					0.165
Low	24	43.5	24	33	
High	29	52.5	64	53	
Missing	2	4			
CD138 of the cancer cells					
Negative	12	22	13	25	0.058
Positive	41	74	64	51	
Missing	2	4			
CD117 in the stroma					
Negative	36	65	47	49	0.305
Positive	17	31	44	35	
Missing	2	4			

NR: not reached; NCSLC: non-small cell lung cancer.
Bold values indicate $p < 0.05$

Table 3
Result of Cox regression analysis summarizing prognostic factors with $P < 0.10$.

Variable	Hazard ratio	95% Confidence interval	*P*
Stromal CD4/CD8			**<0.001**[*]
↑CD4+/↑CD8+	1.000		
Other CD4+/CD8+ combination	1.842	(0.404–8.390)	0.430
↓CD4+/↓CD8+	21.123	(3.860–115.584)	**<0.001**
Stromal CD1a			
Low	2.454	(0.969–6.213)	0.058
High	1.000		

None of the clinicopathologic variables emerged as statistically significant during Cox regression analysis.
Bold values indicate $p < 0.05$
[*] Overall significance as a prognostic factor.

Though only shown in univariate analysis, we found that a higher expression of stromal CD1a+ DCs confer an increased DSS when compared to low expression for patients treated with PORT. DCs are professional antigen presenting cells, who can process and present tumor associated antigens and thereby activate adaptive immune cells [28]. Radiation-induced tumor cell death may be associated with the production of maturation signals for DCs [29].

Teitz-Tennenbaum et al. observed that the efficacy of DC immunotherapy was enhanced by radiotherapy [30]. Increasing DC infiltration though immunotherapy could therefore be a potential strategy to improve survival in PORT treated patients.

In conclusion, we have shown that low CD4/CD8 expression is an independent negative prognostic factor in surgically resected NSCLC treated with PORT. Though our results are striking, they should be considered with caution, as the number of included patients is low. Nevertheless, further studies are pivotal in order to elucidate the potential significance of CD4/CD8 expression as a predictive marker in adjuvantly irradiated NSCLC.

Conflicts of interest statement

None declared.

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